Purification, Characterization, and Stereochemical Analysis of a C−C Hydrolase: 2-Hydroxy-6-keto-nona-2,4-diene-1,9-dioic Acid 5,6-Hydrolase

Winnie W. Y. Lam and Timothy D. H. Bugg*

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

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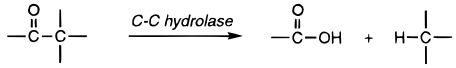
ABSTRACT: 2-Hydroxy-6-keto-nona-2,4-diene-1,9-dioic acid 5,6-hydrolase (MhpC) from *Escherichia coli* has been purified to near homogeneity from an overexpressing strain of *E. coli*. The purified enzyme is a 29 kDa dimeric protein requiring no cofactors for catalytic activity. The enzyme has a K_m of 2.1 μM and a k_{cat} of 36 s^{-1} for its natural substrate and shows high selectivity for the propionate side chain of the substrate. The stereochemical course of the MhpC reaction was elucidated by conversion of protiosubstrate in ^2H_2O and conversion of deuteriated substrate in ^1H_2O, revealing that the reaction proceeds with overall replacement of a succinyl moiety by a proton from water in the H-5_E position, with retention of regiochemistry. Isotope exchange was also observed in the H-5_Z position of the product, which was rationalized by enzyme-catalyzed exchange of ^2H into C-5 of the substrate from ^2H_2O. These data are consistent with a reversible keto-enol tautomerization taking place as the first step of the enzyme mechanism.

Hydrolytic cleavage of carbon−carbon bonds is a comparatively rare enzymatic process, yet this transformation is involved in a key step of extradiol cleavage pathways for the bacterial degradation of aromatic compounds. Oxidative cleavage of catecholic intermediates by non-heme-iron(II)-dependent dioxygenases yields ring fission products containing ϵ-keto dienol functional groups, which are substrates for carbon−carbon bond hydrolysis by a family of C−C hydrolase enzymes (Scheme 1).

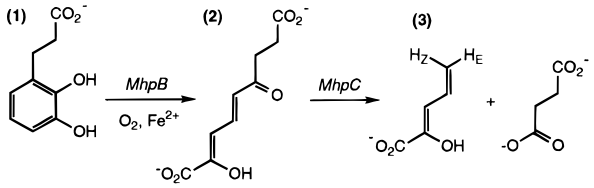
Characterization of this family of C−C hydrolases and their associated chemistry has been limited. The XylF hydrolase and two other isofunctional hydrolases on the catechol *meta*-cleavage pathway have been purified from strains of *Pseudomonas putida* and preliminary biochemical characterization reported (Bayly & Berardino, 1978; Duggleby & Williams, 1986). The BphD hydrolase on the biphenyl degradative pathway has also been purified from *Pseudomonas cruciviae* (Omori et al., 1986), but no mechanistic studies have been reported on these enzymes to date.

A pathway responsible for the degradation of phenylpropionic acid has been identified in *Escherichia coli* (Burlingame & Chapman, 1983). The pathway proceeds via oxidative cleavage of 2,3-dihydroxyphenylpropionic acid (**1**) by a non-heme-iron(II)-dependent dioxygenase MhpB[1] followed by hydrolytic cleavage of the dienol ring fission product 2-hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid (**2**) by hydrolase MhpC to give succinic acid and 2-hydroxypenta-2,4-dienoic acid (**3**) (Scheme 2). As part of a program to investigate the molecular mechanisms of these enzymes, we have overproduced and purified the dioxygenase MhpB (Bugg, 1993). Using purified MhpB, we were able to generate the dienol substrate for hydrolase MhpC, which was found to exist in the *trans transoid* conformation as shown in Figure 2 (Lam & Bugg, 1994).

