How Do Enzymes Activate Oxygen without Inactivating Themselves?

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ABSTRACT

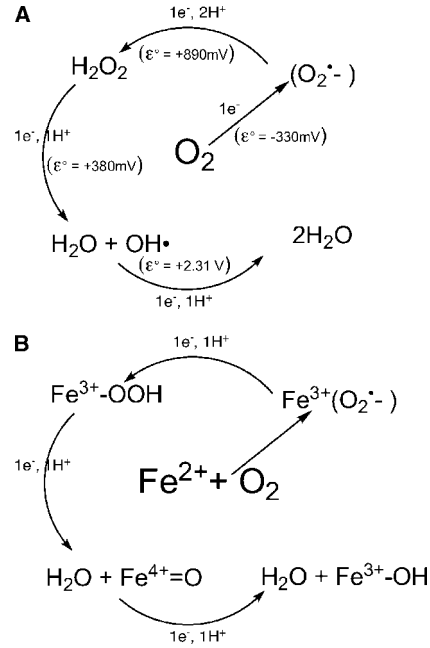
Detailed analyses of the oxidative half-reactions of glucose oxidase and soybean lipoxygenase provide insight into Nature's solution to the "trouble with oxygen". Coupled with studies of other O_2-activating enzymes, two key features emerge. The first is the predominance of a rate-limiting transfer of the first electron transfer to O_2, with subsequent electron and proton transfers occurring in rapid steps. The second feature is the identification of non-metal binding sites and channels for O_2. These permit a controlled reactivity of oxygen to generate the desired regio- and stereochemical products, while minimizing deleterious side reactions.

Introduction

The evolution of multicellular, eukaryotic life forms on the planet Earth is intimately linked to the great oxidation event that occurred *ca.* 0.5×10^9 years ago, at which time atmospheric O_2 accumulated to levels close to its current value of 20%.[1] The stress on pre-existing organisms by the rise in this "toxic" gas was, apparently, more than balanced by the enormous energy available from the reduction of O_2 to water via the oxygenic respiratory chain.

Although O_2 can exist in two spin states, singlet and triplet, the singlet state is sufficiently reactive with organic material that it is rapidly depleted,[2] leaving behind the dominant, ground-state diradical of O_2. This feature of atmospheric O_2 impacts both its relative kinetic inertness and the necessity to activate O_2 via a series of one-electron transfer reactions. As illustrated in Scheme 1A, stepwise electron transfer to O_2 will first lead to superoxide ion, followed by hydrogen peroxide, hydroxyl radical, and, finally, 2 mol of water.[3] With the exception of the first electron transfer process, electron transfer is intimately tied to proton transfers, and these reactions can be formally viewed as proton-coupled electron transfer processes. The emergence of aerobic life was linked to the recruitment of transition metals for enzymatic catalysis of O_2 reactions, with such metals (dominantly copper and iron) playing a role as both electron donor and Lewis catalyst. As illustrated in Scheme 1B for the case of iron,[4] the involvement of a metal center in O_2 activation can alter the nature of reactive oxygen intermediates, with the consequence of the accumulation of species that have longer lifetimes and more controlled reactivity than those formed in the absence of a metal site.

Scheme 1. The Oxidative Cycle, for the Four-Electron Reduction of O_2 to H_2O, in the Absence (A) and Presence (B) of Iron.



The issue of controlled reactivity of reduced oxygen intermediates is certain to underlie the evolutionary success of aerobic life. In this Account, we address whether there are any "rules" that have allowed oxygenic cells to reductively activate O_2 via free radical intermediates while evading extensive oxidative inactivation to themselves. Some systems may solve this conundrum by simply synthesizing high levels of replacement protein; however, this is unlikely to be a general strategy for cellular viability. While metal-containing systems may be expected to have inherently greater control over their reactive intermediates through the formation of discrete complexes, a very large number of enzymes use organic cofactors [e.g., flavins,[5] pterins,[6] and quinones[7]] as redox catalysts in O_2 activation. It is of considerable interest to investigate the catalytic strategies used within these classes of enzymatic reactions.

This laboratory has spent many years developing a set of kinetic tools for investigating oxygen reactivity in enzymes with the goals of understanding the rate-determining steps in O_2 activation and discerning any basic principles that may govern such reactions. In addition to the intellectual satisfaction that comes from understanding how biological catalysts function, such principles may be of great value in the design of new oxidation catalysts and in enhancing the robustness of existing O_2-consuming systems.

Judith Klinman received her A.B. and Ph.D. degrees from the University of Pennsylvania. She began her independent research career at the Institute for Cancer Research, Fox Chase, Philadelphia, prior to moving to the University of California, Berkeley, in 1978. She is currently a faculty member of the Departments of Chemistry and of Molecular and Cell Biology at UCB. Her research interests are focused on protein structure/function and fall into three main areas: protein and peptide-derived cofactors; quantum effects in enzyme reactions and the role of protein dynamics in catalysis; and, the subject of this review, the nature of O_2 activation by enzymes.

