Mechanism of Extradiol Catechol Dioxygenases: Evidence for a Lactone Intermediate in the 2,3-Dihydroxyphenylpropionate 1,2-Dioxygenase Reaction

Jonathan Sanvoisin, G. John Langley, and Timothy D. H. Bugg*

Department of Chemistry, University of Southampton Highfield, Southampton, U.K. SO17 1BJ

Received February 21, 1995

The oxidative cleavage of catechols by non-heme iron-dependent dioxygenase enzymes is a key step in the bacterial degradation of naturally-occurring and man-made aromatic compounds.[1] Two classes of catechol dioxygenases are found: iron(III)-dependent intradiol dioxygenases, which cleave the carbon-carbon bond between the two hydroxyl groups, and iron(II)-dependent extradiol dioxygenases, which cleave a carbon-carbon bond adjacent to the two hydroxyl groups.[1] Despite extensive spectroscopic studies on these enzymes[2] and the determination of the crystal structure of protocatechuate 3,4-dioxygenase,[3] only limited data are available regarding the mechanism of carbon-carbon bond cleavage. A dioxetane intermediate was originally proposed for the intradiol enzyme catechol 1,2-dioxygenase based on $^{18}O_2$ labeling studies;[4] however, more recently an anhydride intermediate has been proposed for the intradiol class, formed by a Criegee rearrangement.[5] In view of the key environmental significance of the catechol dioxygenases and the absence of mechanistic information regarding the extradiol enzymes, we have initiated a study of the mechanism of iron(II)-dependent 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB) from *Escherichia coli*. Here we report evidence from ^{18}O labeling studies and analogue synthesis for a lactone intermediate.

MhpB catalyzes the extradiol cleavage of 2,3-dihydroxyphenylpropionate (**1**) to give the dienol ring fission product **2**,[6] which is then a substrate for hydrolytic cleavage by hydrolase MhpC (Figure 1). Both MhpB and MhpC from *E. coli* have been overproduced and purified to near homogeneity.[7] Possible mechanistic schemes for MhpB are illustrated in Figure 2. We propose that after binding of the substrate catechol and dioxygen by the active site iron(II), electron transfer yields a semiquinone-superoxide-iron(II) complex. Carbon-oxygen bond formation between the semiquinone and superoxide, for which there is some precedent from model systems,[8] could then take place to give two possible peroxy species. Either peroxy species could then react via either intramolecular ring closure, to give a dioxetane intermediate (**5**), or Criegee rearrangement, to give an unsaturated lactone (**6**). Either **5** or **6** could then be cleaved to give product **2**. Chemical precedent for 1,2-rearrangements

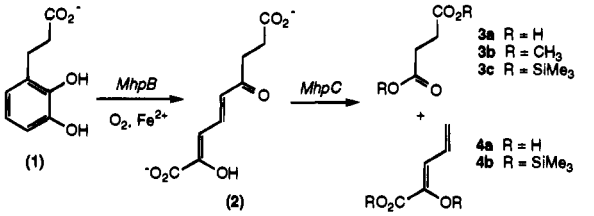

Figure 1. Reactions catalyzed by dioxygenase MhpB and hydrolase MhpC. Structures of dienols **2** and **4a** have been previously confirmed by NMR spectroscopy.[6] Derivative **3b** formed on treatment of **3a** with diazomethane. Derivatives **3c** and **4b** formed on treatment of **3a** and **4a**, respectively with bis(trimethylsilyl)acetamide.

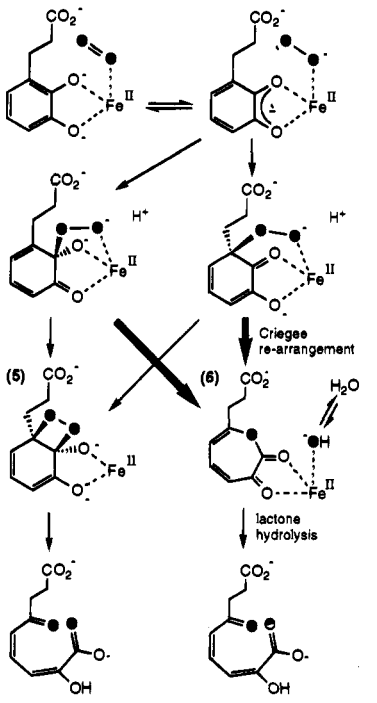

Figure 2. Possible mechanisms for the MhpB reaction, illustrating the putative dioxetane (**5**) and lactone (**6**) intermediates, and the anticipated fate of $^{18}O_2$ via each intermediate. The two possible Criegee rearrangements are indicated with bold arrows. Protons are drawn next to the two peroxy species to indicate that acid catalysis would be required for the Criegee rearrangements.[9]

of peroxy species dictates the need for a good leaving group for O-O bond cleavage,[9] thus, it is likely that acid catalysis would be required for either of the proposed Criegee rearrangements (Figure 2).

