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Biochemical and Biophysical Research Communications 338 (2005) 590–598
BBRC
www.elsevier.com/locate/ybbrc

Review

Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases ☆,☆☆

David P. Ballou [a,*], Barrie Entsch [b], Lindsay J. Cole [a]

[a] *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606, USA*
[b] *School of Biological, Biomedical and Molecular Sciences, University of New England, Armidale, NSW 2351, Australia*

Received 13 September 2005
Available online 26 September 2005

Dedicated to Professors Osamu Hayaishi and the late Howard S. Mason in celebration of their important discovery, 50 years ago, of oxygenase enzymes.

Abstract

Flavoprotein monooxygenases are involved in a wide variety of biological processes including drug detoxification, biodegradation of aromatic compounds in the environment, biosynthesis of antibiotics and siderophores, and many others. The reactions use NAD(P)H and O_2 as co-substrates and insert one atom of oxygen into the substrate. The flavin-dependent monooxygenases utilize a general cycle in which NAD(P)H reduces the flavin, and the reduced flavin reacts with O_2 to form a C4a-(hydro)peroxyflavin intermediate, which is the oxygenating agent. This complicated catalytic process has diverse requirements that are difficult to be satisfied by a single site. Two general strategies have evolved to satisfy these requirements. *para*-Hydroxybenzoate hydroxylase, the paradigm for the single-component flavoprotein monooxygenases, is one of the most thoroughly studied of all enzymes. This enzyme undergoes significant protein and flavin dynamics during catalysis. There is an *open* conformation that gives access of substrate and product to solvent, and a *closed* or *in* conformation for the reaction with oxygen and the hydroxylation to occur. This *closed* form prevents solvent from destabilizing the hydroperoxyflavin intermediate. Finally, there is an *out* conformation achieved by movement of the isoalloxazine toward the solvent, which exposes its N5 for hydride delivery from NAD(P)H. The protein coordinates these dynamic events during catalysis. The second strategy uses a reductase to catalyze the reduction of the flavin and an oxygenase that uses the reduced flavin as a substrate to react with oxygen and hydroxylate the organic substrate. These two-component systems must be able to transfer reduced flavin from the reductase to the oxygenase and stabilize a C4a-peroxyflavin until a substrate binds to be hydroxylated, all before flavin oxidation and release of H_2O_2. Again, protein dynamics are important for catalytic success.

Keywords: Flavoprotein hydroxylase; Monooxygenase; Flavin hydroperoxide; Protein dynamics; Two-component flavin-dependent monooxygenase; Halogenase

The wide variety of flavoprotein monooxygenases

An important class of flavoproteins is the monooxygenase family, which uses NAD(P)H and O_2 as co-substrates. Single-component flavoprotein monooxygenases, such as *p*-hydroxybenzoate hydroxylase and phenol hydroxylase, play important roles in soil detoxification processes by hydroxylating a variety of aromatic and aliphatic compounds. One of the first studied enzymes of this class is salicylate hydroxylase, discovered by Hayaishi et al. [1]. Related flavoprotein monooxygenases in liver microsomes

☆ Financial support was received from the U.S. Public Health Service (Grant GM 647111 to D.P.B.) and the Australian Research Council (A 09906323 to B.E.).
☆☆ *Abbreviations:* FMO, flavin monooxygenase; PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; HPAH, *p*-hydroxyphenylacetate hydroxylase; PAH, *p*-hydroxyphenylacetate; RebH, oxygenase responsible for halogenating tryptophan in the biosynthesis of rebeccamycin; PrnA, tryptophan-7-halogenase from *Pseudomonas fluorescens*; WT, wild type PHBH.
* Corresponding author. Fax: +1 734 763 4581.
E-mail address: dballou@umich.edu (D.P. Ballou).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.081

(FMO) catalyze the oxygenation of a wide variety of nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatom-containing chemicals, including many drugs, in coordination with cytochromes P450 [2], and also in the regulation of the biosynthesis of auxin in plants [3]. Some other flavoprotein monooxygenases include human squalene monooxygenase (cholesterol biosynthesis) [4], ubiB, an oxygenase involved in ubiquinone biosynthesis [5], and ornithine-N^5-oxygenase [6], involved in biosynthesis of siderophores in microorganisms. In addition, there are flavoprotein monooxygenases that carry out Baeyer–Villiger oxygenations on aldehydes and ketones [7,8]. Recently, a number of two-component flavin-dependent monooxygenases have been described; these enzyme systems use a flavin reductase to produce reduced flavin that diffuses or is delivered to a monooxygenase component. Such two-component flavin-dependent enzyme systems are also involved in diverse biological reactions as mentioned above for single component enzymes.

