Exploring the catalytic mechanism of the extradiol catechol dioxygenases
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Introduction
The extradiol catechol dioxygenases catalyse the oxidative cleavage of dihydroxy-aromatic substrates using non-haem iron(II) as a cofactor and dioxygen as cosubstrate [1]. The extradiol dioxygenases cleave the carbon–carbon bond adjacent to two phenolic hydroxy groups, whereas a separate family of iron(III)-dependent intradiol dioxygenases cleave the carbon–carbon bond situated between two adjacent hydroxy groups. These families of enzymes are found in bacterial catabolic pathways used for the breakdown of aromatic compounds, which occur predominantly in soil bacteria such as Pseudomonas [2]. The ability of micro-organisms to degrade man-made aromatic compounds is unique, and is being exploited for the bioremediation of contaminated soil and groundwater [3]. Thus there is considerable interest in understanding the molecular mechanisms employed by these non-haem iron-dependent enzymes.

A pathway responsible for the degradation of phenylpropionic acid has been identified in Escherichia coli which proceeds via extradiol cleavage of 2,3-dihydroxyphenylpropionic acid (1) by 2,3-hydroxyphenylpropionate 1,2-dioxygenase (MhpB) [4]. The meta-ring fission product (2) is then further cleaved by a C–C hydrolase enzyme, MhpC, to give succinic acid and 2-hydroxypenta-dienoic acid (3), as shown in Figure 1. Compound (3) is then converted by hydratase and aldolase enzymes into pyruvate and acetaldehyde [4].

Biochemical characterization of dioxygenases MhpB and MpcI
The mhpB and mhpC genes have previously been mapped by Burlingame to minute 8 of the E. coli chromosome, immediately after the lac operon [5], and auxotrophic mutants deficient in phenylpropionic acid utilization constructed [6]. Using an auxotrophic mhpB strain LW366, it was possible to subclone the mhpB gene from Clarke-Carbon plasmid pLC20-30 by complementation. The mhpB gene was subcloned on to a 5.5 kb DNA fragment, which was inserted into vector pBR322. Since this DNA fragment also contained a regulatory gene responsible for induction of the phenylpropionate pathway genes, induction of this plasmid with phenylpropionic acid gave 20-fold overexpression of MhpB activity [7].

In common with many other dioxygenase enzymes, MhpB enzyme activity was unstable in crude extracts. However, MhpB activity could be substantially stabilized by the addition of 10% ethanol, 10% glycerol and 0.5 mM iron(II) to the

Figure 1
Reactions catalysed by E. coli MhpB and MhpC.

Abbreviations used: MhpB, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase; MhpC, 2-hydroxy-6-ketona-2,4-diene-1,9-dioic 5,6-hydrolase; MpcI, metapyrocatechase 1.

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purification buffer. Purification by either precipitation or chromatographic methods gave an inactive apoenzyme, which could be fully re-activated by treatment with iron(II) and a reducing agent such as ascorbate. The apoenzyme was purified by hydrophobic interaction and gel-filtration chromatography to give purified enzyme of specific activity 48 units/mg [7]. The purified enzyme showed a subunit molecular mass of 36 kDa by SDS/PAGE, and a native molecular mass of 134 kDa by gel filtration, indicating that the enzyme exists as a tetramer, as found for other extradiol dioxygenases such as catechol 2,3-dioxygenase [8].

The E. coli mhpB gene has recently been sequenced [10], confirming the N-terminal sequence obtained for purified enzyme, and revealing 58% sequence identity with the inferred amino acid sequence of a catechol 2,3-dioxygenase (MpcI) from Alcaligenes eutrophus [9]. Neither amino acid sequence shows very similar substate specificity for a range of extradiol dioxygenases such as catechol 2,3-dioxygenase [8].

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**Mechanism of extradiol aromatic ring cleavage**

The extradiol oxidative cleavage reaction is a complex multistep reaction. Much is required of the enzyme: activation of dioxygen; activation of an aromatic ring; carbon–carbon bond cleavage; and insertion of both atoms of molecular oxygen. Yet the enzyme utilizes only a non-haem iron(II) cofactor for this sequence of events. How might this reaction proceed?