Scheme 1: Generalized Reaction Scheme for C−C Hydrolases



Scheme 2: Reactions Catalyzed by Dioxygenase MhpB and Hydrolase MhpC



We anticipated that the mechanism of MhpC would involve a keto-enol tautomerization to generate a keto-intermediate (**4**) containing an electron sink for carbon−carbon bond cleavage, followed by fragmentation of the C−C bond and generation of the dienol product (**3**) (Scheme 3). The existence of keto-enol isomerases on other aromatic degradation pathways has been reported (Whitman et al., 1991, 1992), thus we wished to establish whether there was a single enzyme responsible for the MhpC reaction, whether any cofactors were required, and whether there was a discrete keto-intermediate (**4**) as proposed. Here we report the purification and characterization of MhpC and analysis of its stereochemistry and mechanism by ^2H-labeling approaches. A communication describing the identification of the dienol substrate and product of MhpC and preliminary stereochemical results has been previously reported (Lam & Bugg, 1994).

MATERIALS AND METHODS

Strains and Plasmids. Overproduction strain W3110/pTB9 containing a 5.5 kb *Eco*R1/*Cla*I DNA insert was obtained as previously described (Bugg, 1993). Succinyl

* Author to whom correspondence should be addressed. Tel: 01703-593816. Fax: 01703-593781. E-mail: tdb@soton.ac.uk.

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[1] Abbreviations: MhpB, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase; MhpC, 2-hydroxy-6-keto-nona-2,4-diene-1,9-dioic acid 5,6 hydrolase.

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MhpC Ser Tyr Gln Pro Gln Thr **Glu** *Ala Ala* **Thr Ser** *Arg* **Phe** *Leu* Asp *Val* Glu

BphD Met Ser Glu Leu Asn **Glu** *Ser Ser* **Thr Ser** *Lys* **Phe** *Val* Thr *Ile* Asn

FIGURE 1: Amino acid sequence alignment for N-terminal sequence of MhpC against the inferred N-terminal sequence of C−C hydrolase BphD from the *Pseudomonas* sp. KKS102 biphenyl catabolic pathway. Conserved residues are indicated in bold type; functionally conserved residues are indicated in italics.

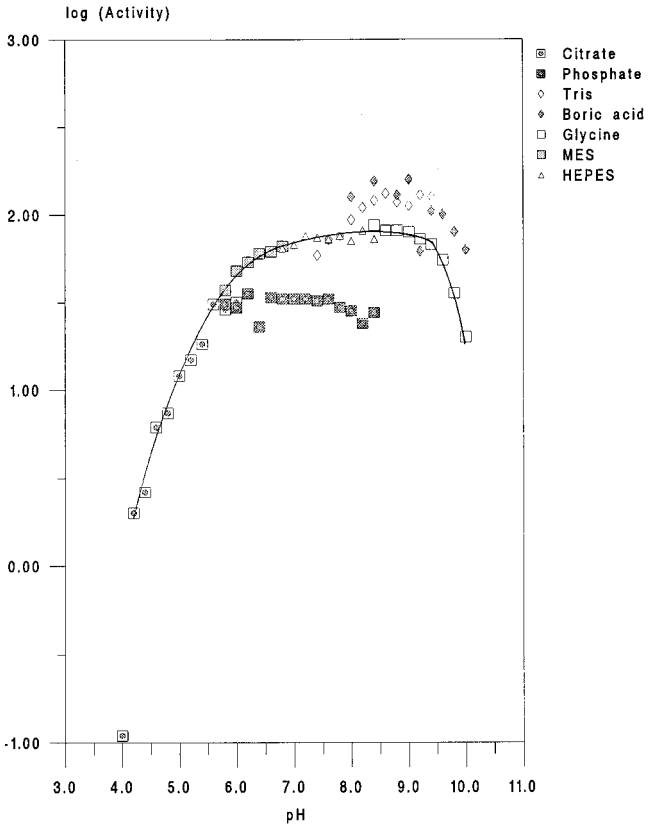
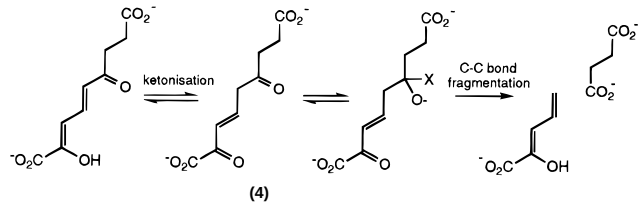


FIGURE 2: pH/rate profile of MhpC. Buffers made up as described in Materials and Methods.

