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# Oxygen Activation in Flavoprotein Oxidases: The Importance of Being Positive

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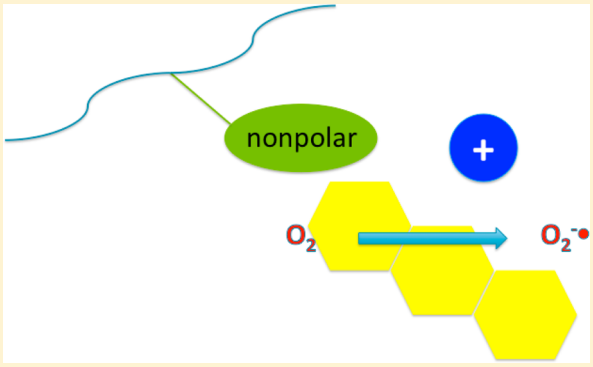
**ABSTRACT:** The oxidation of flavin hydroquinones by $O_2$ in solution is slow, with second-order rate constants of ~250 $M^{-1}$ $s^{-1}$. This is due to the obligatory, single-electron transfer that initiates the reaction being thermodynamically unfavored and poorly catalyzed. Notwithstanding considerations of $O_2$ accessibility to the reaction site, its desolvation and geometry and other factors that can also contribute to further rate acceleration, flavoprotein oxidases must activate $O_2$ for reaction with flavin hydroquinones to be able to achieve the 100−1000-fold rate enhancements typically observed. Protein positive charges have been identified in glucose oxidase, monomeric sarcosine oxidase, *N*-methyltryptophan oxidase and fructosamine oxidase that electrostatically stabilize the transition state for the initial single electron transfer that generates the $O_2^{-\bullet}$/flavin semiquinone radical pair. In choline oxidase despite the presence of three histidines in the active site, the trimethylammonium group of the reaction product provides such an electrostatic stabilization. A nonpolar site proximal to the flavin C(4a) atom in choline oxidase has also been identified, which contributes to the geometry and desolvation of the $O_2$ reaction site. The relevance of $O_2$ activation by product charges to other flavoprotein oxidases, such as for example those catalyzing amine oxidations, is discussed in this review. A nonpolar site close to the flavin C(4a) atom and a positive charge is identified through structural analysis in several flavoprotein oxidases. Mutagenesis has disclosed nonpolar sites in $O_2$-reducing enzymes that utilize copper/TPQ or iron. It is predicted that classes of $O_2$-reducing enzymes utilizing other cofactors also contain a similar catalytic motif.




## INTRODUCTION

Flavins are among a limited number of organic and inorganic biocatalysts that can effectively reduce dioxygen ($O_2$) to superoxide ($O_2^{-\bullet}$), hydrogen peroxide ($H_2O_2$), or water ($H_2O$).[1,2] In solution, the flavin-assisted reduction of $O_2$ is autocatalytic and yields nonstoichiometric amounts of $O_2^{-\bullet}$ and $H_2O_2$.[2] The reaction is slow, primarily because the initial single-electron transfer from the singlet flavin hydroquinone to the triplet $O_2$ is catalyzed poorly.[2] In flavin-dependent enzymes, $O_2$ reduction can be accelerated by 3−4 orders of magnitude or, alternatively, be abated, demonstrating control and modulation of flavin reactivity by the protein microenvironment.[2,3] $O_2$ reactivity in flavin-dependent monooxygenases and oxidases, which are defined respectively as yielding $H_2O$ and $H_2O_2$ from $O_2$ reduction,[2] is considerably enhanced, with second-order rate constants typically in the $10^5$−$10^6$ $M^{-1}$ $s^{-1}$ range.[2,4−7] In contrast, flavoprotein dehydrogenases suppress to various extents the reactivity of the flavin hydroquinone with $O_2$, with the limiting cases of "true" dehydrogenases that are completely unreactive toward $O_2$.[2,3,8] Besides exhibiting poor $O_2$ reactivity, dehydrogenases typically yield $O_2^{-\bullet}$.[2]

A general consensus has emerged that a positively charged group is required to activate $O_2$ in flavin-dependent enzymes.[1] Mechanistic studies by Klinman and co-workers have elucidated the exquisite details of $O_2$ activation by flavoproteins and demonstrated that a histidine acts as an electrostatic catalyst in glucose oxidase.[9−11] A lysine residue has been shown to be the site of $O_2$ activation in a number of flavoprotein oxidases through mutagenic and mechanistic investigations.[9,12−14] In choline oxidase, mechanistic and mutagenic investigations have established that $O_2$ activation is not provided by any of the three active site histidines present in the active site, but rather by the nonionizable, positive charge on the ammonium moiety of the product of the enzyme-catalyzed reaction.[15−19] This Current Topic summarizes our current understanding of $O_2$ activation in flavoprotein oxidases, with an emphasis on the importance of positive charges harbored on substrates rather enzymes.

