Non-coordinative binding of O_2 at the active center of a copper-dependent enzyme

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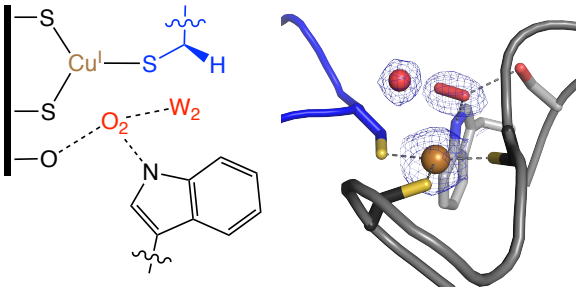
Abstract. Molecular oxygen (O_2) is a sustainable oxidation reagent. O_2 is strongly oxidizing but kinetically stable and its final reaction product is water. For these reasons learning how to activate O_2 and how to steer its reactivity along desired reaction pathways is a longstanding challenge in chemical research.[1] Activation of ground-state diradical O_2 can occur either via conversion to singlet oxygen or by one-electron reduction to superoxide. Many enzymes facilitate activation of O_2 by direct fomation of a metal-oxygen coordination complex concomitant with inner sphere electron transfer. The formylglycine generating enzyme (FGE) is an unusual mononuclear copper enzyme that appears to follow a different strategy. Atomic-resolution crystal structures of the precatalytic complex of FGE demonstrate that this enzyme binds O_2 juxtaposed, but not coordinated to the catalytic Cu^I. Isostructural complexes that contain Ag^I instead of Cu^I or nitric oxide instead of O_2 confirm that formation of the initial oxygenated complex of FGE does not depend on redox activity. A stepwise mechanism that decouples binding and activation of O_2 is unprecedented for metal-dependent oxidases, but is reminiscent of flavin-dependent enzymes.



Introduction

Reduction of O_2 to superoxide with Cu^I as electron donor has been proposed as common first step in O_2-activation by mononuclear and noncoupled binuclear copper enzymes.[2] Because transfer of the first electron is energetically difficult under standard conditions, it must be coupled to thermodynamically favorable steps, and the reduced oxygen species must be stabilized by the electrostatic structure of the protein environment. In copper-dependent enzymes this requirement is achieved by coupling the reduction of O_2 with the formation of a Cu^{II}-superoxide coordination complex. To minimize the energy barrier for this process the cofactor is usually coordinated by nitrogen and oxygen ligands arranged in a geometry that stabilizes Cu^{II} relative to Cu^I, and allows binding of O_2 with minimal reorganization of the coordination sphere.[1a, 1b, 2c, 3] Facilities that enable this strategy have been observed in the structures of several copper enzymes. Lytic polysaccharide monooxygenases (LPMO) coordinate copper by a histidine brace that provides three nitrogen ligands in a T-shaped geometry. The fourth site of the square planar coordination sphere is left open for O_2.[2c, 4] In the peptidyl-glycine-α-hydroxylating monooxygenase (PMH) the tetrahedral coordination sphere of copper includes two histidines, a methionine and a loosely bound water molecule which can be displaced by O_2.[2b, 5] Structural and spectroscopic evidence also implicates a tetrahedral coordinated copper with three nitrogen ligands and a water as O_2-activating center in the particulate methane monooxygenase (pMMO).[6] Heme and non-heme iron enzymes also use preformed O_2-coordination sites,[7] and fit well into the view that O_2-activation via inner-sphere metal-to-O_2 electron transfer is a general strategy of metal-dependent oxidases. In the following we discuss evidence that the copper-dependent formylglycine generating enzyme (FGE) may not follow this common theme. Instead, FGE coordinates Cu^I in a thiol-rich environment and does not immediately form a Cu^{II}-superoxide upon O_2-binding.

