Gating Effects of Component B on Oxygen Activation by the Methane Monoxygenase Hydroxylase Component*

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Component B (MMOB) of the soluble methane monoxygenase (MMO) system accelerates the initial velocity of methane oxidation by up to 150-fold by an unknown mechanism. The active site of MMO contains a diferric, hydroxyl-bridged diiron cluster located on the hydroxylase component (MMOH). This cluster is reduced by the NAD(P)H-coupled reductase component to the diferrous state, which then reacts with O₂ to yield two reaction cycle intermediates sequentially termed compounds P and Q. The rate of compound P formation is shown here to be independent of O₂ concentration, suggesting that an MMOH-O₂ complex (compound O) is (Irreversibly) formed before compound P. Compound Q is capable of reacting with hydrocarbons to yield the MMOH-product complex, compound T. It is shown here that MMOB accelerates catalysis by increasing -1000-fold the rate of O₂ association and reaction with diferrous MMOH leading to compound P.

Modeling of the single turnover reaction in the presence of substoichiometric MMOB suggests that MMOB also accelerates the compound P to Q conversion by -40-fold. Due to this O₂-gating effect of MMOB, either compound Q or T becomes the dominant species during turnover, depending upon the substrate concentration and type. Because these are the species that either react with substrate (Q) or release product (T), their buildup maximizes the turnover rate. This is the first direct role in catalysis to be recognized for MMOB and represents a novel method for oxygenase regulation.

Methane monooxygenase (MMO) catalyzes activation of O₂ for the oxidation of methane to methanol (1, 2).

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\text{CH}_4 + O_2 + \text{NADH} + H^+ \rightarrow \text{CH}_3\text{OH} + H_2O + \text{NAD}^+ \]

Many other hydrocarbons serve as adventitious substrates (3). The soluble form of MMO isolated from Methylosinus trimethylporum OB3b consists of three independent components; a 245-kDa hydroxylase (MMOH), a 40-kDa reductase (MMOR), and a 15-kDa protein termed component B (MMOB) (4). The roles of MMOR and MMOH in catalysis have been defined in a straightforward manner. Each of the two αβγ-protopomers of MMOH contains a hydroxyl-bridged diiron cluster that is essential for O₂ activation and hydrocarbon oxidation and thus appears to be at the active site of the enzyme (2, 7, 4–11). MMOR contains FAD and a [2Fe-2S] cluster and serves to transfer electrons from NADH to the MMOH active site cluster (4, 12, 13). In contrast to the well defined roles of these components, that of MMOB has been more difficult to determine.

MMOB contains no metals, prosthetic groups, or cofactors. Nevertheless, in past studies, we have shown that MMOB is active in: (i) increasing the rate of the steady state reaction up to 150-fold (15); (ii) perturbing the spectroscopic features of MMOH (15, 16); (iii) shifting the redox potential values of MMOH (17); (iv) altering the product distribution for complex substrates that can be hydroxylated in more than one position (6); and (v) varying the rate of product formation when MMOH functions through a H₂O₂ shunt (6, 18). It has also been postulated that MMOB plays roles in increasing the rate of electron transfer and in efficiently coupling substrate oxidation to NADH consumption (14, 19–21). Together, these studies suggest that MMOB alters the structure of MMOH in a way that accelerates and potentially regulates the reaction. However, the basis for these effects is unknown.

Recently, we have shown that the catalytic cycle of MMOH proceeds through several discrete intermediates (22, 23). In an initial step, the diferric resting state of the MMOH diiron cluster is reduced to the diferrous state. This state reacts with O₂ to form an intermediate termed compound P (formally a peroxy adduct), which then spontaneously converts to a yellow species termed compound Q. Compound Q appears to be the species that reacts directly with substrates to yield the terminal enzyme-product complex (compound T) (22). In the presence of MMOB at low substrate concentrations, these intermediates form at progressively decreasing rates. This has allowed some of them to be trapped and characterized (23–25).

In this study, we have investigated the effect of MMOB on the kinetics of compounds P and Q formation and decay. We show that a major new role for MMOB is to greatly increase the rates of formation of these intermediates. Our findings provide the first direct demonstration of the kinetic basis for the essential regulatory role of MMOB in establishing efficient catalysis.

EXPERIMENTAL PROCEDURES

Materials—Bacterial growth and purification of MMO from M. trimethylporum OB3b, as well as enzyme activity assays, were as reported previously (4, 26). MMOH exhibited specific activity in the range of 600–900 nmol/min/mg for furan turnover. The buffer for all experiments was 100 mM MOPS, pH 7.7. All reagents were of the highest grade available and were obtained from Sigma, Aldrich, or EM Scientific. Water was glass distilled. Anaerobic sample handling techniques have been described previously (4, 5).

