Journal of Inorganic Biochemistry 134 (2014) 118–133

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Focused review

Controlled oxidation of aliphatic C–H bonds in metallo-monooxygenases: Mechanistic insights derived from studies on deuterated and fluorinated hydrocarbons

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ARTICLE INFO

Article history:
Received 27 October 2013
Received in revised form 6 January 2014
Accepted 11 February 2014
Available online 21 February 2014

Keywords:
C–H activation
Enzyme catalysis
Fluorinated substituents
Isotopomers
Metalloprotein
Monooxygenase

ABSTRACT

The control over the regio- and/or stereo-selective aliphatic C–H oxidation by metalloenzymes is of great interest to scientists. Typically, these enzymes invoke host–guest chemistry to sequester the substrates within the protein pockets, exploiting sizes, shapes and specific interactions such as hydrogen-bonding, electrostatic forces and/or van der Waals interactions to control the substrate specificity, regio-specificity and stereo-selectivity. Over the years, we have developed a series of deuterated and fluorinated variants of these hydrocarbon substrates as probes to gain insights into the controlled C–H oxidations of hydrocarbons facilitated by these enzymes. In this review, we illustrate the application of these designed probes in the study of three monooxygenases: (i) the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath), which oxidizes straight-chain C_1–C_5 alkanes and alkenes to form their corresponding 2-alcohols and epoxides, respectively; (ii) the recombinant alkane hydroxylase (AlkB) from *Pseudomonas putida* GPo1, which oxidizes the primary C–H bonds of C_5–C_{12} linear alkanes; and (iii) the recombinant cytochrome P450 from *Bacillus megaterium*, which oxidizes C_{12}–C_{20} fatty acids at the ω-1, ω-2 or ω-3 –CH positions.

1. Introduction

With the recent impetus toward greener chemistry, chemo-enzymatic synthesis has undertaken increasing significance and attention [1–6]. We can learn much from nature to develop new catalysts in the laboratory, even based on new chemistry, as has been shown in biological oxidations [7–11]. Enzymes definitely have much to offer on how to control chemical synthesis. Aside from regio- and stereo-selectivity, there are other advantages provided by enzymes. First, some reactions can be carried out at the interface between polar and non-polar environments. This feature of interfacial chemistry allows the protein molecules to come in contact with the substrates more effectively, bringing substrates into the hydrophobic pocket of the enzyme where the active site is confined but without the interference from competing reactions. The effective concentration of the substrates is relatively higher due to their hydrophobic nature and the enzyme can readily reach a higher velocity (V_{max}) for the catalysis at lower substrate concentrations based on the Michaelis–Menten kinetics. Second, enzyme-based chemical conversions can be easily scaled up through the fermentation process, and the reactions can be carried out on whole-cell platforms without the interference from other side products [12,13]. The overall conversion process is robust and efficient. Once the C–H activation occurs at a given carbon atom of the substrate molecule, the synthetic strategy for successive steps can be straightforward.

Monooxygenases for the aliphatic C–H oxidation are enzyme systems that harness molecular oxygen (O_2) at the catalytic center to mediate oxidation of hydrocarbon substrates [14–22]. Typically, one of the two oxygen atoms in the O_2 is transferred to the substrate either to activate a C–H bond in the aliphatics, or the π-system in the vinylic or aromatic compounds. The other oxygen atom is reduced to a molecule of water with the assistance of two protons. (Eq. 1)

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \quad (1)$$

In our laboratory, three monooxygenases are currently under in-depth study to elucidate the molecular basis for their functions: (i) the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) [23–27]; (ii) alkane hydroxylase (AlkB) from

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http://dx.doi.org/10.1016/j.jinorgbio.2014.02.005
0162-0134/

Pseudomonas putida GPo1 [28–31]; and (iii) cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* [32–34]. These three enzyme systems exhibit different functions and possess different metal active sites to mediate the chemistry.

