

Radical Intermediates in Monooxygenase Reactions of Rieske Dioxygenases

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Rieske *cis*-diol forming dioxygenases are among the most versatile of nature's oxidizing catalysts and are often employed to initiate the biodegradation of recalcitrant environmental pollutants.¹ The natural substrates for these enzymes are unactivated aromatic compounds. These are converted to nonaromatic *cis*-dihydrodiols with the incorporation of both oxygen atoms from O₂ in a reaction coupled to the oxidation of NADH.

Naphthalene 1,2-dioxygenase is a Rieske dioxygenase that catalyzes the oxidation of its name substrate to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene.^{1b} The enzyme system (NDOS) consists of an FAD and Fe₂S₂ cluster-containing reductase (NDR), a ferredoxin (NDF), and an α₃β₃ oxygenase (NDO) containing a Fe₂S₂ Rieske cluster and a mononuclear iron center in each α subunit. NDOS exhibits a broad substrate range for aromatic *cis*-dihydroxylation.^{1c} Moreover, it catalyzes monooxygenase reactions with a diversity rivaling cytochrome P450 (P450) and methane monooxygenase (MMO).^{1b,c,2}

The mechanism for oxygen insertion by Rieske dioxygenases is unknown for both the di- and monooxygenation reactions. Concerted mechanisms as well as those enlisting cation or radical intermediates have been proposed.^{1d,3} Here, we examine the mechanism of the monooxygenase reaction of NDOS using the diagnostic probes norcarane (**1**) and bicyclohexane (**6**). These give rearranged or ring-expanded products when radical or cation intermediates, respectively, are formed.⁴ For radical intermediates, the ratio of unrearranged to rearranged products allows estimation of the radical lifetime. Failure to observe reorganized products suggests a concerted mechanism or a short-lived intermediate. It is shown here for the first time that Rieske dioxygenases catalyze monooxygenase reactions by formation of a substrate radical intermediate. These results may hold implications for the mechanism of dioxygenation by this family.

The oxidation of **1** and **6** yielded the *endo*- and *exo*-2-alcohols **2a**, **2b**, **7a**, and **7b**, respectively, with minor amounts of the 3-alcohols **3** and **8**.⁵ The radical rearrangement products of each probe (**5** and **10**) formed in substantial yield, representing 62% of the product in the case of **1**. The lifetimes of the radical intermediates formed during the oxidation of **1** (11 ns) and **6** (18 ns) are in close agreement despite a 10-fold difference in inherent rearrangement rates of the radicals formed (2 × 10⁸ and 2.9 × 10⁷ s⁻¹, respectively). This strongly supports similar radical rebound mechanisms. No detectable product from cation rearrangement was observed for **1**, while the minor amount from **6** (**9**) mirrors that observed in the Barton ester radical abstraction control reactions; thus, it is attributed to radical rearrangement through internal bond cleavage. No desaturation products were observed.

Table 1. Product Distribution from Probe Oxidation by NDOS

probe conditions	2-endo product	2-exo product	3-ol product	cation product	radical product	radical lifetime
norcarane						
1	2a	2b	3	4	5	
multiple turnover	19.6%	10.6%	7.9%	0%	61.8%	10.6 ± 1.6 ns
single turnover	16.3%	7.3%	7.1%	0%	69.2%	14.6 ns
bicyclohexane						
6	7a	7b	8	9	10	
multiple turnover	10.1%	51.7%	~2% ^a	3.9%	32.3%	18.0 ± 3.0 ns

^a This product elutes in a position that is not fully resolved from that of the 2-*endo* product, so a small amount may be produced.

Past studies have shown that the stoichiometrically reduced NDO component alone can turn over once to yield *cis*-dihydrodiol products.^{3a} As shown in Table 1, reduced NDO is also capable of promoting a single turnover (STO) monooxygenase reaction of **1**. The distribution of products of the reaction is similar to that observed during multiple turnover of NDOS. The product from rearrangement of a radical intermediate (**5**) is observed in 69% yield, and there is no evidence for a cation intermediate or desaturation products. This STO reaction shows that the products do not arise from secondary oxidation reactions.

