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Oxygen Reactivity in Flavoenzymes: Context Matters

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Abstract

Many flavoenzymes – oxidases and monooxygenases – react faster with oxygen than free flavins do. There are many ideas on how enzymes cause this. Recent work has focused on the importance of a positive charge near N5 of the reduced flavin. Fructosamine oxidase has a lysine near N5 of its flavin. We measured a rate constant of 1.6×10^5 $M^{-1}s^{-1}$ for its reaction with oxygen. The Lys276Met mutant reacted with a rate constant of 291 $M^{-1}s^{-1}$, suggesting an important role for this lysine in oxygen activation. The dihydroorotate dehydrogenases from *E. coli* and *L. lactis* also have a lysine near N5 of the flavin. They react with O_2 with rate constants of 6.2×10^4 $M^{-1}s^{-1}$ and 3.0×10^3 $M^{-1}s^{-1}$, respectively. The Lys66Met and Lys43Met mutant enzymes react with rate constants that are nearly the same as the wild-type enzymes, demonstrating that simply placing a positive charge near N5 of the flavin does not guarantee increased oxygen reactivity. Our results show that the lysine near N5 does not exert an effect without an appropriate context; evolution did not find only one mechanism for activating the reaction of flavins with O_2.

Keywords

Dihydroorotate dehydrogenase; Fructosamine oxidase; Oxygen reactivity; Oxygen activation; Flavin

Flavins in solution, when reduced, react with molecular oxygen. However this reaction is not very fast. The reaction is slow because spin inversion is required for the reaction of a singlet (reduced flavin) with a triplet (O_2) to form singlet products, oxidized flavin and H_2O_2 [1, 2]. This reaction occurs by electron transfer from reduced flavin to O_2 yielding a caged radical pair – superoxide anion and semiquinone - which collapses to the C4a-hydroperoxide, which decomposes into H_2O_2 and oxidized flavin (Figure 1).

Flavoproteins can be categorized based on their reactivity with O_2 and the products that are formed from their reactions [3]. Reduced oxidases react rapidly with O_2 to give oxidized enzyme and H_2O_2 [3]. Reduced monooxygenases react rapidly to form the flavin hydroperoxide, which either oxygenates a substrate or eliminates H_2O_2 [4]. Although the radical pair in Figure 1 is thought to be an intermediate in oxidases and monooxygenases, it has not been observed, with the possible exception of one enzyme [5], suggesting that forming the radical pair is rate-determining. Dehydrogenases usually react slowly with O_2, sometimes more slowly than free flavins, to give a mixture of H_2O_2 and superoxide anion [6]. There is considerable interest in understanding how reduced flavoenzymes control oxygen

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Materials and methods S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

reactivity [6]. Structures of flavoproteins have been studied in an attempt to uncover clues for the basis of the acceleration of the reaction. At this time there is no clear understanding of how structures may explain oxygen reactivity.

There are many ideas on how enzymes can speed the reaction of reduced flavins with O_2. These either focus on controlling the access of O_2 to the flavin, or on stabilizing O_2^- in the caged radical intermediate. Access has been proposed to be controlled by channels through which O_2 would approach the flavin, facilitating their encounter [7, 8]. Recent molecular dynamics studies suggested that the protein matrix guides oxygen to a spot over C4a of the flavin for the reaction in some enzymes [9, 10]. Crystal structures of L-galactono-γ-lactone dehydrogenase (GALDH), which belong to the vanillyl-alcohol oxidase family, reveal that an alanine is near the C4a position of the flavin; it reacts poorly with O_2 [11]. Replacing the alanine residue of GALDH with a glycine increased oxygen activation by 400-fold, suggesting that the bulk of the alanine side-chain inhibits the flavoprotein dehydrogenase from reacting with molecular oxygen.

Ideas that consider increasing reactivity emphasize ways in which the protein could stabilize the obligate semiquinone–superoxide intermediate, especially by considering the polarity of the local environment in which O_2^- would form [12]. Lewis acid catalysis could promote superoxide formation [13, 14]. For example, glucose oxidase has two histidines near the flavin that appear to be important for oxygen activation. The positive charge of one of the histidines stabilizes the developing negative charge of O_2^-. The positive charge that activates oxygen is thought to be located on the product bound to the active site in choline oxidase [15].

