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Review

Mechanisms of reduced flavin transfer in the two-component flavin-dependent monooxygenases

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ABSTRACT

Two-component flavin-dependent enzymes are abundant in nature and are involved in a wide variety of biological reactions. These enzymes consist of a reductase which generates a reduced flavin and a monooxygenase that utilizes the reduced flavin as a substrate for monooxygenation. As reduced flavin is unstable and can be oxidized by oxygen, these enzymes must have a means to efficiently coordinate the transfer of the reduced flavin such that auto-oxidation can be minimized. Various types of experiments and methodologies have been used to probe the mode of reduced flavin transfer. Results from many systems have indicated that the transfer can be achieved by free diffusion and that the presence of one component has no influence on the kinetics of the other component. Contradicting results indicating that the transfer of the reduced flavin may be achieved via protein–protein mediation also exist. Regardless of the mode of reduced flavin transfer, these enzymes have a means to control their overall kinetics such that the reaction rate is slow when the demand for oxygenation is not high.

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Introduction

Flavin-dependent enzymes are indispensible for biological oxidation. Flavins in the forms of FAD[1], FMN and riboflavin are among the most common redox mediators in catabolic and anabolic processes [1]. Reductases and dehydrogenases catalyze the electron transfer between organic reductants and electron acceptors. Oxidases mediate electron transfer from reductants to oxygen while oxygenases oxygenate organic substrates by using a reductant and oxygen as co-substrates [2–5]. Knowledge of flavin-dependent enzyme mechanisms has a wide impact on various applications in biomedicine and biotechnology.

As flavins serve as an electron mediator, the enzyme-bound (covalently or non-covalently linked) flavin cofactor is commonly cycled between the oxidized and reduced states (Fig. 1A). The overall reaction for most flavin-dependent enzymes consists of a

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[1] *Abbreviations used:* FMN, flavin mononucleotide; $FMNH^-$, reduced flavin mononucleotide; FAD, flavin adenine dinucleotide; $FADH^-$, reduced flavin adenine dinucleotide; HPA, *p*-hydroxyphenylacetate; HPAH, *p*-hydroxyphenylacetate hydroxylase; C_1, the reductase component of HPAH from *Acinetobacter baumannii*; C_2, the oxygenase component of HPAH from *Acinetobacter baumannii*; HpaC, the reductase component of HPAH from *E. coli* and *Pseudomonas aeruginosa*; HpaB, the oxygenase component of HPAH from *E. coli*; HpaA, the oxygenase component of HPAH from *Pseudomonas aeruginosa*; ActVB, the reductase component of two-component enzymes involved in actinorhodin biosynthesis; ActVA, the oxygenase component of two-component enzymes involved in actinorhodin biosynthesis; SsuE, the reductase component of alkanesulfonate monooxygenase; SsuD, the oxygenase component of alkanesulfonate monooxygenase; StyB, the reductase component of styrene monooxygenase from *Pseudomonas* sp. VLB120; SMOB, the reductase component of styrene monooxygenase from *Pseudomonas putida*; StyA, the oxygenase component of styrene monooxygenase from *Pseudomonas* sp. VBL120; StyA1, the oxygenase component of styrene monooxygenase from *Rhodococcus opacus* 1CP; SMOA, the oxygenase component of styrene monooxygenase from *Pseudomonas putida*; NSMOA, the N-terminally histidine-tagged SMOA; StyA2B, the oxygenase of styrene monooxygenase fused to an NADH-flavin oxidoreductase; TftD, the oxygenase component of chlorophenol 4-monooxygenase (from *Burkholderia cepacia* AC1100); TftC, NADH:flavin adenine dinucleotide oxidoreductase from *Burkholderia cepacia* AC1100; PheA2, flavin reductase of phenol hydroxylase from *Bacillus thermoglucosidasius* A7; FRET, FÖrster resonance energy transfer; BRET, bioluminescence resonance energy transfer; YFP, yellow fluorescence protein; cyt *c*, cytochrome c; DCIP, dichlorophenol indophenol; MD, menadione; Fre, NAD(P)H-flavin oxidoreductase from *E. coli*; FRG, NAD(P)H-dependent flavin reductase; FRP, NADPH-dependent flavin reductase; LuxG, NADH–FMN oxidoreductase; LuxAB, luciferase.