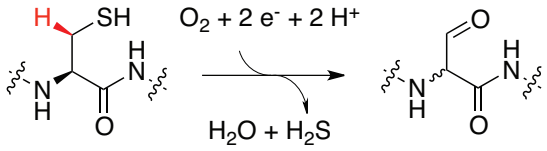


Figure 1. FGE-catalyzed oxidation of peptidyl-cysteine to formylglycine (fGly) is initiated by abstraction of the pro-(R)-β-hydrogen atom from the substrate (red).

FGE catalyzes O_2-dependent oxidation of peptidyl-cysteines in specific client proteins to formylglycine (fGly, Figure 1).[8] This post-translational modification is essential for the activation of arylsulfatases.[9] The physiological relevance of this modification is evidenced by clinical observations that reduction of FGE activity in humans leads to multiple sulfatase deficiency, which is a rare but devastating genetic disorder.[8] The ability of FGE to introduce aldehyde functions has also been recognized as a tool for site-specific modification of recombinant proteins.[9-10] Meanwhile, the catalytic mechanism of this enzyme remained a complete mystery until the discovery that its activity depends on copper as an integral redox cofactor.[11] The crystal structure of FGE from *Thermomonospora curvata* (*tc*FGE) in complex with silver (*tc*FGE:Ag^I, 1.7 Å, PDB: 5NXL) and from *Streptomyces coelicolor* in complex with copper (*sc*FGE:Cu^I, 2.2 Å, PDB: 6MUJL) demonstrated that the metal cofactor is bound in a linear bis-cysteine coordination site.[12] This coordination mode is unusual for O_2-activating enzymes but is reminiscent of high-affinity copper chaperones that stabilize copper as Cu^I.[1a, 13] More recently, we also crystallized *tc*FGE in complex with Cu^I and a peptide substrate (*tc*FGE:Cu^I:S, 1.04 Å, PDB: 6S07, substrate: Abz-ATTPL**C**GPSRASILSGR, Abz: o-aminobenzoic acid).[14] This structure showed that the cysteine of the substrate joins the coordination sphere forming a tris-thiolate copper complex with trigonal planar geometry (structure **0**, Figure 2). Since this species still provides no preformed coordination site for O_2, the question as to how this metal site would initiate redox chemistry remained unanswered.

Results and discussion

Structure of *tc*FGE:Cu^{I}:S:O_2. To address this question, we soaked crystallized *tc*FGE:Cu^{I}:S in a solution containing high concentrations of O_2 ($[O_2] \leq 5$ mM). This supersaturated O_2 solution was generated by reacting chlorite dismutase with sodium chlorite.[15] This enzyme has been shown to convert chlorite exclusively to O_2 and Cl^- with no observed side reactions.[16] The soaked crystals were frozen and analyzed by synchrotron X-ray diffraction at 100 K. Two data sets collected from crystals that were soaked for 20 s could be solved to a resolution of 1.8 Å (*tc*FGE:Cu^{I}:S:O_2, structure **1a**, Figure 2) and 1.4 Å (**1b**, Figure S1). The structures of **1a** and **1b** are essentially identical with the anaerobic complex (structure **0**, r.m.s.d = 0.146 for 2441 atoms),[14] except for one subtle but significant difference. In the anaerobic complex the closest non-coordinating neighbor of the metal is crystallographic water 1, as evidenced by a spherical residual electron density (3.3 Å, Cu-O).[14] In the oxygenated complex **1a** this position is occupied by an ellipsoid electron density (Figure S2) that is best modelled as two linked heavy atoms (denoted as O^1 and O^2). The high resolution of structure **1b** (1.4 Å) invites further analysis of the ellipsoid electron density in the active site. Modelling the unbiased omit map with O_2 as the guest gave the best fit (Figure S3), compared to models for water and hydrogen peroxide which left positive, or, respectively negative difference electron densities. Neither of the heavy atoms approaches the metal close enough for a coordinative interaction (3.1 Å, Cu-O^1; 3.4 Å, Cu-O^2). Instead, the two atoms are in hydrogen-bonding distance to the side chains of Trp228 (2.8 Å, N_{Trp}-O^1) and Ser266 (2.9 Å, O_{Ser}-O^1), and to crystallographic water 2 (2.7 Å, to O_{W2}-O^2). The distance to the sulfur atom of the copper ligand Cys269 is also shorter (2.9 Å, S_{Cys}-O^2) than the expected van der Waals distance (3.3 Å), indicating an attractive interaction between this residue and the guest. Most notably – and in contrast to the structures of other O_2-bound metalloenzymes – the new guest binds juxtaposed, not coordinated to the metal center.[2b, 7a, 17]

