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Review

Control of catalysis in flavin-dependent monooxygenases

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ABSTRACT

Flavoprotein monooxygenases reduce flavins, speed their reaction with oxygen, and stabilize a C4a-oxygen adduct long enough to use this reactive species to transfer an oxygen atom to a substrate. The flavin–oxygen adduct can be the C4a-peroxide anion, in which case it reacts as a nucleophile. The protonated adduct – the C4a-hydroperoxide – reacts as an electrophile. The elimination of H_2O_2 competes with substrate oxygenation. This side-reaction is suppressed, preventing the waste of NAD(P)H and the production of toxic H_2O_2. Several strategies have been uncovered that prevent the deleterious side-reaction while still allowing substrate hydroxylation.

Introduction

Reduced flavoenzymes react with oxygen. For many flavoenzymes, this is a deleterious side-reaction and is often suppressed by the protein, but oxidases and monooxygenases have evolved to use O_2 as a physiological substrate. Monooxygenases (*a.k.a.* hydroxylases) oxygenate a substrate by taking advantage of the high reactivity of O_2 reduced to the peroxide level. Oxygenation of a substrate cleaves the oxygen–oxygen bond and produces water. Monooxygenases activate O_2 by synthesizing a covalent flavin–oxygen adduct, the C4a-(hydro)peroxide. The electrophilic or nucleophilic reactions of the peroxyflavin intermediate parallel those of organic peroxides used by synthetic chemists. A number of mechanistically diverse reactions comprise the catalytic cycles of monooxygenases; several strategies for promoting and controlling these reactions are now evident. Conformational changes appear to be generally important in coordinating critical reactions and stabilizing labile intermediates. This brief review describes the inherent chemical constraints on flavin–oxygen chemistry, some enzymatic strategies for dealing with these constraints, and examples of enzymes that use this chemistry for various biological tasks.

Flavin–oxygen reactions

Flavin hydroquinones (2-electron reduced flavins) react in solution relatively slowly with O_2 despite the extremely favorable free energy change (Fig. 1). For instance, the bimolecular rate constant for the reaction of reduced tetraacetyl riboflavin is $\sim$250 M^{-1} s^{-1}, about seven orders of magnitude lower than the diffusion-limited collision frequency [1]. This is because total electron spin must be conserved in chemical reactions. The ground state of O_2 is unusual because it is a triplet – it has two unpaired electrons. Most organic molecules are singlets – all their electrons are paired. Conservation of spin in the reaction of oxygen with a singlet requires two unpaired electrons in the products, thus forming superoxide and an organic radical. Most organic radicals are very unstable, and superoxide is not exceedingly stable either, so their formation is unfavorable, preventing rapid reactions. However, flavin radicals – the semiquinones – have some stability in solution, allowing the slow reaction of O_2, although not at rates fast enough to support biochemistry without catalysis. The stabilities of intermediates such as flavin and oxygen radicals can be altered enormously by the protein environment. Consequently, most flavin monooxygenases react with oxygen 2–4 orders of magnitude faster than free flavins.

The superoxide–semiquinone pair, caged in aqueous solvent upon formation, reacts by radical coupling before the intermediates can diffuse apart [2]. An oxygen–carbon bond forms between superoxide and the isoalloxazine at C4a, a site of high spin-density in the neutral semiquinone [3]. The resulting C4a-peroxide anion is protonated in water, forming the C4a-hydroperoxide. Hydrogen peroxide is rapidly eliminated in a buffer-catalyzed reaction by the deprotonation of N5 and the protonation of the oxygen of the leaving group to form oxidized flavin. In fact, the hydroperoxide has not been seen in the pathway because the rate-determining step at attainable oxygen concentrations is the initial bimolecular reaction. The hydroperoxide, generated in water in pulse-radiolysis experiments, eliminates H_2O_2 with a rate constant of 260 s^{-1},

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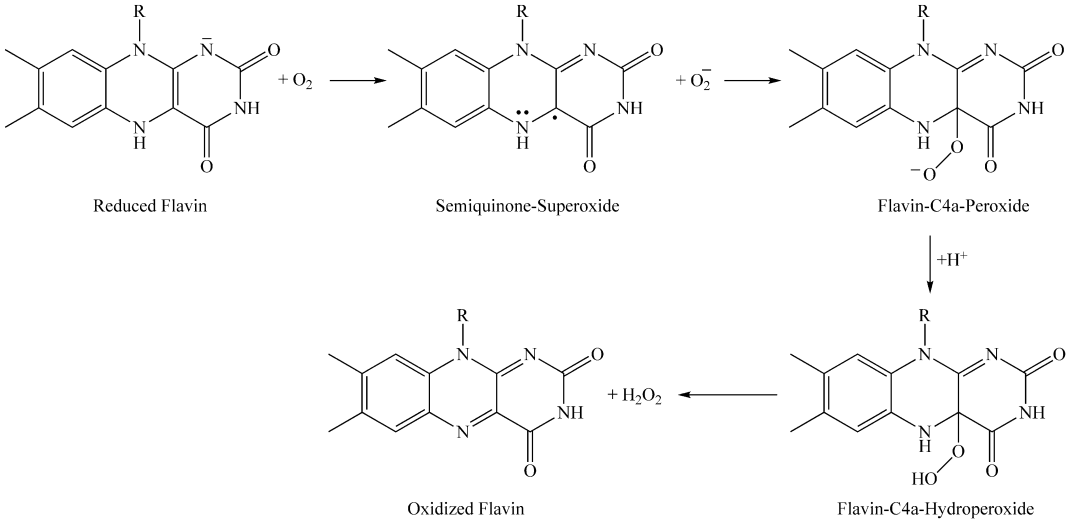