Scheme 3: Putative Mechanistic Scheme for MhpC Illustrating Keto-Intermediate (**4**)[a]



[a] X is either OH (from attack of H_2O) or an active site nucleophile.

CoA synthetase overproduction strain HB101/pGS202 containing the *E. coli sucC* and *sucD* genes (Buck & Guest, 1989) was a gift of Prof. J. Guest (University of Sheffield).

Chemicals. 2,3-Dihydroxyphenylpropionic acid was synthesized from 2,3-dimethoxycinnamic acid (Blakley & Simpson, 1963). *p*-Nitrophenylsuccinate was prepared using a literature method (Baddar & El-Assal, 1950). Ethyl 2-hydroxy-penta-2,4-dienoate was synthesized from allyl magnesium bromide and diethyl oxalate (Rambaud et al., 1988). Data for ethyl 2-hydroxy-penta-2,4-dienoate follows. ^{1}H NMR (270 MHz, $CDCl_3$): δ_H 6.77 (1H, dt, J = 10.3, 10.3, 17.4 Hz, H-4), 6.20 (1H, d, J = 10.3 Hz, H-3), 5.39 (1H, dd, J = 2.1, 17.4 Hz, H-5$_Z$), 5.23 (1H, dd, J = 2.1, 10.3 Hz, H-5$_E$), 4.30 (2H, q, J = 7.1 Hz, CH_3CH_2O), 1.33 (3H, t, J = 7.1 Hz, CH_3CH_2O) ppm; ^{13}C NMR (67.5 MHz, $CDCl_3$): δ_C 165.7, 139.6, 129.9, 120.2, 112.4, 62.4, 14.3 ppm.

Preparation of Ring Fission Product 2-Hydroxy-6-keto-nona-2,4-diene-1,9-dioic Acid (**2**). MhpB apoenzyme (50 units, 50 mL), purified as previously described (Bugg, 1993), was reactivated by incubation with freshly made-up solutions of 100 mM ammonium iron(II) sulfate (2.0 mL) and 100 mM sodium ascorbate (2.0 mL) on ice for 5 min. The reactivated holoenzyme was then added to a solution of 2,3-dihydroxyphenylpropionic acid (0.1 g) in 50 mM Tris-HCl buffer, pH 8.5 (500 mL), giving rise to the bright yellow color of the ring fission product. The mixture was stirred for 20 min at room temperature, followed by acidification with 2 M HCl to pH 1.0 and extraction with ethyl acetate (3 × 50 mL). The combined extract was washed with brine (20 mL), dried over sodium sulfate, and concentrated at reduced pressure to give a yellow solid. The crude product was dissolved in 50 mM potassium phosphate buffer, pH 8.0 (5.0 mL), and loaded onto a DEAE-Sephadex A-50 column (2.5 × 10 cm), which had been equilibrated with the same buffer. The column was then eluted at 0.3 mL/min with 50 mM potassium phosphate, pH 8.0 (30 mL), followed by the same buffer containing 0.1 M NaCl (30 mL), which eluted the unreacted 2,3-dihydroxyphenylpropionic acid. Further elution with the same buffer containing 0.2 M NaCl (30 mL) eluted the yellow ring fission product. Elution was completed at 0.6 mL/min using the same buffer containing 1 M NaCl (100 mL). Fractions showing high absorbance at 394 nm, which were free of 2,3-dihydroxyphenylpropionic acid by thin layer chromatography (R_f = 0.76, 1:9 acetic acid/ethyl acetate), were pooled, acidified with 2 M HCl to pH 1.0, and extracted with ethyl acetate (3 × 20 mL). The combined extract was dried over sodium sulfate and concentrated at reduced pressure to give the ring fission product (**2**) as a yellow solid (90 mg, 76% yield). ^{1}H NMR (270 MHz, d_6-acetone): δ_H 7.64 (1H, dd, J = 11.4, 15.8 Hz, H-4), 6.41 (1H, d, J = 15.8 Hz, H-5), 6.36 (1H, d, J = 11.4 Hz, H-3), 4.65 (1H, br), 2.94 (2H, t, J = 6.7 Hz), 2.61 (2H, t, J = 6.7 Hz) ppm. ^{13}C NMR (67.5 MHz, d_6-acetone): δ_C 198.3, 174.0, 165.4, 147.3, 135.7, 130.8, 109.6, 35.6, 28.1 ppm.

