New insight into cofactor-free oxygenation from combined experimental and computational approaches

Soi Bui and Roberto A Steiner

Molecular oxygen (O₂), in spite being a potentially strong oxidant, typically displays very poor reactivity with organic molecules. This is largely due to quantum chemical reasons as O₂ in its ground state is a diradical (³O₂) whilst common organic substrates are in a singlet state. For this reason catalysis involving O₂ as a reactant is typically mediated by enzymes containing redox metal and/or organic co-factors. Cofactor-independent oxygenases (and oxidases) are therefore intriguing enzymes from a fundamental viewpoint. This review looks at recent advances that have been made in understanding of this class of intriguing biocatalysts highlighting the power of an inter-disciplinary approach involving structural biology, spectroscopy and theoretical methods.

Address
Randall Division of Cell and Molecular Biophysics, King’s College London, New Hunt’s House, Guy’s Campus, London SE1 1UL, United Kingdom

Corresponding author: Steiner, Roberto A (roberto.steiner@kcl.ac.uk)

Introduction

The incorporation of one or both atoms of molecular oxygen (O₂) into organic substrates is catalyzed by monooxygenases and dioxygenases, respectively. These enzymes, collectively known as oxygenases [1], play a key role in the metabolism of aromatic amino acids [2], fatty acids [3,4], sugars [5], and vitamins [6], as well as in the biosynthesis of collagen [7]. Oxygenation reactions are also used for the degradation of various endogenous and exogenous organic compounds [8], whilst some bacteria employ oxygenases to facilitate the breakdown of molecules that are environmental pollutants [9]. Furthermore, oxygenases can catalyze enantiospecific reactions making them attractive for the production of chiral chemicals, although their industrial application is not without practical problems [10].

From a mechanistic perspective, the task of oxygenases is a difficult one because molecular oxygen in its normal ‘resting’ state (the form present in the air) is a diradical with a triplet ground-state electronic structure (³O₂) that is not reactive toward the vast majority of singlet-state organic substrates. To deal with this limitation of quantum chemical nature, oxygenases typically rely on transition metals or reduct oxygen cofactors for catalysis [11,12]. A remarkable group of oxygenases, however, can catalyze O₂-incorporation reactions in a cofactor-independent manner [14]. Cofactor-free oxygenases (and oxidases) are therefore intriguing from the viewpoint of fundamental enzymology. In this review we will briefly summarize recent advances in our mechanistic understanding of this fascinating group of enzymes.

Cofactor-free oxygenases

Structural information is available for various cofactor-independent oxygenases including bacterial carbon monoxide-forming 2,4-dioxygenases that degrade quinolones [15], the vancomycin biosynthetic DpgC dioxygenase [16,17], Streptomyces coelicolor ActVA-Orf6 monoxygenase involved in polyketide-tailoring [18], bioluminescent coelenterazine luciferases [19–21], and RhCC from Rhodococcus jostii RHA1 whose exact biological role is still unknown [22]. Their three-dimensional structures show that cofactor-independent oxygenation is enabled by a variety of folds and quaternary arrangements (Figure 1). Whilst bacterial carbon monoxide-forming 2,4-dioxygenases (Figure 1a) and Renilla coelenterazine luciferase (Figure 1j) are monomeric enzymes belonging to the α/β-hydrolase fold superfamily, DpgC, ActVA-Orf6 and RhCC are assembled into oligomers. Interestingly, Oplophorus luciferase (Figure 1k) that catalyzes the same reaction as Renilla monoxygenase, relies on a completely different architecture that is similar to those of fatty acid-binding proteins (FABs). The oligomeric ActVA-Orf6 (Figure 1g), that oxidizes 6-deoxydihydrokalafungin to...
Dihydrokalafungin in the biosynthesis of the polyketide antibiotic actinorhodin, is a homodimer in which each protomer displays a ferredoxin-like fold whilst DpgC is a hexamer (Figure 1b) formed by individual chains whose C-terminal region that contains the active site shows homology to the crotonase family of enoyl-CoA isomerase/dehydratases. DpgC is unique as there are no other examples of redox chemistry from this enzyme class. The recently identified RhCC monooxygenase (Figure 1n), is a trimer that belongs to the tautomerase superfamily characterized by a structural β-α-β fold. Like DpgC, RhCC is the first oxygenase identified within this superfamily.

