

Review

A theoretical study of the dioxygen activation by glucose oxidase and copper amine oxidase

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Abstract

Glucose oxidase (GO) and copper amine oxidase (CAO) catalyze the reduction of molecular oxygen to hydrogen peroxide. If a closed-shell cofactor (like FADH₂ in GO and topaquinone (TPQ) in CAO) is electron donor in dioxygen reduction, the formation of a closed-shell species (H₂O₂) is a spin forbidden process. Both in GO and CAO, formation of a superoxide ion that leads to the creation of a radical pair is experimentally suggested to be the rate-limiting step in the dioxygen reduction process. The present density functional theory (DFT) studies suggest that in GO, the creation of the radical pair induces a spin transition by spin orbit coupling (SOC) in O₂⁻(rad), whereas in CAO, it is induced by exchange interaction with the paramagnetic metal ion (Cu(II)). In the rate-limiting step, this spin-transition is suggested to transform the O₂⁻(rad)–FADH₂⁺(rad) radical pair in GO and the Cu(II)–TPQ (triplet) species in CAO, from a triplet (T) to a singlet (S) state. For CAO, a mechanism for the O–O bond cleavage step in the biogenesis of TPQ is also suggested.

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1. Introduction

Molecular oxygen is required by organisms with aerobic metabolism as the terminal electron acceptor in respiration and as a reagent for direct biochemical synthesis. Despite its powerful oxidizing character, reactions of oxygen with reducing substrates are usually slow at ambient temperatures [1]. Aerobic bacteria, algae, yeasts, plants and small animals cover their oxygen supply by diffusion [2]. In larger animals, diffusion became insufficient with increasing metabolic rates and a decreasing ratio of body surface to body volume. Animals therefore developed oxygen transport systems and oxygen carrying proteins—the most recent inventions of nature in its utilization of oxygen. Oxygen is a stable biradical with two unpaired electrons generating the electronic triplet ground state $X^3\Sigma_g^-$ of the O₂ molecule. Triplet dioxygen has a very sluggish kinetic reactivity but is capable of reacting with a strong thermodynamic driving force. The higher energy and greater reactivity of singlet than triplet O₂ is a major contributing factor in maintaining the present level of triplet dioxygen in the atmosphere. A

concerted insertion of the ground state dioxygen into organic (diamagnetic) molecules is a spin-forbidden process. Biological systems activate triplet dioxygen for controlled chemical synthesis via electron transfer and proton transfer reduction. Dioxygen generally undergoes reactions in a stepwise manner via formation of free radical intermediates with one unpaired electron [3,4]:



After the production of the first diamagnetic species (Eq. (3)), the following steps do not depend on spin correlation in the radicals:



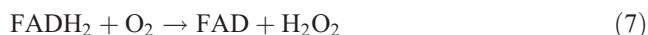
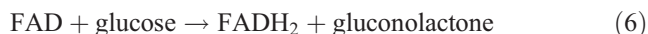
Although dioxygen is a strong oxidant at pH 7 (when it is a concerted four-electron transfer agent), it is a weak one-

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electron oxidant [4]. In terms of the reduction potential, the limiting step is the first electron transfer to O₂. Electron sources in the enzyme adequate for the reduction of dioxygen will produce all the other reduced forms via reduction (Eq. (1)), hydrolysis and disproportionation steps (Eqs. (2)–(5)).

One important aspect of Eqs. (1)–(5) is the coupling of proton and electron transfer steps emphasized in numerous studies [3,4]. Another important aspect of dioxygen activation has so far not been addressed sufficiently: if there is a closed-shell cofactor, which is an electron source for the reduction of dioxygen, production of the closed shell species is spin-forbidden. After the electron transfer step (Eq. (1)), the radical pair M⁺(rad) . . O₂⁻(rad) is produced in the triplet state, where M is the electron-donor. The proton transfer (Eq. (2)) does not change the spin multiplicity of the electronic system, but at the stage of the hydrogen peroxide production (Eq. (3)), the system is in a ground singlet state. Thus, the triplet → singlet (T → S) spin transition has to occur somewhere during this transformation. Dioxygen activation has been studied in two different enzymes: (1) glucose oxidase (GO), which uses only an organic cofactor (FADH₂) for dioxygen activation, and (2) copper amine oxidase (CAO), which utilizes both a copper center and an organic cofactor (reduced form of TPQ) for the dioxygen activation.

