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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 O₂ 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 (H₂O₂), 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 mono-oxygenases, 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.
kₚ: 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 (H₂O₂).
Oxidative half-reaction: the part of a redox–enzyme catalytic cycle in which the enzyme reoxidizes. More specifically, in the case of a flavoenzyme, it refers to the reoxidation of reduced flavin by oxygen (oxides) 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 O₂ 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 H₂O₂ elimination from C4a-hydroperoxyflavin without oxygenation of the substrate.

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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).

**Primming 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 D₂O 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 H₂O₂. 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 ¹⁸O₂ 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 (kₘₐₓ) [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.
also resulted in large (from 550- to 2500-fold) decreases in the reoxidation rate constants of the enzyme bound reduced flavins [20,21]. A positive charge around the flavin binding site was also shown to be a key feature for enhancing the oxygen reactivity in choline oxidase, illustrating an interesting variation on this theme [22]. Instead of using a positive charge from residues located near the flavin ring, it is the trimethylammonium group of the reaction product that provides the positive charge to generate an electrostatically favorable environment for the reaction of the reduced cofactor with oxygen [22]. However, as usual in biology, overgeneralizations should be avoided. Indeed, replacing a positively charged Lys located near the flavin N5 with noncharged residues in dihydroorotate dehydrogenase does not result in a significant decrease of the oxidation rates [21] (Figure 2b). For mouse polyamine oxidase, pH-dependent studies indicate that the neutral (rather than the positively charged) form of an active site Lys is required for more efficient flavin oxidation [23] (in this case, the positively charged product might function in oxygen activation). As properly indicated by Palffy and coworkers [21], the context (i.e., the environment of the flavin) matters, and so each enzyme and its reactivity with oxygen should be analyzed for its specific properties. However, many in-depth studies, both in the past few years and less recently [13], clearly indicate that electrostatics (particularly enrichment of positive charges in proximity of the N5 locus of the flavin) fine-tune the reaction with oxygen. This is fully consistent with the envisioned formation of the flavin semiquinone–superoxide radical pair, which, with the experiments on glycolate oxidase, has begun to gain experimental support [15]. These ideas can now be discussed also from a structure versus dynamics perspective, as outlined in the next section.

**A molecular view**

The accumulating evidence in support of the electrostatically tuned electron transfer that primes the reaction of reduced flavin with oxygen has logically raised questions about the molecular mechanisms underlying this initiating step of the oxygen–flavin reaction. Does this step require direct contact between dioxygen and the cofactor? If yes, is there a preferred or even specific locus on the flavin ring for the cofactor–oxygen reaction? Or can the electron transfer be mediated by elements of the protein matrix as is the case in other types of electron transfer reactions? Can the mode of execution of this step be at the heart of the functional differences between oxidases and monooxygenases? The structural enzymology of the oxygen interactions with flavoenzymes has been an active subject of many recent studies, which have used a combination of molecular dynamics simulations, site directed mutagenesis, and structural and kinetics experiments. The first example was the investigation of oxygen diffusion into the active sites of two enzymes, the oxygenase component of p-hydroxyphenylacetate hydroxylase (also known as C3) and alditol oxidase [24,25]. It was revealed that molecular oxygen diffuses through multichannel pathways that funnel the gas into a specific site near the flavin C4a position [25]. Similar features were also highlighted by computational studies on other oxygen utilizing enzymes such as D-amino acid oxidase [26] and Lys-specific histone demethylase 1 [27]. These findings were corroborated by mutating residues that were shown by the simulations to play a role in gating oxygen access to the flavin. A Phe-to-Trp substitution in p-hydroxyphenylacetate hydroxylase decreased the reactivity of the reduced enzyme with O2 by ~20-fold, and a Gly-to-Val substitution in D-amino acid oxidase decreased its activity ~100-fold [25,26]. Most insightful results were obtained from the investigation of L-galactono-1,4-lactone dehydrogenase. An Ala-to-Gly substitution targeting a residue adjacent to the flavin N5-C4a locus drastically increased oxygen activity, practically converting the enzyme from being a dehydrogenase (poorly reacting with oxygen) to an oxidase capable of efficiently using oxygen as electron acceptor [28]. Another very insightful study was performed on aryl-alcohol oxidase. In this case, substitution of a flavin interacting Phe with Ala resulted in a ~120-fold decrease in oxidation rate, whereas substitution of the same residue with a bulkier Trp yielded a remarkable 2-fold increase in oxidation rate [29] (Figure 3). A similar
observation was made in choline oxidase, in which substitution of an active site Val with Ala led to a 50-fold decrease in reaction rate of reduced flavin with oxygen [30]. These seemingly contradictory findings nicely outline the two sides of the problem. Oxygen has to reach the flavin and its access must not be obstructed or hampered by residues that, by being in direct contact with the cofactor, can represent a physical barrier for oxygen (e.g., Ala-to-Gly substitution in L-galactono-1,4-lactone dehydrogenase). However, an increase in hydrophobicity coupled to a physically more confined environment around the oxygen reacting site can help to guide and position O2, facilitating its reaction with reduced flavin (e.g., Phe-to-Trp substitution in ary1-alcohol oxidase).