| Figure 1 | CO-forming 2,4-dioxygenases | DpgC dioxygenase | ActVA-Orf6 monooxygenase | Luciferases monooxygenases | RhCC monooxygenase |
|----------|-----------------------------|------------------|--------------------------|---------------------------|------------------|
| (a)      | 2WJ3 / monomer              | (d)              | (g)                      | (j)                       | (n)              |
| (b)      | reaction catalysed           | (e)              | (h)                      | (i)                       | (o)              |
| (c)      | proposed peroxide intermediate| (f)              |                          |                           | (p)              |

Cofactor-independent oxygenases. Cartoon representations of cofactor-independent oxygenases (a, d, g, j, k, n) with schematics of the chemical reaction they catalyze (b, e, h, i, o), and proposed peroxide intermediates (c, f, i, m, p). Structural models are drawn to scale with their PDB code and oligomeric state indicated. Individual chains in the cartoon models are represented in shades of grey, except for (a) that is shown in green. The blue circles in the reaction schemes highlight the protons that are abstracted from the different substrates leading to their activation for reactivity with molecular oxygen (O2). Oxygen atoms derived from O2 are shown in red.
Carbon monoxide (CO)-forming cofactor-independent 2,4-dioxygenases. (a) Sequence alignment of various bacterial proteins exhibiting high similarity to *A. nitroguajacolicus* Ru61a 1-H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (HOD). Residues are highlighted in different shades of sequence conservation.
Bacterial cofactor-free dioxygenases involved in the breakdown of N-heteroaromatic quinoline derivatives

Our current mechanistic understanding of cofactor-independent oxygenation is largely based on studies performed on the bacterial carbon monoxide-forming 1-H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (HOD) from *Arthrobacter nitrogauciacolicus* Rü61a. HOD and its homologous 1-H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO) from *Pseudomonas putida* 33/1 were among the first cofactor-free dioxygenases reported in the literature [23]. They are approximately 31-kDa monomeric enzymes that catalyze the O2-dependent cleavage of the N-heteroaromatic ring of quinoline derivatives with concomitant formation of carbon monoxide (Figure 1b). This reaction is chemically identical to that catalyzed by the metal-dependent quercetin dioxygenase [24–26]. However, neither HOD nor QDO are related in sequence to the latter enzyme. HOD and QDO share ~37% sequence identity and constitute a separate family of cofactor-free dioxygenases of the α/β-hydrolase fold superfamily [27]. A BLAST search reveals a number of bacterial proteins with high sequence similarity to HOD/QDO that are likely to be involved in similar breakdown reactions (Figure 2a). Although most of these proteins have not been investigated functionally, a recent report indicates that Aqdc1 and Aqdc2 from *Rhodococcus erythropolis* BG43 are competent for the degradation of the *Pseudomonas aeruginosa* quorum sensing molecule 2-heptyl-3-hydroxy-4(1H)-quinoline (PQS, *Pseudomonas* quinoline signal) [28]. Purified recombinant Aqdc1 catalyzes the O2-dependent cleavage of PQS to form N-octanoylanthalenic acid and CO with an apparent *Km* and *kcat* values of 27 μM and 21 s⁻¹, respectively, thus supporting a 2,4-dioxynolytic cleavage like that catalyzed by HOD/QDO [28]. Overall, CO-forming bacterial dioxygenases appear to be able to operate on 3-hydroxy-4(1H)-quinolones bearing alkyl chains of different length at substrate position 2 (~R in Figure 1b) albeit with different effectiveness.