GO is a 130–325 kDa (depending on degree of glycosylation), homodimer flavoprotein consisting of 1 mol of FAD per subunit. GO has been isolated from red algae, citrus fruits, insects, bacteria and molds [5]. The X-ray structure from *Aspergillus niger* has been determined [6]. In the active site, there are two histidine residues (His-516 and His-559) in close proximity to FAD [6,7]. Only one of them (His-516) has been assigned an important role as the general base in the substrate oxidation [7]. Dioxygen can occupy the cavity between the FADH₂ and His-516 moieties. The reaction sequence of GO proceeds in two half-reactions known as the reductive and oxidative half-reactions, described in Eqs. (6) and (7), respectively:



During the reductive half-reaction, two electrons and two protons are transferred to FAD present at the active site. In the oxidative half-reaction, FADH₂ acts as a transducer molecule and provides electrons and protons to dioxygen to yield H₂O₂ and complete the catalytic cycle.

CAOs form a family of redox active enzymes that is present in both prokaryotes and eukaryotes. They catalyze the oxidative deamination of primary amines by dioxygen to form aldehydes, ammonia and hydrogen peroxide. Amine oxidases are homodimers with 70–95 kDa subunits, with each subunit containing a cofactor, 2,4,5-trihydroxyphenylalaninequinone referred to as topaquinone (TPQ), and a

copper ion [8–10]. The crystal structures from *Escherichia coli* (ECAO) [11], pea seedling (PSAO) [12], *Arthobacter globiformis* (APAO) [13] and yeast *Hansenula polymorpha* (HPAO) [14] have been solved. CAOs are proposed to be dual-function enzymes, catalyzing both cofactor biogenesis from an intrinsic, specific precursor tyrosine [15,16] and oxidative deamination of amines. Catalysis requires the TPQ redox cofactor. The active site is deeply buried, and a significant amount of reorientation of the polypeptide is needed when the substrate comes in. The organic cofactor TPQ and copper are in close proximity but are not coordinated to each other. Experiments have shown that the CAOs utilize a two-step, ping-pong type mechanism in its catalytic cycle [17]. The process can be formally divided into reductive and oxidative half-reactions, with the reductive half-reaction being



and with the oxidative half-reaction being



Both the reductive [18] and the oxidative [19] half-reactions have been studied using B3LYP functional calculations. The suggested mechanisms agree well with the ones suggested experimentally. Both the reductive and oxidative half-reactions are suggested to occur in six steps. Intermediates and transition state structures connecting the different intermediates were optimized. In these mechanisms, the copper center is not redox-active and mostly plays a rather minor role. The exception is the spin-forbidden step in the oxidative half-reaction involving dioxygen, where the spin-orbit coupling on the copper center is likely to be important. The copper complex is also involved in a step where one of its water ligands is suggested to donate a proton to the TPQ cofactor. These mechanisms will not be discussed here, but instead the focus will be on the dioxygen activation steps in the biogenesis of TPQ and in the oxidative half-reaction.

2. Computational details

All the calculations were performed using the GAUSSIAN-98 [20] and Jaguar [21] programs. The calculations for the mechanism were performed in two steps. First, an optimization of the geometry was made using the B3LYP method [22–24] with a double zeta d95 basis set for GO and LACVP for CAO. Open-shell systems were treated using unrestricted B3LYP (UB3LYP). All degrees of freedom were optimized and the transition states obtained were confirmed to have only one imaginary frequency of the Hessian. In the second step for GO, the B3LYP energy was evaluated for the optimized geometry using the large 6–311+G(2d,2p) basis set, which includes diffuse functions and two polarization functions on each atom. For CAO, the