Collectively, these recently reported experiments start to provide an exciting account and visualization of the tuned reaction of oxygen in a flavoenzyme active site. A clearly emerging theme is that the reaction is primarily affected by changes in the local environment of the flavin N5-C4a locus. This is the site that is directly involved in the reaction with oxygen, and changes in its environment and accessibility are the central (although, obviously, not the only) factors that control reactivity with oxygen and the outcome of the reaction.

**Housing a highly reactive intermediate: stabilization of C4a-(hydro)peroxyflavin at the flavin N5 position**

As discussed so far, the oxygen reaction starts with a single electron transfer at the flavin N5-C4a locus to generate a radical pair between superoxide and flavin semiquinone (Figure 1). However, subsequent steps diverge depending on enzyme type and, in many cases, also on enzyme substrate. Monoxygenases form and stabilize (for up to hours) the C4a-(hydro)peroxyflavin adduct, which is used to oxygenate a substrate. By contrast, most oxidases directly produce hydrogen peroxide without detectable intermediates, possibly because either a second electron transfer occurs directly or the C4a-hydroperoxyflavin forms but decays very rapidly, impeding its kinetic detection (Figure 1). Considering their properties, monoxygenases are the obvious system of choice for probing oxygen reaction with and activation by flavoenzymes. Indeed, the past few years have witnessed terrific progress, including structural determination and enzymological characterization of a variety of monoxygenases and an oxidase that is capable of stabilizing C4a-hydroperoxyflavin.

**Two-component monoxygenases: flavin as a diffusible oxygen-activating substrate**

Two-component monoxygenases consist of a reductase component, which generates a reduced flavin that is subsequently transferred via free diffusion or protein–protein mediation to an oxygenase component, where the reaction with oxygen takes place [31]. Thus, the distinguishing feature of these enzymes is that they use reduced flavin as a diffusible substrate, rather than as a prosthetic group, to activate oxygen in substrate monoxygenation. This feature makes them especially suited for dissecting the role of protein residues in modulating flavin reactivity with oxygen [31–35]. Binding of reduced flavin to oxygenase generally marks the initial step of the reaction, in which the binary complex reacts with oxygen to form C4a-hydroperoxyflavin. The intermediate is relatively stable and has a half-life at 4 °C of approximately a few minutes [33–35]. Studies of p-hydroxyphenylacetate hydroxylase have identified a His as a crucial residue for the hydroxylation reaction [36], whereas a Ser was shown to be crucial for C4a-hydroperoxyflavin stabilization [37]. The crystal structure indicates that this Ser is engaged in a hydrogen bond with the flavin N5 atom (Figure 4a) [38]. Its replacement with Ala drastically decreases the intermediate stability by approximately 1400-fold [37]. Many crystal structures of other two-component flavin-dependent oxygenases have been more recently solved and can be compared with the structure of p-hydroxyphenylacetate hydroxylase. Although the similarity between them is generally low, the overall structural folding is well preserved. Above all, the feature of H-bonding interactions between the flavin N5 and a hydroxyl group of Ser or Thr is conserved in all structures, including the oxygenase components of 4-hydroxyphenylacetate 3-monoxygenase from *Thermus thermophilus* (HpaB) [39], chlorophenol 4-monoxygenase from *Burkholderia cepacia* AC1100 (TRD) [40], and the enzyme involved in cholesterol catabolism in *M. tuberculosis* (HsaA) [41]. Hydrogen bonding to the reduced flavin N5-H is crucial for C4a-hydroperoxyflavin formation and stabilization in other systems as well; these systems will be further discussed in the rest of this section.