Although the observed electron densities implicate O_2 as the guest, we also examined the possibility that the diatomic guest may be something else. Specifically, superoxide or hydrogen peroxide may emerge from reductive dissociation of a copper:oxygen adduct during exposure to high doses (6 – 9 MGy) of 12.4 keV X-ray photons.[18] Synchrotron irradiation is notorious for inducing critical changes in the redox-sensitive centers of metalloenzymes,[19] with the result that the observed structures no longer correspond to the oxidation state of the crystallized species.[7c, 20] To explore the possibility that structure **1a/1b** was induced by intensive irradiation, we collected additional diffraction data sets from FGE crystals using 2 to 500-fold lower irradiation doses (3 MGy – 17 kGy). These data revealed no changes in the electron density maps (Figure S4). Hence, if structure **1a/1b** is formed by photoreduction or photooxidation, the responsible reaction would have to occur with rare if not unprecedented efficiency.[2b, 19, 21] More likely, the observed electron density represents the unreacted complex of FGE with Cu^{I}, the substrate and O_2.

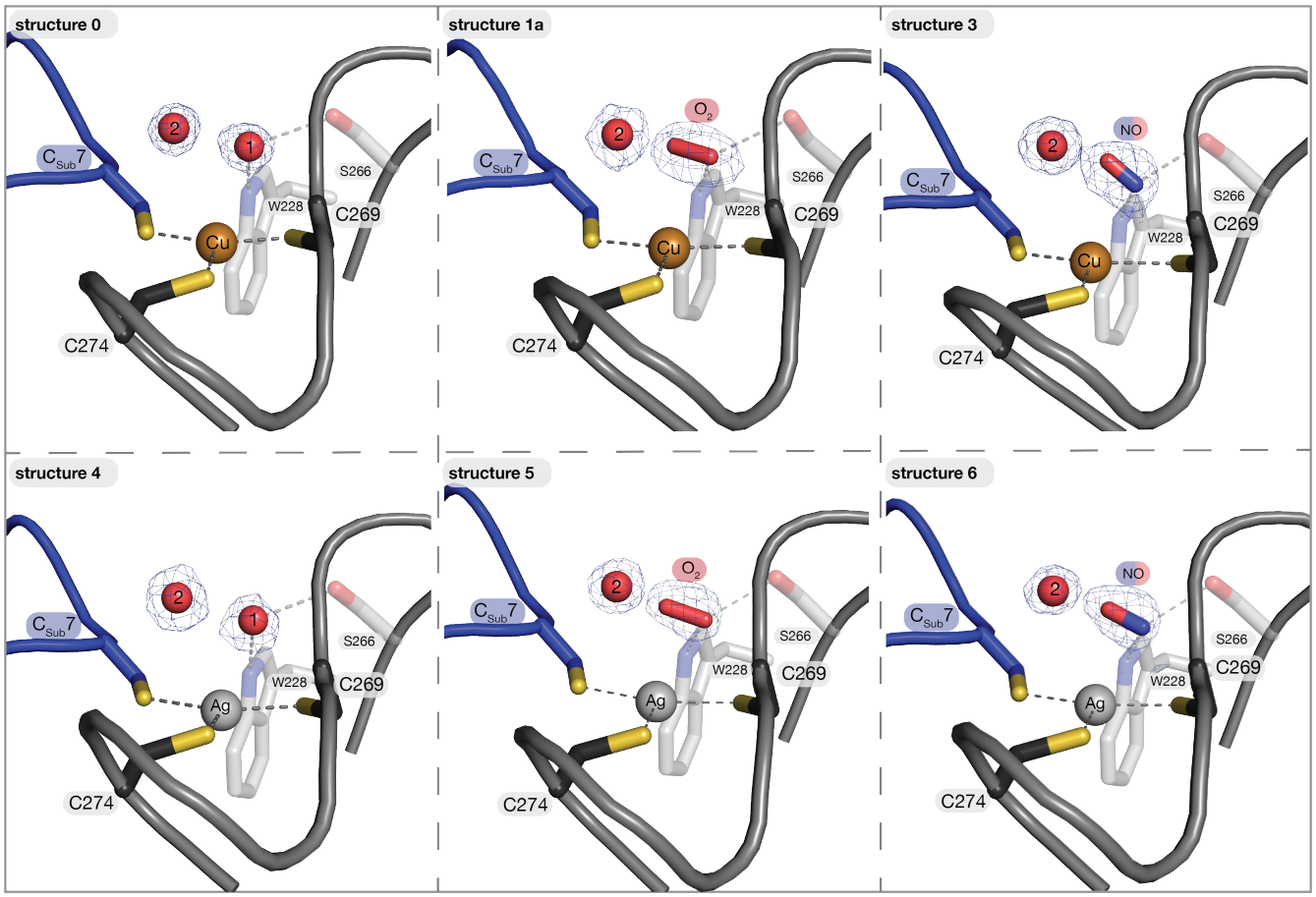


Figure 2. Panel 1 and 2: Active site view of *tc*FGE:Cu^{I}:S (structure **0**),[14] *tc*FGE:Cu^{I}:S:O$_2$ (**1a**), *tc*FGE:Cu^{I}:NO (**3**), *tc*FGE:Ag^{I}:S (**4**), *tc*FGE:Ag^{I}:S:O$_2$ (**5**), and *tc*FGE:Ag^{I}:S:NO (**6**). 2m|F$_o$|-D|F$_c$| maps are contoured at σ-level = 1.2. Active site residues Trp228, Ser266, Cys269 and Cys274 from *tc*FGE (gray) and Cys7 from the peptide substrate (blue) are depicted in stick representation. Less populated (> 20 %) alternative positions of Cys274 and the metal in structures **3**, **5** and **6** are shown in Figures S6 and S9. Data collection and refinement statistics of the diffraction data are summarized in Tables S2, S3 and S4. Distance between the closest guest and the metal (Cu^{I} or Ag^{I}): 3.3 Å (H$_2$O_1, structure **0**); 3.1 Å (O$_2$, structure **1a**); 3.0 Å (NO, structure **3**); 3.1 Å (H$_2$O_1, structure **4**); 3.0 Å (O$_2$, structure **5**); 2.9 Å (NO, structure **6**).