Stopped-flow Absorption Spectroscopy—Single turnover reactions by MMOH were monitored using a stopped-flow apparatus (Update In-

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1 The abbreviations used are: MMO, methane monooxygenase; MMOH, MMO hydroxylase component; MMOR, MMO component B; MMOR, MMO reductase component; MOPS, 3-(N-morpholino)propanesulfonic acid.

2 Compound P is termed compound L or the peroxy intermediate for MMOH isolated from Methylococcus capsulatus Bath (24, 25).
The Effect of MMOB on Oxygen Reactivity with Diferrous MMOH—Past catalytic studies have shown that MMOH reacts rapidly with O₂ only when in the diferrous state (4). The concentration of this state is conveniently monitored using an EPR signal near g = 16 (5, 26). Fig. 1 shows the time course of the disappearance of diferrous MMOH at 4°C with addition of O₂. The first order rate constant for the disappearance of diferrous MMOH was 22 ± 2 s⁻¹, confirming our earlier observations (22) (Fig. 1, □). Without MMOB, the rate constant decreased approximately 100-fold to 0.023 ± 0.003 s⁻¹ (Fig. 1, ●). This rate constant would be limiting in the catalytic cycle of reconstituted MMO for many substrates including methane (turnover number = 0.17 s⁻¹ at 4°C). This activation toward reaction with O₂ is the first direct evidence for a specific role that MMOB plays during the catalytic cycle.

Oxygen Concentration Dependence—The rate of disappearance of the g = 16 EPR signal for diferrous MMOH in buffer were found to be independent of O₂ concentration in the range of 300–700 μM within experimental error (data not shown). This suggests that a fast, effectively irreversible O₂ association step precedes the formation of compound P. We will term this new Michaelis complex between diferrous MMOH and O₂ compound O.

Effect of MMOB on the Compound Q Time Course in the Absence of Substrate—In the presence of MMOB, compound Q (λₚ₃₃ = 430 nm) forms (k ~ 1 s⁻¹, 4°C) and decays (k ~ 0.05 s⁻¹, 4°C) in the catalytic cycle following the disappearance of the diferrous MMOH, compound O, and compound P (22). Without MMOB, compound Q is difficult to detect during single turnover reactions, indicating that the rate of at least one reaction in its formation or decay has been significantly changed. Fig. 2 (solid lines) shows the effect of the ratio of MMOB to MMOH concentration on the time course of compound Q. In this study, we find: (i) the maximum observed amount of compound Q increases in close proportion to the relative amount of MMOB in the reaction mixture; (ii) the rate of MMOB to MMOH sites is stoichiometric, the compound Q concentration first builds rapidly, then plateaus for a period of time, and finally decays at a rate almost independent of MMOB concentration; (iii) the duration of the plateau decreases as the ratio of MMOB to MMOH sites increases.

Effect of MMOB on Product Yield During a Single Turnover—The time course of the propylene oxidation during a single turnover of diferrous MMOH in buffer was rapidly mixed with O₂-saturated buffer (1:1) at 4°C. The MMOB-MMOH diiron cluster ratio was as shown in the figure. Time courses of the reactions were monitored at 430 nm, which is maximal for compound Q (solid lines). At a ratio of 1:1 MMOB-MMOH sites, the time course was essentially superimposable on the 2:1 curve shown and is omitted here for clarity. The dotted line represents the simulation of the kinetic model shown in Fig. 4.
model that accounts well for the data is shown in Fig. 4, and the results of a simulation based on this model are presented in Fig. 2 (dotted lines). The right and left sides of the model in Fig. 4 represent the pathways of the single turnover reaction of MMOH ± MMOB, respectively. Rate constants for most of these steps have been measured during the current or previous study (22). An equilibrium binding step between MMOH and MMOB is shown to occur at each step of the model. The MMOB complexes with diphosphoryl MMOH have been detected and their dissociation constants determined (15, 17). The product yield data reported in Fig. 3 suggests that it is possible to oxidize diphosphoryl MMOH without product formation. The ratio of the rates for productive and nonproductive pathways is fixed by the observed product yields. We have modeled this “leak” as a breakdown of compound P in the absence of direct evidence for the species involved.\(^3\) The information now in hand fixes the values of the rates or relative rates of many steps of the model (indicated in boldface italic in Fig. 4). Trial and error methods were used to find the remaining values to best simulate the experimental observations. From this simulation, we find that a satisfactory fit to the data requires: (i) much higher rates of compound P and compound Q formation in the presence of MMOB; (ii) little effect of MMOB on compound Q decay; and (iii) a significant increase in affinity between MMOB and the MMOH as the cycle progresses toward diphosphoryl MMOH. In this way, some compound Q is quickly formed by the rapid oxidation of the diphosphoryl MMOH-MMOMOB complex, but its concentration then plateaus as MMOB is sequestered in the high affinity diphosphoryl MMOH-MMOMOB complex. As shown in Fig. 2, the result of the simulation reproduces the major features of the data. Most significantly, the model correctly predicts the dependence on MMOB of the maximum amount of compound Q formed as well as the occurrence of a plateau in the compound Q time course for substoichiometric MMOB. Consequently, we believe that the basis of this model is correct. However, values for constants determined by trial and error for a simulation of this complexity are unlikely to be unique, and additional measurements of individual rate and equilibrium constants will be required before further refinement of the model can be made. Also, other, more complicated aspects of the MMOH-MMOMOB interaction may have to be taken into consideration in order to fully account for the data, including: (i) the formation of a secondary complex consisting of MMOH and 2 MMOB (15) and (ii) the effect of hysteresis in the structural change of MMOH caused by MMOB (6).

