Lactone synthesis activity in a site-directed mutant of an extradiol catechol dioxygenase enzyme

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A H115Y site-directed mutant of extradiol catechol dioxygenase MhpB catalyses an intramolecular lactonisation reaction upon its natural substrate.

The non-heme iron-dependent catechol dioxygenases catalyse the oxidative cleavage of catechol substrates, as part of bacterial catabolic pathways used for the degradation of aromatic compounds in the soil.[1] There are two classes of enzyme: the intradiol dioxygenases (Fig. 1a) which utilise a non-heme iron(III) cofactor, yield *E*,*E*-muconic acid products; and the extradiol dioxygenases (Fig. 1b) which utilise a non-heme iron(II) cofactor, yield 2-hydroxy-6-ketohexa-2,4-dienoic acid products.[1]

The factors controlling the choice of intradiol *vs.* extradiol cleavage pathways are not fully understood. The active sites of the extradiol dioxygenases contain a mononuclear iron(II) centre, ligated by two histidine and one glutamic acid ligand; the active sites of intradiol dioxygenases contain a mononuclear iron(III) centre, ligated by two histidine and two tyrosine residues.[1] Mechanistic studies on 2,3-dihydroxyphenylpropionate (DHP) 1,2-dioxygenase from *Escherichia coli* have established the intermediacy of a seven-membered lactone intermediate,[2] formed by Criegee rearrangement of a proximal hydroperoxide intermediate.[3] We have previously proposed that the same hydroperoxide intermediate is formed in both the extradiol and intradiol catechol dioxygenase mechanisms, which re-arranges either *via* alkenyl or acyl migration respectively in the two families.[3,4]

Recent studies indicate that acid–base catalysis is essential for extradiol cleavage. Studies of a biomimetic model reaction for extradiol cleavage have revealed a requirement for an active site base, which generates the catecholate monoanion, and an active site proton donor, for acid catalysis of the Criegee rearrangement.[5] Groce and Lipscomb have reported that replacement of His-200 in *Brevibacterium fuscum* 3,4-dihydroxyphenylacetate 2,3-dioxygenase by Phe gives a mutant enzyme capable of intradiol cleavage

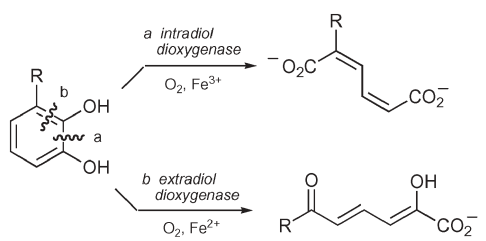

Fig. 1 Reactions catalysed by intradiol and extradiol dioxygenases.

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activity.[6] In the X-ray crystal structure of *Sphingomonas* protocatechuate 4,5-dioxygenase,[7] a Class III extradiol catechol dioxygenase,[8] there are two additional acid–base residues within 4 Å of the iron(II) centre: His-195 and His-127. We have recently found that replacement of the corresponding His-179 or His-115 in *E. coli* MhpB by Ala or Gln leads to loss of extradiol dioxygenase activity, and that mutation of nearby residues leads to effects upon acid–base catalysis.[9] In two Class III extradiol dioxygenases, 2-aminophenol 1,6-dioxygenase[10] and 3-hydroxyanthranilate dioxygenase,[11] His-127 (found as His-115 in *E. coli* MhpB) is replaced by tyrosine. We have therefore investigated the replacement of this residue by Tyr, and in this communication we report the observation that the H115Y site-directed mutant catalyses a novel lactone synthesis reaction, rather than extradiol cleavage.

Previous studies have shown that *E. coli* MhpB can be expressed as an *N*-terminal maltose binding protein fusion, without loss of activity (k_{cat} 50 sec^{-1}, K_m 0.05 mM) compared with the native enzyme (k_{cat} 29 sec^{-1}, K_m 0.026 mM). The H115Y mutant *mhpB* gene was expressed as an *N*-terminal MBP fusion protein, which was purified to near homogenity by amylose affinity chromatography. Incubation with the natural substrate 2,3-dihydroxyphenylpropionic acid gave none of the extradiol product (λ_{max} 394 nm), but instead showed time-dependent formation of a new product with λ_{max} 275 nm. The rate of formation of the new product was proportional to enzyme concentration, and showed Michaelis–Menten kinetics (see Fig. 2). Analysis by Lineweaver–Burk plot gave values of K_m = 1.4 mM and a specific activity of 3.4 U/mg. This product was not observed using other site-directed mutants of H115 and H179.