The structures of both HOD and QDO have been determined by X-ray crystallography [15**] (Figure 2b). As expected on the basis of comparative sequence analysis [29] these dioxygenases belong to the α/β-hydrolase fold superfamily. This is a group of enzymes that rely on a nucleophile-histidine-acidic residue triad to hydrolyze typically C–O, C–N, C–C bonds although a large array of reaction types can be catalyzed by members of this versatile family [30*]. Currently, CO-forming bacterial dioxygenases and the bioluminescent coelenterazine *Renilla* luciferase monooxygenase are the only oxygenases known to employ the αβ-hydrolase scaffold and its catalytic machinery for O2-dependent catalysis. HOD and QDO, like most enzymes of the αβ-hydrolase fold family, display a two-domain structure constituted by a 'core' domain and a 'cap' domain (Figure 2b). The core domain folds in the canonical αβ hydrolase architecture consisting of a mostly parallel, eight-stranded β sheet surrounded on both sides by α helices (only the second β strand is antiparallel) with the central β sheet featuring a left-handed superhelical 90° twist. The cap domain, formed by four α helices is positioned between β6 and αD. Both domains contribute to delineate the active site cavity. However, only the core domain hosts the nucleophile-histidine-acidic residue catalytic triad, which in the case of HOD is formed by Ser101-His251-Asp126 (Figure 2a,b). A Ser-His-Asp composition is the most common form of the triad [27]. Occasionally, the Ser nucleophile is replaced by an Asp or Cys residue, as in the case of *Renilla* luciferase where the triad is Asp-His-Asp [19].

**Substrate activation**

A common step in cofactor-independent oxygenation is the initial deprotonation of the bound organic substrate [14,31]. The H⁺ ion abstracted in various cofactor-independent-catalyzed reactions is highlighted in Figure 1 (blue circle). The structure of HOD in complex with its natural substrate QND (~R = –CH₃ in Figure 1b) obtained under anaerobic conditions reveals that the organic molecule binds in the active site between the core and the cap domains (Figure 2c) with the substrate’s O3 atom at H-bond distance to the Nε2 atom of the triad’s H251 (2.6 Å) [15**]. This interaction affords the deprotonation of the substrate’s hydroxyl group by the His-Asp subset of the triad, essential for substrate activation. Site-directed mutagenesis experiments have shown that replacement of H251 by an alanine (H251A) reduces *kcat* for QND dioxygenation by several orders of magnitude essentially abolishing catalytic activity [15**,32,33]. Also, replacement of the...
Cofactor-free oxygenation

Figure 3

(a) 1S catalytic dyad

ΔG (kcal/mol)

catalytic dyad

direct attack mechanism

charge transfer (CT) mechanism

(b)

Current Opinion in Structural Biology
acidic residue of the triad (D126A) yielded a strongly impaired variant. Consistent with this, density functional theory (DFT) calculations show that the 3OH proton is transferred to His251 in the wild-type system, but it remains on the substrate in the HODH251A and HODH126D systems [33]. The nucleophile S101 appears to be dispensable for catalysis as S101A replacement only impacted negatively on QND binding as judged by an increase in the $K_m$ constant. The idea of a nucleophile-independent mechanism is further supported by the lack of sequence conservation for this residue whilst the histidine-acidic dyad is strictly invarient (Figure 2a). Kinetic analysis under anoxic transient-state conditions indicates that substrate deprotonation is not rate-limiting as suggested by the 15-fold lower $k_{cat}$ (20 °C) compared with $k_H$ (5 °C) at low pH [33]. Overall, the His-Asp dyad is essential for substrate deprotonation and its consequent activation toward O2.

How does the activated substrate react with O2?

Mechanistic concepts borrowed from the field of flavin-dependent oxygenation [34,35] led to the plausible hypothesis that the activated (carbonyl)anion possesses a sufficiently low thermodynamic potential to allow the transfer of an electron to O2 to generate a substrate radical and superoxide anion [32]. This charge-transfer mechanism is shown for HOD catalysis on the left-hand side of Figure 3a. Following formation of the (S*-O•*) pair (1R_CT in Figure 3a) radical-radical recombination would lead to a C2-(hydro)peroxide (1I1). To address experimentally the nature of the compounds involved in the reaction with O2 a recent elegant study used the cyclic hydroxylamine spin probe 1-hydroxy-3-methoxy carbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) in the HOD-catalyzed reaction [36**]. Using a HOD variant in which the W160 residue that forms part of the active site cavity (Figure 2e) is substituted by an alanine (HODW160A) the authors were able to detect significant amounts of CM-nitroxide radical by electron paramagnetic resonance spectroscopy. This variant also released the proposed peroxide intermediate (Figure 1c), which was reduced to the corresponding alcohol and characterized by NMR spectroscopy. These data were interpreted as evidence for two key intermediates in the catalytic mechanism: a substrate radical and a substrate (hydro)peroxide. However, no significant amounts of CM-nitroxide radical were detected when wild-type HOD was used in the reaction. A possible reason for this is that the wild-type protein is able to efficiently shield its active site, whereas the mutant protein may have a more open or more flexible active-site pocket thus allowing access to the CMH probe [36**].