The simulation predicts that neither compound Q nor compound P will accumulate in high levels in the absence of MMOB. Accordingly, we have recently observed using Mössbauer spectroscopy that for \(^{57}\)Fe-enriched diphosphoryl MMOH exposed to saturating O\(_2\) and frozen after 35 s at 4 °C, diphosphoryl and diphosphoryl MMOH were the only major species present and that these occurred in approximately the 1:1 ratio predicted by the model for that time.\(^4\) In contrast, in the presence of MMOB, the model predicts that compounds P and Q will accumulate in \(\geq 70\%\) yield at different times during the first few seconds of the reaction because their formation rates are much faster than their decay rates. Past Mössbauer studies have shown that these compounds do form in high yield (23–25) at the predicted times.

**Significance for Turnover Rate and Efficiency—**We have shown previously that substrates react directly with compound Q to form an MMOH-product complex (compound T) and that the rate of this reaction depends linearly on both the compound Q and substrate concentrations (22). The results shown in Fig. 2 and the model in Fig. 4 indicate that the effect of MMOB is to increase the rate of formation of compound Q, thereby maximizing the rate of product formation when substrate is present. Conversely, the rate of product formation is expected to be slow in the absence of MMOB because compound Q is slowly formed. These predictions are confirmed by the good fit to the data presented in Fig. 3 (solid lines) based on the model shown in Fig. 4 with the addition of a new step in which compound Q reacts with substrate to form product. The model predicts that the yield of product from a single turnover will be independent of the type of substrate added, which was shown here to be approximately the case for propylene and nitrobenzene.

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\(^3\) The “leak” is unlikely to occur at compound Q because different substrates with different rate constants for reaction with compound Q would then be expected to exhibit greatly different yields, which is not the case. Compound Q appears to be a Michaelis complex between diphosphoryl MMOH and O\(_2\), and is thus also unlikely to break down directly to oxidized MMOH. Note that MMOB appears to increase the rates of both the formation of compound Q and the “leak” to similar extents. Thus, these processes may represent a branch in the same reaction.

\(^4\) Y. Liu, K. Kauffmann, J. C. Nesheim, E. Münck, and J. D. Lipscomb, unpublished results.
CONCLUSION

It has been shown here that MMOB plays a specific role in MMO catalysis by increasing the rate of compound P formation by as much as 1000-fold. It is likely that the rate of conversion of compound P to compound Q is also increased at least 40-fold. This has the effect of shifting the rate-limiting step in catalysis either to the reaction of substrates with compound Q to form enzyme-bound product or to the product release step, depending upon the specific substrate utilized. Together these effects maximize the rate of turnover and minimize the possibility that an activated species prior to compound Q in the catalytic cycle will react with adventitious substrates or the enzyme itself.

The physical basis for the enhancement in the reaction rate with O₂ remains unknown. It may be that it derives from the lower redox potential of the MMOH-MMOB complex, which would be expected to increase the affinity of the diiron cluster for O₂ and perhaps facilitate the O=O bond-breaking chemistry (17). Alternatively, the effect may derive from an increase in the O₂ accessibility of the active site or the diiron cluster through a conformational change in accord with previous observations demonstrating such changes (6, 15, 17).