Structure of redox-inactive complexes. The proposed binding mode of O_2 in a hydrophilic pocket juxtaposed to a redox active metal is very unusual. This picture is at odds with the general view that symmetric diatomic molecules preferentially bind to hydrophobic pockets.[22] Nevertheless, specific solvation of O_2 in hydrophilic pockets is well precedented by several crystallographic studies of O_2-consuming enzymes (Table S5). Secondly, the proposition that FGE binds O_2 without coordination implicates that the precatalytic complex forms without redox reaction. To test this idea, we examined the structures of redox inactive analogs of this complex. First, we soaked anaerobic crystals of *tc*FGE:Cu^{I}:S in a solution containing nitric oxide (NO, [NO] ≤ 2 mM).[23] Because this molecule is similar to O_2 in many ways, it is commonly used as an O_2-analog for structural studies,[24] However, NO is an even weaker electron acceptor than O_2.[25] Despite this key difference, NO-soaked crystals contained almost identical electron densities as the oxygenated samples (structure **3**, Figure 2). Specifically, the residual ellipsoid electron density in the active site suggests that NO binds in the same way as O_2. In a complementary experiment we examined the structure of *tc*FGE in which at least 98 % of Cu^{I} is replaced with Ag^{I}, as inferred by X-ray fluorescence spectroscopy (Figure S6). The *tc*FGE:Ag^{I}:S complex crystallizes in the same crystal form, and adopts exactly the same structure (structure **4**) as *tc*FGE:Cu^{I}:S (**0**), confirming the conclusion based on NMR spectroscopy that Ag^{I} is a reliable structural substitute for Cu^{I} in the resting state of FGE.[14] Soaking of *tc*FGE:Ag^{I}:S with O_2 or NO again formed structures (**5** and **6**) that are indistinguishable from their copper-containing analogs (**1** and **3**, Figure 2). The observation