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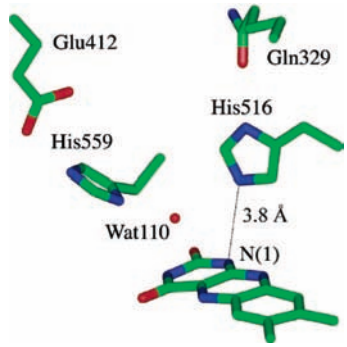


FIGURE 1. Scheme of active site residues in glucose in GO. Oxygen is expected to diffuse near or at the site occupied by Wat110.

Glucose Oxidase as a Prototype for the Catalytic Strategy of Non-metallo-oxidases

Glucose oxidase (GO) has been studied for many decades by a variety of techniques that include X-ray crystallography,[8] NMR,[9] electrochemistry,[10] and steady-state and pre-steady-state kinetics.[11,12] The non-covalently bound flavin adenine dinucleotide (FAD) cofactor of GO can be exchanged with FAD analogs of varying reduction potentials, allowing structure–reactivity correlations to be pursued.[13] GO is an exceptionally robust enzyme, facilitating both basic mechanistic investigations and its application to diagnostic kits (e.g., as a glucose sensor).[14]

As with virtually all flavin-dependent oxidases, the net reaction catalyzed by GO can be broken down into two half-reactions that describe first the oxidation of the substrate, eq 1a and subsequently the reduction of O_2 to hydrogen peroxide, eq 1b:

$$E\text{–}Fl_{ox} + S \rightarrow E\text{–}Fl_{red} + P \qquad (1a)$$

$$E\text{–}Fl_{red} + O_2 \rightarrow E\text{–}Fl_{ox} + H_2O_2 \qquad (1b)$$

where Fl_{ox} and Fl_{red} represent the oxidized and reduced forms of flavin and S and P represent glucose and gluconolactone, respectively. The presence of two coupled, but irreversible steps greatly facilitates the study of the oxidative half-reaction in GO and related reactions. In such instances, monitoring the reaction at low O_2 concentrations leads to the rate parameter $k_{cat}/K_m(O_2)$, eq 1b, which is independent of the reaction of the alternate substrate with enzyme, eq 1a. This property of $k_{cat}/K_m(O_2)$, which includes all steps from O_2 binding up through the first irreversible step of O_2 chemistry, allows O_2 reactivity to be probed independent of rate-limiting steps that may reside elsewhere in the reaction scheme.

The relationship of the bound FAD to active site residues in GO is illustrated in Figure 1. In the course of reduction by substrate via a hydride ion transfer to the N-5 position of the FAD, the cofactor is converted to a delocalized anion at the N-1 position. Although the pK_a at this position in the reduced flavin is near neutrality in solution, the enzyme active site has perturbed the pK_a, such that the reduced flavin remains an anion down to pH 5.[9] This feature is certainly related to the fact that there are two histidines in close proximity, His516 and His559, that when protonated may be expected to lower the pK_a of the bound reduced flavin below the functional pH

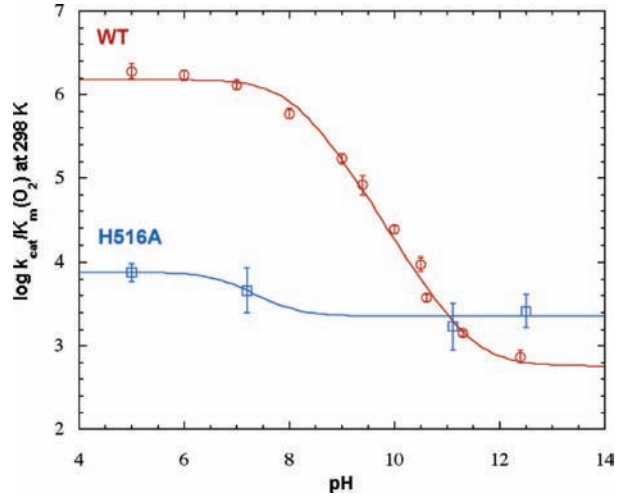


FIGURE 2. The pH dependence of GO for the wild-type (WT) enzyme and for H516A.

range. Given the charged nature of the active site, it is perhaps not surprising that O_2 has never been demonstrated to bind to the active site of active GO. Kinetic investigations under single turnover conditions indicate a second-order reaction between the O_2 and reduced enzyme,[11] leading to the conclusion that the K_d for O_2 lies well above the maximal achievable O_2 concentration in solution, *ca.* 1 mM at ambient pressure.

The reduction of O_2 to H_2O_2 involves an uptake of two electrons (from the reduced flavin) and two protons (at least one of which is expected to derive from one of the active site histidines of Figure 1). The pH profile of $k_{cat}/K_m(O_2)$ for GO, Figure 2, indicates a pK_a = 8.1[15] that is somewhat elevated from that expected for a free histidine, consistent with an electrostatic interaction between a protonated histidine and the anionic flavin. Given the stability of GO at high pH, it has been possible to go to a high enough pH to show that the rate actually levels off, rather than going toward zero in the high alkaline regime.[15] This is an unusual result that indicates two forms of GO with greatly differing reactivity toward O_2 ($k_{cat}/K_m(O_2) = 1.5 \times 10^6$ M^{-1} s^{-1} at low pH and 5.7×10^2 M^{-1} s^{-1} at high pH). One notable feature is that the rate measured at the high pH is close to the rate of reaction of free flavin with O_2,[16] that is, the enzyme remains active at high pH though without any visible rate acceleration.