Fig. 1. The reaction of reduced flavin with O_2. The initial step in the reaction of reduced flavins with O_2 is a single-electron transfer to form a solvent-caged semiquinone–superoxide pair. Radical recombination gives the flavin-C4a-peroxide anion, followed by protonation to the hydroperoxide, and elimination of H_2O_2.

much larger than the pseudo-first order rate constant of ~0.5 s^{-1} that could be obtained with high oxygen concentrations [4].

Extensive model chemistry has shown that hydroperoxyflavins react like other organic hydroperoxides [2]. Alkylating N5 of flavin models prevents the rapid elimination of hydrogen peroxide, allowing their reactivity with substrates to be studied. The isoalloxazine moiety attached to the proximal peroxy–oxygen is electron-withdrawing, polarizing the O–O bond and activating it for heterolytic cleavage. Thus nucleophiles such as amines or sulfides attack the distal oxygen of the hydroperoxide and displace the flavin oxide leaving group. Brønsted analysis suggests that a partial charge of about −0.4 develops on the proximal oxygen in the transition state.

Tasks and challenges for monooxygenases

Effectively harnessing the reactive potential of the peroxyflavin requires the promotion of a number of reactions, and, just as importantly, the inhibition of side-reactions. Peroxyflavins are synthesized by first reducing oxidized flavin with a pyridine nucleotide, followed by the reaction with O_2. Once synthesized, the peroxyflavin oxygenates the substrate; often this substrate must be activated. After oxygen transfer, the flavin hydroxide eliminates water to form oxidized flavin. The wildly different transition states in the catalytic cycle – hydride transfer, O_2 activation, substrate oxygenation, and water elimination – require different stabilizing interactions from the protein. Hydroxylases spread the chemistry across the protein using coordinated conformational changes. While monooxygenases promote these diverse reactions, they also prevent the elimination of H_2O_2 from the peroxy adducts by blocking the access of solvent to N5 and sometimes speed the competing oxygen transfer reaction. Thus monooxygenases balance many chemical requirements, making them the most complex of the "simple" flavoenzymes.

The biological benefit of successful monooxygenation is (usually) a hydroxylated compound used in metabolism or signal transduction. Failed attempts not only deprive organisms of a valuable metabolite, but also waste reduced pyridine nucleotides and generate toxic hydrogen peroxide. Thus monooxygenase evolution must be guided by selective pressure to develop control mechanisms assuring that committing NAD(P)H results in successful hydroxylation. Assessing the appropriateness or presence of the substrate is difficult because several reaction steps intervene between the reduction of the flavin and the transfer of oxygen from the hydroperoxide. Two strategies have evolved to prevent wasteful NAD(P)H oxidase activity – "bold" and "cautious". "Cautious" monooxygenases require a hydroxylatable substrate to be bound in order to allow rapid flavin reduction. Thus hydroperoxide formation from the reduced enzyme is triggered by the presence of the substrate to be hydroxylated. Enzymes employing this strategy stabilize the hydroperoxide from wasteful elimination only moderately, relying on hydroxylation to out-compete the side-reaction. "Bold" monooxygenases allow rapid flavin reduction and subsequent hydroperoxide formation regardless of the presence of a hydroxylatable substrate, but effectively protect the flavin hydroperoxide from elimination. These enzymes stall the catalytic cycle until a competent substrate is encountered, again preventing futile NAD(P)H oxidase activity. The reactions and control mechanisms outlined above are implemented in different ways by different enzymes. The large number of permutations of catalytic control, reactivity of the hydroperoxide, and protein structures precludes an extensive discussion of every type of hydroxylase. What follows are examples selected either because they illustrate general hydroxylase paradigms, or they represent interesting biological applications of monooxygenase chemistry.

Aromatic hydroxylases – "Cautious" hydroxylases

p-Hydroxybenzoate hydroxylase

p-Hydroxybenzoate hydroxylase (PHBH)[1] is the most extensively studied flavoprotein monooxygenase. Its mechanism is understood in unparalleled detail and illustrates the chemistry shared by all aromatic hydroxylases, as well as some features that are uniquely adapted to PHBH. PHBH is a homodimer of 45 kDa subunits and uses a non-covalently bound FAD as a prosthetic group. It catalyzes the hydroxylation of *p*-hydroxybenzoate (pOHB) to 3,4-dihydroxybenzoate at the expense of NADPH and O_2 [5–7]. PHBH is one of the many microbial hydroxylases involved in lignin catabolism.

The catalytic cycle of PHBH consists of two half-reactions (Fig. 2). In the reductive half-reaction, the oxidized FAD prosthetic

[1] *Abbreviations used:* PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; KMO, kynurenine 3-monooxygenase; MICAL, Molecule Interacting with CasL; EGCG, epigallocatechin-3-gallate; CH, calponin homology; CRMP, collapsing response mediator protein; FMO, flavin-dependent monooxygenase.