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Oxidative Enzymatic Alkene Cleavage: Indications for a Nonclassical Enzyme Mechanism

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Enzymatic alkene cleavage by incorporation of two oxygen atoms is described to occur either following a dioxygenase[1,2] or a monooxygenase[3] mechanism. In case of a monooxygenase mechanism, one oxygen atom in the cleavage product(s) originates from water and one from molecular oxygen. On the other hand, both oxygen atoms of one molecule O_2 are incorporated in the dioxygenase pathway via a dioxetane or related 1,2-dioxo-cyclic intermediate. We report here that mechanistic investigations of the alkene cleavage by our recently identified enzyme preparation from the fungus *Trametes hirsuta* (Scheme 1) implied a further, not yet described, mechanism.

In our previous study, a monooxygenase pathway was already excluded,[4a] since employing $^{18}O_2/H_2O^{16}$ as well as $^{16}O_2/H_2O^{18}$ showed that both oxygen atoms incorporated during the cleavage of indene **1** originate from molecular oxygen. Furthermore, we could show that neither an epoxide nor a diol was an intermediate in a possible multiple-step pathway, since none of them was transformed to the final cleavage product and none of them was detected during the reaction course. Since attempts to identify the involved enzyme are still ongoing,[5] another possible multiple-step cleavage pathway involving two enzymes had to be excluded: namely that one enzyme transforms O_2 to superoxide which then reacts further with the substrate. Such an uncoupling of the oxygen reduction step from the product formation step has previously been reported for a catechol dioxygenase.[6] Performing the alkene cleavage experiment in the presence of superoxide dismutase had no effect on the cleaving activity; thus, the existence of free superoxide could be excluded for the *Trametes*-catalyzed alkene cleavage. Therefore, a dioxygenase pathway seemed the most likely one involving a dioxetane intermediate for the cleavage of the styrene-type substrates. To check this, experiments were performed to identify indirectly the dioxetane intermediate by reducing it to the corresponding vicinal diol, following an example from literature employing NADH.[7] A possible dioxetane intermediate should be stable enough to allow such a reaction.[8] However, no diol could be detected. Although this can be attributed also to a, for example sterical hindrance, it forced us to undertake further experiments.

Subsequently, to get a possible hint for the assumed dioxetane intermediate in the dioxygenase pathways, the cleavage of indene was performed by employing a $^{16}O_2/^{18}O_2$ gas mixture. Since we have already shown that only molecular oxygen is incorporated,

Scheme 1. Oxidative Alkene Cleavage of Indene

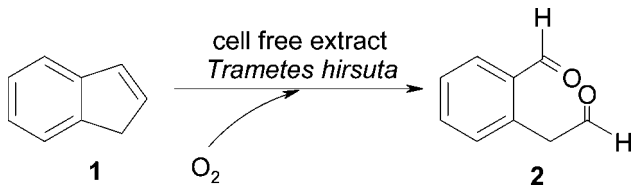
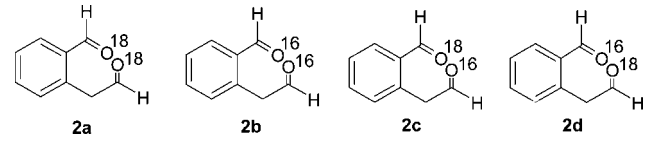


Table 1. Molecular Weight Distribution of Cleavage Products **2** in the Presence of $^{16}O_2$ and $^{16}O_2/^{18}O_2$

	$^{16}O_2$		$^{16}O_2/^{18}O_2$ = 60:40	
m/z	abundance	intensity[a] (%)	abundance	intensity[a] (%)
145	553	16.0	191	3.0
146	2257	65.4	115	1.8
147	3449	100.0	5561	88.9
148	388	11.2	6255	100.0
149	–	–	2544	40.7
150	–	–	2207	35.3
151	–	–	580	9.3
152	–	–	403	6.4

[a] Relative intensities are correlated to the area of the mass with highest abundance

only dilabeled **2a** and unlabeled **2b** products should be obtained in case of a dioxetane intermediate, but no monolabeled product such as **2c** or **2d**.



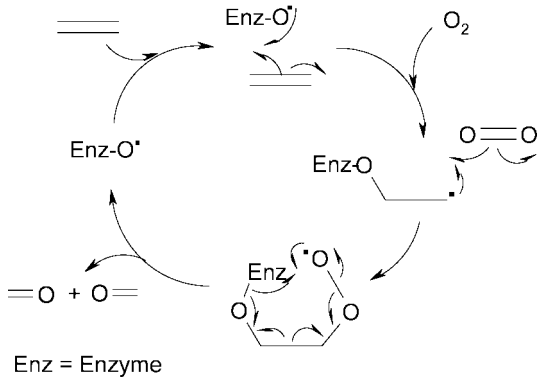
Performing the cleavage of indene **1** with a $^{16}O_2/^{18}O_2$ mixture (60:40) within 225 min showed that actually monolabeled products (**2c/2d**) were formed beside **2a** and **2b**. Careful computational analysis of the MS-pattern obtained (Table 1) by the computer program IsoPat2[9] permitted an estimate of the amount of each labeled and unlabeled species.