standard LACV3P** basis set of triple zeta quality including one polarization function on each atom was used together with an ECP [25] for the copper atom. Zero-point vibrational effects and thermal effects were added based on B3LYP calculations using the same basis set as for the geometry optimization. In the case of GO, the dielectric effects from the surrounding environment were obtained using the CPCM polarizable conductor model (Cosmo) [26], where the solvent cavity is formed as a surface of constant charge density of the solvated molecule. The default isodensity value of 0.0004 au is used. The radii of the solvent molecules were taken from the parameters for water. The United Atom Topological Model [27] was used to build the cavity around each heavy atom and the radius of each atomic sphere was determined by multiplying the van der Waals radius by a scaling factor of 1.2. For CAO, the self-consistent reaction field method as implemented in Jaguar [28,29] was used to evaluate the solvent corrections by employing the LACVP basis set. A probe radius of $R = 1.40 \text{ \AA}$ corresponding to the water molecule was chosen. In both cases, the dielectric constant was set equal to 4, which corresponds to a dielectric constant of about 3 for the protein and 80 for the water medium surrounding the protein [30]. Since models with the same charge were used throughout the present study, the relative dielectric effects were found to be rather small and not very sensitive to the method used or to the value chosen for the dielectric constant. The relative energies discussed below are Gibbs free energies, where all the effects described above are added. Normal errors of using B3LYP and different aspects of modeling enzyme active sites are described in recent reviews [31–33].

3. Dioxygen activation by GO

The oxidative half-reaction (Eq. (7)) has been studied [34] by the density functional theory (DFT) using the hybrid B3LYP functional [22–24] for the model shown in Fig. 1. The reaction is calculated to be exothermic by 7.2 kcal/mol without including zero-point, thermal and dielectric effects.

From the B3LYP calculations, the following mechanism is suggested. In the first step, an electron is transferred from the reduced cofactor FADH₂ to dioxygen occupying the small cavity between the N1 and N^ε atoms. The optimization from the O₂...FADH₂ starting point leads to the creation of the FADH₂⁺(rad)...O₂⁻(rad) ion–radical pair. It is concluded from the DFT calculations that the electron transfer occurs without any (enthalpic) activation barrier in this model. This does not necessarily mean that the formation of the radical pair is exergonic since there should be a large loss of entropy involved when the nearly free O₂ becomes bound. The reason for such an easy electron transfer is that the electron affinity (EA) of dioxygen in the presence of a protonated histidine is extremely high, 118.2 kcal/mol from the calculations including dielectric effects. In comparison, the EA of free O₂ in gas phase is

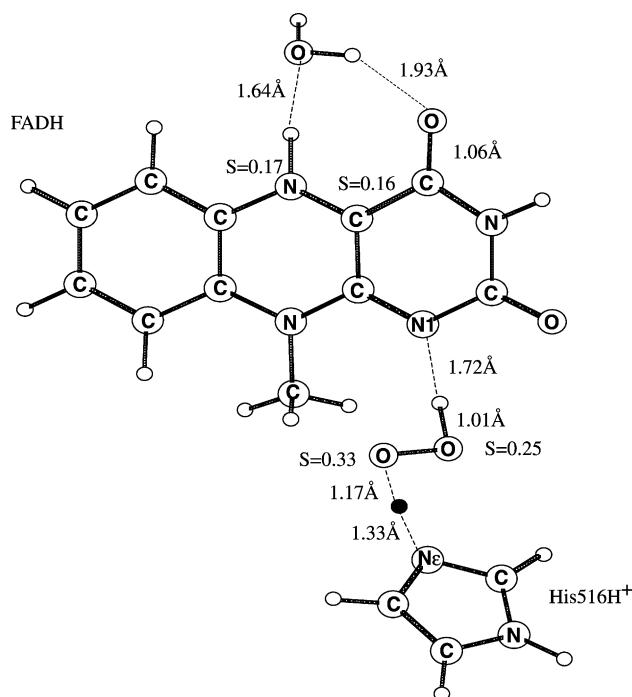


Fig. 1. Optimized transition state for formation of hydrogen peroxide in glucose oxidase.