It was anticipated that intermediates **5** and **6** could be distinguished by $^{18}O_2$ labeling experiments, since cleavage of dioxetane **5** would necessarily yield two atoms of ^{18}O in the product, whereas formation of lactone **6** would be accompanied by formation of either water or iron(II)-bound hydroxide ion, which might exchange with solvent to give incomplete ^{18}O labeling in the carboxylate position of **2**. By the same analysis, some ^{18}O incorporation from $H_2{}^{18}O$ into the carboxylate position of **2** might be expected via lactone **6**. Treatment of **2** with MhpC would then allow the determination of the ^{18}O content in both the C-6 ketone and the C-1 carboxylate of **2** by mass spectral analysis of the MhpC products **3a** and **4a**, respectively.

2,3-Dihydroxyphenylpropionate (**1**) was incubated with re-activated MhpB and MhpC, purified as previously described,[6,7] in 100 mM Tris buffer at pH 8.0 under 100% $^{16}O_2$, or 99% $^{18}O_2$ or in media containing 50% $H_2{}^{18}O$. The products succinic acid (**3a**) and 2-hydroxypentadienoic acid (**4a**) were isolated,

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Table 1. ^{18}O Incorporation into **3b**, **3c**, and **4b**[a]

	3a, predicted % ^{18}O via		**3b** obsd intensity				**3c** obsd intensity				**4a**, predicted % ^{18}O via		**4b** obsd intensity			
	5	**6**	*m/z* 147	*m/z* 149	*m/z* 151	% ^{18}O	*m/z* 247	*m/z* 249	*m/z* 251	% ^{18}O	**5**	**6**	*m/z* 258	*m/z* 260	*m/z* 262	% ^{18}O
isotopic abundance			99.0	1.0	0.0		91.2	8.6	0.2				91.0	8.8	0.2	
$^{16}O_2/H_2{}^{16}O$			96.3	3.7	–		85.7	14.3	–				92.8	7.2	–	
99% $^{18}O_2/H_2{}^{16}O$	99	99	39.8	53.3	6.9	57	9.1	82.4	8.5	91	99	<99	12.3	80.0	7.7	86
$^{16}O_2$/50% $H_2{}^{18}O$	50[b]	50[b]	48.1	44.0	7.9	50[b]	49.6	42.5	7.9	46[b]	0	>0	78.5	21.5	–	15

[a] Predicted and observed values of incorporation of one ^{18}O atom are given, with the observed mass spectral intensities for unlabeled and ^{18}O-labeled samples, expressed as a percentage of the sum of the intensities. Accurate peak intensities shown were obtained by selected ion monitoring of the relevant peaks. Atom % ^{18}O incorporation into **3b**, **3c**, and **4c** was deduced after subtraction of the theoretical natural abundance M + 2, calculated from the isotopic abundances shown. Enzymatic conversions were carried out in a 2.0 mL total volume of degassed Tris buffer (100 mM, pH 8.0) containing 2,3-dihydroxyphenylpropionic acid (2.5 mM), equilibrated with $^{18}O_2$ at STP. Degassed MhpC (5 units) and MhpB (1–2 units reactivated with 1 mM ascorbate and 1 mM iron(II) ammonium sulfate) were added, and the reaction was stirred for 15 min. Products were obtained by acidification and extraction into ethyl acetate. The evaporated residue was treated with either an ethereal solution of diazomethane or an excess of bis(trimethylsilyl)acetamide in diglyme, prior to GC/MS analysis. [b] ^{18}O incorporation due to MhpC rather than MhpB in these cases.

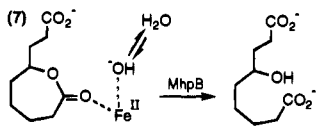
treated with either diazomethane or bis(trimethylsilyl)acetamide, and subjected to GC/MS analysis. The observed mass spectral intensities for **3b**, **3c**, and **4b** are shown in Table 1, together with the expected incorporation via intermediates **5** and **6**.

The unlabeled sample of dimethyl succinate (**3b**) gave an M + H peak at *m/z* 147 under NH_3 chemical ionization conditions. The corresponding spectrum of **3b** from the $^{18}O_2/H_2{}^{16}O$ incubation indicated an incorporation of 57% of one atom of ^{18}O. The trimethylsilyl derivative **3c** afforded a major fragment ion of *m/z* 247 (M − CH_3) when analyzed under EI conditions; when isolated from the $^{18}O_2/H_2{}^{16}O$ incubation, **3c** showed a *m/z* 249/247 ratio consistent with 91% incorporation of one atom of ^{18}O. The small peak observed at *m/z* 251 is consistent with the natural abundance of ^{13}C, ^{2}H, ^{29}Si, etc. These data indicate that ^{18}O is substantially incorporated by MhpB into the C-6 ketone of product **2**, although the discrepancy between the incorporation into **3b** and **3c** suggests that there are variable amounts of nonenzymatic exchange in the C-6 ketone of **2** prior to MhpC conversion.

