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Probing enzyme-mediated oxidation reactions in crystallo*

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Abstract: Reaction intermediates define the chemical steps of transformation from substrates to products, and capture of intermediates along catalytic pathways remains one focal point in the studies of enzymatic mechanisms. Performing enzymatic reactions in protein single crystals has been shown to be very effective in trapping and identifying unstable intermediates. In this critical review, we provide several examples of in crystallo approaches to trap reactive oxidation intermediates, thereby allowing the studies of enzymatic oxidation mechanisms.

Keywords: AlkB family DNA/RNA repair enzymes; oxidation in crystallo; oxygenase; reaction mechanisms.

INTRODUCTION

Enzymes catalyze transformations of diverse chemical structures with specificity and selectivity. A central task in elucidating enzymatic mechanism is to structurally characterize the intermediates along the reaction coordinate. Yet the reactive and short-lived nature of intermediates requires either ultra-fast technique or methods that accumulate stabilized intermediates for characterization purposes. An ultimate goal is to "visualize" intermediates while introducing minimal perturbations.

X-ray crystallography is one solution to visualize such actual intermediates [1]. When enzymatic reactions are performed within crystalline enzymes, the well-defined active site environment and confined enzymatic dynamics may help to preserve and trap otherwise fleeting intermediates. For example, the ultra-high-resolution structures (~1.1 Å) of wild-type and mutant D-2-deoxyribose-5-phosphate (DRP) aldolase complexes with DRP identify the postulated covalent carbinolamine and Schiff base intermediates in the aldolase mechanism [2]; and multiple structures of bacteriorhodopsin—a small protein from the cell membrane of a salt-loving microorganism that pumps protons out of cells—solved at different intermediate states even provide the complete motion picture of the proton within bacteriorhodopsin [3].

In fact, this approach is no different than the synthesis and characterization of labile species in the cavities of the self-assembled cages and capsules [4–6]. It has been shown that self-assembled tetrahedral container molecules, which form in water from simple organic subcomponents and iron(II) ions, are able to render normally air-sensitive white phosphorus not only air-stable but also water-soluble [5]. In this particular case, such stabilization is achieved through constriction of individual P_4 molecules within the hydrophobic hollows of the container molecules; addition of oxygen atoms to P_4 molecules would result in molecules too big for the containers.

On the other hand, before embarking on an experiment that aims to characterize enzymatic intermediates in crystallo, one must test to make sure that the chemical transformations performed by the

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enzyme in solution take place in a crystal as well. Also remember that the same reaction may have a very different kinetic profile now. Once the reaction has been confirmed, one then needs to carefully design a proper triggering strategy to initiate the reaction in the crystalline condition. Three main triggering strategies have been used: diffusion of small molecules, UV–vis light illumination, and X-ray irradiation [7]. Combined with the triggering strategies, data collection methods can also be classified into three main categories: real-time crystallography (often Laue diffraction) that aims to catch intermediates while they appear and disappear (in real time), monochromatic radiation with stabilized intermediates, and steady-state strategies that are based on kinetic equilibrium. While real-time crystallography seems most elegant, it is often technically very challenging [8,9]. On the other hand, substantial efforts have been made to the latter two strategies. For example, to alter the reaction coordinates in order to stabilize and accumulate (or to elongate the lifetime of) intermediates, both physical methods (often by changing temperature) and chemical methods (by changing pH, introducing modification to the enzyme, substrate, or cofactor, etc.) have been widely explored. In addition, experiments (e.g., spectroscopic studies) can often be designed to optimize the condition to allow accumulation of intermediates of interest in the crystalline conditions. Overall, regardless of the data collection methods selected, the aim is to collect a full data set before a particular intermediate disappears.

DISCUSSION

Oxygenase-mediated chemical transformations: Significance

In chemistry, oxidation is the loss of electrons or an increase in oxidation state. It is also without doubt an essential process of life; for example, electron transfers occur in numerous metabolic processes, including respiration, glycolysis, photosynthesis, etc. To efficiently carry out oxidation reactions, nature has evolved distinct classes of enzymes which are termed "oxidoreductases", i.e., enzymes catalyzing the transfer of electrons from one molecule to another. Well-known examples of oxidoreductases—just to name a few—include "chloroplast ferredoxins" (involved in the photophosphorylation reactions of photosynthesis), cytochromes (generally referred to as membrane-bound hemeproteins), and catalase (which is found in nearly all organisms that are exposed to oxygen and catalyzes the decomposition of harmful hydrogen peroxide to water and dioxygen).

A large subclass of oxidoreductase—termed "oxygenase"—performs oxidation of a substrate by transferring an oxygen atom from the dioxygen molecule. Oxygenases also play critical roles in a cell: among the most important oxygenases are the cytochrome P450 oxidases, histone and nucleic acid demethylases, protein hydroxylases, etc. Two types of oxygenases exist: monooxygenase and dioxygenase, depending on the number of oxygen atom(s) enzymatically transferred to the substrate. Oxygenase is not to be confused with oxidase, which is used to describe any enzyme that uses dioxygen molecule as electron acceptor and reduces dioxygen to water or hydrogen peroxide.

Oxygenase-mediated reactions are diverse

Nature has evolved distinct chemistry designs for different oxygenases to fulfil their biological oxidation tasks. Metal ions are often present in the active site of an oxygenase, with copper and iron being the most commonly found. The metal ions can be assembled in either mononuclear fashion or metal-cluster fashion; several representative metallo-oxygenases are shown in Fig. 1. Owing to the diversities of the active sites, the chemistry catalyzed by these enzymes is consequently complex and diversified; so are the intermediates involved in the corresponding oxidation reactions. Thus, while oxygenases within a certain subclass could share key reactive intermediates, a unified theme of dioxygen activation is not likely and, hence, oxidative reaction mechanisms will need to be studied in a case-by-case manner.