A more recent study put forward an alternative hypothesis for the generation of the peroxide intermediate. On the basis of a combined kinetic, spectroscopic and DFT computational work that takes advantage of available structural information Hernandez-Ortega et al. [37**] proposed a novel reaction mechanism that does not involve a formal single electronic transfer to dioxygen. The calculations indicate that the 1R_CT complex is unable to form spontaneously by electron transfer, and, consequently it is expected that any reactivity of O2 will come from dioxygen and not from the stabilization of a radical pair. This is proposed to occur with C2 atom of the activated substrate (1S•+) reacting directly with 3O2 to form a C=O=O bond as a triplet-state intermediate peroxide (1I1) (direct attack mechanism in Figure 3a). The latter compound then undergoes an inter-system crossing leading to (1I1). The reaction energy profile for the direct attack mechanism and subsequent catalytic steps is shown in Figure 3b. Following the formation of (1I1), attack of the peroxide on the carbonyl function affords the formation of the endoperoxide (1I2), which then decomposes with the formation of significantly stabilized carbon monoxide and anthranilate derivative products (1P). Overall, the formation of (1I1) encounters the highest barrier along the reaction trajectory (Δ$G^\ddagger$ = 17.4 kcal mol$^{-1}$ in a solvent model), and hence is proposed to be the rate limiting step for HOD catalysis. This in agreement with transient-state kinetic studies that show that oxygen-dependent steps are rate-limiting for overall catalysis. Additionally, kinetic and computational analyses show that the W160A substitution decreases the activity dramatically ($k_{cat}$/$K_m$ is 12,500 ± 600 s$^{-1}$ M$^{-1}$ and 2.4 ± 0.3 s$^{-1}$ M$^{-1}$ for wt HOD and HODW160A, respectively) with energy profiles for the corresponding DFT model showing increased barriers for $^3$TS1 and $^3$TS3 [37**]. Thus, the W160A replacement could therefore have an effect on the O2 activation mechanism.

Peroxide intermediate

The formation of peroxide intermediates is central to the mechanism proposed for essentially all cofactor-independent oxygenases (Figure 1). Yet, their structural elucidation has proven difficult. Urate oxidase (UOX) is a tetrameric cofactor-independent oxidase [14,38] that catalyzes the breakdown of urate to 5-hydroxisourate (5-HIU) with the latter compound further degraded.
Figure 4

Visualization of the peroxide intermediate formed in the oxygenation step catalyzed by the cofactor-independent oxidase UOX. (a) Reaction catalyzed by UOX. The peroxide intermediate has been trapped using the natural substrate UA and also using its 9-methyl derivative (MUA). (b) Anaerobic UOX:MUA complex. MUA (like UA) binds at the interface between two subunits (shown in yellow and pink) of the UOX tetramer. Red spheres represent water molecules. H-bonds are shown as broken yellow lines. (c) Upon exposure to O2, the UOX–MUA complex reacts to generate a C5-(hydro)peroxide (5-PMUA). (d) The peroxide is specifically radiolyzed at low X-ray dose under cryoconditions generating O2. (e) In-crystallo Raman spectroscopy supported by QM/MM calculations show that the band at 605 cm⁻¹ is a 5-PMUA ‘signature’. (f) Online in-crystallo Raman spectroscopy reveals the specific dose-dependent decrease of the 605 cm⁻¹ 5-PMUA ‘signature’ band.
to allantoin (Figure 4a). On the basis of UV–visible spectroscopic evidence, early mechanistic studies suggested that a peroxide adduct (5-PIU) is generated initially by the dioxygenation of the urate dianion (UD) \[39\]. In subsequent steps H\(_2\)O\(_2\) release and H\(_2\)O attack at position 5 would form 5-HIU. How UOX exactly contributes to the generation of UD is currently not clear. A mechanism of general base catalysis enabled by a Thr-Lys dyad has been proposed \[40\] and a neutron diffraction study on the aerobic chloride-inhibited UOX–substrate complex suggested that UD might be generated from the deprotonation of the iminol tautomer of UA \[41\].

