Review

The enigmatic reaction of flavins with oxygen

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The reaction of flavoenzymes with oxygen remains a fascinating area of research because of its relevance for reactive oxygen species (ROS) generation. Several exciting recent studies provide consistent mechanistic clues about the specific functional and structural properties of the oxidase and monooxygenase flavoenzymatic systems. Specifically, the spatial arrangement of the reacting oxygen that is in direct contact with the flavin group is emerging as a crucial factor that differentiates between oxidase and monooxygenase enzymes. A challenge for the future will be to use these emerging concepts to rationally engineer flavoenzymes, paving the way to new research avenues with far-reaching implications for oxidative biocatalysis and metabolic engineering.

Flavins and oxygen: a fundamental and multipurpose redox reaction

Oxidation of reduced flavin by O_2 is one of the most fascinating reactions in biochemistry. Flavins [generally in the form of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN); see Glossary] are versatile redox cofactors, capable of receiving up to two electrons from reducing substrates and conveying them to electron acceptors [1]. Currently, more than 100 000 protein sequences deposited in the NCBI database are classified as flavin-dependent enzymes. These proteins orchestrate networks of redox reactions to serve the cellular physiological needs. Oxygen is readily available in aerobic organisms, and therefore it is commonly employed as electron acceptor in flavoenzyme catalysis to generate hydrogen peroxide (H_2O_2), which is emerging as a key cell signaling molecule [2]. Flavoenzyme oxidases are also among the most active generators of ROS, the prime examples of this being the NADPH oxidases [3], mitochondrial Complex I [4], and the monoamine oxidases [5]. They are important for cellular redox metabolism and homeostasis, and many flavin-dependent oxidases are well-known drug targets; for instance, a mycobacterial protein involved in cell wall biosynthesis (decaprenylphosphoryl-β-D-ribose 2′-epimerase) is a flavin-dependent enzyme that has recently been targeted as a strategy to kill *Mycobacterium tuberculosis* [5–7].

A separate class of flavoenzymes, the flavoprotein monooxygenases, uses oxygen to oxidize reduced flavin, but initially forms a quasi-stable C4a(hydro)peroxyflavin, which can be considered an activated form of oxygen that is capable of incorporating a single oxygen atom into an organic substance [8] (Figure 1). The reactions catalyzed by these oxygenating enzymes often feature an outstanding degree of chemo, regio-, and/or stereospecificity, which make flavoprotein monooxygenases very promising for biocatalytic applications related to synthesis of valuable compounds [9–11].

How the reduced cofactor in flavin-dependent enzyme reacts with oxygen, a hydrophobic molecule by nature, has been one of the most controversial and actively investigated enigmas in enzymology and cofactor biochemistry [12,13]. Reaction rates of reduced flavin with oxygen are

Glossary

C4a-(hydro)peroxyflavin: a reactive flavin species that is typically generated during the oxidative half-reaction of monooxygenases (Figures 1 and 5).
Flavin-dependent enzyme: an enzyme that uses flavin either as a cofactor or substrate (it might not constitutively bind the flavin molecule).
Flavins: vitamin B2 (riboflavin) and its derivatives that serve as reactive groups in redox enzymes. They are generally found in the forms of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).
Flavoenzyme: an enzyme that uses a constitutively bound flavin molecule as a prosthetic group.
k_{ox}: the rate constant for the reaction of reduced flavin with oxygen; that is, the oxidative half-reaction, which is generally measured by stopped-flow techniques.
Monooxygenase: an enzyme that incorporates an oxygen atom into a substrate using molecular oxygen as an oxygen donating substrate, and an external reductant such as NAD(P)H.
Oxidase: an enzyme that oxidizes a substrate using molecular oxygen as an electron acceptor to generate hydrogen peroxide (H_2O_2).
Oxidative half-reaction: the part of a redox–enzyme catalytic cycle that leads to enzyme reoxidation. More specifically, in the case of a flavoenzyme, it refers to the reoxidation of reduced flavin by oxygen (oxidases) or other electron acceptors (dehydrogenases).
Reactive oxygen species (ROS): oxygen containing reactive chemical species that are under the spotlight of current research in biology because of their involvement in ageing, senescence, and pathological conditions such as cancer and neurodegeneration. ROS include radical (e.g., superoxide, hydroxyl radical) and nonradical (e.g., hydrogen peroxide, ozone) compounds.
Reductive half-reaction: the part of a redox–enzyme catalytic cycle in which the enzyme (typically its cofactor/prosthetic group) is reduced by a substrate.
Spin inversion: at room temperature, molecular oxygen is in the triplet state. This implies that chemical reactions of O_2 with organic molecules such as the flavin are spin-forbidden. For this reason, the oxidation by molecular oxygen of the two electron reduced flavin is thought to occur through two consecutive electron transfer steps.
Uncoupled reaction: a reaction that occurs in monooxygenases due to H_2O_2 elimination from C4a-hydroperoxyflavin without oxygenation of the substrate.