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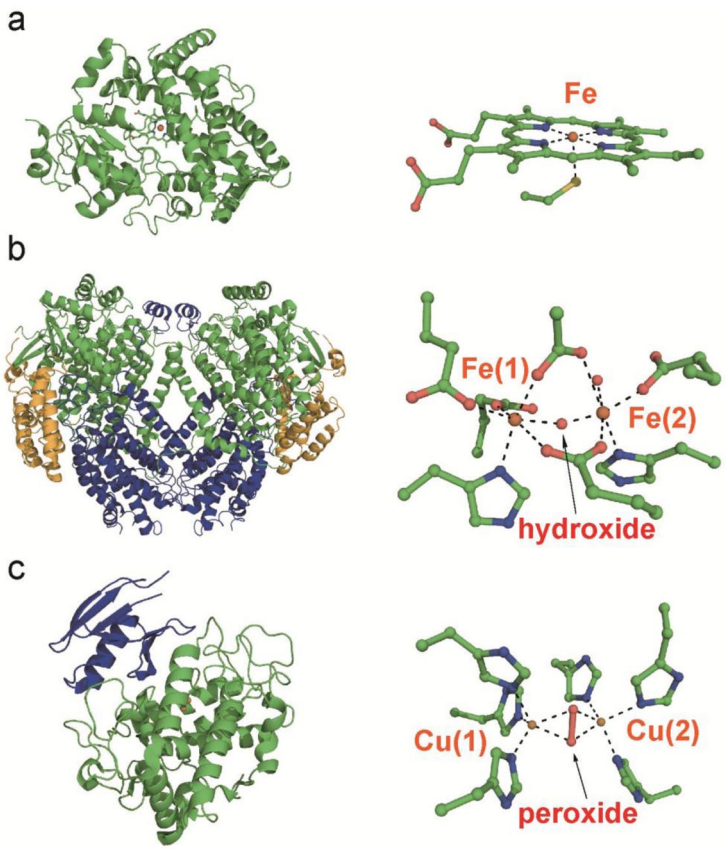

Fig. 1 Oxygenases use different active-site metal ions to perform various oxidation reactions. (a) The overall structure of human cytochrome P450 3A4 (PDB code: 1W0E). A heme is bound by the protein in order to carry out the oxidation of its various substrates. (b) A bacterial methane monooxygenase (1MMO) utilizes a di-iron center to hydroxylate methane. The three subunits (α, β, and γ) are colored in green, blue, and orange, respectively. (c) The complex structure (1WX4) of a tyrosinase and cappie protein ORF378 (blue); a di-copper center is used.

The subject of this critical review concerns oxygenases and the oxidative transformations catalyzed correspondingly (or oxygenase-mediated chemical reactions), rather than summarizes the very rich and diverse knowledge of oxidoreductases. As mentioned above, owing to the fact that reaction mechanisms of oxygenases differ substantially from one class to another, several representative examples of successful intermediate-entrapment for several types of oxygenases will be highlighted and discussed. These oxygenases all play critical roles in various important biological processes; therefore, elucidation of their mechanism will contribute to our understanding of the fundamentals of life.

Catalytic cycle of P450cam

The cytochrome P450 superfamily is a large and diverse group of heme-binding monooxygenases. They catalyze the oxidation of various organic compounds, including metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotic substances such as drugs and other toxic chemicals. While activation of inert C–H bonds has attracted enormous attention in the organic chemistry community very recently, nature has already designed the P450 enzymes to catalyze the stereospecific

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hydroxylation of nonactivated hydrocarbons at physiological temperature. The detailed mechanism used by these enzymes to activate C–H bonds remained elusive.

Schlichting and co-workers chose P450cam, which catalyzes the regio- and stereospecific hydroxylation of camphor to 5-*exo*-hydroxycamphor, as their protein target since P450cam is one of the best characterized P450 proteins [10]. Three intermediates in the catalytic pathway of P450cam were captured with their trapping techniques and cryo-crystallography: a five-coordinate iron(II) camphor complex **3**, a six-coordinate iron(II)-O_2 complex **4**, and an activated oxygen complex **5** (Fig. 2). Careful