Preparation of [3,5-$^2H_2$]-Ring Fission Product. Ring fission product (**2**, 5 mg) was dissolved in 100% deuteriated potassium phosphate buffer,[2] pH 8.0 (0.7 mL), left for 5 h at room temperature, and analyzed directly by ^{1}H NMR spectroscopy. Complete disappearance of the signals corresponding to H-3 and H-5 was observed, together with the appearance of a singlet at 7.72 ppm corresponding to H-4. Samples were lyophilized and used directly for enzymatic conversion. ^{1}H NMR (270 MHz, ^2H_2O): δ_H 7.72 (1H, s, H-4), 3.06 (2H, t), 2.66 (2H, t) ppm.

Assays of MhpC. Hydrolase MhpC was routinely assayed by monitoring the decrease in absorbance at 394 nm due to the consumption of the ring fission product (ϵ = 19 150 M^{-1} cm^{-1} at pH 8.0) in 50 mM Tris-HCl buffer (pH 8.0).

[2] Deuteriated 50 mM potassium phosphate buffer was prepared by lyophilization of 0.5 M potassium phosphate buffer pH 8.0 (100 μl) and redissolving the solid residue in ^2H_2O (1.0 mL).

Table 1: Purification Scheme for MhpC[a]

	volume (mL)	activity (units)	protein (mg)	specific activity (units/mg)	purification (-fold)
crude extract	59	1221	962	1.3	1.0
25−50% ammonium sulfate	15	727	291	2.5	1.9
phenyl agarose pool	30	524	153	3.4	2.6
Q sepharose pool	20	433	37	11.7	9.0
Sephacryl S-300 pool	8	110[b]	4.4	25.0	19
MonoQ Pool	4	103	2.8	36.8	28
phenyl superose pool	2	12[c]	0.16	75.0	57

[a] One unit of enzyme activity corresponds to the activity required to convert 1 μmol of substrate per minute. [b] Most active fraction which was used for subsequent purification steps. [c] Most active fraction.

Succinate release assay carried out using the colorimetric phosphate detection assay previously described (Lanzetta et al., 1979). A typical assay involved addition of MhpC (0.001−0.01 unit) to a solution of **2** (0.2 mg) in 50 mM Tris-HCl, pH 8.0 (3.0 mL), and withdrawal of 100 μL aliquots over a 15 min period. Each aliquot was transferred to an Eppendorf tube and heated at 100 °C for 5 min to inactivate MhpC. A 20−40 μL aliquot of the boiled solution containing succinate product was treated added to a solution of succinyl CoA synthetase (1 unit), coenzyme A (20 nmol), ATP (100 nmol), and $MgCl_2$ (10 mM) in 50 mM Tris-HCl (pH 7.2) in a total volume of 100 μL. After a 10 min incubation at 25 °C the Malachite green color reagent (800 μL) was added (Lanzetta et al., 1979), and after another 1−2 min, 34% sodium citrate was added (100 μl), and the absorbance at 660 nm measured. Calibration of P_i release was carried out using known concentrations of KH_2PO_4 (1−10 nmol) as standards.

Purification of Hydrolase MhpC. Purification buffer A consists of a degassed stock of 50 mM potassium phosphate buffer (pH 7.0) containing 10% ethanol and 10% glycerol (PEG buffer), to which is added 0.5 mM freshly made-up ammonium iron(II) sulfate prior to use. Purification buffer B consists of a filtered stock of 50 mM Tris-HCl (pH 7.2). Protein concentration was determined using the method of Bradford using bovine serum albumen as standard (Bradford, 1976). Enzyme purity was analyzed on 12% SDS−polyacrylamide gels, according to standard procedures. All purification steps were carried out at 4 °C unless indicated otherwise.