The implication that a single residue may confer all the catalytic activity provided by GO in O_2 reduction has been tested by site-specific mutagenesis, with the mechanistically relevant result also shown in Figure 2. As illustrated, mutation of His516 to alanine gives a rate that is similar to WT enzyme in the high pH regime. Most importantly, the mutated enzyme has almost completely lost the rate acceleration seen in WT at the reduced pH.[15] This result allows assignment of the pK = 8.1 to His516 and indicates that virtually all of the catalytic effect in the GO reduction of O_2 can be assigned to a single protonated histidine!

The formation of H_2O_2 from O_2 is a multistep process, and our understanding of the physical origin of the rate acceleration afforded by His516 is linked to our under-

Table 1. Summary of Impact of Probes of the Reaction of O_2 with Glucose Oxidase

	impact	
perturbant	pH 5	pH 12.5
(1) viscosogen $\frac{k_{cat}/K_m(O_2), (+\text{viscosogen})}{k_{cat}/K_m(O_2), (-\text{viscosogen})}$	~1	~1
(2) D_2O $\frac{k_{cat}/K_m(O_2), (H_2O)}{k_{cat}/K_m(O_2), (D_2O)}$	~1	~1
(3) ^{18}O in O_2 $\frac{k_{cat}/K_m(O_2), (^{16}O–^{16}O)}{k_{cat}/K_m(O_2), (^{18}O–^{16}O)}$	1.027 (0.003)	1.028 (0.004)
(4) accumulation of flavin semiquinone	none	none

standing of which steps limit catalysis. These steps may involve O_2 binding, proton transfer, electron transfer, or a combination thereof. Probes for each process are available and include (i) the impact of solvent viscosogen on $k_{cat}/K_m(O_2)$, (ii) the magnitude of the solvent deuterium isotope effect, and (iii) the magnitude of the discrimination between $^{16}O/^{18}O$ for the reactant O_2 (compare refs 15, 17, and 18). Additionally, spectroscopic studies can indicate whether detectable intermediates accumulate along the reaction path.

In the case of GO, these probes have been applied at both low and high pH, with the results obtained summarized in Table 1. It can be seen that there is no impact of solvent viscosogen, ruling out a rate-limiting binding of O_2 or release of H_2O_2 from the enzyme. Similarly, the measured $k_{cat}/K_m(O_2)$ is independent of solvent D_2O, an unexpected result at first glance, given the importance of protonation at His516 for catalysis. Considerable insight comes from the $^{16}O/^{18}O$ effect, which is near the limit expected (1.03) for superoxide anion formation.[19] Although these ^{18}O kinetic isotope effects are very small, they can be measured quite precisely by collecting unreacted O_2 from reaction samples as a function of percent conversion, quantitatively converting the O_2 to CO_2 and analyzing the isotopic composition of the CO_2 via isotope ratio mass spectrometry.[20]

The data in Table 1 clearly implicate a single electron transfer from reduced flavin to O_2 as the controlling step for $k_{cat}/K_m(O_2)$ under all conditions examined, a conclusion supported by the failure to detect any flavin semiquinone during turnover.[15] Since early NMR experiments had shown that the enzyme-bound flavin persists as an anion down to pH 5,[9] the mechanism will involve the reduction of O_2 by the bound flavin anion between pH 5 and 12.5. How then can the protonation of His516 facilitate this process? Clearly, a charged active site residue is unlikely to enhance the binding of the hydrophobic O_2 molecule, and in any case, there is no evidence for detectable binding of O_2 at any pH. The protonated His516 may act as a proton donor during O_2 reduction, but the absence of a solvent D_2O effect rules out a rate-limiting proton transfer. This points toward the importance of the

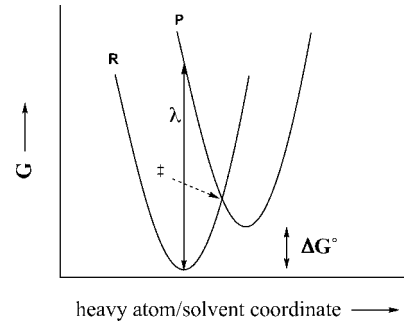


FIGURE 3. Illustration of the parameters controlling $\Delta G^{\ddagger}$ according to Marcus theory for the intersection of the parabolas representing the reactant (R) and the product (P). $\Delta G°$ represents the reaction driving force and λ represents the sum of the inner (λ_{in}) and outer (λ_{out}) reorganization energies.

point charge on the protonated histidine as the origin of the observed catalysis, with the charge being manifest on either the stability of the intermediate superoxide anion, the barrier leading to the superoxide anion, or a combination thereof.

Given the demonstrated rate limitation of $k_{cat}/K_m(O_2)$ by electron transfer from cofactor to O_2, the Marcus theory of electron transfer provides a beautiful context in which to analyze the impact of His516 on catalysis. According to this theory, the barrier to reaction, $\Delta G^{\ddagger}$, can be represented as the point of intersection of two parabolas and is described mathematically in terms of $\Delta G°$, the net reaction driving force, and λ, the environmental reorganization term that describes the degree to which the substrate and its surrounding environment must be deformed into a configuration that reflects the configuration of the product state.[21] These are referred to, respectively, as the thermodynamic and kinetic barriers controlling the electron-transfer process, Figure 3. This simple but powerful conceptual approach has allowed us to dissect the contributions of the enzyme active site to the stabilization of the product superoxide versus the kinetic barrier for its formation.