that both O_2 and NO can displace water 1 from the active site of the *tc*FGE:Cu^I:S and the *tc*FGE:Ag^I:S complexes (**0** and **4**) despite the 10^4-fold excess of water ([O_2], [NO] ≤ 5 mM, [H_2O] = 55.5 M,) is remarkable since the two guests are considered rather weak hydrogen bond acceptors. In the anaerobic complexes (**0** and **4**) water 1 is coordinated by a trigonal planar array of hydrogen bonds from Ser266, Trp228 and water 2.[14] For water this is a suboptimal geometry of interactions. Release of this high energy water may provide the driving force needed to attract O_2 or NO into the hydrophilic pocket.

Reaction *in crystallo*. Finally, we examined the possibility that the species observed in **1a/1b** was trapped only because the crystallized enzyme is restricted from turning over, for example due to crystal packing effects. To this end, we compared the electron densities of crystals soaked with O_2 for 20 s (**1b,** 1.4 Å), 40 – 60 s (**1c,** 1.2 Å) and 90 – 150 s (**1d,** 1.2 Å). Crystals soaked for longer periods (> 3 min) started to crack and no longer diffracted. In contrast, the Ag^I-containing crystals remained intact and still diffracted to a resolution of 1.9 Å even after 1 hour of incubation with O_2. This different behavior indicates that O_2 induced redox-dependent activity in the Cu^I-containing crystals. Inspection of the electron densities of **1c** and **1d** revealed time-dependent conversion of the reactive complex (**1b**) to a new species with a linear bis-thiolate coordinated metal and diminished electron density for residues 4 – 8 on the substrate, including the target of catalysis – Cys7 (Figure S11). The electron density of the guest is also subject to change with the result that the model for O_2 becomes slightly worse in **1c** and **1d**, and the model for water becomes slightly better (Figure S12). The geometry of the linear metal center is identical to that observed for FGE in the absence of substrate.[12] Disconnection from the copper center and the increased flexibility of the peptide is consistent with the interpretation that Cys7 has been converted to fGly. The relative occupancies of the initial and the new species changed with time consistent with about 20 % conversion after 60 s and 35 % conversion after 150 s. This approximate rate is in the same order of magnitude as the rate for *tc*FGE-catalyzed substrate turnover in solution (1.4 min^{-1}).[14] *tc*FGE does not catalyze any other reaction with similar efficiency.[11b] Furthermore, characterization of O_2-incubated crystals by high resolution electrospray ionization mass spectrometry (HR-ESI-MS) confirmed that the crystal-bound substrate was converted to the fGly containing product (Figure S13). Therefore, we conclude that the emerging species in structures **1c** and **1d** is *tc*FGE in complex with the product peptide (*tc*FGE:Cu^I:P).In summary, observation of time-dependent substrate consumption by ESI-MS and crystallography demonstrates that the crystallized enzyme is active.