pMMO is a membrane-bound protein, whose active site is a copper based cluster [7,25–27,35,36]. In addition to methane, its natural substrate, pMMO also oxidizes the secondary C – H bonds particularly at the C-2 positions of C_2–C_5 linear alkanes to their corresponding alcohols, and oxidizes related alkenes to oxiranes [37,38]. The stereo-selectivity for *n*-butane and *n*-pentane is in preference for the formation of (2*R*)-alcohols with enantiomeric excesses (*ee*) of 70(8)% and 80(2)%, respectively [37,39]. However, when propene and 1-butene are used as epoxidation substrates for pMMO, the *ee* of the enzymatic products is only 18% and 37%, respectively, in favor of *S*-configuration. Usually, radical clock compounds containing cyclopropane moiety as mechanistic probes [40,41] are employed to ascertain whether the C – H bond oxidation is involved with the hydrogen abstraction/oxygen rebound chemistry [40,42–44], or occurs by the "oxenoid" or "oxene" insertion reaction mechanism [23,26,43,45]. The direct insertion mechanism involves a low-spin state of the activated metal active site to form C – O bond without a significant barrier according to the two-state hypothesis [44,46–49]. However, since only linear alkanes and alkenes can be oxidized by pMMO, it is not possible to examine the reaction using radical clock compounds. In the case of pMMO, cryptically chiral (*R*)- or (*S*)-[1-^{2}H$_1$,1-^{3}H$_1$]ethanes have been used to examine the degrees of inversion or retention of the C – H bond oxidation that occurred at the sp^3 carbon of the chiral methyl end [50] and to determine the lifetime for the sp^3 or sp^2 radical species formed after hydrogen atom abstraction. The corresponding experimental observations indicate that the hydroxylation mediated by the catalytic site in pMMO takes place with full retention of configuration, suggesting that the C – H bond activation in this system is mediated by a concerted or direct "oxene" insertion reaction mechanism [23,26]. It has been proposed that the active site in pMMO consists of a $Cu^ICu^ICu^I$ tricopper-cluster complex that can be activated by dioxygen to produce a mixed-valence $[Cu^{II}Cu^{II}(\mu\text{–}O)_2Cu^{III}]$ intermediate that harnesses a highly reactive "singlet oxene" for facile concerted insertion into one of the C – H bonds of methane as well as C_2–C_5 small alkanes [7,35,51].

AlkB is also a membrane-associated protein that belongs to the superfamily of particulate alkane hydroxylase (pAHs), but its active site is thought to comprise eight histidine ligands coordinated to a diiron center [16,18,20,21]. This structural feature of the catalytic site is similar to the case of xylene monooxygenase, fatty acid desaturases, fatty acid monooxygenases, steroid oxygenases and aldehyde decarbonylases [52,53] but different from the case of ribonucleotide reductase R2, soluble methane monooxygenase (sMMO), toluene monooxygenase (TMO) and other monooxygenases, in which the diiron clusters are bridged by carboxylate residues with the duplicated motif composed of (D/EXXH) [16,54]. It has been shown that the active reactive intermediate of AlkB that participates in the hydrocarbon oxidation is also a non-heme diiron-oxo complex [29].

The substrates of AlkB, medium chain-length alkanes (C_3–C_{12}), are specifically activated at the primary carbon [28,55–58]. Besides linear chain alkane, AlkB also hydroxylates branched chain alkanes, cyclic alkanes and simple aromatics with linear alkane substituents [59]. AlkB carries out the epoxidation of olefins, sulfoxidation of methyl sulfides, *O*-demethylation of methyl ethers and other similar oxidations [60–62]. Recent results further support that AlkB can facilitate the desaturation of norcarane suggesting that the hydroxylation and desaturation mediated by the histidine-rich diiron system are highly related [30].

With respect to the mechanism of C – H bond activation mediated by the whole-cell overexpressing AlkB or the purified AlkB, there are significant amounts of rearranged products observed in experiments with bicyclo[3,1,0]hexane, norcarane, and the ultra-fast probe, *trans*-2-phenyl-1-methylcyclopropane [30,63]. Generally speaking, the reaction mediated by the non-heme diiron center of AlkB is carried out by radical rebound reaction mechanism, where the radical species after hydrogen atom abstraction can sustain for an unusually long time (1–300 ns) [30,64,65].