Recent studies have shown that a lysine near N5 is important for reactivity of several enzymes with O_2. Monomeric sarcosine oxidase, an enzyme which contains covalently bound flavin, catalyzes the oxidation of sarcosine [16, 17]. Monomeric sarcosine oxidase has a lysine that hydrogen-bonds to a water which hydrogen-bonds to N5 of the flavin [18]. Mutating Lys265 to the neutral methionine decreased the rate constant for the reaction of O_2 by 8,000-fold [19]. Crystallography showed that Cl^- can bind in a pocket, near N5 of the flavin, lysine, and the water, suggesting that this is a pre-organized site for the reaction of O_2 [20]. Another oxidase, N-methyltryptophan oxidase, also has a lysine near N5 of the flavin. Mutating this residue to glutamine decreased the rate constant for the oxygen reaction by ~2,500 fold [21]. These are among the largest decreases in oxygen reactivity seen as an effect of site directed mutagenesis.

These observations show that a lysine near N5 is a key player in oxygen activation in these oxidases and thus sparked our interest in exploring the generality of lysine's ability to accelerate the reaction of O_2. The model-enzymes fructosamine oxidase and dihydroorotate dehydrogenases (DHODs) were studied because they all have lysines near N5 (Figure 2).

Fructosamine oxidase from the fungus *Aspergillus fumigatus* is a flavoprotein oxidase that catalyzes the oxidation of the carbon-nitrogen bond of fructosamines. The amine substrate of the enzyme, formed physiologically by the spontaneous reaction of glucose and amino groups of amino acids, is oxidized to an imine and hydrolyzed non-enzymatically [22].

The reaction of the reduced wild-type fructosamine oxidase with O_2 was studied in stopped-flow experiments. Flavin oxidation was observed by an increase in absorbance at 450 nm without any indication of an intermediate. Observed rate constants varied linearly with O_2 concentrations, giving a bimolecular rate constant of 1.6×10^5 $M^{-1}s^{-1}$ [23]. Fructosamine oxidase has a lysine orthologous to that of monomeric sarcosine oxidase, the residue identified to be important in O_2 reactivity. Lys276 was mutated to methionine. The reduced Lys276Met mutant reacts with O_2 with a rate constant of 291 $M^{-1}s^{-1}$, a 550-fold decrease

(Table 1). Again, there was no indication of an intermediate and the reaction produced H_2O_2 [a]. As with monomeric sarcosine oxidase, a large decrease in reactivity occurred, showing that lysine plays an important role in oxygen-activation in these two oxidases. The location of this positive charge is important. The Lys53Met mutant enzyme, where the lysine is nearly centered over the aromatic ring of the flavin on the *si*-face, reacted with O_2 only 30-fold slower than wild-type [23].

Unrelated enzymes, DHODs, catalyze the oxidation of dihydroorotate to orotate during the only redox reaction in the pyrimidine biosynthetic pathway. DHODs can be categorized into two different classes based on their sequences: Class 1 and Class 2 [24]. The Class 1 and 2 enzymes have different selectivity for oxidizing substrates and are also located in different compartments of the cell. The Class 2 enzymes are mitochondrial membrane proteins while Class 1 enzymes are cytosolic. Class 2 DHODs *in vivo* are oxidized by ubiquinone while Class 1A enzymes are oxidized by fumarate [3].

Class 2 *E. coli* DHOD has a lysine (Lys 66) near N5 of the isoalloxazine of the flavin [26]. The rate constant for the reaction of O_2 with the reduced wild-type enzyme is 6.2×10^4 $M^{-1}s^{-1}$ [25], a value that is similar to that seen in some oxidases [27]. The oxidative half-reaction of the Lys66Met mutant enzyme was studied by mixing the reduced enzyme with buffer containing various concentrations of O_2. The Lys66Met mutant enzyme reacted with a rate constant of 6.2×10^4 $M^{-1}s^{-1}$, no change. Neither the wild-type nor the mutant enzyme formed an observable intermediate during their reactions and each produced H_2O_2 [a].

The Class 1A DHOD from *Lactococcus lactis* also has a lysine (Lys43) near N5 of the isoalloxazine of the flavin [28]. The rate constant for the reaction of O_2 with the reduced wild-type enzyme is 3.0×10^3 $M^{-1}s^{-1}$. The Lys43Met mutant enzyme reacted with a rate constant of 2.6×10^3 $M^{-1}s^{-1}$, only 1.2-fold slower – virtually no change. Again, the removal of the lysine residue near N5 of the flavin had no significant impact. Neither the mutant nor the wild-type enzyme formed an intermediate during their reactions and each produced H_2O_2 [a].