The first step in such an analysis involves a calculation of $\Delta G°$ for GO at both the low and high pH regimes. These driving force values can be derived from half-reduction potentials for the one-electron reduction of enzyme-bound Fl_{ox} to its semiquinone and the one-electron reduction of O_2 to the superoxide anion ($\Delta G° = -F\Delta E°$). The redox potentials for the enzyme-bound flavin in GO ($E°$) follow from previously measured values[10] after correction to represent pH-independent values for oxidation of the anionic form of the reduced flavin cofactor,[15] Table 2. The available reduction potential for superoxide formation from O_2 is for the free species[3] and may be expected to be increased via electrostatic interactions with the protonated His516 at low pH. After correction for the maximum possible stabilization of the superoxide anion by a neighboring point charge, the redox potential for the net reaction at pH 5 is shown to be more favorable by only about 2 kcal/mol, indicating that non-thermodynamic factors must be at play in the observed rate acceleration.[15]

Table 2. Summary of ΔG° and λ Values for Glucose Oxidase

parameter	low pH	high pH
	E - $HisH^+$ - O_2 - $FADH^-$ → E - $HisH^+$ - $O_2^{-\bullet}$ - $FADH^\bullet$	E - His - O_2 - $FADH^-$ → E - His - $O_2^{-\bullet}$ - $FADH^\bullet$
WT: ε° (V vs. NHE)	-0.065	-0.12
ΔG° (kcal/mol)[a]	-1.3	0.92
λ[b]	24 (17) kcal/mol	40 (8) kcal/mol
λ[c]	28 (7) kcal/mol	ND[d]
H516A: λ[b]	40 (12) kcal/mol	ND[d]

[a] Includes an electrostatic stabilization of superoxide anion by the protonated histidine (see text). [b] From the temperature dependence of $k_{cat}/K_m(O_2)$, refs 15 and 22. [c] From the driving force dependence of $k_{cat}/K_m(O_2)$, ref 22. [d] Not determined.

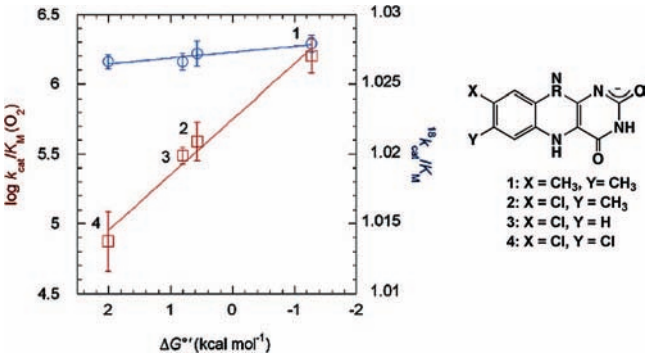


FIGURE 4. Plot of the driving force dependence for rate [($k_{cat}/K_m(O_2)$], red, and the isotope effect on the rate [$^{18}(k_{cat}/K_m)$], blue, in the reaction catalyzed by GO.

Two strategies are available to estimate the contribution of λ to the reaction barrier for GO. In the first instance, the rate is measured as a function of temperature, providing $\Delta G^\ddagger$ ($=\Delta H^\ddagger - T\Delta S^\ddagger$) at both low and high pHs. When these experimental $\Delta G^\ddagger$ values, together with the values for ΔG°, are used, λ is seen to have increased by *ca.* 16 kcal/mol at the high pH, Table 2. Issues that arose regarding these data were the large error bounds for λ at the low pH (considered below) and the appropriate form of the Marcus expression in relating rate to the free energy barrier. Though both adiabatic and non-adiabatic formalisms have been used in treating outer-sphere electron transfer, depending on the degree of electronic coupling between the donor and acceptor,[21] the adiabatic treatment is preferred in the GO case, given the likelihood of close approach between the freely diffusing O_2 and the active site flavin anion. As discussed by Roth et al., the use of a non-adiabatic formalism would have changed the absolute but not the relative values for λ at the pH extrema.[15,22]

The second strategy for the estimation of λ in GO has involved an examination of rate as a function of reaction driving force. GO is a perfect system in which to use this methodology, given its overall robustness under the conditions necessary to remove the native flavin and replace it with analogs of altered redox potential.[22] The structures of the altered flavins are shown in Figure 4, together with a plot of the observed rate for $k_{cat}/K_m(O_2)$ as a function of driving force. The latter was estimated from the measured value for the enzyme-bound flavin in GO, together with measured solution redox potentials for the flavin analogs in relation to the native flavin structure.[22] Very gratifyingly, the value for λ obtained in this way both reduces the uncertainty of this parameter in the low-pH range and confirms the conclusions reached from temperature dependencies alone, Table 2. From a combination of two independent approaches, it can safely be concluded that a single point charge in GO reduces the kinetic barrier by 12–16 kcal/mol.[15,22] This is further corroborated by the data for the mutant enzyme at low pH where the result of a temperature-dependent study shows the same large λ as seen with WT enzyme at the elevated pH.[22]

It is common to express λ_{tot} in terms of the contribution of both λ_{in} and λ_{out}, with λ_{in} reflecting the stretching/bending of bonds within the substrates themselves and λ_{out} reflecting the reorganization of the surrounding medium that is expected to accompany the movement of charge between one reactant and another.[21] By definition, λ_{in} can be concluded to be independent of pH, indicating an impact of pH/charge on the preorganization of the surrounding active site (λ_{out}, which reduces the need for environmental reorganization as the electron moves from the flavin donor to the O_2 acceptor).