only 10.4 kcal/mol [35]. Thus, the presence of the protonated His-516 is critical for the catalytic mechanism of GO. The ionization potential (IP) of FADH₂ is also quite low, 109.8 kcal/mol, from the present calculations including dielectric effects. The zeroth vibrational level of O₂ is almost isoenergetic with the level $\nu = 3$ of the O₂⁻ ion and the Franck–Condon factor $|\langle \nu' = 0 | \nu = 3 \rangle|^2$ is relatively large. The outer sphere reorganization energy should furthermore be small since the electron transfer distance is short. Thus, the electron transfer step should be very fast and is very unlikely to be the rate-determining step in the whole process.

The occurrence of dioxygen at the active site and weak intermolecular (and O–O) vibrations prompt the first electron jump, which leads to the creation of the radical pair FADH₂⁺(rad)...O₂⁻(rad). Once this radical pair has been formed, the triplet → singlet transition must be induced for the subsequent chemistry to take place. When the radical pair is in the singlet state, the FADH₂⁺(rad) transfers its proton from the N1 nitrogen atom to the O₂⁻ radical, which is then followed by an electron-coupled proton transfer to the O₂H radical forming H₂O₂. The transition state for this 2H⁺ + e⁻ transfer process is shown in Fig. 1. The calculated free energy barrier is 6.6 kcal/mol corresponding to a rate of 10⁸ s⁻¹. The reason the process is concerted can be partially understood by the fact that the proton transfer from N1 to the O₂⁻ radical in the singlet RP, O₂⁻(rad)...FADH₂⁺(rad), does not need any activation energy due to the O₂⁻...H⁺ attraction, and also since no spin change is needed. At the same time, the new nascent

singlet radical pair $O_2^{\cdot-}H(rad) \dots FADH^+(rad)$ creates a perfect condition for an electron-coupled proton transfer where the electron comes from $FADH^+(rad)$ and the proton from $His-516H^+$ to the O_2H radical. It has to be stressed that such a simultaneous $2H^+ + e^-$ transfer can only occur in the singlet state of the whole system. It is important to note that while the system undergoes these transformations, it keeps the singlet state both during H atom transfer and the subsequent proton transfer steps.

A molecular Hessian was evaluated for the transition state of the $2H^+ + e^-$ process. The imaginary frequency at the transition state is 567 cm^{-1} . For the optimized transition state shown in Fig. 1, a clear deuterium isotope effect and almost no oxygen isotope effect was found, which is in complete contradiction with the experimental observations. Since the isotope effects do not match what is experimentally observed, the step going over the transition state in Fig. 1 can be ruled out as rate limiting in GO. This is also in agreement with the rate of 10^8 s^{-1} calculated for the process in Fig. 1, compared to the experimental rate of only 10^6 s^{-1} [36]. Since the electron transfer from the cofactor to O_2 was found to occur without any enthalpic activation barrier, the conclusion drawn here is therefore that it is the singlet–triplet transition that is rate limiting. Still, as mentioned above, the resting state for this step is likely to be the unbound O_2 state since this state is favored due to its large entropy. This means that, apart from the Franck–Condon factor in the spin-transition, the change of bond order between unbound O_2 and bound $O_2^{\cdot-}(rad)$ will contribute to the isotope effects. The nature of the spin-transition including a discussion of the observed oxygen isotope effect has been presented recently [34].