According to computational analysis by IsoPat2[9] the most abundant product was the monolabeled dialdehyde **2c/2d** (50.0%), followed by the unlabeled **2b** (39.5%), and finally the bilabeled dialdehyde **2a** (10.5%). Additionally, it was proven by experiments with $^{16}O_2/H_2O^{18}$ that the exchange of the oxygen atoms of **2** with water is negligible within 225 min. This is probably a specific property of product **2** due to sterical hindrance of the benzylic aldehyde moiety; in contrast, the oxygen atom of e.g. benzaldehyde exchanges rapidly in O-labeled water. Therefore the significant amount of monolabeled dialdehyde formed can only be explained if the two atoms of oxygen originate from two different O_2 molecules. It can be concluded that the alkene cleavage catalyzed by the enzyme from *Trametes hirsuta* follows neither a classical dioxygenase mechanism nor a monooxygenase pathway.

First we suspected that the alkene cleavage might be catalyzed by metal oxides, similar to mechanisms involving OsO_4, Ru, Cr, or MnO_4^-.[10] In a partially purified enzyme fraction (HIC)[5] only the metals Mn, Cu, and Mo could be detected by ICP-MS analysis. Oxides of Cu and Mo are not known to cleave alkenes, and the MnO_4^- species is not described to occur with enzymes (too

10.1021/ja8097096 CCC: $40.75

Scheme 2. Proposed Alternative Enzymatic Mechanism for Alkene Cleavage



reactive). Therefore, this possibility involving metal oxides was excluded. Looking to literature, already in 1973 Waddington described a chemical, radical-alkene cleaving mechanism in the gas phase incorporating the oxygen atoms from two different O_2 molecules,[11] and proposed later a catalytic cycle for such an alkene cleavage involving alkoxy radicals.[12] Applying this concept for the enzymatic alkene cleavage led to the following proposal of a catalytic cycle (Scheme 2).

The catalytic cycle starts with an oxy-radical which adds to the C=C bond, forming a carbon radical, and the latter reacts with molecular oxygen. The obtained intermediate rearranges via a six-membered ring to set free again the oxy-radical and the cleaving products. According to this mechanism an ideal 1:1 mixture of $^{16}O_2$/$^{18}O_2$ should lead to a product distribution of 1:2:1 = **2b**:(**2c**+**2d**):**2a** for the cleavage of indene. Generalized, a gas ratio of x:y will lead to a product distribution of x^2:2xy:y^2, which corresponds in case of the employed 60:40 mixture to a ratio of 36:48:16. This fits nicely to the actual measured mass distribution for **2** (39.5:50:10.5) after a reaction time of 225 min. For verification, the alkene cleavage was repeated with a commercial $^{16}O_2$/$^{18}O_2$ mixture (47:53). After 16 h reaction time the sample showed a distribution of **2b**:(**2c**+**2d**):**2a** = 33:47.4:19.6. Due to the extended reaction time, this ratio had to be corrected due to the slow but measurable exchange of the oxygen labels (see Supporting Information). The corrected ratio **2b**:(**2c**+**2d**):**2a** = 24:47.4:28.6 fitted again nicely the theoretical ratio (22.09:49.82:28.09). Considering the rather low isotope effect for oxygen,[13] the possible deviations are within experimental error. Furthermore, it can be expected from the experimental setup, that the actual amount of active enzyme is negligible in comparison to the turnover number achieved, so that a preloading of the enzyme with ^{16}O is not significantly influencing the mass distribution. Therefore, the obtained results strongly support the proposed catalytic cycle.

From the chemical alkene cleavage mechanism for the gas phase proposed by Waddington involving alkoxy radicals, another important aspect can be deduced: at the moment all alkene-cleaving enzymes need a metal like Fe, Ni^{2+}, Cu, or Mn^{2+};[1,2] however, in the catalytic cycle of Waddington no metal was required, suggesting that enzymatic alkene cleavage could also occur by alkoxy radicals without the involvement of a metal in the catalytic cycle.

For radical (cleavage) reactions, enzymes might have a significant advantage over chemical catalysts, namely the radical intermediates[14] are shielded in the active site of the enzyme until the catalytic cycle is completed, therefore minimizing possible side reactions, such as epoxidation, rearrangements, polymerization...

In summary, we have shown that the enzyme preparation of *Trametes hirsuta* cleaves alkenes following neither the classical dioxygenase mechanism nor a monooxygenase mechanism. A catalytic cycle for an alternative enzymatic alkene cleavage was proposed, whereby two oxygen atoms of two different oxygen molecules were incorporated.

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

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