This distinction between λ_{in} and λ_{out} is further emphasized by the measured properties of the ^{18}O kinetic isotope effects.[22] Keeping in mind that the size of the ^{18}O effect is expected to reflect a stretching of the O_2 bond to bring it into a geometry that more closely approximates the superoxo product, a very surprising result is that neither the size of the measured oxygen isotope effects nor their trend with driving force (Figure 4) is compatible with a classical Marcus treatment. As discussed by Jortner and co-workers in the context of non-adiabatic electron-transfer reactions,[23] when the vibrational frequencies for reactants are high in relation to kT (note the stretching frequency for O_2 of 1556 cm^{-1} relative to kT of 200 cm^{-1}), it may be expected that motions within the reactants will occur quantum mechanically. The data for GO, in fact, implicate such a quantum mechanical contribution to λ_{in}. Although there has been much focus in the recent literature on the role of tunneling in enzymatic C–H activation reactions (e.g., refs 24–26), the findings with GO indicate that tunneling behavior may also be expected to arise for enzymatic reactions involving significantly larger nuclei.

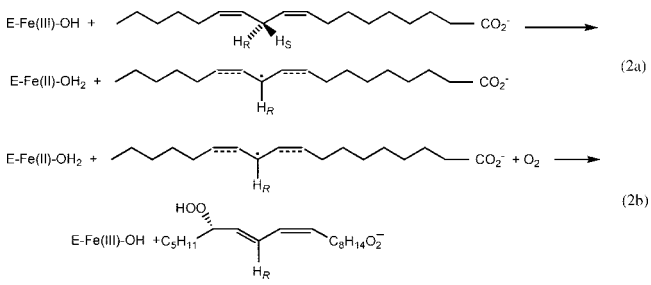
With the detailed analysis of GO outlined above, it is now possible to come to specific conclusions regarding the catalytic strategy in GO and, likely, other enzymes that reduce O_2 in the absence of metal ions. The major feature involves a reduction in the kinetic barrier for O_2 activation in the absence of any significant stabilization of the resulting superoxide intermediate. The fact that the protonated His516 in GO does not lead to a greatly enhanced accumulation of this superoxide anion is likely a result of the placement of this charge such that it has the capability to stabilize both the initial reduced flavin anion in the reactant complex and the superoxide anion in the first intermediate complex. Under such circumstances, the role

of the positive residue becomes focused on its impact on the surrounding environment, such that the heavy atom adjustment that is expected to accompany the electron transfer can be minimized. This elegant and simple solution indicates one way that Nature has found to catalyze a reductive chemical reaction at O_2 in the absence of promoting the accumulation of reactive or toxic intermediates.

Lipoxygenase as a Prototype for Directed O_2 Binding and Reactivity

Lipoxygenases play an essential role in the production of hormones in plants and as mediators of inflammation in mammals.[27] The latter property has made lipoxygenase a target for the discovery and design of anti-inflammatory drugs. The enzyme has been extensively studied from both plant and human sources, with X-ray structures being available from both sources.[28–30]

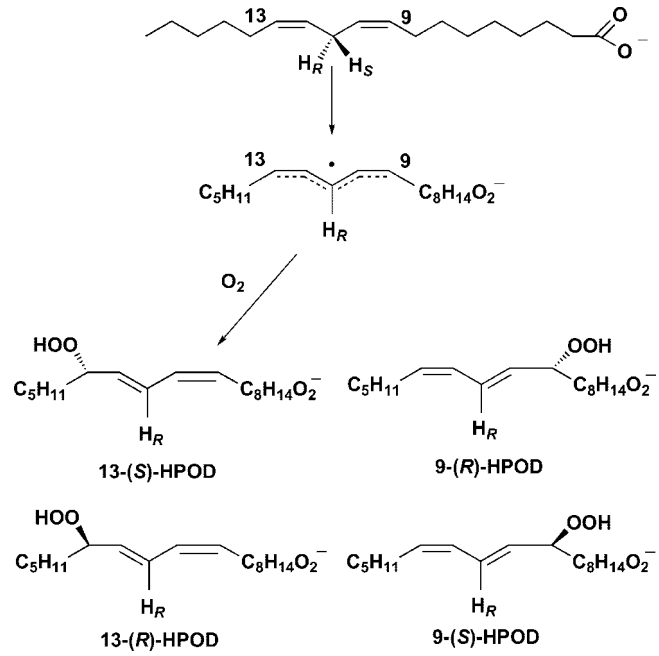
Studies from this laboratory have been focused on the enzyme from soybeans, referred to as SLO. Although structurally and mechanistically very different from GO, the reaction of SLO can be formalized as two half-reactions[31] that involve first the formation of a substrate derived free radical, eq 2a, and subsequent trapping of this radical by molecular O_2 to form the 13-hydroperoxide product, eq 2b.