4. Dioxygen activation in biogenesis of TPQ in CAO

The mechanism for biogenesis of TPQ has been studied recently by B3LYP calculations [37] following essentially a previously suggested scheme [38]. The starting point for this mechanism is when dioxygen binds equatorially and tyrosine axially to the copper center. This structure is found to lead to a Cu(II) complex with an O_2^- ligand and a tyrosyl radical. In the next step, dioxygen attacks the phenol ring and forms a bridging peroxide bond between copper and the phenol. The barrier for this step is 8.4 kcal/mol. At this point, the O–O bond cleavage can begin. The optimized transition state structure is shown in Fig. 2 and the computed barrier is 16.7 kcal/mol. As usual, in a heterolytic O–O bond cleavage, this is accompanied by proton transfers, in this case from the sp^3 carbon of the phenol ring to one of the oxygens of the peroxide. An external water molecule assists in this proton transfer. O–O bond cleavage is driven by an unusually large exothermicity of 46.0 kcal/mol. Shortly after the TS, when the proton has reached the peroxide oxygen, the O–O bond distance starts to increase and the reaction goes to the product without any additional barrier. In the

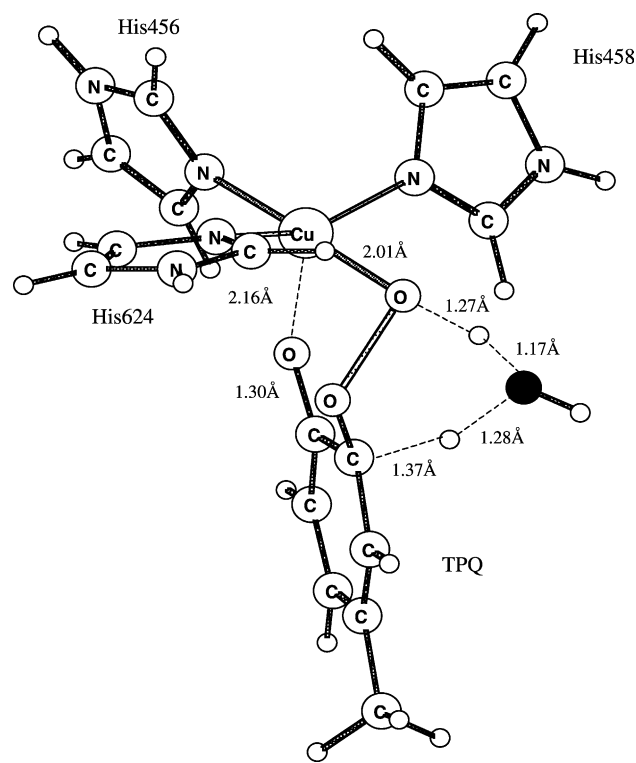
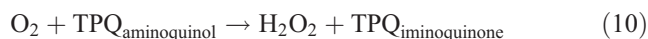


Fig. 2. Transition state for O–O bond cleavage for TPQ biogenesis in CAOs.

next step of TPQ biogenesis, a water molecule attacks the quinone and the hydroxide group adds to the ring while the proton is transferred to one of the oxygens through a few conserved water molecules. A proton on the sp^3 -carbon of the quinone is then transferred to the hydroxide on copper using the same water chain. In the final step, two protons and two electrons on the quinone are removed by adding O_2 , forming H_2O_2 and TPQ.

5. Dioxygen activation in the oxidative half-reaction of CAO

The mechanism for dioxygen activation in the oxidative half-reaction (Eq. (9)) has been studied recently by B3LYP calculations [39]. The reaction sequence of the dioxygen reduction to hydrogen peroxide can be written as



The following mechanism is suggested by the B3LYP calculations. In the first step, as suggested experimentally, dioxygen replaces the axial water (W_a) and binds between the O-2 and O-4 position of the cofactor TPQ [14,40]. Dioxygen binding is accompanied by an electron transfer from the TPQ to dioxygen, which leads to the creation of a $Cu(II) \dots O_2 \dots M^+(rad)$ system, where $M^+(rad)$ represents the semiquinol form of TPQ. This electron transfer step