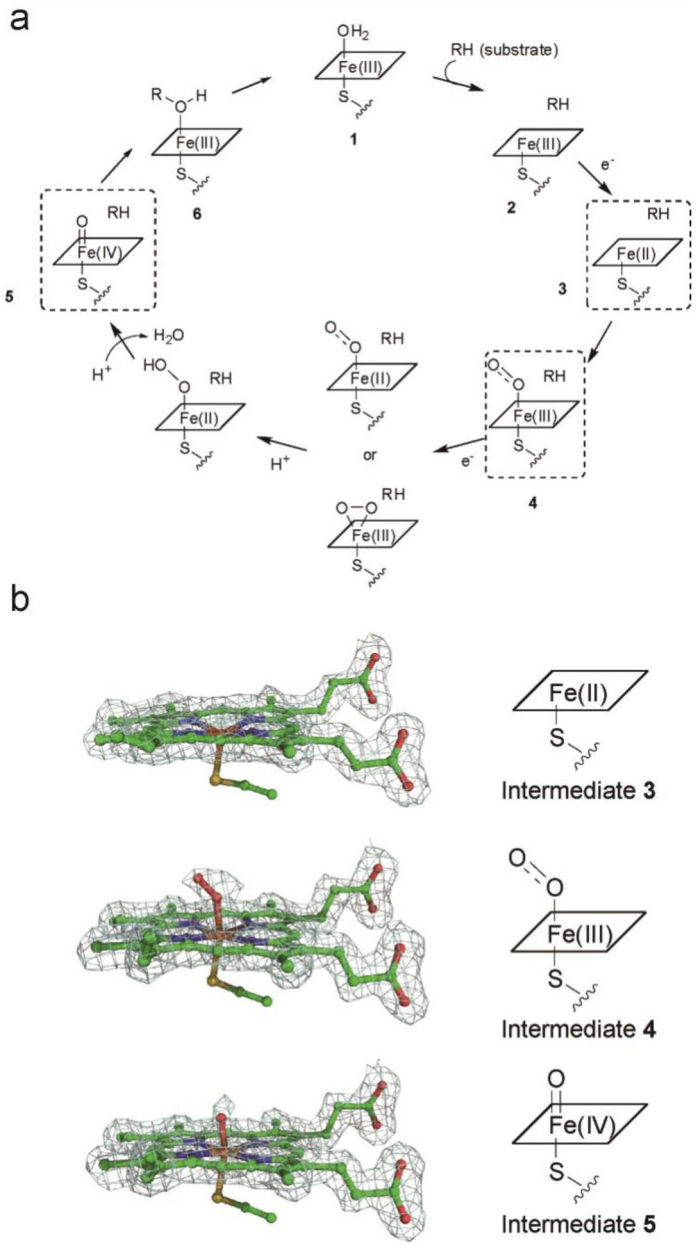

Fig. 2 Mechanistic study of P450cam by Schlichting et al. (a) Catalytic cycle of P450cam, with the intermediates observed in a box. (b) Electron density maps of intermediates **3**, **4**, and **5**.

experimental strategies were designed to allow the accumulation and capture of these previously uncharacterized unstable intermediates: (1) dithionite was first diffused to a previously determined iron(III) camphor complex **2** in order to provide the electron needed to reduce **2** to the ferrous complex **3**; (2) complex **3** was then converted to **4** by exposure to high partial pressure of O_2; and (3) addition of the second electron was added through X-ray radiolysis of water to produce the reactive intermediate **5**, and by warming up these radiolytically treated crystals, a product complex, which matches up with results from earlier studies, was also obtained. It is such a clever (yet technically challenging) stepwise "entrapment" strategy that eventually allows the resolution of the three intermediates.

Side-on binding of dioxygen to iron by naphthalene dioxygenase

While heme-iron proteins represent a large number of oxygenases, non-heme iron enzymes also catalyze a wide range of important oxidation reactions [11], paralleling those of the heme systems. In fact, the active sites of these non-heme enzymes are often more difficult to study partly because that they do not exhibit the spectral features of the porphyrin ring. Studies have established a highly conserved 2-His-1-carboxylate (Asp or Glu) facial triad motif to be the ligands that coordinate the catalytically essential metal ion [12,13].

Bacterial Rieske non-heme iron dioxygenases catalyze the stereospecific addition of dioxygen molecules to aromatic hydrocarbons. One member of the family is the naphthalene dioxygenase, which converts naphthalene to *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. Ramaswamy and co-workers used indole, which is also a substrate of naphthalene dioxygenase, to probe the mechanism of oxygen activation and catalysis of this non-heme iron enzyme [14]. They successfully trapped crystal structures of naphthalene dioxygenase where a dioxygen molecule is bound to the metal iron. What is more surprising is that instead of in an end-on binding mode to the iron, the authors observed side-on binding of the dioxygen (Fig. 3). In a complex with naphthalene dioxygenase and substrate indole, the dioxygen molecule is lined up for an attack on the double bond of the aromatic indole. While chiral arene *cis*-dihydrodiols are interesting targets of enantioselective chemical synthesis, the mechanism used by the naphthalene dioxygenase certainly offers one solution to this problem.

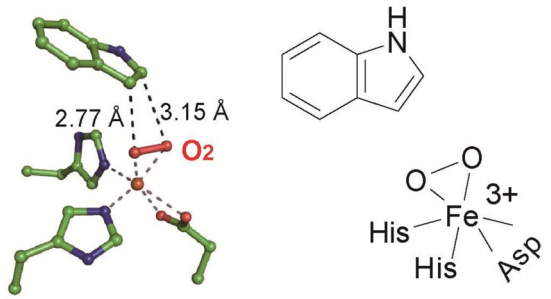

Fig. 3 Binding of naphthalene at the active site of naphthalene dioxygenase. The 2-His-1-carboxylate (Asp or Glu) facial triad motif is shown. Dioxygen molecule, which is binding to the metal iron in a side-on fashion, is colored in red.

Superoxo and alkylperoxo intermediates from an oxidation process mediated by an extradiol ring-cleaving dioxygenase

The homoprotocatechuate 2,3-dioxygenase is also a mononuclear iron enzyme that catalyzes the proximal extradiol ring cleavage of 3,4-dihydroxyphenylacetate to form ring-opened 5-carboxymethyl-2-hydroxymuconic semialdehyde. 4-Nitrocatechol, which is an alternative substrate with slower oxygen activation and insertion steps, was used by Lipscomb and colleagues to study the mechanism of the

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homoprotocatechuate 2,3-dioxygenase [15]. In their experiment, a crystal of the iron(II)-containing homoprotocatechuate 2,3-dioxygenase was soaked with 4-nitrocatechol in a low O_2 environment. Subsequently, a superoxo, alkylperoxo, and bound product intermediates were observed in their crystal (Fig. 4), which consists of four subunits. Interestingly, two of these subunits harbor the alkylperoxo intermediate and the other two subunits have the superoxo and product intermediates, respectively. It was proposed by the authors that the simultaneous presence of three different reaction intermediates in a single-crystalline enzyme with independent active sites can be attributed to the specific packing interactions between different subunits. In these different subunits, subtle conformational differences of the active-site residues contribute to the stabilization of different intermediates.