Notable features of the SLO reaction include (i) the presence of an active site iron center that must be in a +3 valence state in order to react with substrate, (ii) the removal of a hydrogen atom from the C-11 of linoleic acid to form a delocalized (allylic or pentadienyl) free radical as a discrete intermediate, and (iii) the addition of O_2 to a unique position of this delocalized radical to generate a regio- and stereo-specific 13-hydroperoxide product.[31]

The properties of the first half-reaction, eq 2a, have received considerable attention, with the observation of the some of the largest kinetic deuterium isotope effects seen in an enzyme-catalyzed reaction.[32–34] This feature, together with a small enthalpy of activation for protium transfer coupled to a very small temperature dependence for the kinetic isotope effect, indicates a reaction mechanism in which a hydrogen atom tunnels from the substrate donor to a ferric hydroxide acceptor.[33] The efficiency of this wave function overlap is intimately linked to heavy atom motions of both the H-donor and acceptor and the surrounding environment, such that the barrier height reflects these motions rather than the movement

Scheme 2. Illustration of the four possible regio- and stereochemical hydroperoxide products from the radical derived from hydrogenation abstraction at C-11 of linoleic acid.



of the hydrogen. As a result of its aggregate properties, SLO has assumed the role as the "gold standard" for modeling of environmentally coupled hydrogen tunneling in enzyme-catalyzed reactions (cf. refs 35–37).

Investigators have puzzled for many years as to the origin of the high specificity of the subsequent reaction of the substrate-derived free radical with O_2. Initially, it had been postulated that lipoxygenases catalyze the formation of an organometallic intermediate between the fatty acid substrate and the active site ferric center, [38] with this complexation giving rise to the observed regio- and sterochemical course of the second half-reaction, eq 2b). Given the preponderance of evidence in support of an outer-sphere, proton-coupled electron-transfer reaction between substrate and the iron center, the control of the O_2 reaction must lie elsewhere. For most of the history of investigations of O_2 reactivity, it has been assumed that O_2 binds via a partitioning into the hydrophobic interior of the target protein, with such partitioning being aided by the natural breathing modes of the protein. This paradigm for O_2 binding most likely originated with early studies in which high pressures of O_2 were shown to quench the fluorescence of a buried fluorophore in proteins that do not normally react with O_2.[39] If such indiscriminate behavior were to occur in the SLO reaction, the expectation is that O_2 would be free to attack multiple positions within the substrate-derived free radical, leading to a multitude of products, Scheme 2.

Insight into the specificity of the O_2 reaction in SLO comes from site-specific mutagenesis studies in which five active site hydrophobic residues have been altered to smaller side chains. The targeted side chains are highlighted in Figure 5 as Leu546 and Leu754, which lie on opposite faces of the reactive carbon of a modeled

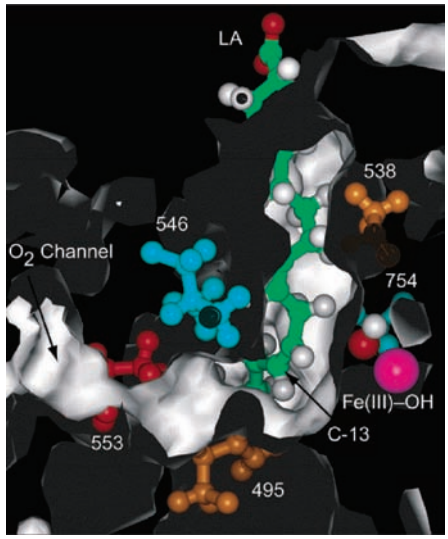


FIGURE 5. Active site of SLO with bound substrate (linoleic acid, in green), illustrating the five residues that were mutated to alanine (Ile553, Leu546, Leu754, Gln495, and Ile538).

Table 3. Impact of Active Site Mutation in SLO on the Stereo- and Regiospecificity of the Hydroperoxide Product (HPOD)[a]

	% HPOD			
enzyme	13(*S*)	3(*R*)	9(*S*)	9(*R*)
WT-SLO	95	3	<1	2
Ile^{553}→Ala	97	1	<1	<1
Gln^{495}→Ala	95	2	1	2
Ile^{538}→Ala	93	3	2	3
Leu^{546}→Ala	85	2	3	10
Leu^{754}→Ala	62	12	10	16
lle^{553}→Phe	95(2)	2	1	2

[a] Reference 40.

complex of SLO with linoleic acid, and Ile538, Gln495, and Ile553, which reside at longer distances from this reactive carbon.[40] The data in Table 3 show very clearly that reduction in size of the latter class of side chains has a small, likely minimal, impact on the stereochemical outcome of the O_2 reaction. In marked contrast, decreasing the size of either Leu546 or Leu754 leads to alternative products in a mechanistically insightful pattern. In the case of Leu546 to alanine, one major new product is formed (the 9(*R*) lipid hydroperoxide), which is the expected product for reaction of O_2 from the same face of the substrate-derived radical as occurs in WT enzyme. This implicates Leu546 as a physical barrier between the entering O_2 and the 9-position of the radical intermediate. The Leu754 to alanine mutation is even more damaging, allowing reaction at both the 9(*R*)-position and the 9- and 13-positions on the opposite face of the radical intermediate [13(*R*)- and 9(*S*)-products]. It appears that the presence of both Leu546 and Leu754 maintains an active site environment that constrains the reaction of O_2 to the desired position.[40]