In the presence of $^{16}O_2/H_2{}^{18}O$, the data for dimethyl succinate (**3b**) indicated 100% incorporation of one atom of ^{18}O and 15% incorporation of two atoms of ^{18}O (corrected for 50% ^{18}O in media). Similarly, the sample of **3c** from the $^{16}O_2/H_2{}^{18}O$ incubation showed 91% incorporation of one atom of ^{18}O and 9% incorporation of two atoms of ^{18}O. The quantitative incorporation of one atom of ^{18}O confirms that hydrolase MhpC inserts an oxygen atom from H_2O into **3a**, consistent with its anticipated mechanism.[6] The low level of incorporation of two atoms of ^{18}O is consistent with a small amount of nonenzymatic exchange in the C-6 ketone of **2** prior to MhpC cleavage, consistent with the less than quantitative incorporation into **3b** and **3c** from $^{18}O_2$.

The dienol product **4a** was converted into a stable trimethylsilyl derivative (**4b**), for which a molecular ion was observed at *m/z* 258 under EI conditions. Analysis of **4b** obtained from $^{18}O_2/H_2{}^{16}O$ incubations indicated an incorporation of 86% of one atom of ^{18}O, indicating that MhpB also substantially labels the C-1 carboxylate position of **2** from $^{18}O_2$. The corresponding data for the $^{16}O_2/H_2{}^{18}O$ incubation show 21.5% M + 2 from 50% $H_2{}^{18}O$, which when subtracted from background natural abundance corresponds to 30% incorporation of one atom of ^{18}O into **4b**.[10] Analysis of the fragmentation pattern of **4b** confirmed that the ^{18}O label was present in the carboxylate oxygens in both cases, since the ^{18}O content of the fragment at *m/z* 215/217 due to loss of CH_3 + CO was halved in each case.[10] Incorporation of ^{18}O label into the carboxylate of **4a** from $H_2{}^{18}O$ is consistent only with formation of the lactone intermediate **6**.

The proposed intermediacy of lactone **6** and an iron(II) hydroxide species which is exchangeable with solvent implies that this enzyme should possess a catalytic ability for lactone hydrolysis. This hydrolytic partial reaction was examined by synthesis of a saturated seven-membered lactone analogue (**7**) as an alternate substrate for MhpB-catalyzed hydrolysis.[11]



Incubation of **7** with reactivated MhpB in 100 mM Tris buffer at pH 8.0 resulted in the time-dependent production of the corresponding hydroxy acid over a 15 min period by HPLC analysis. No hydrolysis was observed in the absence of enzyme, with unactivated apoenzyme, or with enzyme reactivated by iron(II)/ascorbate which had been subsequently inactivated by heat treatment.[7] Thus, dioxygenase MhpB does indeed possess lactone hydrolase activity which is dependent on the iron(II) cofactor.

The combination of the ^{18}O labeling data and the hydrolysis of the synthetic lactone argue strongly in favor of lactone intermediate **6** rather than dioxetane **5**. These data do not establish the identity of the putative peroxy intermediate preceding **6**, which could either be a C-1 peroxy species which undergoes acyl migration or a C-2 peroxy species which undergoes alkenyl migration (Figure 2). The relatively low level of exchange of the extruded ^{18}O label at the stage of **6**, together with the iron(II) dependence of the lactone hydrolysis reaction, suggests that the active site iron(II) mediates the hydrolysis of **6** via an iron(II) hydroxide species, analogous to that proposed for aconitase.[12] Similarly low levels of exchange of enzyme-bound iron(II) [^{18}O]hydroxide in the fumarase reaction have recently been reported.[13]

Acknowledgment. This work was supported by the Biomolecular Sciences panel of the Biotechnology and Biological Sciences Research Council (Grant GR/H55406).

Supporting Information Available: Original mass spectral data and characterization data for the compounds involved in the lactone hydrolysis reaction described herein (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(10) Accurate peak intensities and homogeneity of ^{18}O-labeled MS peaks were confirmed by selected ion monitoring analysis of the GC/MS data for each *m/z* species.

(11) Lactone **7** was synthesized by alkylation of cyclohexanone pyrrolidine enamine with methyl 3-bromopropionate, followed by Baeyer–Villiger oxidation with *m*-chloroperbenzoic acid and alkaline hydrolysis with aqueous sodium hydroxide in overall 59% yield. HPLC analysis on Bio-Rad HPX87C organic acids column at 0.6 mL/min in 0.005 M sulfuric acid: Retention times: lactone **7**, 72 min; hydroxy acid product, 29 min.

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