Stereochemistry of the reaction catalysed by 2-hydroxy-6-keto-6-phenyl-hexa-2,4-dienoic acid 5,6-hydrolase (BphD)

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The stereochemical course of the reaction catalysed by C-C hydrolase BphD from *Burkholderia xenovorans* LB400 occurs with replacement of a benzoyl group by hydrogen with overall retention of stereochemistry.

C-C hydrolase enzymes catalyse the hydrolytic cleavage of a carbon–carbon bond adjacent to a ketone, yielding a carboxylic acid product.[1] A class of C-C hydrolases are found on bacterial *meta*-cleavage pathways responsible for the degradation of aromatic compounds in soil.[2] Amino acid sequence alignments of these enzymes,[3] and structure determination of hydrolase BphD from *Rhodococcus* RHA1,[4] have revealed that they are members of the αβ-hydrolase family, containing a Ser-His-Asp catalytic triad.

Mechanistic studies of C-C hydrolase MhpC, on the phenylpropionic acid catabolic pathway of *Escherichia coli*, have established that the reaction occurs with enzymatic insertion of the H-5$_E$ hydrogen of the product 2-hydroxypentadienoic acid, and replacement of a succinyl group with overall retention of stereochemistry.[5,6] Pre-steady state kinetic analysis of MhpC has provided evidence for a keto-intermediate,[7] which is attacked by water *via* a general base-catalysed mechanism.[8] We have recently established a synthetic route to the aryl *meta*-ring fission intermediates on the biphenyl catabolic pathway, responsible for the degradation of polychlorinated biphenyls.[9] The availability of synthetic intermediates on this pathway enables mechanistic studies of C-C hydrolase BphD, which bears 50% amino acid sequence identity to *E. coli* MhpC. Here we report the elucidation of the stereochemistry of the BphD-catalysed reaction (Fig. 1), and pre-steady state kinetic analysis.

2-Hydroxy-6-keto-6-phenyl-hexa-2,4-dienoic acid was synthesised by Heck coupling of 1-phenyl-prop-2-en-1-ol and ethyl 3-bromo-2-acetoxy-propenoate.[9] C-C hydrolase from *Burkholderia xenovorans* LB400[10,11] was expressed as an N-terminal His$_6$ fusion protein, and purified to near homogeneity (specific activity 11.8 u/mg). Steady-state kinetic parameters for (His)$_6$-BphD were determined to be K_M 2.0 μM and k_{cat} 6.5 s^{-1}, similar to reported values for native BphD.[11] Enzymatic conversion in 50 mM potassium phosphate buffer, pH 8.0, followed by extraction of the unstable 2-hydroxypentadienoic acid product ($t_{1/2}$ 5–10 min[12]), gave the ^{1}H NMR spectrum shown in Fig. 2A, identical to that of the MhpC enzymatic product.[6] The separation of the H-5$_E$ hydrogen (5.42 ppm) and H-5$_Z$ hydrogen (5.18 ppm) allows the determination of stereochemistry by ^{1}H NMR spectroscopy. Attempted enzymatic conversions of substrate in ^2H_2O resulted in very rapid ^{2}H exchange in the substrate dienol, obscuring the stereochemical determination. Therefore, the deuteriated substrate was prepared by exchange of H-5 and H-3 of the dienol substrate, by incubation of substrate in ^2H_2O. Conversion of 10 mg of the deuteriated substrate in ^1H_2O by 8 units of BphD, and direct monitoring of the reaction by ^{1}H NMR spectroscopy, gave the spectrum shown in Fig. 2B. Integration of the ^{1}H signals for H-5$_E$ (89%) and H-5$_Z$ (31%, see Table 1) shows that the H-5$_E$ hydrogen is inserted by BphD. There is 31% exchange of the H-5$_Z$ hydrogen, similar to that observed for MhpC,[6] which is due to partial release of the ketonised reaction intermediate, followed by non-specific re-enolisation in solution. Therefore, the stereochemistry of

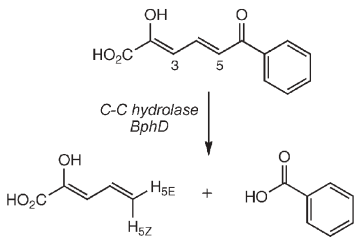

Fig. 1 Reaction catalysed by C-C hydrolase BphD. H-3 and H-5 exchanged with ^{2}H prior to reaction in ^1H_2O.

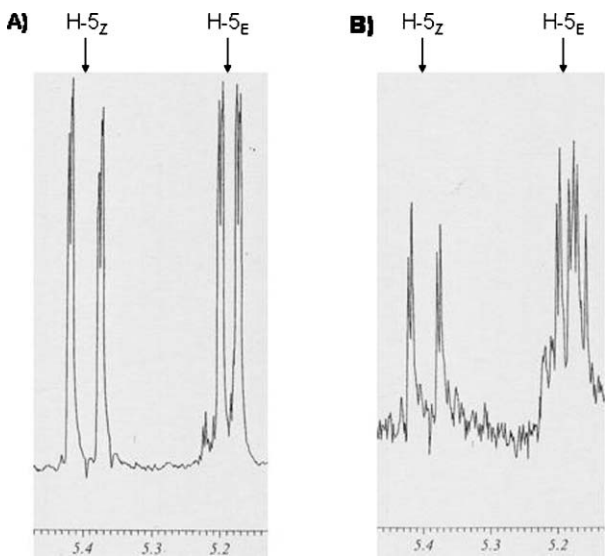

Fig. 2 Part of ^{1}H NMR spectra for (A) conversion of ^{1}H-RFP in ^1H_2O, (B) conversion of ^{2}H-RFP in ^1H_2O.

