# Conversion of Extradiol Aromatic Ring-Cleaving Homoprotocatechuate 2,3-Dioxygenase into an Intradiol Cleaving Enzyme

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The iron-containing aromatic ring-cleaving dioxygenases that catalyze cleavage of catechol and its analogues fall into two classes according to the redox state of the active site metal.[1]
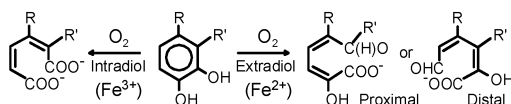
Fe^{2+}-containing dioxygenases catalyze extradiol ring cleavage to yield yellow muconic semialdehyde adducts, while the Fe^{3+}-containing dioxygenases catalyze intradiol ring cleavage to yield colorless muconic acid adducts. Each class catalyzes cleavage of a range of substrate analogues, but the presence of the vicinal OH functions (or rarely, one OH and one NH_2 group) is essential, and the cleavage site exhibits high fidelity. There are no examples of extradiol dioxygenases cleaving a substrate with only two adjacent hydroxyls in an intradiol manner.[2] In contrast, the presence of a methyl group adjacent to the vicinal hydroxyls induces extradiol cleavage by some intradiol dioxygenases.[3] Nevertheless, the fidelity of the cleavage site of this dioxygenase class for all other substrates has also proven to be absolute.

The basis for this ring fissure regiospecificity is intriguing, especially because a similar iron−peroxy−substrate intermediate is thought to lead to ring cleavage in each class.[1,4] Some possible origins include the following: (i) the oxidation state of the iron, (ii) attack of activated oxygen at different ring carbons,[1] (iii) formation of equatorial versus axial peroxy aromatic ring adducts,[5] (iv) the form of activated oxygen generated, (v) the trans influence of the endogenous iron ligands (unique for each class),[6−8] or (vi) the effect of second sphere residues. In the past, this question has been probed by model and theoretical studies.[5,9−11] Here, we address this question by mutating a second sphere residue near the iron site of the extradiol-cleaving homoprotocatechuate (HPCA) 2,3-dioxygenase (2,3-HPCD). It is shown that the mutation of H200 to Phe (H200F) switches the ring cleavage from extradiol to intradiol for the alternative substrate 2,3-dihydroxybenzoate (2,3-DHB; R = H, R′ = COOH), the first such change in specificity observed for an extradiol dioxygenase.

2,3-HPCD isolated from *Brevibacterium fuscum* catalyzes the O_2-dependent proximal extradiol cleavage of HPCA to form α-OH-δ-carboxymethyl *cis,cis*-muconic semialdehyde as illustrated above (R = CH_2COOH, R′ = H).[12] Structural studies[13] show that H155, H214, and E267 are iron ligands and the other three ligand sites are occupied by O_2 and HPCA during catalysis consistent with other dioxygenases.[6,7] HPCA is proposed to chelate the iron, but only one of the OH functions is ionized in the complex.[14] Spectroscopic[12] and crystallographic[4] studies of enzyme−substrate−NO complexes suggest that small molecules including O_2 bind to the iron opposite E267. Given this orientation, several residues are in positions that could influence O_2 and HPCA binding. One of these is the
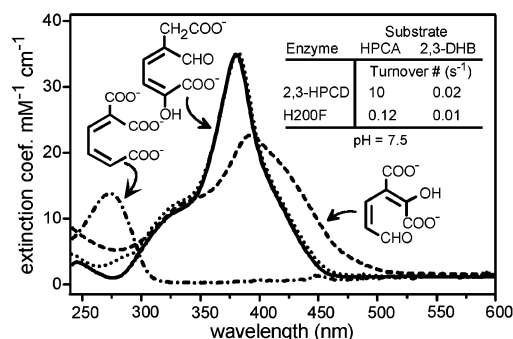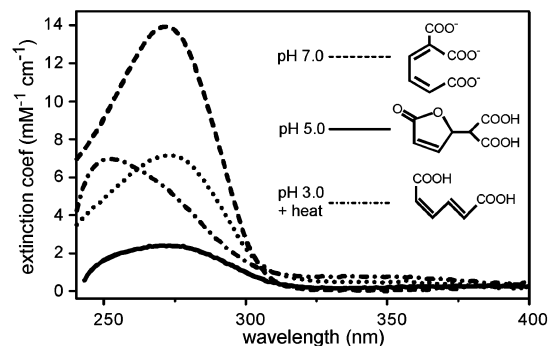
Figure 1. Optical spectra of cleavage products at pH 7.5. HPCA cleaved by 2,3-HPCD (—) or H200F (····) (spectra nearly superimposed). 2,3-DHB cleaved by 2,3-HPCD (- - -) or H200F (-·-·).

conserved[7,15] residue H200 (4.0 and 3.2 Å from the Fe^{2+} and HPCA OH^{C4}, respectively). We have speculated that a base might be required to deprotonate the second OH of HPCA to promote the Criegee rearrangement of the peroxy intermediate, leading to O insertion into the ring.[14] Accordingly, Sato et al. have reported structural comparisons between 2,3-dihydroxybiphenyl (2,3-DHBP) 1,2-dioxygenase (BphC) and the BphC-2,3-DHBP and BphC-2,3-DHBP-NO complexes that indicate the equivalent of 2,3-HPCD H200 (H194) may serve as the base in addition to supplying a proton to the peroxy intermediate during O−O bond cleavage.[4]