The above data provide compelling evidence of a role for specific side chains in guiding O_2 to its destination, with the corresponding implication of a discrete pathway for O_2 to partition from bulk solvent to the active site. The high-resolution X-ray structure for SLO[28] had intimated

Scheme 3. Summary of the minimal number of steps required for the oxidative half-reaction of SLO. These include diffusion of O_2 to the protein (k_1, k_{-1}), movement of O_2 through the protein (k_2, k_{-2}), combination of O_2 with the substrate-derived radical (k_3, k_{-3}), hydrogen atom abstraction from the Fe(II)–OH_2 by the hydroperoxyl-radical intermediate (k_4, k_{-4}), and dissociation of the product hydroperoxide (k_5).

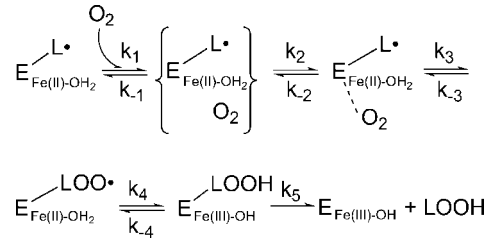


Table 4. Comparison of Kinetic Parameters for WT and Ile553Phe in SLO with Linoleic Acid (LA) as Substrate[a]

	WT SLO	Ile553→Phe
k_{cat} (s^{-1})	230 ± 15	102 ± 8
K_m(LA) (μM)	18 ± 3	19 ± 14
$k_{cat}/K_m(O_2)$ (μM^{-1} s^{-1})	21 ± 1	0.96 ± 0.08
SKIE[b]	0.98 ± 0.1	1.06 ± 0.08
^{18}O KIE[c]	1.0115 ± 0.0013	1.0105 ± 0.0008

[a] Reference 18. [b] Solvent deuterium kinetic isotope effect. [c] ^{18}O kinetic isotope effect.

that such a pathway could exist, Figure 5, where a putative O_2 channel is shown to intersect the substrate channel at an angle of 90°. At this juncture, it is valuable to step back and formulate the minimum number of steps necessary for the reaction of O_2 with SLO, Scheme 3. As shown, this is expected to involve initial diffusion of O_2 to the protein (k_1 and k_{-1}), movement of O_2 through the protein to its targeted binding site (k_2 and k_{-2}), combination of the ground-state triplet O_2 with the preformed substrate radical (k_3 and k_{-3}), transfer of a hydrogen atom from the active site ferrous–water to form the lipid hydroperoxide and ferric hydroxide (k_4 and k_{-4}), and, finally, release of the product lipid peroxide to solvent (k_5).

A number of probes have been applied to SLO in order to specify the degree to which each of the above summarized steps contribute to the rate-controlling step in the O_2 reactivity of SLO. These include the investigation of the impact of solvent viscosogens and solvent D_2O, as well as the measurement of ^{18}O kinetic isotope effects on $k_{cat}/K_m(O_2)$. Analogous to GO, as well as many other O_2-consuming enzymes (see below), neither solvent viscosogen nor D_2O was found to have any impact on reactivity,[18] Table 4, eliminating a number of possible rate-limiting steps from Scheme 3. These include either the binding of O_2 or the release of product peroxide from the enzyme, as well as a hydrogen atom transfer from the ferrous–water to the hydroperoxyl-radical intermediate. Support for the remaining step, the combination of O_2 with the substrate-derived radical, as the rate-determining step comes from the measured ^{18}O kinetic isotope effect, which is clearly non-unity, Table 4.

With these data in hand, it is now possible to examine the role of specific active site side chains in the O_2

reaction, in particular Ile553, which forms a putative barrier from the solvent channel to the bound substrate radical (cf. Figure 5). As already shown in Table 3, reduction of the size of Ile553 has no significant impact on the distribution of products. While an increase in bulk at this position, Ile553 to phenylalanine, likewise has no impact on the nature of the products, it has a highly specific impact on the $k_{cat}/K_m(O_2)$, Table 4. When either k_{cat} or k_{cat}/K_m(LA) is used as the control, it can be seen that Phe553 has, approximately, a 2-fold impact on these measured rate constants. By contrast, the parameter $k_{cat}/K_m(O_2)$ has been reduced 20-fold. The measured ^{18}O kinetic isotope effect with Phe553 is within experimental error of that seen with the wild-type enzyme, Table 4, implicating a similar rate-limiting step that involves a decrease in bonding to oxygen. A simple explanation for an unchanged ^{18}O kinetic isotope effect, together with a 20-fold decrease in rate, is that the chemical reaction of O_2 with substrate is "gated" at position 553: passage of O_2 through the "kink" that is generated at position Ile553 is controlled by protein breathing modes, with the probability of these modes decreasing in the Ile553→Phe mutation.[18]

The aggregate data, both kinetic and structural, for SLO point toward a picture for O_2 reactivity in which (i) the O_2 binds via a discrete channel, which directs its movement through a specific region of the protein; (ii) this prebound O_2 undergoes further diffusion past a constriction in the channel at position 553, facilitated by a protein motion(s) that permits O_2 to pass into the substrate-binding pocket; and (iii) the combination of O_2 with position C-13 of the delocalized substrate radical occurs in a rate-limiting step to form the one-electron-reduced hydroperoxyl radical.