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Table 1 Integration for H-4, H-3, H-5_Z, H-5_E of product 2-hydroxypentadienoic acid from conversion of ^{2}H-labelled substrate in ^1H_2O by BphD *vs.* MhpC

	H-4	H-3	H-5_Z	H-5_E
BphD	1.00	0.15	0.31	0.89
MhpC[6]	1.00	0.02	0.30	1.00

the BphD-catalysed reaction occurs with replacement of the benzoyl substituent by hydrogen with overall retention of stereochemistry.

In order to examine the kinetic mechanism for the BphD-catalysed reaction, pre-steady state kinetic analysis of the enzyme-catalysed reaction was carried out at 30.3 μM substrate and 30.3 μM BphD in 50 mM potassium phosphate buffer pH 8.0. Observation at 430 nm gave a single exponential curve for substrate consumption (k = 9.4 s^{-1}). Observation of product appearance at 270 nm also gave a single exponential curve (k = 10.2 s^{-1}). Therefore, only a single step kinetic mechanism is observed for BphD, whereas a two-step kinetic mechanism is observed for MhpC, comprising a fast initial keto–enol tautomerisation, followed by rate-limiting C–C cleavage.[7] This result implies that the initial ketonisation step is much slower, and rate-limiting, in the BphD catalytic cycle.

In order to probe further the relative energy barriers in the BphD reaction, the solvent kinetic isotope was measured in 100% ^2H_2O. A value of 1.76 ± 0.02 was measured on v_{max}, higher than the value of 1.42 measured previously for MhpC.[6] The higher solvent kinetic isotope is consistent with the initial tautomerisation step being rate-limiting, since keto–enol tautomerisation involves proton transfer with an active site base. The slower tautomerisation by BphD may reflect the greater resonance stabilisation of the aryl substrate for BphD, or a lesser degree of substrate destabilisation than in MhpC.[7]

The availability of a crystal structure for BphD from *Rhodococcus*,[4] together with these mechanistic data, allows us to propose a more detailed catalytic mechanism for the BphD-catalysed reaction, shown in Fig. 3. The substrate is predicted to bind with the C-1 carboxylate interacting with Arg-188 at the bottom of the active site, and with the C-6 carbonyl positioned between (and beneath) the sidechains of His-263 and Ser-110. Our previous observation that BphD is able to process a reduced substrate containing a secondary alcohol at C-6,[13] together with mechanistic studies on C-C hydrolase MhpC,[6–8] implies that C–C cleavage proceeds *via* a general base mechanism, not a nucleophilic mechanism. His-263 appears to be responsible for both keto–enol tautomerisation and deprotonation of the catalytic water molecule, since there are no other acid–base residues in the vicinity of the active site.[4]

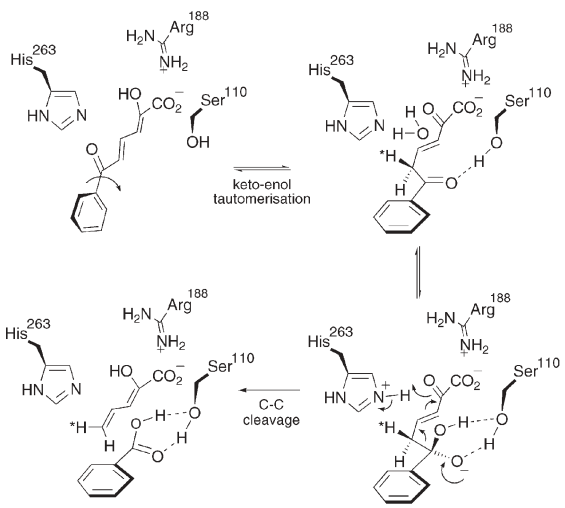

Fig. 3 Proposed catalytic mechanism for C-C hydrolase BphD, illustrating the reaction stereochemistry.

There are two stereochemical mechanisms that could give rise to insertion of the H-5_E hydrogen: either protonation at the C-5 *proS* hydrogen, followed by C–C cleavage onto the *re* face of the 3,4-double bond; or protonation at the C-5 *proR* hydrogen, followed by C–C cleavage onto the *si* face.[5] The orientation of the bound substrate, in relation to Ser-110 and His-263, requires that the scissile C5–C6 bond must rotate towards Ser-110, and therefore that C–C cleavage occurs onto the *re* face. Twisting of the dienol substrate towards Ser-110 would facilitate protonation at C-5 at the *proS* hydrogen by His-263, hence the mechanism shown in Fig. 3 would result in the observed labelling of the H-5_E position.

In summary, we have found that the stereochemistry of the reaction catalysed by C-C hydrolase BphD occurs with insertion of the H-5_E hydrogen, and overall replacement of a benzoyl group by hydrogen with retention of stereochemistry. This is the same stereochemical course as C-C hydrolase MhpC from *E. coli*, but the two enzymes show different kinetic behaviour under single turnover conditions, implying that keto–enol tautomerisation is rate-limiting in this enzyme. The availability of a synthetic route to the BphD substrate will allow a more detailed examination of the catalytic roles of His-263 and Ser-110 in this enzyme, which will be reported in due course.

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