Cytochrome P450 BM3 is an iron heme enzyme [32,66]. The iron heme center of P450 BM3 consists of a 6-coordinate ferric iron heme with an axial thiolate and an axial water molecule as ligands (Fig. 1). When the hydrophobic substrate interacts with the P450 BM3 to expel water molecules within the hydrophobic pocket, the active site is converted to a 5-coordinate ferric iron heme (pathway *a* in Fig. 1) [66–69]. The resulting high-spin ferric intermediate has a high redox potential and is readily reduced to ferrous heme by taking the electron(s) from NADPH mediated by the FAD/FMN cofactors (pathway *b* in Fig. 1). The iron heme center is then poised for binding dioxygen, and upon receiving additional electrons, the high valent ferryl oxo species, namely compound I (Cpd I), is formed (pathway *c* in Fig. 1) [32,44,70–73]. The formation of Cpd I plays the central role in the hydrogen atom abstraction/radical rebound reaction or O-atom insertion mechanism for the controlled C – H activation in the catalysis (pathway *d* in Fig. 1)[9,43–45,70,74].

Cytochrome P450 BM3 from *B. megaterium* comprises a water-soluble monooxygenase (BMP domain) fused with a FAD/FMN reductase (BMR domain) [75] that accepts reducing equivalents from NADPH to activate O_2 at the heme iron and mediates the hydroxylation of saturated and unsaturated fatty acids with a chain length of 12 to 20 carbons at their sub-terminal ends [76]. Because of its high yield of heterologous over-expression in *Escherichia coli*, profound turnover efficiency, and catalytic promiscuity, P450 BM3 has been extensively engineered *via* site-directed or random mutagenesis for the activation of small alkanes, fine chemical conversions, as well as pharmaceutical lead optimization [4,32,77–82].

To study the hydroxylation reaction mechanism mediated by cytochrome P450 BM3, a series of cyclopropyl fatty acids that are similar to the natural substrates of cytochrome P450 BM3, *i.e.*, C_{12}–C_{16} fatty acids, have been developed as the mechanistic probes [83–85]. It is shown that the oxidation of C_{13}, C_{15} and C_{17} cyclopropyl fatty acids mediated by P450 BM3 results in less than 3% rearranged products that come from the radical intermediates. In addition, no product that come from the cation intermediate is observed [83–85]. The estimated rebound rate constants (k_t) are in the range of 2–3 $\times$ 10^{10} s^{-1}. Moreover, it is also shown that fatty acid derivatives with the mono-substituted cyclopropane even yield no rearranged products. The outcomes of these experiments either indicate that the mechanistic probes are not sensitive enough to produce significant or detectable amounts of rearranged products, or implicate the involvement of low-spin state of Cpd I with its low kinetic barrier for the C – O bond formation in the hydroxylation of these fatty acid substrates by cytochrome P450 BM3 according to the two-state model [44,46,48], *i.e.*, the hydroxylation is mediated preferably by the direct "oxenoid" or "oxene" insertion mechanism [43,45]. A portion of the products obtained from C_{13} cyclopropyl fatty acids, the shortest fatty acid (lauric acid) to serve as a good substrate of P450 BM3, have further been identified to emerge from the cation intermediate during the hydroxylation of these substrates [83].

The most obvious similarity among these enzyme systems is that they all bear high-valent metal active sites as well as hydrophobic substrate-binding pockets to carry out the controlled C – H bond activation of hydrocarbons [11,26,32,38,66,86,87]. Regardless, how each system refuels the electrons consumed to perform functional catalytic cycles is the issue of major significance.

To validate mechanisms accounting for the substrate specificity as well as regio- and stereo-selectivity of these enzymes, many molecular manipulation techniques can be deployed to create structural and functional variations to elucidate the molecular machinery used in the aliphatic C – H oxidation. For instance, to manipulate the enzyme itself, one extensively used technique is site-directed or random mutagenesis. The substrates can be manipulated by deuteration and fluorination to