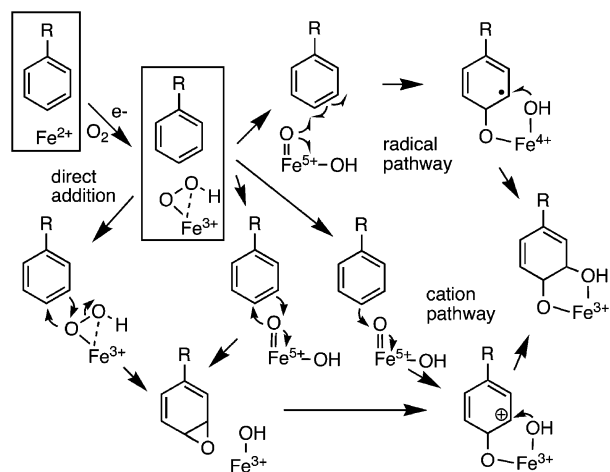
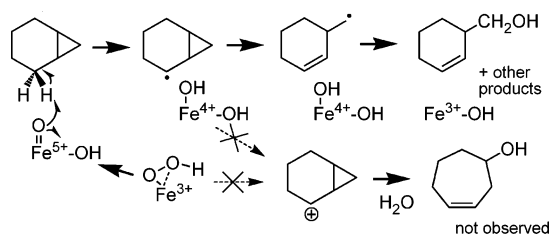
Mechanistic theory for the Rieske dioxygenases has been advanced recently by several observations. First, X-ray crystallography has revealed that NDO forms a side-on bound Fe(III)-hydroperoxy substrate intermediate at the mononuclear iron site.⁶ Second, the STO experiments have shown that the chemical reaction can be carried out without NDR and NDF. Both metal centers of NDO are oxidized during the STO, revealing the sources of the two reducing equivalents required by the stoichiometry of the reaction.^{3a,b} This stoichiometry shows that the reactive species is at the oxidation level of the observed Fe(III)-hydroperoxy species. Third, it has been shown that the resting, oxidized NDO can also catalyze the reaction if supplied with H₂O₂, which contributes both of the oxygen atoms and the two required electrons.^{3c} This observation also supports the proposal that the Fe(III)-hydroperoxy species is a key intermediate. Finally, the first iron chelate biomimetic complexes have been synthesized that carry out *cis*-dihydroxylation reactions.^{7a,b}

Computational studies based on the observations listed above have revealed an extensive set of possible oxygen insertion mechanisms.^{1d,3h,i,7c} It was concluded that protonation of the initially formed peroxy adduct is required to form a reactive species, but from this point, many paths are possible, as shown in Scheme 1.^{1d,3h,i} If the reactive species is the Fe(III)-hydroperoxy species itself,

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Scheme 1. Mechanisms Proposed for Rieske Dioxygenases**Scheme 2.** Mechanism of Norcarane Oxygenation by NDO

the most likely intermediate is a cation after an initial concerted O–O bond cleavage and oxygen insertion step. Alternatively, if the O–O bond cleavage occurs as a precursor to substrate attack to yield an Fe(V)–oxo–hydroxo species, either cationic or radical intermediates could ensue.

Like the dioxygenase reactions, the monooxygenase reactions could be envisioned to proceed either directly from the Fe(III)–hydroperoxy species or after formation of a high-valent Fe(V)–oxo–hydroxo species. As shown in Scheme 2, if an intermediate forms, it is likely to be a cation for the Fe(III)–hydroperoxy species and a radical for the high-valent Fe(V)–oxo–hydroxo species. This point has been widely discussed in recent monooxygenase literature.⁸ As for MMO, concerted addition or the occurrence of very short-lived intermediates is also possible.⁹

Our results unequivocally show that monooxygenation reactions of two alicyclic probes by NDO occur via radical intermediates, supporting the formation of a high-valent intermediate before the insertion reaction.¹⁰ The lifetime of the radical is comparable to those observed for many non-heme monooxygenases, such as the long-chain hydrocarbon oxidizing AlkB family, but much longer than those observed for P450 and MMO.^{4c} Also, in contrast to the reactions of the latter two enzymes, the NDO-catalyzed reaction yields little or no cation-derived ring expansion products, suggesting that the high-valent intermediate formed is unlikely to abstract a second e[−] from the radical intermediate (Scheme 2).

It remains unclear whether the dioxygenation reaction also proceeds through the high-valent species. However, the apparent ability of the enzyme to form this species shows that its participation is possible. This is contrary to the computational studies that concluded that the energy for formation of high-valent species is slightly too high for it to materially participate in the dioxygenation reaction.^{3h,i} Reaction through the high-valent intermediate would

unify the results of the enzyme and *cis*-diol forming biomimetic compound studies. The reactions of the latter are thought to proceed through a high-valent intermediate based on the pattern of ¹⁸O solvent exchange.^{7a,b} Early ¹⁸O studies showed that O from O₂ is incorporated with high fidelity in the dioxygenase reaction,¹¹ but some exchange has been noted in the peroxide shunt reaction.^{3c} Thus, O–O bond cleavage may precede oxygen insertion as expected for the high-valent intermediate.

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Supporting Information Available: Additional methods and characterization of products (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (5) NDO (25 μM), NDR (25 μM), and NDF (250 μM) were reacted with **1** or **6** plus NADH (300 μM) in 0.05 M MES buffer, pH 6.9 plus 0.1 M NaCl under an air atmosphere at 23 °C for 30 min. **1** and **6** are turned over by NDOs at about 1/6 the rate of naphthalene at saturation when determined by O₂ uptake. Complete methods are given in the Supporting Information. In the absence of NDO or NADH, no product was detected.
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