takes place without any enthalpy barrier. A few attempts were made to calculate the reactant with triplet O_2 in the presence of the full model of TPQ, but all these attempts failed. Whenever O_2 comes close to the reduced TPQ, an electron transfer from TPQ to O_2 takes place. This does not mean that the formation of the radical pair is exergonic since there should be a large loss of entropy involved when the nearly free O_2 becomes bound. The overall reaction (Eq. (10)) is a spin-forbidden process. $TPQ_{\text{aminoquinol}}$, H_2O_2 and $TPQ_{\text{iminoquinone}}$ are all singlets, and O_2 is a triplet. At the stage of hydrogen peroxide production, the system has to undergo a triplet(T) \rightarrow singlet(S) spin transition. Experimentally, the presence of the Cu metal center is estimated to enhance the rate of the oxygen reduction by 10^4 fold [41] and its substitution with the diamagnetic Ni^{2+} and Zn^{2+} ions in HPAO resulted in substantially reduced catalytic activity. However, on substituting Cu^{2+} with paramagnetic Co^{2+} , the enzyme activity was not altered [41]. After the electron transfer step, the $O_2^{\cdot-}(\text{rad}) \dots M^+(\text{rad})$ radical pair (RP) produced is still in a triplet state. In the next step, as suggested experimentally [42], superoxide crosses over a barrier of 8.2 kcal/mol and binds to the copper. This step is endothermic by 2.6 kcal/mol. On replacing Cu^{2+} with Zn^{2+} , the calculations show that superoxide binds equally well to zinc. This result combined with the experimental observations mentioned above strongly suggest that apart from providing an electrostatic stabilization to the superoxide species, the paramagnetic metal has a specific role in the mechanism. Once the $Cu-O_2^{\cdot-}(\text{rad})$ bonded species is formed, a proton transfer from O-2 and a hydrogen atom transfer from the O-4 site of the cofactor take place in a concerted manner. This is at least partly in agreement with experiments that also suggest that the first proton transfer takes place from the O-2 site of the cofactor [42]. This concerted $2H^+ + e^-$ transfer leads to the formation of singlet H_2O_2 and the triplet form of the cofactor TPQ. Due to the formation of the triplet TPQ, this step is quite endothermic by 12.9 kcal/mol. At this juncture, a $Cu(\text{doublet}) \dots TPQ(\text{triplet})$ species is formed. After the formation of this species, a spin transition takes place due to the weak exchange between Cu and TPQ, and singlet TPQ is formed. This step is calculated to be exothermic by 29.1 kcal/mol. Thus, on the basis of model calculations [39] and the above experimental observations, the presence of a paramagnetic metal can be implicated in the T \rightarrow S spin transition in the cofactor TPQ. The T \rightarrow S transition in cofactor TPQ is suggested to be the rate-limiting step in the entire oxidative half-reaction. As mentioned above, entropy effects favoring free O_2 are likely to make the unbound O_2 state the resting state for the rate-limiting spin transition, which can explain the large oxygen isotope effects found experimentally [7,42]. The extremely complex mechanism of H_2O_2 formation and the experimentally measured oxygen isotope effect [42] related to the rate-limiting T \rightarrow S transition will be discussed in a future study [39].

6. Summary

The present study of dioxygen activation in GO and CAO can be summarized as follows. In GO and in the oxidative half-reaction of CAO, dioxygen accepts two protons and two electrons from the cofactor to yield H_2O_2 , whereas in the biogenesis of TPQ, the O–O bond cleavage takes place. In both cases, endergonic binding of dioxygen is followed by a spontaneous electron transfer without any enthalpy barrier. In GO, the triplet \rightarrow singlet nonradiative transition (a spin flip) is induced by a relatively strong spin orbit coupling (SOC), which originates from SOC in the superoxide moiety of the RP. This is suggested to be the rate-limiting step [34]. In the oxidative half-reaction of CAO, the T \rightarrow S spin transition in the cofactor TPQ induced by the presence of a paramagnetic metal is suggested to be the rate-determining step [39]. Both in GO and CAO, these results are supported by the presence of significant $^{18}O/^{16}O$ isotope effects [7,42]. Since the resting state in both cases are likely to be one where O_2 is not yet bound, the change of bond order between bound $O_2^{\cdot-}(\text{rad})$ and unbound O_2 will cause the large isotope effect, but Franck–Condon factors in the spin-transition will also contribute. In addition to the already known information, calculations have provided new insights about the catalytic function of these enzymes.

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