## $O_2$ REACTION WITH FLAVIN HYDROQUINONES IN SOLUTION

The reduction of $O_2$ to $H_2O_2$ by flavin hydroquinones is thermodynamically favorable due to the high redox potential of the $O_2/H_2O_2$ couple ($E_{m7}$ = +0.36 V),[20] which is 0.57 V more positive than the $E_{m7}$ value of −0.21 V for the oxidized/

**Received:** February 17, 2012
**Revised:** March 19, 2012
**Published:** March 20, 2012

   dx.doi.org/10.1021/bi300227d | *Biochemistry* 2012, 51, 2662−2669

hydroquinone flavin in solution.[21] In spite of such a large thermodynamic drive, the reaction occurs sluggishly due to the diradical nature of the most stable state of $O_2$ in air and solution, where the two unpaired electrons in the $\pi^*2p$ orbitals have parallel spins.[22] Since pairs of electrons in the atomic and molecular orbitals of nonradical species like flavin hydroquinones have opposite spins, they cannot fit in the vacant spaces of the $\pi^*2p$ orbitals of $O_2$.[22] Thus, the initial transfer of an electron from flavin hydroquinone to $O_2$ to generate $O_2^{-\bullet}$ and flavin semiquinone, followed by spin inversion of the resulting radical pair, is an obligatory initial step in the reaction of flavin hydroquinones with $O_2$ (Figure 1).[1,2,23] This almost

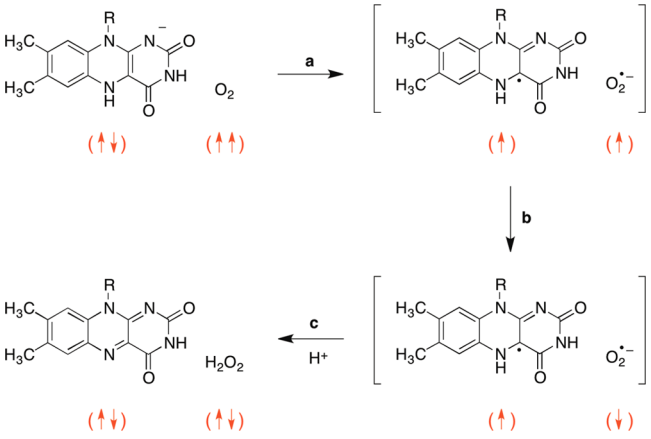



**Figure 1.** Reaction mechanism of flavin hydroquinone oxidation with $O_2$. After initial transfer of an electron from the reduced flavin to $O_2$ (a) generating $O_2^{-\bullet}$ and flavin semiquinone, spin inversion of the resulting radical pair (b) allows for further reaction to yield oxidized flavin and hydrogen peroxide (c). The quantum spins of the flavin and oxygen species are indicated in red. Step c may proceed through the formation of a transient C(4a)-(hydro)peroxyflavin (not shown here) as observed in pyranose 2-oxidase,[66] or through an outer sphere second electron transfer (shown) as proposed for glucose oxidase.[9]

entirely abates the thermodynamic drive for the reaction, because the $E_{m7}$ values for the couples $O_2/O_2^{-\bullet}$ and flavin hydroquinone/semiquinone are estimated at −0.16 V and −0.10 V, respectively.[24] Accordingly, the two-electron $O_2$ reduction by flavins in solution is slow, with a second-order rate constant of 250 $M^{-1}$ $s^{-1}$ determined at pH 6.5.[23] Notwithstanding considerations of $O_2$ accessibility to the reaction site,[25−29] its desolvation and geometry,[12,25,30] and other factors such as flavin stereochemistry that also can contribute to further rate acceleration,[3,25] in flavoprotein oxidases $O_2$ must be activated for reaction with flavin hydroquinones. Thus, stabilization of the transition state for the initial, obligatory electron transfer that generates the $O_2^{-\bullet}$/flavin semiquinone radical pair is required to attain the rate accelerations for flavin oxidation typically observed in flavoprotein oxidases.

## ■ $O_2$ ACTIVATION IN FLAVOPROTEIN OXIDASES

Current understanding of $O_2$ activation for reaction with reduced flavoproteins derives primarily from mechanistic and mutagenic investigations of glucose oxidase using steady state kinetics, solvent viscosity, and solvent and $^{18}O$ kinetic isotope effects.[9−11] Detailed reviews of $O_2$ activation in glucose oxidase are available,[1,31] and the topic will be only summarized here. Briefly, the pH dependence of the second-order rate constant for reaction of flavin hydroquinone with $O_2$ ($k_{cat}/K_{oxygen}$) determined by using steady state kinetics identifies limiting values of 1 500 000 $M^{-1}$ $s^{-1}$ at low pH and 600 $M^{-1}$ $s^{-1}$ at high pH, with a p$K_a$ value of 8.1.[9] The value at high pH is similar to the value of 250 $M^{-1}$ $s^{-1}$ reported for the reaction of free flavin hydroquinone in solution,[23] indicating minimal, if any, $O_2$ activation by the protein. Instead, there is a 2500-fold enhancement of $O_2$ reduction at low pH. Significant $^{18}O$ isotope effects (i.e., ∼1.03) and lack of solvent kinetic isotope effects are observed on $k_{cat}/K_{oxygen}$ both at pH 5.0 and 12.5, demonstrating rate-limiting electron transfer, but not proton transfers, in the two-electron reduction of $O_2$ by the enzyme-bound flavin.[9] When the active site His516 is substituted with alanine, accounting for removal of the imidazole side chain, there is a ∼200-fold decrease in the $k_{cat}/K_{oxygen}$ value at low pH, while the associated $^{18}O$ isotope effect remains significant with a value of 1.02.[9] These data establish the protonated, positively charged His516 as being largely responsible for the electrostatic stabilization of the transition state for the first electron transfer occurring between the flavin hydroquinone and $O_2$ in the active site of glucose oxidase.[9]