Mechanistic interpretation. Taken together, the structural similarity of *tc*FGE in complex with redox-active and redox-inactive metals and ligands provides evidence that initial formation of the precatalytic complex does not require transfer of an electron from Cu^I to O_2. The competing explanation that structure **1a** is an X-ray artefact, namely a product of photoreduction, is difficult to reconcile with the complete independence of the observed structure from the applied irradiation dose over almost three orders of magnitude. Another alternative explanation is that O_2-soaking at room temperature generated enzymes with copper:O_2 adducts, which dissociated to the observed noncovalent arrangement of O_2 and Cu^I as the temperature dropped in liquid nitrogen. It is indeed plausible that the non-covalent complex and the adduct are in equilibrium and that this equilibrium depends on temperature. However, if direct coordination of O_2 to Cu is essential for binding, the redox-inactive complexes should not have formed with similar efficiency under the same conditions. Based on these considerations we conclude that structure **1a** represents a native state of FGE. Because the trigonal planar tris-thiolate copper complex in the anaerobic complex of *tc*FGE (**0**) does not provide a preformed coordination site for O_2, the non-coordinative complex with O_2 emerges as a necessary intermediate in the catalytic cycle (red frame, Figure 4).[14]

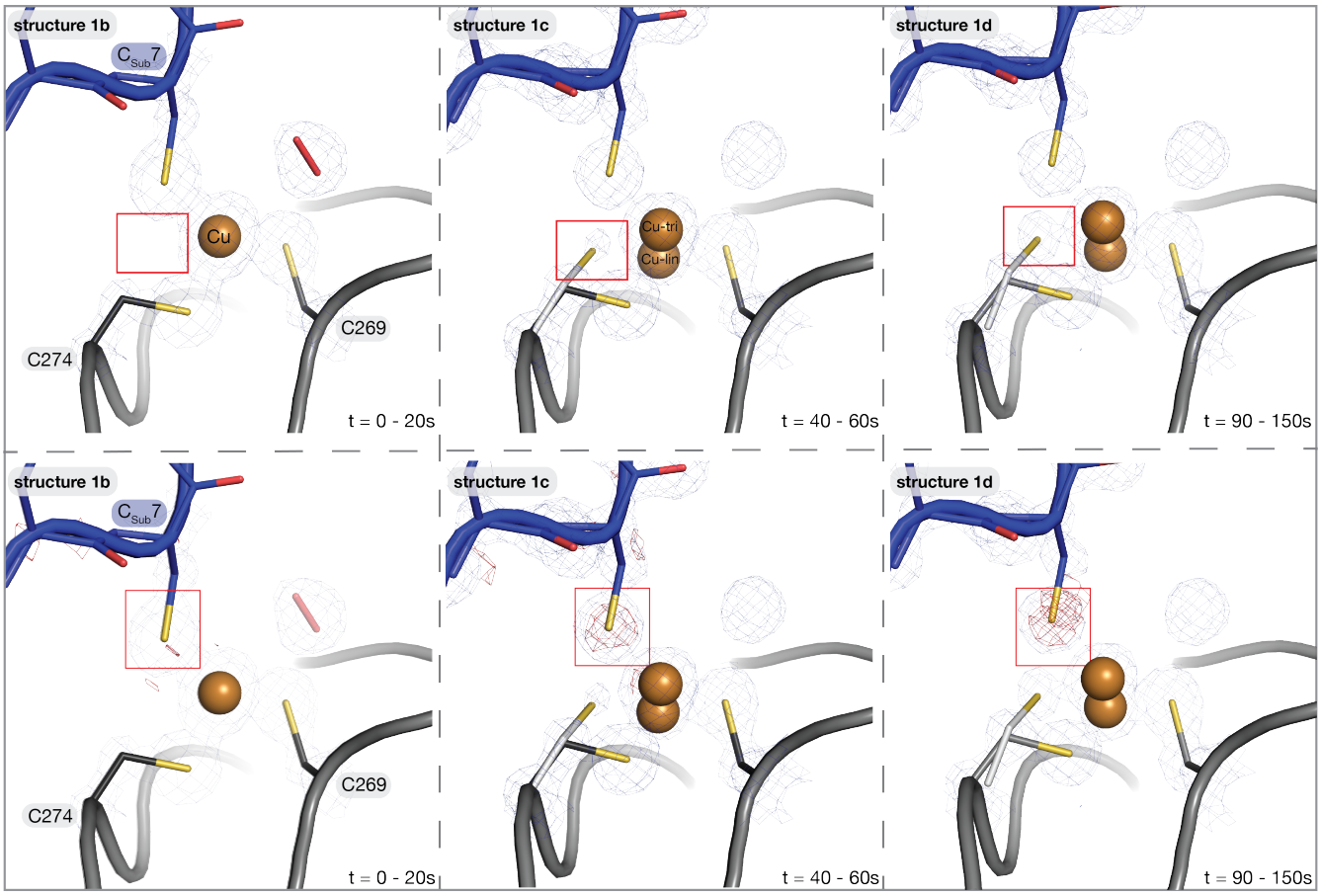


Figure 3. Panel 1: Electron densities of *tc*FGE:Cu:S crystals soaked with O_2-containing buffer for < 20s (**1b**), 40 – 60 s (**1c**) and 90 – 150s (**1d**). 2m|F_o|-D|F_c| maps are contoured at σ-level = 1.8. Panel 2: same view as in panel 1 highlighting the diminishing electron density at Cys7 in red. m|F_o|-D|F_c| maps are contoured at σ -level = 3.0). For a view on the entire peptide see Figure S10. The time-dependent changes of the ellipsoid electron density of the guest are visualized in Figure S12.