We believe that the “gating” effect of MMOB on O₂ reactivity described here is its major role in catalysis. Many other effects of MMOB documented thus far can be accounted for in the context of an accelerated reactivity with O₂ that maximizes the efficiency of catalysis. In other multicomponent oxygenases, regulation of catalysis has been shown to be effected by factors such as: (i) specificity between components for electron transfer (see, for example, Ref. 28); (ii) substrate gating of electron transfer through coupling of redox potential to binding free energy (29); and (iii) requirement for specific component complexes for catalysis of the O₂ bond-breaking reaction (30). The regulation of catalytic efficiency through oxygen gating appears to be a novel role for an accessory protein in an oxygenase mechanism. A comparable role is likely to be played by the component B from MMO systems isolated from other bacteria and perhaps also by the similar small protein components from other, newly recognized oxygenases that use dinuclear iron clusters in catalysis (31).

REFERENCES

1. Dalton, H. (1980) Adv. Appl. Microbiol. 26, 71–87
2. Lipscomb, J. D. (1994) Annu. Rev. Microbiol. 48, 371–399
3. Higgins, I. J., Best, D. J., and Hammond, R. C. (1988) Nature 336, 561–564
4. Fox, B. G., Froland, W. A., Dege, J. E., and Lipscomb, J. D. (1989) J. Biol. Chem. 264, 10023–10033
5. Fox, B. G., Surerus, K. K., Münck, E., and Lipscomb, J. D. (1988) J. Biol. Chem. 263, 10553–10566
6. Froland, W. A., Andersson, K. K., Lee, S.-K., Liu, Y., and Lipscomb, J. D. (1992) J. Biol. Chem. 267, 17588–17597
7. 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, 3688–3703
8. Rosenzweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1993) Nature 366, 537–543
9. DeWitt, J. G., Bentsen, J. G., Rosenzweig, A. C., Hedman, B., Green, J., Pilkington, S., Pappaanthymis, G. C., Dalton, H., Hodgson, K. O., and Lippard, S. J. (1993) J. Am. Chem. Soc. 115, 9219–9235
10. DeRose, V. J., Liu, K. E., Kurtz, D. M., Hoffman, B. M., and Lippard, S. J. (1993) J. Am. Chem. Soc. 115, 6440–6441
11. 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
12. Lund, J., Woodland, M. P., and Dalton, H. (1985) Eur. J. Biochem. 147, 297–305
13. Lund, J., and Dalton, H. (1985) Eur. J. Biochem. 147, 291–296
14. Green, J., and Dalton, H. (1989) Biochem. J. 259, 167–172
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. Paulsen, K. E., Liu, Y., Fox, B. G., Münck, E., Stankovich, M. T., and Lipscomb, J. D. (1994) Biochemistry 33, 713–722
18. Andersson, K. K., Froland, W. A., Lee, S.-K., and Lipscomb, J. D. (1991) New J. Chem. 15, 411–415
19. Lund, J., and Dalton, H. (1985) J. Biol. Chem. 260, 15795–15801
20. Liu, K. E., and Lippard, S. J. (1991) J. Biol. Chem. 266, 12836–12839
21. Liu, K. E., and Lippard, S. J. (1991) J. Biol. Chem. 266, 12836–12839; Correction (1991) J. Biol. Chem. 266, 24899
22. Lee, S.-K., Nesheim, J. C., and Lipscomb, J. D. (1993) J. Biol. Chem. 268, 21569–21577
23. Lee, S.-K., Fox, B. G., Froland, W. A., Lipscomb, J. D., and Münck, E. (1993) J. Am. Chem. Soc. 115, 6450–6451
24. Liu, K. E., Wang, D., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1994) J. Am. Chem. Soc. 116, 7465–7466
25. Liu, K. E., Valentine, A. M., Qui, D., Edmondson, D. E., Appelman, E. H., Spira, T. G., and Lippard, S. J. (1995) J. Am. Chem. Soc. 117, 4997–4998
26. Fox, B. G., Froland, W. A., J. Biol. Chem. 268, 12836–12839; Correction (1991) J. Biol. Chem. 266, 24899
27. Hendrich, M. P., Münck, E., Fox, B. G., and Lipscomb, J. D. (1990) J. Am. Chem. Soc. 112, 5861–5865
28. Mason, J. R., and Cambre, R. (1992) Annu. Rev. Microbiol. 46, 277–305
29. Sligar, S. G., and Gunsalus, I. C. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3906–3910
30. Lipscomb, J. D., Sligar, S. G., Namtev, M. J., and Gunsalus, I. C. (1976) J. Biol. Chem. 251, 1116–1124
31. Fox, B. G., Shanklin, J., Al, J., Loehr, T. M., and Sanders-Loehr, J. (1994) Biochemistry 33, 12776–12786
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