Emerging Views of O_2 Reactivity in Proteins

Though the nature of the O_2-derived products formed in GO and SLO is very different, both reactions appear to operate via rate-limiting addition of the first of two electrons to O_2, and in neither case is proton transfer rate-determining. At this juncture, fairly detailed studies have been completed with a number of other O_2-consuming enzymes as well. These include dopamine β-monooxygenase (DβM) and peptidylglycine α-amidating monooxygenase (PHM),[41] tyrosine hydroxylase (TH),[42] methane monooxygenase (MMO),[43] copper amine oxidase (CAO),[44,45] and cytochrome P-450.[46] DβM and PHM appear to be in a class of their own, in that the activation of O_2 is a reversible process and tightly coupled to the C–H activation of substrate.[41] In all other O_2-activating enzymes studied thus far within this laboratory, an irreversible step takes place during O_2 activation prior to subsequent active site chemistry.

Among the remaining enzymes studied, all contain metals at their active site, iron in the case of TH, MMO, and cytochrome P-450 and copper in the case of the CAOs. In principle, this distinguishes these enzyme systems from GO and SLO, in that the metal ion can both assist and control the O_2 reactivity. Of considerable interest in the context of the O_2 cycle summarized in Figure 1, analyses of ^{18}O kinetic isotope effects in TH,[42] MMO,[43] and P-450[46] are consistent with a similar rate-limiting transfer of the first electron to the O_2 for the parameter $k_{cat}/K_m(O_2)$. This is, perhaps, as expected given the large inherent differences in thermodynamic driving force for superoxide formation versus the formation of subsequent O_2-derived intermediates, Scheme 1A. For TH, there has been some ambiguity as to the origin of this first electron, that is, whether this comes from the reduced pterin or Fe^{2+}; in the original study of ^{18}O isotope effects, it was proposed that pterin is the initial reductant rather than the metal center.[42] In certain ways, this is quite analogous to the reaction proposed for O_2 reactivity in the eukaryotic CAOs where the reduced cofactor reduces O_2 to superoxide ion in close proximity to an active site cupric ion, with the latter providing electrostatic stabilization.[44,45] An important distinction between TH and eukaryotic CAOs is that the metal does not appear to change its valence during catalytic turnover in the CAO reaction, with the reduced cofactor providing both the electrons and protons needed to form the hydrogen peroxide product.

A second surprise in studies of the oxygen half-reaction of the eukaryotic CAOs has been the identification of a discrete, non-metal O_2 binding pocket that is positioned adjacent to both the reduced cofactor and the active site Cu^{2+} and is comprised of hydrophobic side chains. Site-specific mutagenesis has identified a methionine (634) as a key residue within the pocket of the CAO from *Hansenula polymorpha*, with $k_{cat}/K_m(O_2)$ correlating with the size of the side chain at this position.[47] Many features remain unexplained, which include the reason for a better correlation of $k_{cat}/K_m(O_2)$ with the size than the hydrophobicity of the side chain of 634, as well as the channel that leads from the solvent to the O_2 binding pocket. Xenon binding studies have identified a tight-binding xenon adjacent to the Met634 pocket, termed the O_2 "anteroom".[48] This leads naturally to the idea that movement of O_2 from its anteroom to the reactive O_2 pocket in CAO may be gated by protein breathing modes, analogous to the behavior proposed above for the oxidative portion of the SLO reaction.

Conclusions

A number of initially unexpected properties for O_2 activation in enzymes have emerged from studies conducted over the last decade. The first is the predominance of evidence for a rate-limiting transfer of the first electron to O_2 for the parameter $k_{cat}/K_m(O_2)$, with the subsequent transfer of the electrons and protons needed for product formation occurring in rapid steps. This is fully compatible with the inherent chemical properties of O_2, whereby the thermodynamics for the stepwise uptake of electrons by O_2 indicate a highly unfavorable first electron transfer. The enzymatic strategy for increasing the rate of this first electron transfer has been shown very clearly in the GO

system to reside within the kinetic barrier, such that the resulting bound superoxide ion undergoes relatively little (or possible no) stabilization relative to the free species. This is a very satisfying result, showing how an enzyme can catalyze the formation of a potentially reactive and toxic species without increasing the lifetime of such a species at the enzyme active site. Despite the potential toxicity of reaction intermediates derived from O_2, the inherent redox characteristics of O_2 make it possible to minimize deleterious side reactions in biological reactions. O_2 is a remarkable gas, indeed!

The second key feature to result from studies of the enzymology of oxygen is the implication of both discrete channels and non-metal binding pockets for O_2 that increase the likelihood of the correct regio- and stereochemical outcome of its reaction. This flies in the face of the historical belief that O_2 would diffuse randomly though a protein, in response to breathing modes that would provide the gas with access to interior, hydrophobic regions. It is, on reflection, not surprising that enzymes will have evolved discrete O_2 channels and pockets, since only in this manner can the O_2 reactivity be controlled, especially in the cases where it is undergoing activation either in the complete absence of a metal ion or via an outer-sphere electron transfer reaction from the metal center.

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