Site directed mutagenesis coupled with mechanistic and kinetic studies have shown that a lysine residue is primarily responsible for $O_2$ activation in an increasing number of flavoprotein oxidases. In monomeric sarcosine oxidase, replacement of Lys265 with methionine results in the 8000-fold decrease of the rate constant for flavin oxidation determined using stopped-flow spectrophometry from 283 000 $M^{-1}$ $s^{-1}$ to 35 $M^{-1}$ $s^{-1}$.[14] In *N*-methyltryptophan oxidase, replacement of Lys259 with glutamine decreases the rate constant for flavin oxidation from 213 000 $M^{-1}$ $s^{-1}$ to 87 $M^{-1}$ $s^{-1}$.[13] In fructosamine oxidase, substitution of Lys276 with methionine yields a mutant enzyme where the rate constant for flavin oxidation is 290 $M^{-1}$ $s^{-1}$ as compared to 160 000 $M^{-1}$ $s^{-1}$ of the wild-type enzyme.[12] These studies establish the importance of protein positively charged groups for $O_2$ activation in the active sites of flavoprotein oxidases.

## ■ $O_2$ ACTIVATION IN CHOLINE OXIDASE

Choline oxidase has three histidine residues and no lysines or arginines in the active site (Figure 2). However, $O_2$ activation is

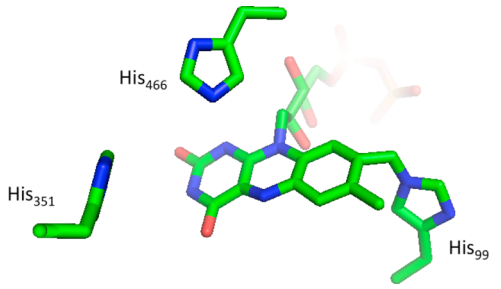
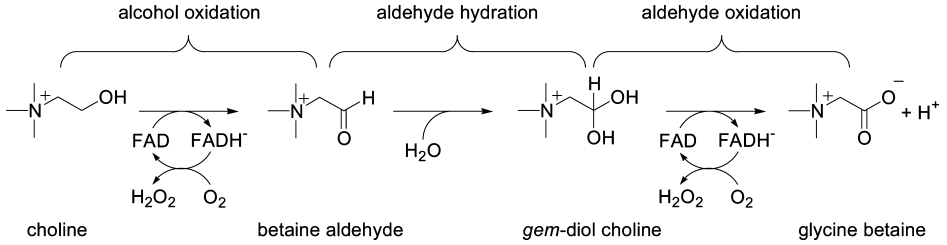



**Figure 2.** The three histidines in the active site of choline oxidase. The structure of the Ser101Ala variant of the enzyme is shown, from the Protein Data Bank entry 3NNE.[35]

catalyzed by the nonionizable, positively charged trimethylammonium group of the product generated in the oxidation of choline catalyzed by the enzyme, betaine aldehyde. The experimental evidence in support of this alternate mode of $O_2$ activation is presented below.

**Biophysical Properties.** Choline oxidase from *Arthrobacter globiformis* has been extensively studied in its mechanistic,

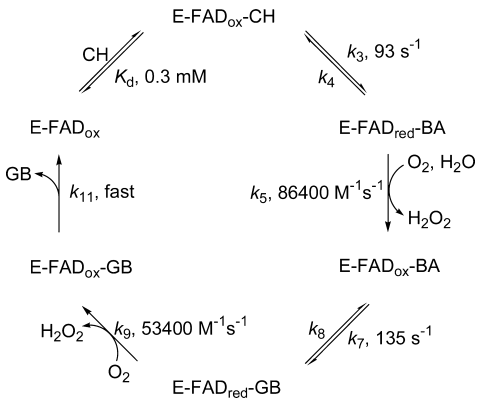
**Scheme 1. Two-Step, Four-electron Oxidation of Choline Catalyzed by Choline Oxidase**