**Single-component monoxygenases with long-lived C4a-(hydro)peroxyflavin**

Flavoenzyme monoxygenases such as Baeyer–Villiger monoxygenases, flavin-containing monoxygenases, and N-hydroxylating monoxygenases feature a remarkable property: they require the presence of a bound NADP⁺ in their oxidative half-reaction to stabilize C4a-hydroperoxyflavin that can persist even up to hours before decay. When these enzymes are reduced by chemical agents such as dithionite, their reaction with oxygen directly leads to hydrogen peroxide without any monoxygenation. This ‘moonlighting’ function of NADP(H) as both electron donor
A moving flavin for a short-lived C4a-hydroperoxyflavin

Another class of widely studied flavoprotein monooxygenases mostly catalyze hydroxylation of aromatic compounds [1,48,49]. They are also known as members of the p-hydroxybenzoate hydroxylase (PHBH). In these enzymes, formation of C4a-hydroperoxyflavin can only be detected in the presence of substrates [1,48,49]. In the absence of substrate, elimination of H₂O₂ from C4a-hydroperoxyflavin occurs rapidly. As shown by the extensive studies on p-hydroxybenzoate hydroxylase, these enzymes exhibit a very complex mechanism of function based on a so-called ‘moving’ flavin. The cofactor ring adopts at least two conformations along the catalytic cycle, one for reduction by NADPH and one that is active in substrate hydroxylation. For a more comprehensive analysis, the reader is referred to very fine reviews on the p-hydroxybenzoate hydroxylase mechanism of function [48,50] and to the more recent studies on two other family members, 2-methyl-3-hydroxyphenylalanine-5-carboxylic acid monooxygenase [49,51] and the ReBC oxygenase, which is involved in rebeccamycin biosynthesis [52]. Here, we emphasize that, although this is an unusual context given the complexity of the moving flavin mechanism, a general feature emerges from the analysis of this monooxygenase family: a small cavity exists in front of the flavin C4a atom, which is the reactive site for oxygen to form the (not so stable) C4a-hydroperoxyflavin involved in substrate hydroxylation.

Pyranose 2-oxidase: a link between oxidases and monooxygenases

Pyranose 2-oxidase oxidizes pyranose sugars, such as d-glucose, at the C2 position and uses oxygen as electron acceptor [53,54]. This enzyme can be thought of as an informative outlier: it is a unique case of an oxidase enzyme that forms and stabilizes a well-characterized C4a-hydroperoxyflavin [55]. The crucial factor for stabilization of the intermediate has been revealed by site directed mutagenesis and kinetic isotope effect studies. Substitution of a Thr residue that interacts with the flavin N5 atom abrogates the characteristic C4a-hydroperoxyflavin stabilization, so that flavin oxidation and hydrogen peroxide production occurs without any evident intermediate, as is typically the case for oxidases [56,57]. Interestingly, mutations targeting the His that covalently binds to the flavin do not perturb this aspect of the enzymatic reaction [58]. Thus, C4a-hydroperoxyflavin seems to exquisitely depend on the fine environment around the flavin C4a-N5 locus, whereas the seemingly drastic removal of the flavin–protein covalent linkage has little effect. Indeed, the crucial component appears to be the hydrogen bond to the flavin N5 atom by the Thr side chain. Taking advantage of the enzyme being an oxidase in which the flavin can be reduced by d-glucose and that the active site is rather excluded from outside solvent, specific deuterium labeling at the flavin N5-position in pyranose 2-oxidase was carried out and gave insightful information regarding the stability of the C4a-hydroperoxyflavin [59]. In this study, the kinetics of the N5-deuterated enzyme and solvent isotope effects clearly showed that the bond breakage of N5-H controls the overall process of H₂O₂ elimination and thus the stability of C4a-hydroperoxyflavin (Figure 5). Similar to the studies with monooxygenases, this result beautifully...
A proper approach makes the difference

What are we learning from these recent studies? The first key feature, which is consistently supported by structural, mutagenesis, and computational studies, is that the oxygen reaction in flavoenzymes occurs through a direct contact between oxygen and the flavin N5-C4a locus (Figures 1 and 5). As a consequence, it is the environment around this locus that primarily and most heavily controls the reaction and determines its outcome. In this context, comparison of monooxygenases and oxidases indicates a clear differentiating feature: monooxygenases display a well-defined cavity located above the C4a atom. An excellent example is given by p-hydroxyphenylacetate hydroxylase, which has a small spherical cavity that separates the C4a atom and the substrate atom undergoing hydroxylation (Figure 4a). The volume of this cavity consistently matches the volume of molecular oxygen [38]. This concept is supported by the NADP(H)-dependent enzymes of the Baeyer–Villiger (flavin-containing) monooxygenase family, in which the ribose–nicotinamide group of NADP+h defines a niche that seems perfectly suited for hosting molecular oxygen (Figure 4b). Thus, although in the framework of different contexts and varying implementations, the occurrence of an oxygen binding and reacting site is a feature that is common to proteins that stabilize C4a-hydroperoxyflavin. An element that seems to be crucial for intermediate stabilization in these enzymes is the presence of a group that hydrogen bonds to the flavin N5 position. One idea that is finding experimental support [56, 59] is that protection of the N5 is crucial to hamper an intramolecular proton transfer between the N5 and an oxygen of the peroxide adduct (Figure 5a). Such a proton transfer could also be mediated by the solvent or protein environment. N5-deprotonation can be expected to inevitably cause the collapse of the intermediate and release of hydrogen peroxide.