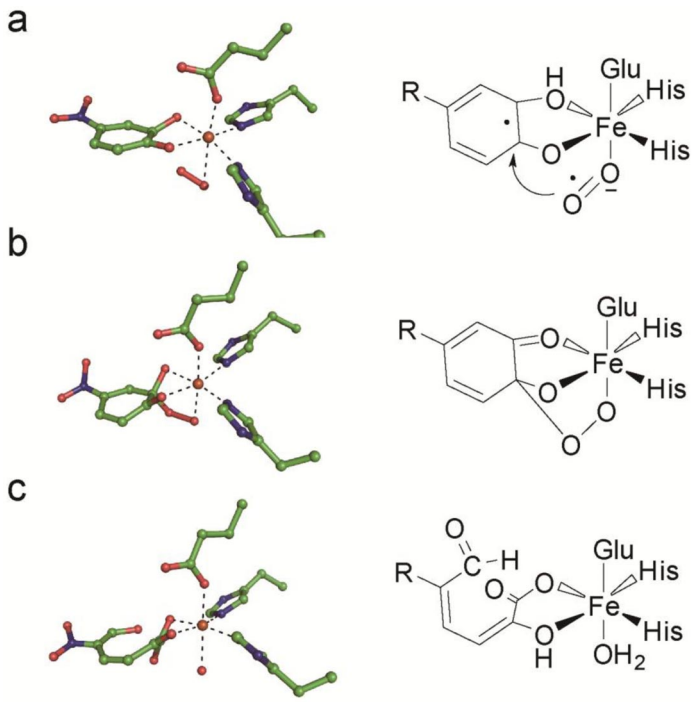

Fig. 4 Intermediates trapped during the oxidation reaction catalyzed by extradiol ring-cleaving dioxygenase. (a) The superoxo intermediate. (b) The alkylperoxo intermediate. (c) The bound product intermediate.

In the superoxo intermediate structure, the dioxygen molecule is also found to be in a side-on binding fashion to the center iron (Fig. 4). The substrate 4-nitrocatechol chelates the iron with both of its hydroxyl groups, and the aromatic ring is found to be puckered, rather than being planar. This is consistent with the electron transfer from the 4-nitrocatechol substrate to the dioxygen molecule, which is now assigned as a superoxo species. In the alkylperoxo structure, the key substrate-alkylperoxo-iron(II) intermediate is observed. Such species has been predicted but was structurally characterized for the first time by the authors, which unambiguously defines the major steps of chemical transformations mediated by the extradiol ring-cleaving dioxygenase.

Reaction cycle of isopenicillin *N* synthase

Isopenicillin *N* synthase (IPNS) catalyzes the biosynthesis of isopenicillin *N* (IPN), the precursor of all penicillins. Two ring closure reactions were proposed to be the key steps: an initial formation of a four-

membered β-lactam followed by an iron(IV)-oxo-mediated closure of a five-membered thiazolidine ring. Two compounds were used in the X-ray crystallography experiments by Baldwin and colleagues: a natural substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) tripeptide and a substrate analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-L-*S*-methyl-cysteine (ACmC) [16]. As anticipated, a bicyclic product complex IPNS-iron(II)-IPN was observed with the fast ACV complex, and a monocyclic intermediate stage was captured with the substrate analogue (Fig. 5). The observation of these mono- and bicyclic structures supports the two-stage reaction mechanism and also hints at the involvement of a high-valency iron(IV)-oxo species. Again, the successful experimental design requires anaerobic crystal growth and subsequent oxygen exposure to promote reaction initiation in crystallo.

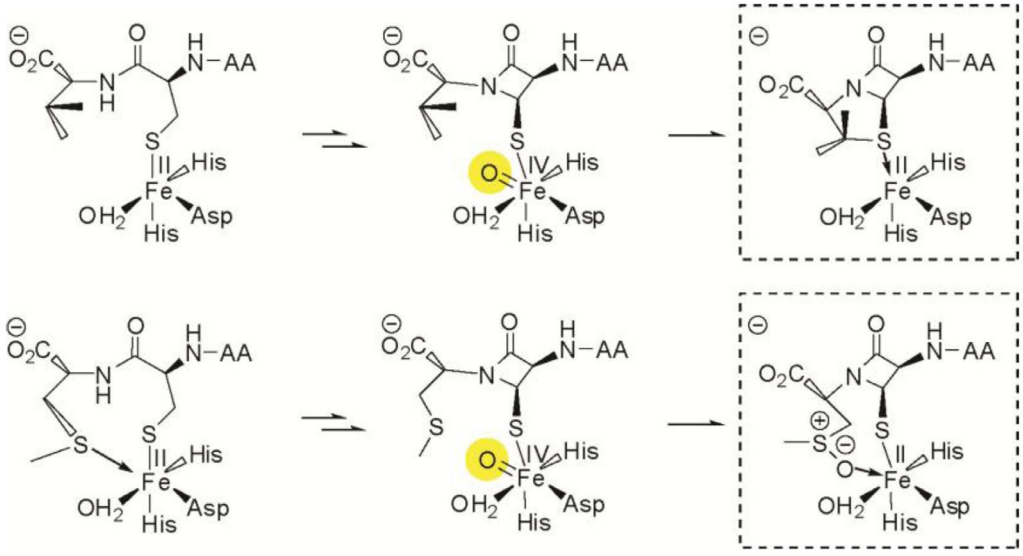

Fig. 5 Mono- and bicyclic structures (boxed) formed by IPNS, and the proposed iron(IV)-oxo species highlighted in yellow.