Recently, X-ray crystallography combined with online in-crystallo Raman spectroscopy has provided direct evidence for a C5(S)(hydro)peroxide in the initial cofactor-independent dioxygenation stage of the UOX-catalyzed reaction \[42**\]. Using 9-methyl uric acid (MUA) as substrate, electron density maps at (near)-atomic resolution unambiguously reveal that exposure of the anaerobic UOX–MUA complex (Figure 4b) to O\(_2\) results in MUA conversion into its C5(S)-peroxo derivative (5-PMUa) with clear pyramidalization at C5 (Figure 4c). Non-resonant Raman spectra recorded from crystals of anaerobic UOX–MUA and UOX–PMUa complexes show that the spectral region centered at 600 cm\(^{-1}\) reports on changes resulting from the oxygenuation reaction (Figure 4c). Upon MUA peroxidation a distinct band develops at 605 cm\(^{-1}\) (blue trace) whilst the shoulder at 597 cm\(^{-1}\) in the UOX-MUA complex (green) disappears. Quantum mechanics/molecular mechanics (QM/MM) calculations predict a band at 600 cm\(^{-1}\) (experimental 605 cm\(^{-1}\)) for the 5PMUA (hydro)peroxide resulting form a set of modes involving C5–Op1 bond stretching and C5–Op1–Op2 bending coupled to ring distortions.

Remarkably, the C5–Op1 bond is susceptible to selective radiolysis at very low X-ray doses (Figure 4d,f). Rupture of the C5–Op1 bond is accompanied by the loss of pyramidalization at C5 leading to a planar organic structure while a diatomic molecule 1.2 Å long, consistent with O\(_2\), is liberated and trapped above it (Figure 4d). Dioxygen produced in situ adopts a well-defined position with its molecular axis 3.15 Å above the flat organic molecule and both O1 and O2 atoms interacting with N24(N82) at distances of 3.19 Å and 3.09 Å, respectively, whilst the T37(Oy1) atom is closest to O2 at 2.65 Å. This is consistent with results obtained using room-temperature O\(_2\) pressurization (40 bars) in the presence of the AZA inhibitor \[43\]. Online Raman analysis shows that peroxide rupture causes the 605 cm\(^{-1}\) ’signature’ band to selectively decrease in a dose-dependent manner (Figure 4f) consistent with QM/MM calculations that assign this band to the stretching of the C5–Op1 bond.

Conclusions

Interdisciplinary approaches are central to modern enzymology. Structural biology techniques in combination with steady-state and transient-state kinetics, theoretical quantum chemistry calculations, and advanced solution and in-crystallo spectroscopic measurements have provided important insight into how cofactor-independent oxygenases work. Although not discussed here, the use of molecular dynamics simulations is also uncovering specific pathways and access points for O\(_2\) in this class of oxygenases that are common to different types of proteins \[44**\] and often dynamic in nature.

The extensive work carried out on the bacterial cofactor-free 2,4-dioxygenases HOD and studies on various other cofactor-independent oxygenases convincingly suggest that the generation of a substrate (carbanion) is a prerequisite for O\(_2\) reactivity. Thus, these enzymes apparently utilize the intrinsic reactivity of carbanions toward electrophiles as a general catalytic concept \[14\]. Although the exact mechanism with which O\(_2\) reacts with the activated substrate requires further study, the work of Hernandez-Ortega et al. \[37**\] provides a novel perspective on this reaction by proposing a direct attack to generate a triplet-state peroxide that immediately collapses into a triplet-state. It will be interesting in the future to see whether this novel concept can be generalized to other similar catalytic systems. Finally, using a combination of (online and offline) Raman-assisted X-ray crystallography and theoretical calculations Bui et al. \[42**\] provided unambiguous evidence for a C5-peroxide intermediate in the initial dioxygenation step in UOX cofactor-independent catalysis. Additionally, selective radiolysis afforded exquisite insight into the elusive dioxygen position in the ternary E–S–O\(_2\) complex. This experimental information will guide further calculations aimed at addressing O\(_2\) reactivity in this system. The recent advances on cofactor-independent oxygenases discussed here well exemplify the necessity of synergizing experimental and computational approaches in mechanistic enzymology.