The first mechanistic issue is activation of dioxygen. There is considerable precedent for the activation of dioxygen by bivalent metal ion complexes as a metal(III)–superoxide intermediate [11]. We considered the intermediacy of superoxide likely in the light of a published model reaction for the extradiol cleavage reaction involving potassium superoxide in DMSO [12]. Furthermore the other substrate, catechol, is also capable of one-electron redox chemistry: single-electron oxidation of catechol gives a stable semiquinone species, and further single-electron oxidation gives an ortho-quinone. Thus iron(II) might mediate single-electron transfer reactions between catechol and dioxygen. In support of this hypothesis, a rhodium(III)–catecholate complex prepared by Bianchini et al. [13] gave on exposure to dioxygen a metal–semiquinone–superoxide complex. Furthermore, a similar iridium(III)–catecholate complex was found on exposure to dioxygen to give a complex containing a peroxy bridge attached covalently to the carbocyclic ring [14]. We believe that these precedents provide clues to the early steps in the extradiol dioxygenase mechanism.

Our hypothesis for the MhpB reaction mechanism is shown in Figure 2. A series of elegant ESR experiments carried out by Arciero and Lipscomb [15] on protocatechuate 4,5-dioxygenase has revealed that the non-haem iron(II) cofactor in this extradiol dioxygenase binds both catecholic hydroxy groups of the substrate, and binds dioxygen. Electron transfer from iron(II) to dioxygen would give a transient iron(III)–superoxide intermediate. Further electron transfer from catechol to iron(III) gives a semiquinone–iron(III)–superoxide intermediate. Recombination of superoxide with semiquinone gives one of two possible peroxy adducts (5) or (6).

There are then two quite different mechanisms for carbon–carbon bond cleavage. The first mechanism proposed for these enzymes by Hayashi [16] involved intramolecular closure of a similar peroxy intermediate to give a dioxetane intermediate (7), which could readily fragment to give the desired product incorporating both oxygen atoms from dioxygen. The second mechanism involves a Criegee rearrangement similar to that proposed for the intradiol enzymes by White et al. [17]. Migration of the carbon–carbon bond adjacent to the peroxy bridge on to an electron-deficient oxygen would give an unsaturated lactone intermediate (8). The departing oxygen atom could stay bound to the iron(II) cofactor as iron(II) hydroxide, and could thus participate in
Figure 2
Possible mechanistic schemes for the MhpB-catalysed reaction, showing the incorporation of $^{18}$O label from H$_2^{18}$O via lactone (8)

<table>
<thead>
<tr>
<th>Diagram</th>
<th>Description</th>
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<tbody>
<tr>
<td><img src="image.jpg" alt="Diagram" /></td>
<td>1. $^{18}$O incorporation from H$_2^{18}$O via lactone (8)</td>
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<tr>
<td><img src="image.jpg" alt="Diagram" /></td>
<td>2. Derivatization of lactone (8)</td>
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The hydrolysis of the lactone to give the desired product (see Figure 2).

In order to distinguish between the dioxetane (7) and lactone (8) intermediates, we investigated the incorporation of $^{18}$O$_2$ and H$_2^{18}$O into the reaction products. If the reaction proceeds through the dioxetane intermediate then both atoms of $^{18}$O$_2$ must be incorporated into product, whereas via the lactone intermediate one might expect some exchange of the iron(II)–hydroxide intermediate with solvent water, resulting in incomplete incorporation into the C-1 carboxylate of the product. Similarly, no incorporation would be expected from H$_2^{18}$O via the dioxetane mechanism, but some incorporation might be observed via the lactone mechanism (see Figure 2).

Attempts to derivatize the ring fission product for mass spectral analysis were unsuccessful, thus the ring fission product was further converted into succinate and compound (3) using purified hydrolase MhpC. Derivatization of succinic acid as either its dimethyl or di(trimethylsilyl) ester gave stable derivatives for GCMS, which showed 57 and 91% incorporation respectively of one atom of $^{18}$O from $^{18}$O$_2$, as expected via either mechanism. Product (3), although very unstable, was successfully converted into its di(trimethylsilyl) derivative, which gave a molecular ion of m/z 258 by GCMS analysis. In the presence of H$_2^{18}$O, this derivative gave 86% incorporation of $^{18}$O; however, in the presence of H$_2^{16}$O, crucially 30% incorporation of $^{18}$O was observed, which can only be explained by the lactone intermediate [18].

The intermediacy of a seven-membered lactone was further investigated by the preparation of a saturated seven-membered lactone analogue. Incubation of this analogue with re-activated MhpB led to the time-dependent production of the hydrolysis product, verifying that this dioxygenase enzyme possesses lactone hydrolase activity [18].