Oxidative demethylation mechanism of the AlkB family enzymes

The *Escherichia coli* AlkB family proteins use a mononuclear iron(II) center (chelated by the 2-His-1-carboxylate facial triad), O_2, and 2-ketoglutarate (2KG) to perform the oxidation of some of the unwanted methyl groups on DNA bases (Fig. 6) [17]. Its human homologue ABH2 guards the mammalian genome against 1-methyl adenine (1-meA) damage [18], ABH3 may repair methylated RNA damage [19], and FTO is a key factor in regulating energy homeostasis and obesity [20,21]. In addition, within this iron(II)/2KG-dependent subfamily, the JHDM proteins are engaged in the human epigenetic regulation by catalyzing the oxidative demethylation of methylated histones [22]. Thus, elucidation of the oxidation mechanisms of these proteins will not only contribute to our understanding of oxidation reactions designed by nature, but also provide mechanistic basis to potentially perturb these processes for human health (e.g., to design mechanism-based inhibitors and potentially drugs, and others).

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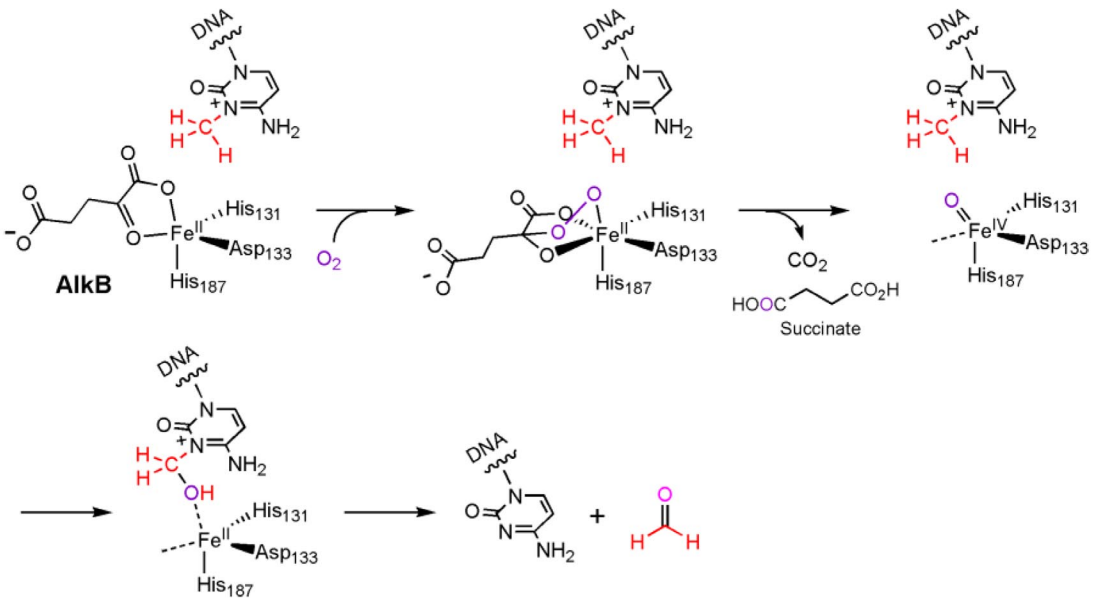

Fig. 6 Oxidative DNA/RNA repair mediated by AlkB.

Challenges to study the AlkB-mediated oxidation mechanism at atomic level

The AlkB proteins bind their substrates weakly. For example, the dissociation constant of *E. coli* AlkB to a substrate-containing DNA is estimated to be within the high nanomolar to low micromolar range [17,23]. Secondly, these proteins display only modest specificity to their substrates. Take ABH2 as an example, the protein shows only a modest two-fold increase when binding to a 1-meA-containing DNA compared to a regular piece of DNA [24]. Both of these make it challenging to obtain a homogeneous complex for structural and functional study. Hence, a new strategy must be employed to overcome these obstacles.

A chemical disulfide cross-linking approach to stabilize protein/DNA complex

The disulfide cross-linking approach was first developed by Verdine and co-workers to characterize structures of transient intermediates and complexes displaying multiple recognition modes [25]. In short, the disulfide cross-link is a covalent bond between the side chain of a cysteine residue and a thio-tethered modified DNA base (Fig. 7a). The cysteine residue is engineered to a proper site on the protein, and the alkanethiol-tether is synthetically incorporated into DNA.

How to install such a cross-link is crucial. Ideally, an engineered covalent bond should mimic a native interaction, for instance, a hydrogen bond, between the protein and its DNA substrate, in order to trap the protein-DNA complex in its native-like conformation (Fig. 7b). Our biochemical analysis has identified residue D135, which forms a hydrogen bond to the exocyclic amino group of 3-methyl cytosine (or 3-meC, an efficient AlkB substrate) [24,26,27]. Subsequently, the AlkB D135C mutant was prepared, and it was found to cross-link well with synthetic DNA probes that contain a thio-tethered cytosine (C*).