biochemical, and structural properties through a variety of techniques including rapid and steady state kinetics, pH and kinetic isotope effects, temperature effects, mutagenesis, and X-ray crystallography.[5,18,19,30,32−40] The enzyme contains FAD covalently linked to the N(3) atom of His99 through its C(8) methyl group (Figure 2).[39] It catalyzes the four-electron, two-step oxidation of choline to glycine betaine with formation of an enzyme associated aldehyde intermediate that predominantly stays bound at the enzyme active site in turnover (Scheme 1).[41] The enzyme-bound flavin in the oxidized state shows maximal absorbance at 359 and 452 nm (pH 8.0), whereas the flavin hydroquinone has a well-defined absorbance maximum at 356 nm, which is indicative of the anionic form of the reduced flavin.[42] The anionic hydroquinone is stabilized throughout the accessible range of pH in which the enzyme is stable (pH 6.0−10.0).[43] The enzyme has a high affinity for sulfite (i.e., $K_d$ = 0.05 mM, pH 7.0) and stabilizes the anionic flavin semiquinone upon anaerobic reduction with dithionite.[42,43] These features point to the presence of a delocalized negative charge on the reduced flavin N(1)−C(2)=O atoms, which is electrostatically stabilized by His466 as suggested by biochemical studies on an enzyme variant devoid of side chain at position 466 (His466Ala).[17,43]

**Kinetic Mechanism.** The minimal steady state kinetic mechanism of choline oxidase is illustrated in Scheme 2 and includes two reductive half-reactions with the choline substrate and the aldehyde intermediate, each followed by an oxidative half-reaction with $O_2$.[41,42,44] Both oxidative half-reactions occur with the organic product of the preceding reductive half-reaction still bound at the active site. The order of the kinetic steps involving substrate binding, catalysis, and product release is unaltered in the pH range 5.0−10.0, between 10 and 45 °C, upon substituting active site residues by mutagenesis or using substrate analogues.[15−19,30,33−35,37,39,45,46] In the wild-type and mutant variants substituted at His99, Ser101, His351, Val464, His466, and Asn510, the overall turnover of the enzyme with choline is limited by the steps in which hydride ions are transferred from the choline alkoxide and the aldehyde intermediate to the flavin, as indicated by stopped-flow and steady state kinetic data.[17−19,33,35,40] Anaerobic reduction of the enzyme-bound flavin with choline in a stopped-flow spectrophotometer occurs without observable transient species.[44] This pattern is maintained in the reductive half-reactions of all of the active site mutant variants that have been investigated with choline or in the wild-type enzyme with the choline analogue 3,3-dimethyl-butanol (DMB).[18,19,30,32−34,37,39,40] Thus, choline oxidase cycles in turnover between the oxidized and hydroquinone states and $O_2$ reacts with the flavin hydroquinone.

**Scheme 2. Minimal Steady State Kinetic Mechanism of Choline Oxidase[a]**




[a]Values for individual rate constants determined using steady state and rapid kinetics approaches are for pH 10.0.[44] E, enzyme; $FAD_{ox}$, oxidized flavin; $FAD_{red}$, flavin hydroquinone; CH, choline; BA, betaine aldehyde; GB, glycine betaine.

**His466.** His466 is fully conserved in the glucose-methanol-choline oxidoreductase superfamily, which includes choline oxidase, glucose oxidase, cholesterol oxidase, methanol oxidase, pyranose 2-oxidase, aryl-alcohol oxidase, and other flavin-dependent enzymes that oxidize a variety of unrelated alcohols.[47] It is equivalent to His516 of glucose oxidase and is located 4.2 Å from both the flavin N(1) and C(4a) atoms (Figure 2). On the basis of these observations, one would expect His466 to have a prominent role in the activation of $O_2$ for reaction with the flavin hydroquinone.

Two mutant variants where His466 is replaced with alanine or aspartate were prepared, purified, and characterized.[17,43] The His466Asp enzyme is devoid of enzymatic activity, incorporates substoichiometric amounts of FAD primarily not covalently bound to the protein, stabilizes the neutral hydroquinone upon anaerobic reduction with choline, and promptly denatures upon titration with glycine betaine.[43] These data indicate that the introduction of a protein negative charge close to the flavin N(1)−C(2) atoms yields defective flavinylation of the protein and complete loss of enzymatic activity. The His466Ala enzyme, instead, shows a 60-fold decrease in $k_{cat}$, allowing for a mechanistic investigation of the role of His466.[17] Substrate and solvent kinetic isotope effects and pH profiles of the kinetic parameters and of the partial rescuing of the enzymatic activity by imidazolium demonstrate that His466 modulates the electrophilicity of the flavin and the polarity of the active site and stabilizes the transition state for choline oxidation.[17] However, the second-order rate constant for reaction of the flavin hydroquinone with $O_2$ ($k_{cat}/K_{oxygen}$) determined for the His466Ala enzyme is only 1.5-times lower than that of the wild-type enzyme (Table 1).[17] This rules out His466 as actively participating in $O_2$ activation for the reaction with the flavin hydroquinone in choline oxidase.