Conflict of interest statement

Nothing declared.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Yamamoto S: The 50th anniversary of the discovery of oxygenases. IUBMB Life 2006, 58:248-250.
2. Fitzpatrick PF: Mechanism of aromatic amino acid hydroxylation. Biochemistry 2003, 42:14083–14091.
3. Hamberg M, Ponce de Leon I, Rodriguez MJ, Castresana C: Alpha-dioxygenases. Biochem Biophys Res Commun 2005, 338:169-174.
4. Haeggstrom JZ, Funk CD: Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem Rev* 2011, 111:5866-5898.

5. Frandsen KE, Simmons TJ, Dupree P, Poulsen JC, Hemsworth CJ, Ciano L, Johnston EM, Tovborg M, Johansen KS, von Freiesleben P et al.: The molecular basis of polysaccharide cleavage by lytic polysaccharide monoxygenases. *Nat Chem Biol* 2016, 12:298-303.

6. Wyss A: Carotene oxygenases: a new family of double bond cleavage enzymes. *J Nutr* 2004, 134:2465-2505.

7. Milyarjhu J: Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol* 2003, 22:15-24.

8. Meunier B, de Visser SP, Shaik S: Mechanism of oxidation reactions catalyzed by cytochrome p450 enzymes. *Chem Rev* 2004, 104:3947-3980.

9. Furukawa K: Oxygenases and dehalogenases: molecular approaches to efficient degradation of chlorinated environmental pollutants. *Biosci Biotechnol Biochem* 2006, 70:2335-2348.

10. van Beilen JB, Duets WA, Schmid A, Witholt B: Practical issues in the application of oxygenases. *Trends Biotechnol* 2007, 25:170-177.

11. Pau MY, Lipscomb JD, Solomon EI: Substrate activation for O2 reactions by oxidized metal centers in biology. *Proc Natl Acad Sci U S A* 2007, 104:18355-18362.

12. Kovaleva EG, Lipscomb JD: Crystal structures of Fe2+ dioxygenase superoxy, alkylperoxy, and bound product intermediates. *Science* 2007, 316:453-457.

A remarkable example of in-crystallo trapping of reaction intermediates in the non-heme Fe2+-dependent homoprotocatechuate 2,3-dioxygenase. Three different intermediates in the O2 activation and insertion reactions of this extradiol-ring-cleaving dioxygenase were visualized including the predicted key substrate-alkylperoxy-Fe2+ intermediate.

13. Bugg TDH: Dioxygenase enzymes: catalytic mechanism and chemical models. *Tetrahedron* 2003, 59:7075-7101.

14. Fetzner S, Steiner RA: Cofactor-independent oxidases and oxygenases. *Appl Microbiol Biotechnol* 2010, 86:791-804.

15. Steiner RA, Janssen HJ, Roversi P, Oakley AJ, Fetzner S: Structural basis for cofactor-independent dioxygenation of N-heteroaromatic compounds at the alpha/beta-hydrolase fold. *Proc Natl Acad Sci U S A* 2010, 107:657-662.

This article describes the crystal structures of alpha/beta-hydrolase fold cofactor-independent dioxygenases (HOD and QDO) in their native state as well as the structure of HOD in complex with its natural 1-H-3-hydroxy-4-oxoquinoline substrate, its N-acetylglutaminyl reaction product, and chloride as dioxygen mimic.

16. Widboom PF, Fielding EN, Liu Y, Bruner SD: Structural basis for cofactor-independent dioxygenation in vancomycin biosynthesis. *Nature* 2007, 447:342-345.

17. Fielding EN, Widboom PF, Bruner SD: Substrate recognition and catalysis by the cofactor-independent dioxygenase DpgC. *Biochemistry* 2007, 46:13994-14000.