The presence of an engineered cross-link in the enzymatic site disturbs the native chemical transformations in the active site. To eventually trap an oxidation-competent complex, the cross-link has to be moved outside of the enzymatic pocket (Fig. 7c). This is accomplished through careful investigation of the active-site cross-linked structures, which were solved with the installed disulfide bond, because interactions outside of the active sites are available and can be converted to distal cross-link [24]. AlkB S129C protein was then prepared, and it was found, as expected, to cross-link with high efficiency to

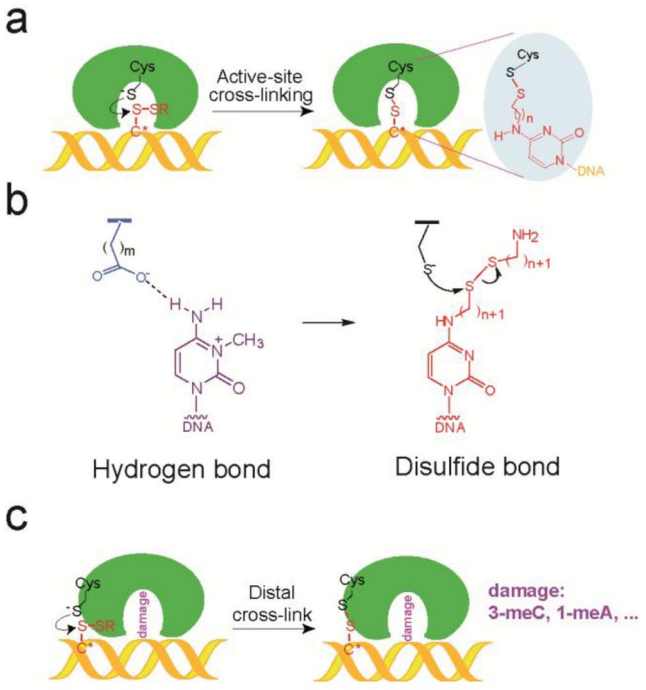

Fig. 7 Concept of chemical cross-link. (a) Active-site cross-link. (b) Cross-link converts a hydrogen bond to a covalent bond. (c) Distal cross-link.

dsDNA probes that contain 1-meA and C*. Similarly, when the conserved residue G169 of one of AlkB's human homologues, ABH2, was mutated to cysteine, high yield of cross-linking was also observed [16]. In such structures, the 3-meC substrate is anticipated to be recognized in the intact active sites of AlkB, thus forming homogenous while functional complexes (Fig. 7c).

Capturing oxidative demethylation intermediates

To elucidate the detailed mechanism of the AlkB family demethylases, we performed oxidation reactions in crystallo: an oxidation-competent AlkB-DNA complex was first crystallized under anaerobic conditions, and then such crystals were exposed to dioxygen in order to initiate oxidation reactions (Fig. 8) [28]. This approach is similar to the use of porous coordination networks by supermolecular chemists to trap transient intermediates of organic chemical transformations. The enzyme-active sites provide nanoscale reaction vessels that may allow isolation of otherwise transient intermediates.

Indeed, a glycol **1** (from 1,N^6-etheno adenine, or εA) and a hemiaminal **2** (from 3-methyl thymine, or 3-meT, which is repaired at a slower rate than 3-meC) intermediates are captured during the AlkB-mediated oxidative demethylation (Fig. 8). The observation of the glycol intermediate **1** firmly confirms the epoxidation demethylation mechanism of εA by AlkB and the hemiaminal intermediate **2** is the first species of this type ever observed in oxidative demethylation of nucleic acids. A zwitterionic intermediate **3** (from 3-meC) is also proposed, based on crystallographic observations. To further support the zwitterionic structure, we made a 3-meC analog, 3-deazamethyl cytosine, through multistep chemical synthesis, and showed that this analog can also be oxidized through the same mechanism (Figs. 9a,b). Quantum mechanical/molecular mechanical (QM/MM) calculations of these structures again proved the proposed intermediates (Fig. 9c). With neutral 3-meT, the intermediate **2** is a relatively "stable" hemiaminal as compared with that derived from 3-meC. Protonation at the O4 atom of **2** initiates bond migration and decomposition of **2** to liberate formaldehyde and yield the final intact thymine base (Fig. 10). In contrast, hydroxylation of 3-meC yields 3-hydroxymethyl cytosine, which readily

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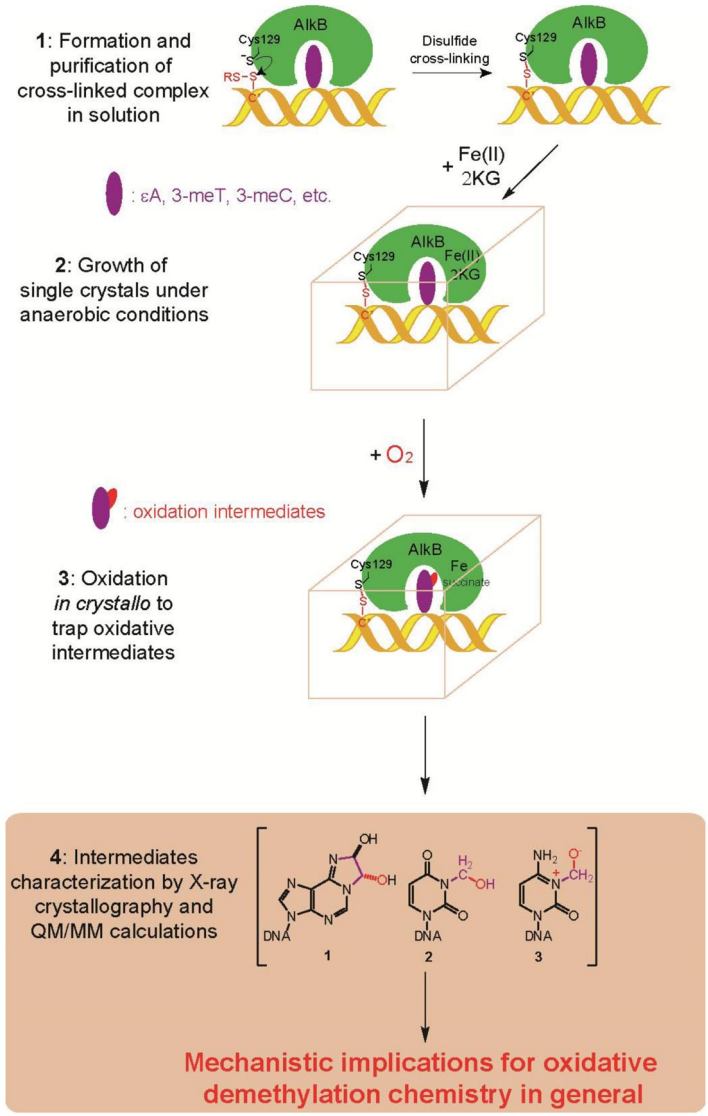