A 5 L culture of W3110/pTB9 was grown for 16 h at 30 °C with aeration in Luria broth containing 100 μg/mL ampicillin, 0.02% phenylpropionic acid, and 20 μM ammonium iron(II) sulfate. The cells were harvested by centrifugation for 10 min at 4400*g* and resuspended in buffer A (70 mL). Cell lysis was carried out by passage through a cell disrupter (5 psi), and cell debris was removed by centrifugation for 20 min at 27000*g*. Powdered ammonium sulfate was added to the crude extract to 25% saturation (144 g/L), the suspension stirred for 1 h, and centrifuged for 20 min at 12000*g*. To the supernatant was slowly added ammonium sulfate (157 g/L) to 50% saturation, the suspension stirred for 1 h, and centrifuged for 20 min at 12,000 x *g*.

The resulting pellet was resuspended in buffer A containing 1 M ammonium sulfate (10 mL), and the suspension loaded onto a phenyl agarose column (Sigma, 5.0 × 1.0 cm). The column was eluted at 0.5 mL/min with a gradient (200 mL) of buffer A containing decreasing ammonium sulfate concentration from 1.0 to 0 M. Fractions containing dioxygenase MhpB activity eluting at 0.2−0.0 M NaCl were pooled and stored at 4 °C. Fractions containing MhpC activity eluting at 0.9−0.7 M NaCl were pooled and dialyzed against buffer B (3 × 2 L).

The dialyzed MhpC pool was then loaded onto a Q Sepharose FPLC column (Pharmacia, 9 × 1.5 cm), and the column eluted at 2.0 mL/min with a gradient (200 mL) of buffer B containing increasing NaCl concentration from 0 to 0.5 M NaCl. Fractions containing MhpC activity eluting at 0.3 M NaCl were pooled and dialyzed against buffer B (2 L). The dialyzed pool was concentrated by loading onto a DEAE-Sephadex A-50 column (5.0 × 1.0 cm) at 0.25 mL/min and eluting with buffer B containing 1 M NaCl, giving a 3 mL pool of activity. This concentrated pool was loaded onto a Sephacryl S300 HR column (Sigma, 83 × 1.6 cm), and the column eluted with buffer B.

The fraction containing the highest MhpC activity was loaded onto a MonoQ FPLC column (Pharmacia HR 5/5) and eluted at 0.8 mL/min with a gradient (50 mL) of buffer B containing increasing NaCl concentration from 0 to 0.5 M. Fractions containing MhpC activity were pooled, and solid ammonium sulfate was added to a final concentration of 1.5 M. This pool was then loaded onto a phenyl superose FPLC column (Pharmacia HR 5/5) which was eluted with a gradient (50 mL) of buffer B containing decreasing ammonum sulfate concentration from 1.5 to 0.75 M. A peak of MhpC activity eluting at 0.8 M was found to be homogeneous by SDS−PAGE. A purification scheme is shown in Table 1.

N-terminal sequencing was carried out on a sample of purified enzyme (60 μg) which had been dialyzed against water (4 × 1l), by the University of Southampton Protein Sequencing Facility.

Native Molecular Mass Determination. Samples of purified MhpC (35 units) were eluted on a Sephacryl S300 HR gel filtration column (83 × 1.6 cm) with purification buffer B, in combination with two of the following protein standards in three separate runs: apoferritin (440 kDa), alcohol dehydrogenase (160 kDa), bovine serum albumen (132 kDa as dimer), ovalbumen (45 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). Fractions collected from the column were assayed for protein concentration and MhpC activity, and the elution volume for each protein was calculated relative to MhpC. The native molecular mass of MhpC was estimated from a linear plot of ln M_r vs (elution volume of standard)/(elution volume of MhpC).

pH/Rate Profile. A range of constant ionic strength buffers were prepared in the following pH ranges at intervals of 0.2 pH unit: sodium citrate-citric acid (4.0−6.0); MES-NaOH (5.8−6.8); potassium phosphate (5.8−8.4); HEPES-NaOH (6.8−8.4); Tris-HCl (7.4-9.4); boric acid-KCl-NaOH (8.0−10.0); glycine-NaOH (8.4−10.0). The pH-dependent extinc-