This cycle starts with binding of the substrate to FGE (**A→ B**, Figure 4). [11c, 12, 14] This step closes the active site and traps water 1 in an unstable position.[14] Release of this high energy water provides the driving force needed to attract O_2 into this hydrophilic pocket (**B → C**). According to the structural evidence presented here, binding of O_2 does not directly initiate redox chemistry. It is therefore not surprising that freeze-quenched samples of *tc*FGE in the presence of substrate and O_2 did not produce any EPR signatures.[11c] On the other hand, using stopped-flow UV-vis spectroscopy, Solomon, Bertozzi and coworkers observed a transient chromophore that may signal the presence of a copper-oxygen adduct.[12b] This preliminary observation together with structural evidence that *tc*FGE can accommodate a bivalent metal (Cd^{II}) in an tetrahedral coordination sphere,[12a] supports a mechanistic model that includes formation of a Cu^{II} in complex with a reduced oxygen species (**C → D**), either as the hydrogen atom abstracting reagent (**D → E**), or as an intermediate en route to an even more reactive radical.[11c, 12a] An interesting question that remains is as to what induces ligation of O_2 to the metal center? Since all atomic ingredients are present in the precatalytic complex, thermal activation is left as the only possible trigger, either by providing the required activation energy to generate a less stable intermediate, or by stabilizing an alternative enzyme conformation that allows a subsequent intermediate to accumulate.