Fig. 8 Trapping AlkB-mediated oxidative intermediates in crystallo.

deprotonates to form the more stable zwitterionic intermediate **3**. Since the positively charged cytosine base is a much better leaving group than the neutral thymine at physiologic pH, the collapse of zwitterion **3** to cytosine and formaldehyde is expected to have a lower energetic barrier. This may partially explain the much faster repair of 3-meC by AlkB as compared to 3-meT [29–30]. In conclusion, the chemical nature of these intermediates can profoundly affect the reaction mechanism, reaction rate, and substrate specificity.

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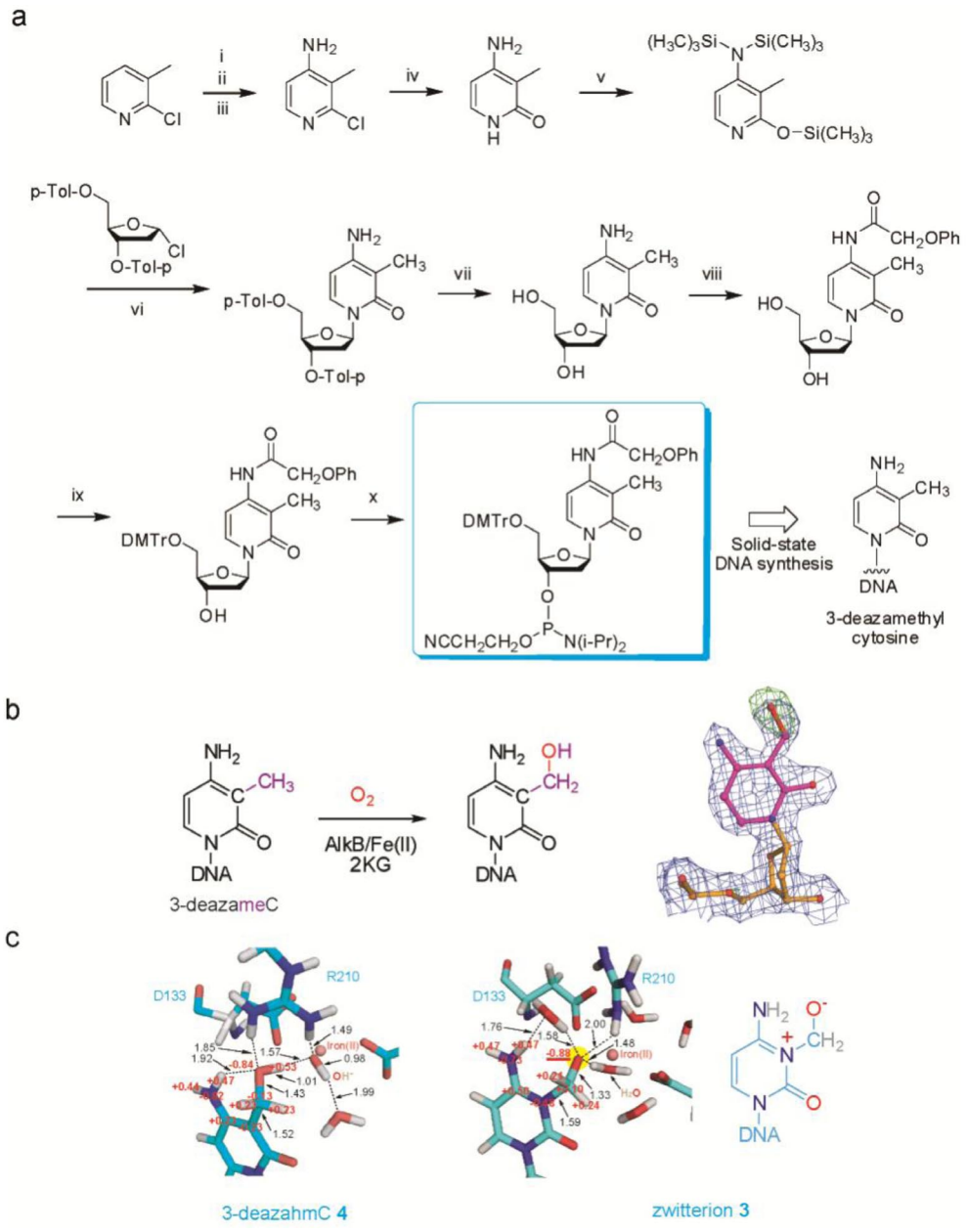

Fig. 9 A combination of chemical synthesis (a), X-ray crystallography (b), and QM/MM calculations (c) is used to reveal the chemical identity of the observed oxidative intermediates.