dx.doi.org/10.1021/bi300227d | Biochemistry 2012, 51, 2662−2669

**Table 1. Second-Order Rate Constants for $O_2$ Reaction of Choline Oxidase in Steady State Kinetics**

| enzyme variant | substrate | pH | $k_{cat}/K_{oxygen}$ ($M^{-1}$ $s^{-1}$) | references |
|---|---|---|---|---|
| wild-type | choline | 6.0−10.0 | 90,000 | 44 |
| | DMB | 6.0−10.0 | 1,200 | 15 |
| His466Ala | choline | 7.0 | 55,000 | 17 |
| His351Ala | choline | 10.0 | 70,000 | 19 |
| His99Asn | choline | 10.0 | 124,000 | 18 |
| Val464Ala | choline | 5.0−10.0 | 1,700 | 30 |
| | DMB | 8.0 | 31 | 30 |

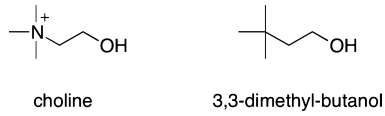
**His351.** His351 is present in the active site of choline oxidase, in a position equivalent to His559 of glucose oxidase (Figure 2). Although located 7 Å from the flavin C(4a) atom, His351 may contribute to $O_2$ activation, possibly through the action of intervening water molecules.

A mutant variant where His351 is replaced with alanine was contructed and investigated in its biochemical and mechanistic properties.[19] Similar to His466, removal of the imidazole side chain on residue 351 results in a 1.2-fold decrease of the $k_{cat}/K_{oxygen}$ value with respect to the wild-type enzyme (Table 1).[19] Consequently, His351 is not required for $O_2$ activation in choline oxidase. Instead, the mechanistic and biochemical data are consistent with His351 participating in substrate binding and positioning, stabilizing the transition state for choline oxidation, and contributing to the overall polarity of the active site by modulating the p$K_a$ of the group that deprotonates choline.[19]

**His99.** A third histidine is present in the active site of choline oxidase at position 99, which is where FAD covalently attaches to the protein (Figure 2). Because of its distance from the flavin C(4a) atom, it appears unlikely that His99 participates in $O_2$ activation. However, it is conceivable that the covalent attachment of the flavin to the protein per se might play a role in $O_2$ activation, possibly by modulating the redox potential of the flavin.[48]

The His99Asn enzyme was prepared, purified, and characterized.[18] The flavin is tightly but not covalently bound to the protein, as expected.[18] Surprisingly, the $k_{cat}/K_{oxygen}$ value is 1.4-times larger than in the wild-type enzyme (Table 1), indicating that His99 and the covalent linkage of the flavin to the protein do not contribute to $O_2$ or flavin activation for reaction. Instead, temperature effects on the rate constant for anaerobic substrate reduction and the associated kinetic isotope effects demonstrate that the FAD-histidyl covalent linkage is important for hydride tunneling in the reaction of choline oxidation.[18] This has been shown to occur in a highly preorganized enzyme−substrate complex where minimal independent movements of the substrate and the flavin are allowed[17,49] (for a recent review see ref 5).

**Substrate.** $O_2$ reacts with the flavin hydroquinone in the enzyme in complex with the product of alcohol oxidation, as suggested by steady state kinetics with choline as substrate (Scheme 2).[41,42,44] When choline is substituted with the uncharged analogue DMB (Figure 3), the $k_{cat}/K_{oxygen}$ is 1600 $M^{-1}$ $s^{-1}$.[15] This represents a 80-fold decrease in the $k_{cat}/K_{oxygen}$ with respect to the value with choline (Table 1).[15] The decrease in the $k_{cat}/K_{oxygen}$ with DMB as compared to choline, along with the minimal effects on $k_{cat}/K_{oxygen}$ of replacing each of the three histidines in the active site of the enzyme, unequivocally establish the positive charge of the organic product formed in turnover as being required for $O_2$ activation




choline 3,3-dimethyl-butanol

**Figure 3.** Structure of choline and its isosteric analogue 3,3-dimethyl-butanol.

for reaction with the flavin hydroquinone. Steric considerations for $O_2$ not being properly positioned or oriented in the active site for reaction with the flavin when the substrate analogue is used can be dismissed because DMB is isosteric with choline (Figure 3). In agreement with $O_2$ activation being exerted by the nonionizable charge of the enzyme-catalyzed product of the reaction rather than a histidine residue, the $k_{cat}/K_{oxygen}$ values of the wild-type enzyme with both choline and DMB are pH-independent between pH 6.0 and 10.0 (Figure 4).[15,18,41]

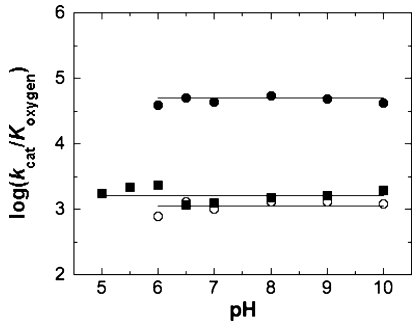



**Figure 4.** pH profiles of the $k_{cat}/K_{oxygen}$ values of choline oxidase wild-type with choline (●) and DMB (○) and Val464Ala enzyme with choline (■) determined at varying concentrations of both organic substrate and $O_2$ at 25 °C. Data are from refs 44 and 15.