Having verified the lactone intermediate (8), our attention turned to the earlier semiquinone intermediate (4). One strategy used to investigate the existence of radical intermediates in enzyme reactions is the use of cyclopropyl radical traps, which utilize the extremely rapid ring opening of the cyclopropylmethyl radical [19]. Since the semiquinone radical proposed for the MhpB-catalysed reaction lies adjacent to the propionate side chain, a cyclopropyl ring could be incorporated into the side chain with a minimal change of substrate conformation. Thus the trans- and cis-substituted cyclopropyl analogues 9a and 9b (see Figure 3) were prepared from 2,3-dimethoxycinnamic acid. Incubation of 9a or 9b with re-activated MhpB gave a new UV absorbance at 405 nm characteristic of a meta-ring fission product, indicating that both analogues were substrates for MhpB. However, no products arising from cyclopropyl ring opening could be detected by HPLC or $^1$H-NMR spec-
troscopy, and no inactivation of MhpB by 9a or 9b was observed [20].

The cyclopropyl-containing ring fission products were found to be substrates, as expected, for hydrolase MhpC; however, analysis of the MhpC reaction products by HPLC revealed more than one peak for the cyclopropyl 1,2-carboxylic acid product. This observation prompted a hypothesis that a radical-promoted isomerization of the cyclopropyl substituents might be taking place via a reversible opening of the cyclopropyl ring during the MhpB reaction (see Figure 3) [20].

In order to test this hypothesis, authentic samples of trans- (10a) and cis- (10b) dimethylcyclopropane 1,2-dicarboxylate were synthesized, and were found to give separable peaks by GCMS with distinctive fragmentation patterns. Incubation of trans- analogue (9a) with MhpB and MhpC followed by treatment with diazomethane gave a mixture of trans- (10a) and cis- (10b) products in a 94/6 ratio, confirming that isomerization was taking place. Conversion of the cis-analogue (9b) by MhpB and MhpC followed by derivatization also gave a mixture of trans- (10a) and cis- (10b) products, this time in a 90/10 ratio, indicating substantial isomerization. Finally, conversion of the trans-analogue (9a) with A. eutrophus dioxygenase MpcI, followed by MhpC and derivatization as before, gave an 85:15 ratio of (10a)/(10b) [20].

These results indicate that an isomerization of the cyclopropyl ring substituents is taking place during the MhpB reaction which is fast compared with the rate of enzymic conversion, and that the ratio of products obtained is dependent on the precise shape of the enzyme active site. Control experiments carried out in $^2$H$_2$O revealed no $^2$H incorporation, thus the isomerization is not consistent with deprotonation/reprotonation of the cyclopropyl ring protons. It therefore appears that the isomerization is due to a reversible cyclopropyl ring opening promoted by the neighbouring semiquinone radical during the MhpB reaction.

In conclusion, these studies provide experimental evidence to support the existence of semiquinone (4) and lactone (8) intermediates in

![Figure 3](image-url)

**Figure 3**

**Cis/trans isomerization of cyclopropyl-containing substrates 9a and 9b via a radical-mediated reversible cyclopropyl ring opening**

The table indicates the ratio of products 10a/10b observed by GCMS analysis after processing with an extradiol dioxygenase (MhpB or MpcI) followed by hydrolase MhpC.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Ratio (10a):(10b)</th>
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<tbody>
<tr>
<td>9a</td>
<td>E. coli MhpB</td>
<td>94:6</td>
</tr>
<tr>
<td>9b</td>
<td>E. coli MhpB</td>
<td>90:10</td>
</tr>
<tr>
<td>9a</td>
<td>A. eutrophus MpcI</td>
<td>85:15</td>
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the extradiol dioxygenase reaction mechanism. The position of attachment of the peroxy bridge remains to be determined: the lactone intermediate could be formed by either acyl migration of a C-1 peroxy intermediate (5) or by alkanyl migration of a C-2 peroxy intermediate (6). Both of these possibilities have precedents in organic chemistry: Baeyer-Villiger oxidation of \( \alpha \)-di-ketones gives anhydride products via acyl migration of a peroxy intermediate \[21\], and alkanyl migration of alkyl hydroperoxides has also been observed \[22\].

The non-haem iron(I\( \text{II} \)) cofactor would therefore seem to fulfil several roles in the proposed catalytic mechanism. 1. It is able to co-ordinate both catechol and dioxygen substrates, bringing the reactive groups into close proximity. 2. It is able to activate both substrates via one-electron transfers. 3. The proposed Criegee rearrangement requires acid catalysis in order to generate a good leaving group for O\( \rightarrow \)O bond cleavage, which would be assisted by iron(I\( \text{II} \)) Lewis acid catalysis. 4. The extruded hydroxide ion is tethered and activated for nucleophilic attack on the lactone intermediate by co-ordination to iron(I\( \text{II} \)). This mechanism therefore highlights the multifaceted catalytic properties of non-haem iron(I\( \text{II} \)).

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Received 19 July 1996

1997