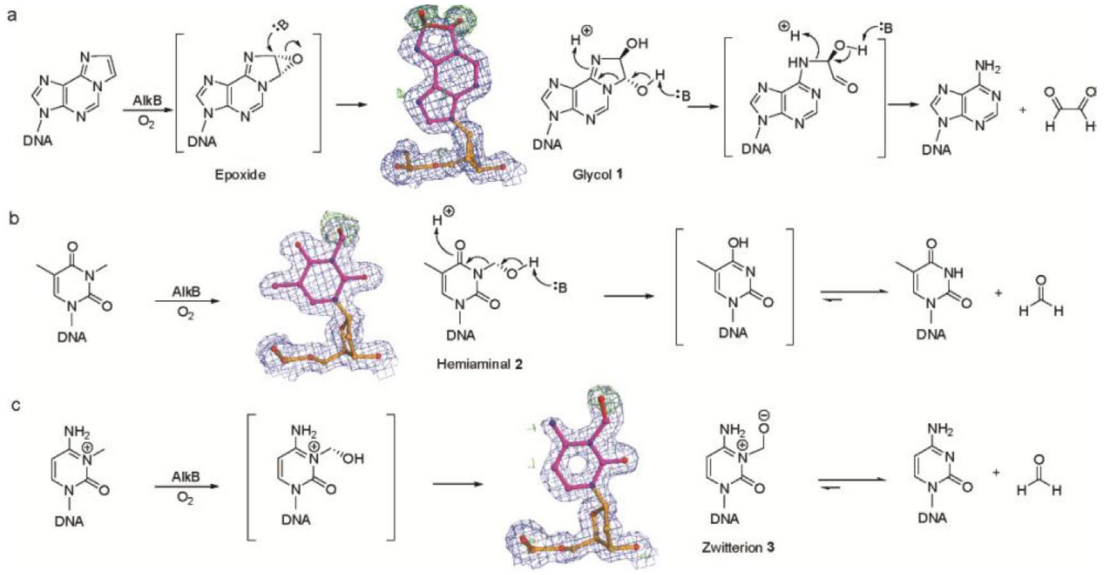

Fig. 10 Proposed demethylation mechanism based on crystallographically observed intermediates.

Future challenges and opportunities

At the very beginning of this critical review, three main triggering strategies to initiate reactions in crystallo and three main data-collection methods are briefly mentioned. Then five specific cases of mechanistic studies of oxidation reactions were summarized; now it will be interesting to examine the triggering and data-collection strategies employed in these examples to extract the principle designs for potential successful experiments in the future. In terms of triggering strategies, diffusion of small molecules is the most widely used method. Since oxygenases require additional cofactors besides the enzymes, it is not surprising to find that one or more cofactor molecules are supplied only at the initiation of the reactions. On the other hand, X-ray radiolysis is utilized once in the case of P450cam, while initiation with UV–vis light is not seen. This result may be due to the fact that these oxygenases are not light-sensitive enzymes and their unique redox chemistry generally requires specific electron-transfer partners. However, comparing diffusion strategy with light initiation method, the latter often has a higher chance of initiating reactions in a homogeneous fashion. Not only can this result give a higher "yield" of intermediates, but it is also particularly important for reactions where multiple intermediates are involved and synchronization is an issue. This area could be where new and smart designs can be explored to study oxygenases from other systems. For instance, photosensitive compounds have been widely used in research; certain caged compounds could also be synthesized to initiate reactions catalyzed by oxygenases as well.

With regard to data-collection methods, monochromatic radiation with stabilized intermediates is mostly found. Different trapping strategies have been employed throughout the examples to achieve the invariant goal: stabilize and accumulate the species of interest. Real-time crystallography is not seen in any of these examples. In the study of P450cam, the authors noted that white radiation would reduce P450cam/O_2 complex, and therefore Laue diffraction method was not used. However, the redox potential at the metal site of oxygenases may also vary; thus, whether or not white radiation would certainly reduce oxidative intermediates needs to be determined for different oxygenase systems. If real-time crystallography is possible, faster intermediates could then be potentially trapped as well. For example, in the case of AlkB family demethylases, the oxygen activation intermediates (Fig. 6) are yet to be struc-

turally characterized. Such intermediates would greatly contribute to our understanding of dioxygen activation by non-heme iron enzymes, if they can be visualized.

CONCLUDING REMARKS

Oxygenases are prevalent in life, and they catalyze numerous critical oxidation reactions with remarkable efficiency and specificity. Elucidation of their oxidation mechanisms can not only enrich our understanding of this fundamentally important chemical reaction, but also enhance our ability to manipulate these processes and design more efficient oxidative reactions for different purposes. A central focus to unravel detailed mechanisms is the structural characterization of the reaction intermediates that define the major chemical steps. Unfortunately, the intermediates are often fleeting owing to the high-efficiency nature of these enzyme-mediated transformations; therefore, delicate experimental strategies have to be designed in order to capture the species of interest.

In this contribution, we review several successful examples of structural characterizations of oxygenase-catalyzed oxidation intermediates. To slow down the conversion from substrates to products, oxidation reactions were performed in crystalline conditions (solid phase) for all the examples given. Special care was also provided in each of the cases by the researchers. Intermediates observed in these studies have provided a detailed picture of their corresponding mechanism.

Of course, the methods to intermediate determination do not have to be confined to X-ray crystallography. For example, spectroscopy is also widely used by researchers in the studies of enzymatic mechanisms [31]. In fact, new and improved tools to study intermediates are very welcome and urgently needed. Our hope is that in the near future our methods will be sufficiently sophisticated to enable the investigations of more reactions of interest.

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