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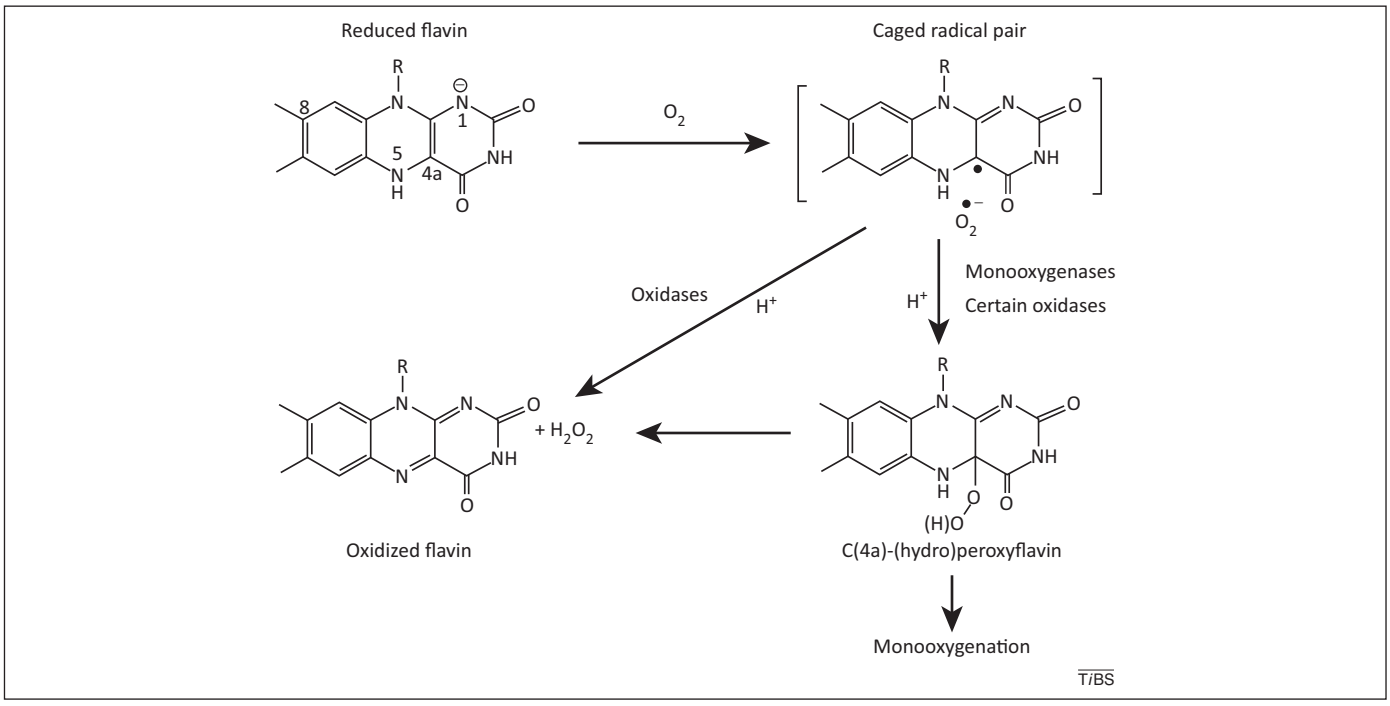