18. Sciara G, Kendrew SG, Miele AE, Marsh NG, Federici L, Malatesta F, Schimppera G, Savino C, Vallone B: The structure of ActIVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. *EMBO J* 2003, 22:205-215.

19. Loening AM, Fenn TD, Gambhir SS: Crystal structures of the luciferase and green fluorescent protein from *Renilla reniformis*. *J Mol Biol* 2007, 374:1017-1028.

20. Schultz LW, Liu L, Cegielski M, Hastings JW: Crystal structure of a pH-regulated luciferase catalyzing the bioluminescent oxidation of an open tetrapyrrole. *Proc Natl Acad Sci U S A* 2005, 102:1378-1383.

21. Tomabechi Y, Hosoya T, Ehara H, Sekine S, Shirouzu M, Inouye S: Crystal structure of nanoKAZ: the mutated 19 kDa component of *Oplophorus* luciferase catalyzing the bioluminescent reaction with coelenterazine. *Biochem Biophys Res Commun* 2016, 470:88-93.

22. Baas BJ, Poddar H, Geersemans EM, Rozeboom HJ, de Vries MP, Permentier HP, Thunnissen AM, Poelarends GJ: Functional and structural characterization of an unusual cofactor-independent oxygenase. *Biochemistry* 2015, 54:1219-1232.

23. Bauer I, Max N, Fetzner S, Lingers F: 2,4-Dioxygenases catalyzing N-heterocyclic-ring cleavage and formation of carbon monoxide. Purification and some properties of 1H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase from *Arthrobacter* sp. Ru61a and comparison with 1H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase from *Pseudomonas putida* 33/1. *Eur J Biochem* 1996, 240:576-583.

24. Jeoung JH, Nianios D, Fetzner S, Dobbek H: Quercetin 2,4-dioxygenase activates dioxygen in a side-on O2–Ni complex. *Angew Chem Int Ed Engl* 2016, 55:3281-3284.

25. Gopal B, Madan LB, Betz SF, Kossiakoff AA: The crystal structure of a quercetin 2,3-dioxygenase from *Bacillus subtilis* suggests modulation of enzyme activity by a change in the metal ion at the active site). *Biochemistry* 2005, 44:193-201.

26. Steiner RA, Kalk KH, Dijkstra BW: Crystal structures of anaerobic enzyme substrate complexes provide insight into the reaction mechanism of the copper-dependent quercetin 2,3-dioxygenase. *Proc Natl Acad Sci U S A* 2002, 99:16625-16630.

27. Nardini M, Dijkstra BW: Alpha/beta-hydrolase fold enzymes: the family keeps growing. *Curr Opin Struct Biol* 1999, 9:732-737.

28. Muller C, Birmes FS, Ruckert C, Kalinowski J, Fetzner S: *Rhodococcus erythropolis* B43 genes mediating *Pseudomonas aeruginosa* quinolone signal degradation and virulence factor attenuation. *Appl Environ Microbiol* 2015, 81:7720-7729.

29. Fischer F, Künne S, Fetzner S: Bacterial 2,4-dioxygenases: new members of the alpha/beta-hydrolase-fold superfamily of enzymes functionally related to serine hydrolases. *J Bacteriol* 1999, 181:5725-5733.

30. Rauverdink A, Kazlauskas RJ: How the same core catalytic machinery catalyzes 17 different reactions: the serine–histidine–aspartate catalytic triad of α/β-hydrolase fold enzymes. *ACS Catal* 2015, 5:6153-6176.

31. Up-to-date review highlighting the versatility of the α/β-hydrolase fold equipped with the Ser–His–Asp catalytic triad.

32. Fetzner S: Oxygenases without requirement for cofactors or metal ions. *Appl Microbiol Biotechnol* 2002, 60:243-257.

33. Freirichs-Deeken U, Rangelova K, Kappl R, Huttermann J, Fetzner S: DIOxygenases without requirement for cofactors and their chemical model reaction: compulsory order temary complex mechanism of 1H-3-hydroxy-4-oxoquinidine 2,4-dioxygenase involving general base catalysis by histidine 251 and single-electron oxidation of the substrate diamin. *Biochemistry* 2004, 43:14485-14499.