**Val464.** In the X-ray structure of the Ser101Ala variant of choline oxidase cocrystallized with the product analogue acetate,[35] a water molecule is sandwiched between the flavin C(4a) atom and the N(3) atom of the side chain of His466, at 3.8 Å from both (Figure 5). The water molecule is localized where $O_2$ is predicted to react with the flavin hydroquinone, as suggested by enhanced-statistics molecular dynamics simulations in alditol oxidase and the monooxygenase component ($C_2$) of *p*-hydroxyphenylacetate hydroxylase showing that multiple diffusion pathways guide $O_2$ to localize close to the flavin C(4a) atom.[25] In this regard, a trapped flavin C(4a)−OH

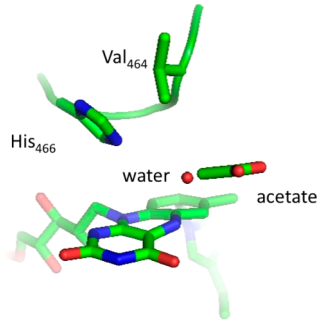



**Figure 5.** Active site of choline oxidase showing Val464. The product analogue acecate was cocrystallized with the protein. A water molecule located between the acetate, His466, Val464, and the flavin C(4a)−N(5) atoms is shown. The structure of the Ser101Ala variant of the enzyme is shown, from the Protein Data Bank entry 3NNE.[35]


dx.doi.org/10.1021/bi300227d | *Biochemistry* 2012, 51, 2662−2669

or C(4a)−OO(H) adduct has been observed by singly crystal spectroscopy and in the X-ray structure of the free wild-type form of choline oxidase devoid of ligands.[38] While to date there has not been evidence for a similar flavin C(4a) adduct in choline oxidase in solution, the X-ray structure is consistent with $O_2$ being guided to the flavin C(4a) atom from the side where Val464 is located. In the active site of choline oxidase, Val464 is spacially adjacent to His466 at a distance of 3.5 Å from the water (Figure 5), suggesting that it may contribute to the localization of $O_2$ for reaction with the flavin hydroquinone. X-ray crystallography, site-directed mutagenesis, and mechanistic approaches have been used to investigate the role of Val464 in catalysis.[30,40]

The mutant enzyme where Val464 is substituted with alanine (PDB code 3LJP; 2.2 Å) is essentially identical to the wild-type enzyme (PDB code 2jbv; 1.86 Å), with an rsmd value of 0.67 Å for 1056 topologically equivalent $C_\alpha$ atoms.[30] The reductive half-reaction is not significantly affected in either the Val464Ala or Val464Thr mutant enzymes, as indicated by anaerobic stopped-flow kinetics on the reduction of the enzyme-bound flavin with choline.[40] Instead, steady state and rapid kinetic analyses of the mutant enzymes, along with enzyme monitored turnover experiments, indicate that the oxidative half-reaction is significantly affected by the mutations of Val464.[30] In the Val464Ala enzyme, the $k_{cat}/K_{oxygen}$ has a pH-independent average value of 1700 $M^{-1}$ $s^{-1}$ (Figure 4), which is 50-fold lower than that of the wild-type enzyme (Table 1).[30] Thus, the nonpolar side chain of Val464 is important for $O_2$ reaction with the flavin hydroquinone, likely by contributing to the geometry and the desolvation of the reaction site where $O_2$ localizes for the subsequent reaction with the flavin.[30] With DMB as substrate for the Val464Ala enzyme, the $k_{cat}/K_{oxygen}$ value further decreases by 55-fold as compared to choline as substrate for the same mutant enzyme (Table 1).[30] A thermodynamic analysis of the effects on $k_{cat}/K_{oxygen}$ of the replacement of valine with alanine in the protein and of choline with DMB as substrate for the Val464Ala enzyme indicates that the two effects are additive.[30] Thus, the function of the nonpolar, aminoacyl side chain that allows $O_2$ localization to the site of reaction and the subsequent $O_2$ activation through the electrostatic effects of the positive charge harbored on the reaction product are independent of each other.

## GENERAL RELEVANCE TO FLAVOPROTEIN OXIDASES

Table 2 summarizes the flavoprotein oxidases for which a positive charge has been established in $O_2$ activation for reaction with flavin hydroquinones by using mechanistic and mutagenic data. To date, histidine, lysine, or the organic product generated in the oxidation reaction have each been shown to act as an electrostatic catalyst. Arginine is notably missing from the list, likely because in the active site of flavoproteins guanido groups typically participate in the binding of substrate carboxylates,[50−54] thereby abating possible electrostatic effects on $O_2$.