Chemical issues

The overall reaction catalyzed by flavoprotein monooxygenases involves three general chemical processes. (A) Reduction of the flavin by NAD(P)H, (B) reaction of the reduced flavin with O_2 to provide a C4a-flavin (hydro)peroxide, which is the oxygenating reagent (it is the peroxide for electrophilic substrates and the hydroperoxide for nucleophilic substrates), and (C) binding, orienting, and activating the substrate for its oxygenation by the C4a-(hydro)peroxide. Each of these three processes has unique requirements and it is not surprising that more than one catalytic active site would be required. Two general strategies have evolved to deal with this complex chemical problem. First, in the case of the single-component flavin monooxygenases, for which *p*-hydroxybenzoate hydroxylase (PHBH) is the model [9–15], it has been found that the isoalloxazine ring of the flavin moves several angstroms and the protein undergoes significant rearrangements in the course of catalysis, so that effectively, there are multiple active sites.

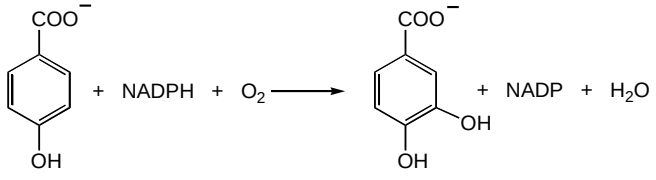
The second approach uses two components to separate the catalytic tasks. This group of two-component hydroxylases has been described mostly in the last decade, and functions by using an oxidoreductase to generate reduced flavin, and another enzyme, the oxygenase, to receive the reduced flavin, and react with O_2 and hydroxylate the substrate. Very little detailed mechanistic information is available on these systems. The two-component systems have no structural or sequence similarities to the one-component enzymes, and thus, these two groups probably evolved independently to carry out identical chemistry. The mechanism of transferring the labile reduced flavin from the reductase to the oxygenase in the two-component systems is not well understood, although recent investigations suggest that for most the reduced flavin diffuses to the oxygenase component quickly before it reacts with oxygen, so that appropriate hydroxylating species can be formed without production of H_2O_2 [16–18].

Reduction of the flavin by NAD(P)H in preparation for its reaction with O_2 occurs by hydride transfer stereospecifically, usually with the pro-*R* hydrogen of the reduced pyridine nucleotide transferring to the N5 of the flavin isoalloxazine [19] in reactions with deuterium isotope effects of 6–10 [20]. Reduction of the flavin is often a critical control point for catalysis by flavoprotein monooxygenases. Thus, for most of the known single-component flavoprotein aromatic hydroxylases, reduction is quite ineffective in the absence of substrate, and this control prevents the wasteful use of NAD(P)H that would produce reactive oxygen species such as H_2O_2. In contrast, controlling the rate of reduction does not regulate the Baeyer–Villiger oxygenases or the mammalian flavin monooxygenases [21–23]. NADPH reduces these enzymes at the same rate in the presence or absence of substrates and the NADP product remains tightly bound. The reduced enzyme-bound flavin then reacts with oxygen to form a C4a-(hydro)peroxyflavin that is quite stable in the absence of substrates; the bound NADP stabilizes the intermediate, which decays slowly over several minutes [21–23]. When substrate is present, reaction with the C4a-(hydro)peroxyflavin occurs quickly to yield oxygenated product [7,8]. Thus, turnover to produce H_2O_2 and oxidized flavin is avoided by a completely different mechanism than with the single-component aromatic hydroxylases.

Aromatic substrate single-component flavoprotein hydroxylases

p-Hydroxybenzoate hydroxylase (PHBH)

PHBH is one of the most thoroughly studied enzymes and is the paradigm for the mechanisms of aromatic hydroxylases. PHBH catalyzes the reaction shown in Scheme 1. The chemistry involving the flavin in the individual reactions of this enzyme is illustrated in Scheme 2. Each of the flavin forms shown in Scheme 2 has a unique absorbance and/or fluorescence signature and can be identified. For example, Fig. 1 shows spectra derived from stopped-flow spectrophotometric studies of various species involved in the reaction of reduced PHBH with oxygen in the presence of the alternative substrate, 2,4-dihydroxybenzoate [24]. In the PHBH mechanism, dynamics involving the protein and flavin are crucial in catalysis [13]. X-ray structures and kinetic analysis of wild type and mutant forms of PHBH [10,25–28] have revealed three conformations that



Scheme 1.