The reactivity of the mutant DHODs shows that lysine near N5 is not guaranteed to be important for oxygen reactivity. If O_2 reacts elsewhere in DHOD, then the Lys66Met substitution should have little affect in the Class 2 enzyme, as observed. The physiological oxidizing substrate of Class 2 DHODs is ubiquinone, which binds near the methyls of the isoalloxazine of the flavin; the reaction occurs by two single-electron transfers [25]. Therefore, Class 2 DHODs have evolved to transiently stabilize the flavin semiquinone. There are two conserved positively charged residues in the ubiquinone binding site, His19 and Arg102 in the *E. coli* enzyme. Conceivably, either of these positive charges could stabilize a developing negative charge if O_2 reacted at this site. Therefore the reactivity of the His19Asn and Arg102Met mutant enzymes was determined. Neither change significantly altered the bimolecular rate constant for the reaction of O_2 (Table 1), eliminating this site as the site where O_2 reacts.

A unifying mechanism for oxygen activation by flavoproteins is being sought by many researchers. Our results show that a single mechanism cannot explain oxygen activation. The lysine near N5, a critical ingredient in oxygen activation for some oxidases, does not exert an effect without an appropriate context – some feature (or features) of the active site allows it to activate oxygen. Aligning the isoalloxazines of the structures of fructosamine

[a]Cytochrome c, which is rapidly reduced by superoxide, was included in oxidation mixtures in order to determine the reduced oxygen species produced by the flavoenzyme. No superoxide was detected.

oxidase and Class 1A and 2 DHODs shows that there is enough space near N5 and the lysine in each to accommodate O_2. DHOD (not an oxidase) reacts with O_2 at virtually the same rate regardless of the presence of a lysine near N5 – it plays no role in oxygen activation. Other structural features must be responsible. Mutagenesis eliminated the quinone binding bucket as the site of the reaction of O_2. The hydrophilic pyrimidine binding site over the *si*-face of the flavin, ringed by hydrogen-bonding side-chains, does not appear to be the site of the reaction of O_2 because the reduced enzyme-orotate complex reacts only about ten-fold slower than the free enzyme (Table 1) [25]. The site of the reaction of O_2 in DHODs remains a mystery. Our results suggest that many mechanisms are possible for oxygen activation by flavoproteins. This is consistent with the variety of structures of flavoproteins that react with oxygen [3]. A small, reactive molecule like O_2, which appeared after the origin of life and flavoenzymes, would be expected to find many sites to react.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DHOD Dihydroorotate dehydrogenase

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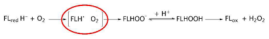

Figure 1.
Mechanism of reaction of reduced flavin with oxygen. It has been proposed that stabilizing the superoxide-semiquinone pair (circled in red) allows rapid reaction with O_2.

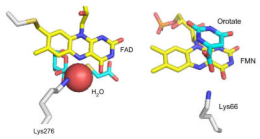

Figure 2.
Fructosamine oxidase (left, from 3dje.pdb) and *E. coli* DHOD (right, from 1f76.pdb) active site structures. The DHOD complex with orotate is shown, but the structure remains the same in its absence. Note that each enzyme has a lysine near N5 of the flavin. The structure of a Class 1A DHOD is not shown, but is essentially identical.

Table 1

Oxidative Half-reaction rate constants.

Enzyme	k_{ox} ($M^{-1}s^{-1}$)	k_{ox} (wild-type)/k_{ox} (mutant)
Wild-type FAOX[a]	1.6×10^5 [b]	
Lys276Met FAOX[a]	291	550
Lys53Met FAOX[a]	5.6×10^3 [b]	29
Wild-type *L. lactis* DHOD	3.0×10^3	
Lys43Met *L. lactis* DHOD	2.6×10^3	1.2
Wild-type *E. coli* DHOD	6.2×10^4 [c]	
Wild-type *E. coli* DHOD•Orotate	5.0×10^3 [c]	12.4 [d]
E. coli Lys66Met	6.2×10^4	1.0
E. coli His19Asn	3.7×10^4	1.7
E. coli Arg102Met	5.9×10^4	1.1

[a] FAOX, fructosamine oxidase.

[b] Ref 23.

[c] Ref 25.

[d] This is k_{OX} (free wild-type)/k_{OX} (orotate complex).