$O_2$ activation by a positive charge on the reaction product is likely of importance for flavoprotein oxidases that oxidize amines, such as for example D-amino acid oxidase and monoamine oxidase. These enzymes typically generate positively charged imino products, which are then released to the bulk solvent and converted to the corresponding keto acids upon reacting with water.[55,56] With D-amino acid oxidase, the rate constants for flavin oxidation determined in a stopped-flow spectrophotometer are 10-times faster in the presence of the imino product of the reaction than in the free enzyme.[57] Moreover, no positively charged protein groups that could serve for $O_2$ activation are present in the active site of the enzyme.[50] These observations are consistent with the product of the enzymatic reaction being important in $O_2$ activation, although they do not demonstrate per se an electrostatic effect, since geometric and desolvation effects arising from the product being bound in the active site of the enzyme cannot be ruled out. Similar 10-fold enhancements in the rate constants for flavin oxidation have been reported for both monoamine oxidase A and B with the enzyme in complex with kynuramine and benzylamine, respectively, as compared to their free form.[58] The active site of monoamine oxidase contains a conserved lysine,[59] but its mechanistic role is not known. Recent studies on a member of the monoamine oxidase enzyme family, polyamine oxidase,[55] are consistent with the active site Lys315 being unprotonated for optimal $O_2$ reactivity and demonstrate that the $k_{cat}/K_{oxygen}$ is minimally affected in the Lys315Met mutant enzyme as compared to the wild-type enzyme.[60] Thus, it appears that polyamine oxidase exploits a strategy different than a protein charge for $O_2$ activation.[60] While lack of a ternary complex of the enzyme with the organic product and $O_2$ in turnover has been proposed for polyamine oxidase,[61] it has not been demonstrated[a].[62] Thereby, the contribution of the reaction product to $O_2$ activation is a viable alternative to the conserved lysine in the polyamine and monoamine oxidase family.

A nonpolar site proximal to the positive charge that activates $O_2$, and similar to that provided by Val464 in choline oxidase, is likely a general feature of flavoprotein oxidases, irrespective of the localization of the charge on the protein or the reaction product. This is because any electrostatic effect on the activation of $O_2$ would be maximized in a nonpolar, desolvated environment rather than in a more polar environment. Moreover, such a nonpolar site is also expected to be a critical feature contributing to the geometry of the site where $O_2$ must localize for subsequent activation and reaction with the C(4a) atom of the flavin hydroquinone. In this respect, analysis of three-dimensional structures suggests the presence of nonpolar side chains in proximity of the flavin C(4a) atom of several flavoprotein oxidases, including for example glucose oxidase (Val560), monomeric sarcosine oxidase (Phe 256), cholesterol oxidase (Leu377), pyranose 2-oxidase (Phe454), monoamine oxidase A and B (Phe352 and Phe343, respectively), polyamine oxidase (Leu375), D-amino acid oxidase (Ile230), and glycolate oxidase (Trp110). These enzymes oxidize alcohols, primary and

**Table 2. $O_2$ Activation by a Positive Charge in Flavoprotein Oxidases**

| enzyme | rate constant ($M^{-1}$ $s^{-1}$) | positive charge | references |
|---|---|---|---|
| choline oxidase | 90,000[a] | reaction product | 15 |
| glucose oxidase | 1,500,000[a] | His516 | 9 |
| monomeric sarcosine oxidase | 280,000[b] | Lys265 | 14 |
| *N*-methyltryptophan oxidase | 210,000[b] | Lys259 | 13 |
| fructosamine oxidase | 160,000[b] | Lys276 | 12 |
| polyamine oxidase | 21,000[a] | not Lys315 | 60 |

[a]Determined by using steady state kinetics. [b]Determined by using rapid kinetics.


dx.doi.org/10.1021/bi300227d | *Biochemistry* 2012, 51, 2662−2669

secondary amines, or α-hydroxy acids, and belong to multiple protein families. While most of them share a PHBH protein fold, glycolate oxidase has an $(\alpha/\beta)_8$ barrel fold, suggesting that the catalytic motif composed of a nonpolar site proximal to the flavin C(4a) atom and a positive charge may have evolved more than once. Nonpolar cavities that completely encapsulate and desolvate $O_2$ have been identified in the X-ray structures of flavoprotein monooxygenases, in which highly reactive C(4a)−OO(H) adducts that form after $O_2$ activation must be stabilized to ensure subsequent hydroxylation of substrates.[25] In oxidases, however, the requirement for nonpolar sites being encapsulated in cavities is not as stringent, since the major obstacle for $O_2$ reduction is the initial activation of $O_2$ to $O_2^{-\bullet}$,[2,24] and destabilization of reaction intermediates is preferred, rather than their stabilization, in order to avoid leaking of reactive oxygen species from the active site of the enzyme.