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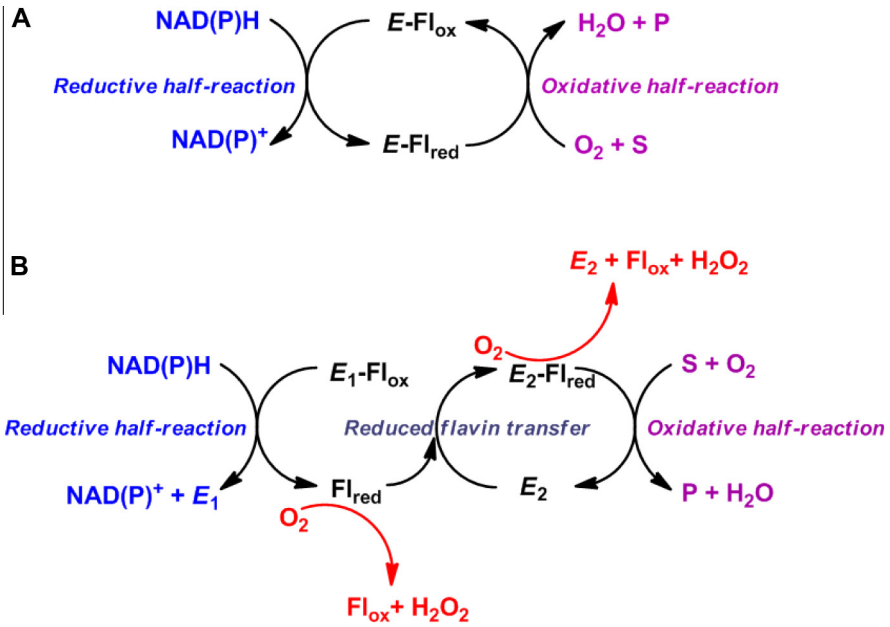

Fig. 1. Catalytic reactions of single-component flavoprotein hydroxylases (A) and two-component flavoprotein hydroxylases (B). The enzyme-bound flavin is reduced by an external reductant (NADH or NADPH) in the reductive half-reaction (blue). The reduced enzyme reacts with oxygen and substrate (S) to form hydroxylated product (P) in the oxidative half-reaction (purple). (A) For single-component enzymes, the oxidized and reduced flavin (Fl_{red}) reside in the same active site. (B) For two-component enzymes, the oxidized flavin (Fl_{ox}) and reduced flavin (Fl_{red}) are transferred between the reductase (E_1) and oxygense (E_2). The red color shows the reaction paths through which oxygen can react with free reduced flavin or *E_2-bound* reduced flavin to form hydrogen peroxide in the uncoupling path without hydroxylation.

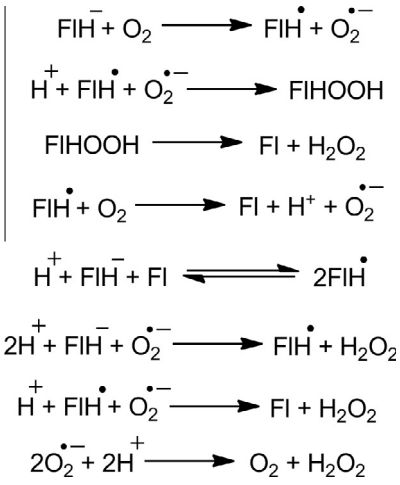

Fig. 2. The reaction of free reduced flavin and oxygen results in hydrogen peroxide, free radicals and reactive oxygen species.

reductive half-reaction where a reductant reduces an oxidized flavin cofactor and an oxidative half-reaction where the reduced flavin is cycled back to the resting oxidized state (Fig. 1A). The flavin cofactor typically remains within the same active site throughout the catalytic cycle, which protects the reduced flavin from nonspecifically reacting with oxygen to generate reactive oxygen species (Fig. 2). Such non-specific reactions would lead to wasteful energy consumption because it would continuously utilize physiological energy sources such as NADH or NADPH.

In addition to the conventional redox scheme described in Fig. 1A, another type of flavin-dependent enzyme in which each half-reaction occurs at different active sites also exists (Fig. 1B). Generally, these enzymes consist of a reductase that catalyzes flavin reduction and an oxygenase that catalyzes oxygenation (Fig. 1B). The first well-known example for this enzyme type is bacterial luciferase, in which the oxygenase component (luciferase) catalyzes the oxygenation of a long chain aliphatic aldehyde, resulting in carboxylic acid with concomitant light emission [6,7]. Since the discovery of bacterial luciferase, it was not until the 1990s that a second oxygenase from *Pseudomonas putida* was reported to catalyze the hydroxylation of *p*-hydroxyphenylacetate by two proteins [8]. From the 2000s onward, many more two-component flavin-monooxygenases have been identified; it has become clear that these enzymes are involved in a wide range of microbial metabolic pathways [3,9]. The lack of discovery of this second type of flavin dependent enzymes in early studies was probably due to the loss of enzymatic activities during purification when the two proteins were separated. It should be noted that up to now, most of these enzymes have only been found in microbes and not in higher organisms. One of the enzymes has been proposed to be involved in pathogenicity of *Mycobacterium tuberculosis* [10]. It will be interesting to see whether knowledge of the ligand binding and reaction mechanisms of these enzymes can pave the way to development of antibiotics in the future.

Overview of two-component flavin-dependent monooxygenases

Four out of eight classes of flavin-dependent monooxygenases identified to date require two proteins for their oxygenation activities [3]. For many systems, it has been shown that external free flavin is not required because the flavin cofactor of the reductase component, can serve as a substrate for monooxygenases after it is reduced by NAD(P)H [11–13]. These enzymes are involved in the metabolism of aliphatic and aromatic compounds, the biosynthesis of natural products, antibiotics and anti-tumor drugs, and have been used as light-emitting bioreporters [3,9].

Since these enzymes are present in mostly aerobic organisms, they must have a means to overcome the auto-oxidation of reduced flavin under aerobic conditions (Figs. 1B and 2) and this

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