The second key feature is the location of the oxygenating cavity that is exhibited by monooxygenases: it is located above the flavin C4a atom, compatible with a ‘face-on’ approach of oxygen with respect to the flavin ring (Figure 5b). As originally pointed out by Schreuder et al. [60], this geometry is entirely consistent with the structural and spatial arrangement required by formation of the oxygen adduct on flavin C4a. This feature contrasts with oxidases, which do not generally display a clearly identified oxygen binding center close to the flavin. However, analyses of oxygen trajectories derived from molecular dynamics simulations, mutagenesis, and crystallographic studies on various systems are now providing some interesting and consistent hints about this issue. In oxidases, oxygen seems to commonly approach the flavin with ‘edge-on’ geometry (Figure 5b). Such spatial arrangement between oxygen and the C4a-N5 atoms of flavin would allow stepwise electron transfer but not formation of C4a-hydroperoxyflavin. Furthermore, it is consistent with the observation that oxidases often function through a ternary complex mechanism in which the reduced flavin reacts with oxygen while the reaction product is bound (often enhancing the reactivity with oxygen, as in the case of D-amino acid oxidase and choline oxidase [22]).

We propose that the ‘face-on’ attack in a well-defined pocket may be a general feature of monooxygenases that directly allows formation of C4a-hydroperoxyflavin. Once the intermediate is formed, C4a-hydroperoxyflavin can be promptly stabilized. In oxidases, the approach of oxygen is less restricted and can be afforded by geometrically less-demanding transient contacts to allow stepwise electron transfer. This does not rule out that certain oxidases might also feature a ‘face-on’ approach, forming C4a-hydroperoxyflavin that can be occasionally detectable in certain systems, such as for pyranose 2-oxidase. Likewise, monooxygenases and hydroxylases can be converted to oxidases if they are modified to decrease their ability to stabilize C4a-hydroperoxyflavin.

Concluding remarks

The reaction of the reduced flavin with oxygen continues to pose several stimulating and intriguing problems from the standpoint of both the chemist and the biologist. The wealth of studies in this area of biochemistry highlight several
emerging features, including the relevance of electrostatics, the fine-tuning of the geometry of the oxygen–flavin interaction, and the roles the protein environment play for the formation and stabilization of reaction intermediates. There are two fundamental challenges to developing a unifying framework for flavoenzyme reactions with oxygen, which, in addition to their inherent interest for enzyme chemistry, also have profound implications in flavoenzyme biology:

i. Why do certain flavoenzymes produce superoxide? Are these enzymes unable to trap superoxide in the active site to allow transfer of the second electron, or are there other unknown chemical or catalytic elements that control this effect? A well-known and medically relevant case for this distinction is given by NADPH oxidases, whose isoenzymes differ in the type of reaction products they produce (superoxide versus peroxide) [3]. Recently, a flavin-linked Env family of sulfhydryl oxidases has also been shown to release superoxide anion during catalysis [61].

ii. Why do certain flavoenzymes (primarily dehydrogenases and electron transferases) react poorly with oxygen, which is outcompeted by other electron acceptors (e.g., flavocytochrome b2 [62] and cellobiose dehydrogenase [63])? Several recent studies described herein provide some clues: residues locally gating N5-C4a might control the accessibility and, therefore, reactivity of the flavin; this is effectively illustrated by 1-galactono-1,4-lactone dehydrogenase and aryl-alcohol oxidases. Furthermore, electrostatic forces clearly play a role in oxygen activation, as shown by several studies in oxidases and monoxygenases [16,22]. Nevertheless, for many systems (e.g., flavocytochrome b2 [62]), the reasons for inefficient reactivity with oxygen remain unclear.

Addressing these questions will eventually open the possibility to rationally modify the reactivity with oxygen and the nature of the resulting products of flavoenzymes. This will offer opportunities to probe and engineer pathways involved in the generation of ROS, and to exploit these enzymes in biotechnology (Box 1).

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