The H200F mutant of 2,3-HPCD was prepared by site-directed mutagenesis.[16] The optical spectra of the products observed for this mutant are compared to those for wild-type (WT) 2,3-HPCD in Figure 1. Both enzymes catalyze the proximal extradiol cleavage of HPCA, although the rate of the reaction using H200F is reduced 99% under saturating conditions (see Figure 1). Interestingly, cleavage of 2,3-DHB proceeds differently depending upon which enzyme is used. It is cleaved exclusively in a distal extradiol[17] position by WT 2,3-HPCD to yield the typical yellow α-hydroxy-muconic semialdehyde product, while H200F yields a UV absorbing product at 271 nm typical of muconic acid products from intradiol dioxygenases.[1]

The product from the H200F-catalyzed reaction with 2,3-DHB was isolated by acidification and extraction with ethyl acetate. The λ_{max} of the product is similar to that reported for the product from 2,3-DHB cleavage by the intradiol cleaving 2,3-dihydroxybenzoate 2,3-dioxygenase from *Tecoma stans*.[18] The synthesis of the putative product, α-carboxy-*cis,cis*-muconic acid, has been reported.[19] The authentic material has a λ_{max} at 271 nm at pH 7 ($\epsilon = 14\,000$ M^{-1} cm^{-1}) shifting to 272 nm ($\epsilon = 6000$ M^{-1} cm^{-1}) at pH 6 and 274 nm ($\epsilon = 920$ M^{-1} cm^{-1}) at pH 5 due to a facile lactonization reaction from the triacid. Mild heating under slightly acidic conditions results in decarboxylation to yield a product with λ_{max} at 262 nm. As shown in Figure 2, each of these spectral shifts is observed for the enzymatic product.

Figure 2. Optical spectra of product formed by H200F as a function of pH. pH conditions and proposed compounds are shown in the figure. A mixture of species occurs at pH 6.0 (•••).

Table 1. ^{1}H NMR Spectral Data for the Cleavage Product of H200F and α-Carboxymuconic Acid

		compound	
		H200F product[a]	α-carboxymuconic acid[b]
chemical shift (δ)	H2	7.30	7.35
	H3	6.38	6.40
	H4	6.04	6.05
coupling constants (Hz)	$^3J_{2,3}$	11.3	11.5
	$^3J_{3,4}$	11.3	11.5

[a] Recorded at 500 MHz in D$_2$O/NaOD. [b] Reference 19.

The ^{1}H NMR spectra of the enzymatic product and the authentic material were found to be comparable as shown in Table 1.[20] The mass spectrum of the enzymatic product revealed the expected mass of 185 for the negative molecular ion from the ring cleavage product. MS−MS of the molecular ion showed major fragments at 141 and 97 consistent with the loss of one and two carboxylates, respectively. The spectral data identify the product of the enzyme reaction as α-carboxymuconic acid, the intradiol cleavage product. This is the first report of the induction of intradiol cleavage by an extradiol dioxygenase.[21]

Several controls show that the product is formed during an enzyme-catalyzed reaction. These include the following: (i) total inhibition by the competitive inhibitor 3-OH phenyl acetate, (ii) lack of effects from the reactive oxygen scavengers SOD and mannitol, and (iii) lack of activity with apo- or heat-denatured H200F.

Extradiol cleavage of either HPCA or 2,3-DHB by WT 2,3-HPCD is dependent on the presence of active site Fe^{2+}. To determine whether there is a favored oxidation state for intradiol cleavage, H200F was allowed to oxidize by standing in air in the presence of 2,3-DHB. At intervals, the iron oxidation state was evaluated by EPR of the enzyme and its NO-complex, and the activity was measured. The intradiol cleavage activity was found to be proportional to the remaining Fe^{2+} in the sample.

H200F was found to oxidize and inactivate ($k_{\text{inact}} = 0.031$ s^{-1}) while turning over HPCA, suggesting a role for H200 in stabilizing the Fe^{2+}−O$_2$ complex to prevent loss of O$_2^{\bullet-}$. This is in accord with the proposed oxygen activation mechanism.[14,22,23]

It is shown here that the ring cleavage reaction of the normal substrate HPCA catalyzed by H200F is slowed relative to that of WT 2,3-HPCD, but the ring cleavage site is unchanged. This suggests that the ability to specifically bind the side chain of HPCA, which is unlikely to be influenced by residue H200, controls substrate binding orientation in the site and that this orientation is an important determinant of the nature of the ring cleavage. On the other hand, when the side chain is truncated to remove this fundamental level of specificity, the effects of the second sphere active site residues on the site of ring cleavage are revealed. Some of the possible determinants of the site of cleavage listed above now seem less important. First, intradiol and extradiol cleavage can apparently both occur with Fe^{2+} as the active site metal in accord with model studies.[11] Second, the same metal ligand set is sufficient for both activities. Third, the fact that the O$_2$ surrogate NO can bind to the iron in the H200F-2,3-DHB complex suggests that O$_2$ is activated by binding to the iron, and thus extra- and intradiol cleavage are likely, at least in this case, to occur from the same form of activated oxygen. The other possible determinants that address the geometry and protonation state of reactive species cannot be fully evaluated without additional mutagenesis and structural studies. However, the fact that H200F can catalyze both intra- and extradiol cleavage in ∼100% regiospecificity depending upon the substrate makes it likely that the geometry of the ternary enzyme−substrate−O$_2$ complex plays an important role. Additionally, the significant decrease in the rate of extradiol cleavage with the loss of a potential acid/base catalyst in position 200 suggests that deprotonation of the substrate or protonation of the peroxy−substrate adduct may be necessary for efficient extradiol cleavage.

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