34. Hernandez-Ortega A, Quesne MG, Bui S, Heuts DP, Steiner RA, Heyes DJ, de Visser SP, Scrutton NS: Origin of the proton-transfer step in the cofactor-free (1H)-3-hydroxy-4-oxoquinoline 2,4-dioxygenase: effect of the basicity of an active site His residue. *J Biol Chem* 2014, 289:8620-8632.

35. Massey V: Activation of molecular oxygen by flavins and flavoproteins. *J Biol Chem* 1984, 259:22459-22462.

36. Mattvei A: To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends Biochem Sci* 2006, 31:276-283.

37. Thierbach S, Bui N, Zapp J, Chhabra SR, Kappl R, Fetzner S: Substrate–assisted O2 activation in a cofactor-independent dioxygenase. *Chem Biol* 2014, 21:217-225.

Elegant work that uses a multi-disciplinary approach supporting a mechanism of charge transfer for the reaction of O2 with the activated substrate in cofactor-free 2,4-dioxygenase. This study also presents evidence for a peroxide intermediate during catalysis.

38. Hernandez-Ortega A, Quesne MG, Bui S, Heyes DJ, Steiner RA, Scrutton NS, de Visser SP: Catalytic mechanism of cofactor-free dioxygenases and how they circumvent spin-forbidden oxygenation of their substrates. *J Am Chem Soc* 2015, 137:7474-7487.
This study combines kinetics, spectroscopic and computational approaches. It proposes that in cofactor-free 2,4-dioxygenases the reaction is initiated by triplet dioxygen and its binding to deprotonated substrate and only thereafter a spin state crossing to the singlet spin state occurs.

38. Colloc’h N, el Hajji M, Bachet B, L’Hermite G, Schiltz M, Prange T, Castro B, Mormon JP: Crystal structure of the protein drug urate oxidase-inhibitor complex at 2.05 Å resolution. Nat Struct Biol 1997, 4:947-952.

39. Kahn K, Tipton PA: Spectroscopic characterization of intermediates in the urate oxidase reaction. Biochemistry 1998, 37:11651-11659.

40. Imhoff RD, Power NP, Borrok MJ, Tipton PA: General base catalysis in the urate oxidase reaction: evidence for a novel Thr-Lys catalytic diad. Biochemistry 2003, 42:4094-4100.

41. Oksanen E, Blakeley MP, El-Hajji M, Ryde U, Budayova-Spano M: The neutron structure of urate oxidase resolves a long-standing mechanistic conundrum and reveals unexpected changes in protonation. PLOS ONE 2014, 9:e86651.

42. Bui S, von Stetten D, Jambrina PG, Prange T, Colloc’h N, de Sanctis D, Royant A, Rosta E, Steiner RA: Direct evidence for a peroxide intermediate and a reactive enzyme-substrate–dioxygen configuration in a cofactor-free oxidase. Angew Chem Int Ed Engl 2014, 53:13710-13714.

In this work near-atomic resolution crystallography supported by in-crystallo Raman spectroscopy and QM/MM calculations show unambiguously that the archetypical cofactor-free uricase catalyzes the first oxygenation step in the degradation of urate via a C5(S)(S)-(hydro)peroxide intermediate. Additionally, low X-ray doses break specifically the intermediate C5–OO(H) bond at 100 K, thus releasing O2 in situ, which is trapped above the substrate radical providing a snapshot of the E–S–O2 complex.

43. Colloc’h N, Prange T: Functional relevance of the internal hydrophobic cavity of urate oxidase. FEBS Lett 2014, 588:1715-1719.

44. Di Russo NV, Condurso HL, Li K, Bruner SD, Roitberg AE: Oxygen diffusion pathways in a cofactor-independent dioxygenase. Chem Sci 2015, 6:6341-6348.

This study uses a combined molecular dynamics and experimental approach to look at O2 access pathways in the cofactor-independent dioxygenase DpgC. Multiple access channels are available, and the architecture of the pathway network can provide regioselectivity and stereoselectivity. Overall, it appears that there are common themes in O2 access that are conserved among very different types of proteins.