The new product from the H115Y mutant was isolated, and was analysed by ^{1}H NMR spectroscopy†. Three downfield signals were observed at 6.92 (t, J = 8.0 Hz), 6.76 (d, J = 8.0 Hz), and 6.70 (d, J = 8.0 Hz) ppm, consistent with a structure containing three adjacent aryl or alkenyl hydrogens. The possibility that the new product was 2-substituted muconic acid **1**, arising from intradiol cleavage,[12] was investigated by synthesis of authentic intradiol cleavage product. Oxidative cleavage of DHP using CuCl–pyridine–methanol gave the muconic acid monomethyl ester,[13] which after alkaline hydrolysis gave muconic acid **1**. Compound **1** showed quite different ^{1}H NMR signals [δ_H 8.11 (d, J = 11.8 Hz), 7.06 (t, J = 11.8 Hz), 6.03 (d, J = 11.8 Hz)], indicating that the isolated product was not due to intradiol oxidative cleavage. Derivatisation of the novel product with bistrimethylsilylacetamide, followed by GC/MS analysis, revealed a new product at *m*/*z* 236, consistent with a mono-silylated derivative **2**, arising from lactone **3**. Hence the novel product was identified as the lactone

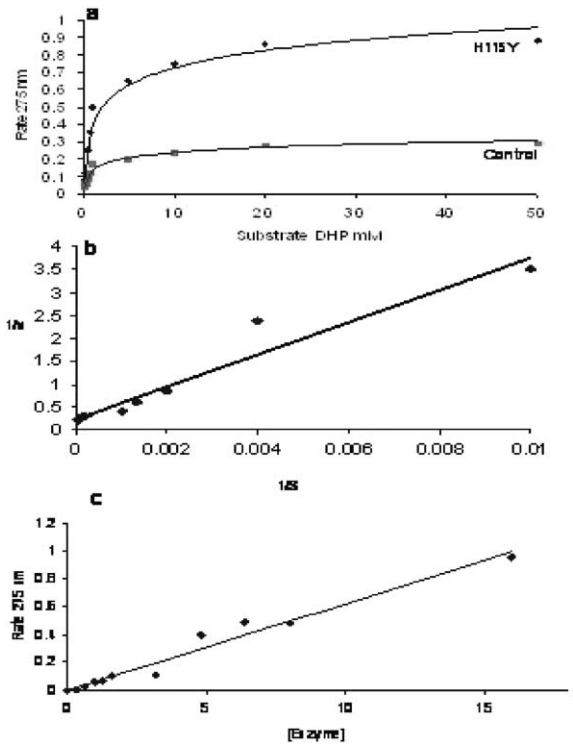
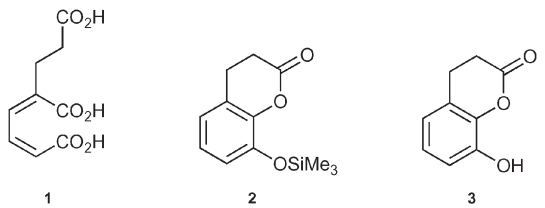


Fig. 2 a. Observed rate at 275 nm *vs*. [DHP] for H115Y and control b. Lineweaver–Burk plot for H115Y mutant c. Rate *vs*. [enzyme] at a fixed concentration of substrate.

arising from cyclisation of the C-2 hydroxyl group onto the propionate sidechain.