Figure 1. Reaction of reduced flavin with oxygen. The reaction starts with transfer of one electron from the reduced flavin to oxygen, to form a caged radical pair between the flavin semiquinone and the superoxide anion. For oxidases, a second electron transfer (associated with a proton transfer) generates oxidized flavin and H_2O_2. For monooxygenases and certain oxidases (e.g., pyranose 2-oxidase), the radical pair collapses to form a C4a-(hydro)peroxyflavin species, which can further perform monooxygenation or H_2O_2 elimination.

immensely different among various flavin-dependent enzymes [1,12,13]. No other organic cofactor displays such a degree of versatility [1]. During the past several years there have been many significant discoveries related to mechanistic models of how flavin-dependent oxidases and monooxygenases control their reactions with oxygen. This biochemical issue is gaining further interest because of its direct connection to ROS physiology, and because of its relevance to oxidative biocatalysis. This review highlights the major concepts emerging from these studies and is organized according to the different stages of the reaction (Figure 1).

Priming the reaction

The fundamental chemistry of the reaction between reduced flavins and oxygen was extensively investigated in the 1970s and 1980s, particularly by the Bruice and Massey groups [12,14]. It is generally believed that flavin and oxygen undergo an initial one electron transfer to generate a radical pair between the neutral flavin semiquinone (one electron reduced) and superoxide radical (Figure 1). This initiating step is chemically required to bypass the spin–inversion barrier, which is inherent to a reaction between molecules that are in singlet (reduced flavin) and triplet (molecular oxygen) states. Because of its intrinsic instability, the radical pair intermediate has never been captured and characterized. Nevertheless, experimental evidence for the occurrence of a species resembling the flavin semiquinone was recently detected by fast kinetics studies of the oxygen-mediated oxidation of glycolate oxidase, performed in D_2O at pH 5.0 [15].

Depending on the enzyme type, the radical pair can give rise to divergent reactions with different products. In flavoenzyme oxidases, the reaction is typically completed by an immediate second electron transfer event to generate the reoxidized flavin and H_2O_2. By contrast, the semiquinone–superoxide radical pair of monooxygenases and certain oxidases collapses to form a spectroscopically well-characterized intermediate, the C4a-(hydro)peroxyflavin, which can then be employed for inserting an oxygen atom into a substrate (Figure 1) [12,13].

Classic work using $^{18}O_2$ to study the solvent kinetic isotope effects on glucose oxidase has shown that the first step of electron transfer in Figure 1 is the rate limiting step of flavin oxidation [16]. In particular, a protonated active site histidine was identified as the crucial positively charged group that contributes to the preorganization energy to facilitate formation of the anionic superoxide radical [16]. Building on this pioneering investigation, many interesting studies on various enzymatic systems have found this electrostatic effect to be a recurrent feature in several flavoprotein oxidases, although with diverse implementations. Monomeric sarcosine oxidase is a landmark example of this concept [17,18]. The crystal structure originally displayed a conserved Lys residue interacting with the flavin N5 atom via a water-mediated hydrogen bond, a feature conserved in many amine oxidases (Figure 2a). Site directed mutagenesis to replace the Lys side chain with Met decreased the rate constant for flavin oxidation by approximately 8000-fold (k_{ox}) [19]. Remarkably, the enzyme retained the ability to oxidize sarcosine; the mutation effectively led to the conversion of an oxidase into a dehydrogenase, because the enzyme was now unable to effectively use oxygen as electron acceptor. Similar mutations with enzymes in the same family such as N-methyltryptophan oxidase and fructosamine oxidase