## GENERAL RELEVANCE TO $O_2$ REDUCING ENZYMES WITH COFACTORS OTHER THAN FLAVINS

Reduction of $O_2$ by enzymes with cofactors other than flavins has been investigated in non-heme-dependent soybean lipooxygenase, iron/pterin-dependent tyrosine hydroxylase, and copper/TPQ amine oxidase.[31] $O_2$ reduction in lipooxygenase begins with a single-electron transfer from a substrate-derived radical,[63] thereby circumventing the requirement that triplet $O_2$ must be activated for reaction as for the case of flavin reactions. Site directed mutagenesis and mechanistic studies have provided evidence that the side chain of Ile553 provides a nonpolar site responsible for $O_2$ binding not at the metal center.[63] With rat tyrosine hydroxylase, $^{18}O$ and deuterium kinetic isotope effects have demonstrated that the overall rate of $O_2$ reduction is determined by a single electron transfer likely involving the enzyme-bound tetrahydropterin, which generates $O_2^{-\bullet}$ and tetrahydropterin radical.[64] Two implications derive from this mechanism: (1) the ferrous iron cofactor likely is the main electrostatic catalyst for $O_2$ activation, but it is not required for $O_2$ localization at the reaction center; (2) $O_2$ would bind to an off-metal site, which has not been identified yet. With bovine serum amine oxidase, $^{18}O$ kinetic isotope effects, enzyme-monitored turnover, and solvent and kinetic isotope effects have shown that $O_2$ activation occurs through a single electron transfer to the enzyme-bound TPQ that generates $O_2^{-\bullet}$/TPQ semiquinone.[31] Steady state kinetics on recombinant amine oxidase from *Hansenula polymorpha* has shown that the enzyme reconstituted with Co(II) turns over with $k_{cat}$ similar to that of the native enzyme containing Cu(II).[31] These data establish that Cu(II) does not change its valence during catalytic turnover, but rather acts as electrostatic catalyst for $O_2$ activation. Site directed mutagenesis, coupled to kinetic and mechanistic studies, has further demonstrated the presence of a nonpolar site proximal to copper, mainly provided by the side chain of Met634, which contributes to the geometry of the site where $O_2$ must localize for subsequent activation.[65]

Besides flavin-dependent oxidases and the enzymes discussed above, a number of enzyme families are known to reduce $O_2$. These include monooxygenases that utilize heme, nonheme iron or iron/pterin, dioxygenases that reduce $O_2$ through heme or nonheme iron, and oxidases that use copper, heme/copper, or molybdenum-pterin as cofactors or that are cofactor-independent. It is predicted that enzymes in these families will contain nonpolar sites proximal to positive charges similar to those identified in flavin- and copper-TPQ/dependent oxidases.

## CONCLUSION

A major hurdle to $O_2$ reduction by flavin hydroquinones is the obligatory, initial single-electron transfer that generates the $O_2^{-\bullet}$/flavin semiquinone radical pair,[2,24] which is thermodynamically unfavorable.[24] Thus, thermodynamic stabilization of the $O_2^{-\bullet}$/flavin semiquinone radical pair and, consequently, of the associated transition state originating from the $O_2$/flavin hydroquinone pair, must occur in order to achieve the rate accelerations typically observed in flavoprotein oxidases and monooxygenases. Evidence indicates that flavoprotein oxidases achieve 100−1000-fold rate accelerations with respect to flavins in solution mainly through electrostatic catalysis, by utilizing a positive charge close to the flavin C(4a) atom.[1] While alternate catalytic strategies that could stabilize the transition state for the obligatory first-electron transfer can be envisioned, such as for example an oxyanion hole with hydrogen bonding interactions to $O_2^{-\bullet}$, evidence in their favor is lacking. To date, the protein positive charges identified in the active sites of flavoprotein oxidases are harbored on histidine or lysine side chains. In choline oxidase, despite the presence of three histidines in the active site, the trimethylammonium group on the product of the enzymatic oxidation of choline, betaine aldehyde, activates $O_2$. Similar strategies for $O_2$ activation in which the charge is carried on the product of the reaction rather than the protein are possibly utilized by other flavoprotein oxidases for which positively charged products have been established. Structural analysis suggests that nonpolar sites close to the flavin C(4a) atom and a positive charge, irrespective of its location in the protein or the product of the oxidation reaction, are likely present in several other flavoprotein oxidases of different families. Similar catalytic motifs have been identified in copper/TPQ or iron-dependent enzymes. Thus, it appears that a catalytic motif composed of a positive charge in a nonpolar environment is present in different protein families that utilize different tools, but similar strategies, to reduce $O_2$. Similar catalytic motifs are predicted in $O_2$ reducing enzymes that contain cofactors other than flavins, copper/TPQ, or iron, or that reduce $O_2$ in a cofactor-independent fashion.

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### Funding

This work was supported in part by NSF-CAREER Grant MCB-0545712 and NSF Grant MCB-1121695 (to G.G.).

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported in part through NSF-CAREER MCB-0545712 and NSF MCB-1121695 from the National Science Foundation. I thank the valuable experimental and intellectual contributions all of the students and postdoctoral research associates who work or have worked in my research group. A heartfelt thank you goes to my collaborators: Drs. Irene Weber, Donald Hamelberg, Markus Germann, Allen M. Orville, Rajeev Prabhakar, Jean-Paul Latgé and Jim Spain.

## ADDITIONAL NOTE

[a]While intersecting lines in Lineweaver–Burk plots demonstrate the existence of a catalytically competent ternary complex,[67,68] parallel lines do not immediately disprove its existence as for the cases in which flavin reduction is practically irreversible.[69,70] With a number of flavoprotein oxidases, but not with mouse polyamine oxidase, product inhibition or steady–steady kinetics at constant ratios of substrate and $O_2$ have been successfully employed to establish the absence or presence of a ternary complex in the kinetic mechanism.[13,61,71−75]

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