1 2 3

Control experiments lacking enzyme, or using boiled enzyme, showed that there is some background non-enzymatic lactonisation of DHP to lactone **3**, due to the presence of 50 μM iron(II) salts in the assay, to the extent of 20–40% of the total observed rate. However, the concentration dependence of the non-enzymatic reaction is linear *versus* [DHP]. Calculation of the second order rate constant for the non-enzymatic reaction at 50 μM Fe^{2+} gives a value of $k = 3.57\ M^{-1}\ s^{-1}$, compared with $k_{cat}/K_m = 2.4 \times 10^5\ M^{-1}\ s^{-1}$ for the enzyme-catalysed reaction.

The enzyme-catalysed lactonisation was found to be selective for Fe^{2+}, no activity being observed using Fe^{3+}, Mn^{2+}, Mg^{2+} or Zn^{2+}. Lactonisation of 2-hydroxyphenylpropionic acid to the corresponding lactone was also observed, but at 50% maximal rate compared to DHP, indicating that the enzyme shows some selectivity towards the DHP substrate.

There is no overall redox change in the lactonisation reaction, thus it is likely that the reaction mechanism involves the iron(II) centre acting as a Lewis acid, as shown in Fig. 3. Rather than ligating the two catechol hydroxyl groups, if the iron(II) cofactor ligates the C-2 hydroxyl and the propionate sidechain, then the

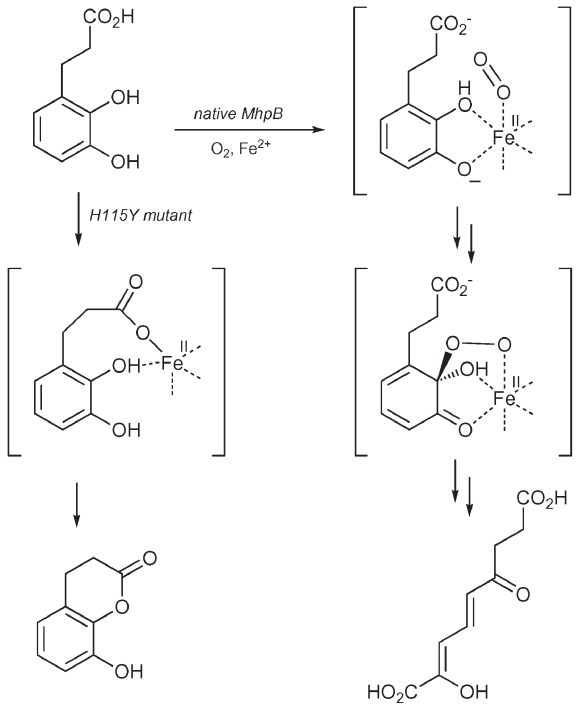


Fig. 3 Reactions catalysed by native and H115Y mutant MhpB.

iron(II) cofactor could activate the carboxyl acid for nucleophilic attack by the C-2 hydroxyl. It appears therefore that His-115 is important in correct positioning of the bound substrate, as well as for acid–base catalysis. Unlike the extradiol cleavage reaction, which is irreversible, lactonisation is a reversible reaction. From the final assay absorbance (in 50 mM potassium phosphate buffer pH 8.0), we estimate the equilibrium position to be 85 : 15 acid : lactone, thus the equilibrium position favours the acid form.

Non-heme iron is known to participate in reversible hydration/dehydration reactions in enzymes such as aconitase,[14] but to our knowledge there is no previous report of a non-heme iron dependent lactonisation activity. Enzyme-catalysed lactonisation has been reported for calcium-dependent paraoxonase,[15] and for lipase-catalysed transformations in organic solvents.[16] The ability of a non-heme iron(II) centre to catalyse a lactonisation reaction demonstrates a novel feature of non-heme iron enzymology.

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Notes and references

† ^{1}H NMR data for **3** (300 MHz, $CDCl_3$): δ 6.92 (1H, t, J = 8.0 Hz), 6.76 (1H, d, J = 8.0 Hz), 6.70 (1H, d, J = 8.0 Hz), 2.88 (2H, t, J = 7.0 Hz), 2.69 (2H, t, J = 7.0 Hz) ppm; GC-MS data for **2**: *m/z* 236 (60%, M^+), 221 (30%, $M - CH_3$), 193 (80%, $M - CH_3 - CO$).

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