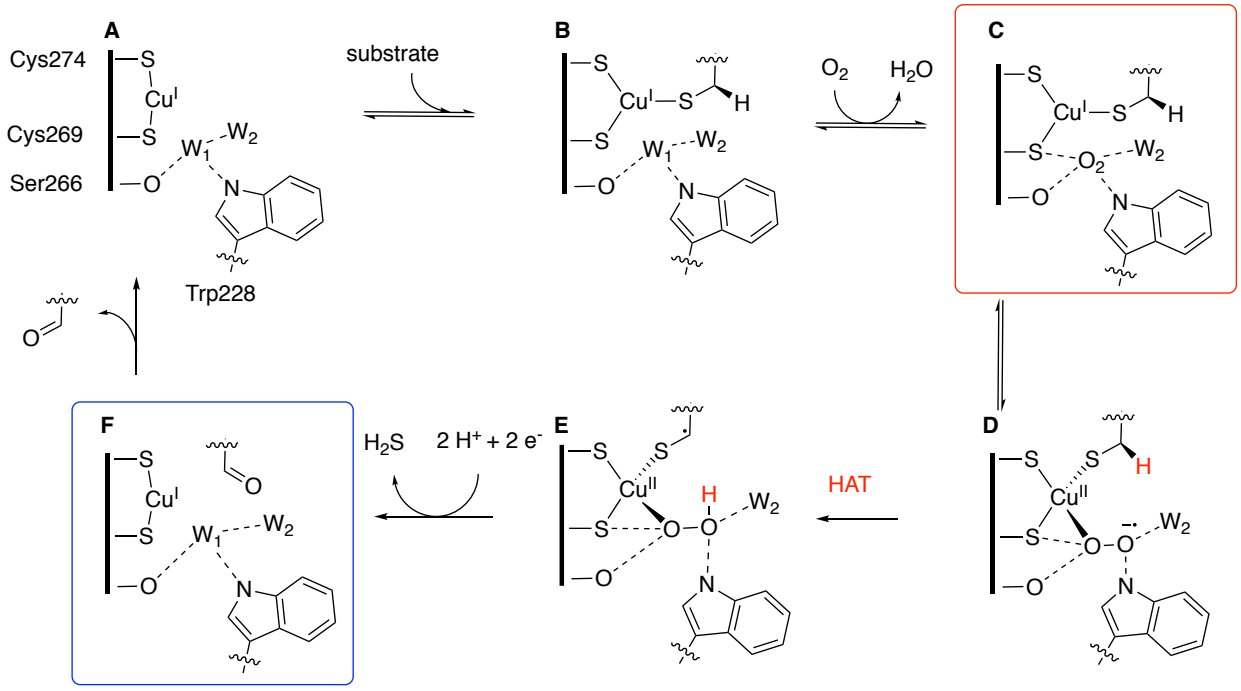


Figure 4. Current mechanistic model for FGE-catalyzed oxidative formation of peptidyl-fGly. Crystallographic water 1 and are denoted as W_1 and W_2. Binding of substrate and O_2 produces the precatalytic complex (framed red). Outer-sphere electron transfer from Cu^I to O_2, formation of a Cu^{II}-superoxide species, followed by abstraction of the the pro-(R)-β-hydrogen atom (red) are predicted steps that may lead to the observed product complex (blue frame). Dashed lines represent hydrogen-bond interactions with the shared proton not shown.

Conclusions

The atomic-resolution structures of *tc*FGE discussed in this report document the fundamental difference between the O_2-activation mechanism of this enzyme and that of other known copper-dependent oxidases. The stable resting states of LPMO and PMH contain a Cu^{II} bound to a nitrogen-dominated coordination sphere. One-electron reduction results in an unstable Cu^I complex that readily combines with O_2 to form a Cu^{II}-superoxide.[26] In contrast, FGE binds its metal with sulfur ligands that stabilize Cu^I. Substrate binding increases the electron density of the copper-sulfur complex and attracts O_2 to a pocket that is lined by hydrogen bond donors. The observations that complex **C** can be trapped by freezing, whereas at room-temperature the enzyme seems to produce a UV-active copper:oxygen species indicate that thermally activated motions may be essential for O_2 activation. Although protein motion is a rarely considered requirement for metal-dependent O_2 activation,[27] our study demonstrates that further examination of the FGE mechanism, by computational and experimental methods, will have to take the dynamic nature of this protein into account. Although FGE is a metal-dependent enzyme, its apparent strategy for O_2 activation is more reminiscent of flavin-dependent oxidases. Similar to FGE, these enzymes bind O_2 in polar pockets juxtaposed to their reduced cofactor flavin. Electron transfer from flavin to O_2 produces a flavin semiquinone and superoxide pair which recombines to form a flavin-oxygen adduct as a key intermediate in the catalytic cycle.[28] The blurring lines between catalysts with organic and inorganic cofactors may provide new inspiration for the design of novel synthetic catalysts or artificial enzymes.

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