Table 2: Structure Specificity of MhpC with Respect to the Propionate Side Chain[a]

side chain	ϵ_{RFP} (M^{-1} cm^{-1}) at pH 8.0	k_{cat} (s^{-1})	K_m (μM)
$CH_2CH_2CO_2H$ (**2**)	19 150 (394 nm)	36	2.1
$CH{=}CHCO_2H$	53 530 (453 nm)	1.1	2.9
$CH_2CH_2CO_2Me$	19 150 (394 nm)	0.9	ND
CH_3	19 800 (388 nm)	0.3	ND
H	21 326 (375 nm)	0.06	ND

[a] Assays carried out by monitoring the decrease in absorbance of the respective ring fission products in 50 mM Tris-HCl buffer (pH 8.0). Extinction coefficients calculated by conversion of equimolar amounts of MhpB substrates using oxygen electrode and calibration of ϵ values using known ϵ_{394} for **2**. ND = not determined.

Table 3: Effect of Group Specific Reagents on MhpC Activity

reagent (concentration)	% activity after 10 min	% activity after 1 h
sodium iodoacetate (5 mM)	94	92
succinic anhydride (5 mM)	96	73
EDC (5 mM)	98	91
DTNB (5 mM)	96	83
p-hydroxymercuribenzoate (5 mM)	41	32
diethylpyrocarbonate (5 mm)	93	88
diethylpyrocarbonate (200 mM)	4	2

[a] Rates determined using continuous UV assay as described in Materials and Methods.

tion coefficients of the ring fission product (**2**) at 317 nm (dienol) and 394 nm (dienolate) were determined by measuring the absorbance of a 0.1 mM solution of **2** in each buffer. MhpC was assayed at 25 °C by monitoring the decrease in absorbance of **2** at 317 nm (for pH 4.0−7.4) or at 394 nm (for pH 7.2−10.0). Activity was calculated using the appropriate values of ϵ_{317} or ϵ_{394} as previously determined.

Group Specific Reagents. Freshly made-up solutions of sodium iodoacetate, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), succinic anhydride, *p*-hydroxymercuribenzoate, 1-ethyl-3-(3-dimethylaminopropyl)carbodidimide methiodide hydrochloride (EDC), and diethylpyrocarbonate in water or ethanol were added to MhpC (2.5 units) in 50 mM Tris-HCl (pH 7.2) to a final concentration of 5−10 mM, and the mixture was kept on ice. Aliquots of the treated enzyme were assayed after 30 s and at intervals up to 4 h after treatment with reagent. In the case of EDC, 10 mM ethylene diamine was included in the incubation. Assays were carried out using both the UV assay and the succinate release assay: data given in Table 3 obtained from UV assays.

Large-Scale Substrate Conversions by MhpC. (1) Conversion in ^1H_2O: Standard Method. MhpC (15 units) was added to a solution of ring fission product (**2**, 10 mg) in 50 mM potassium phosphate buffer pH 8.0 (200 mL). The mixture was stirred at room temperature for 5 min until the absorbance of **2** at 394 nm had disappeared. The mixture was then acidified to pH 1.0 with 2 M HCl, and the products were extracted into ethyl acetate (3 × 100 mL). The combined extracts were washed with brine (20 mL), dried over sodium sulfate, and concentrated at reduced pressure to give a mixture of 2-hydroxypentadienoic acid (**3**) and succinic acid (9.8 mg, 91% yield). Found for succinic acid. ^{1}H NMR (270 MHz, d_6-acetone): δ_H 2.58 (s) ppm. Found for **3**. ^{1}H NMR (270 MHz, d_6-acetone): δ_H 6.78 (1H, dt, J = 10.4, 10.4, 17.4 Hz, H-4), 6.22 (1H, d, J = 10.4 Hz, H-3), 5.41 (1H, dd, J = 2.1, 17.4 Hz, H-5$_Z$), 5.19 (1H, dd, J = 2.1, 10.4 Hz, H-5$_E$) ppm.

(2) Conversion in 90% ^2H_2O. MhpC (15 units, 0.5 mL) was added to a solution of 2 (10 mg) in 50 mM deuteriated potassium phosphate buffer,[2] pH 8.0 (4.5 mL). The remaining procedure is the same as for standard method.

(3) Conversion in 100% ^2H_2O. A solution of MhpC (30 units) in 50 mM Tris-HCl pH 7.2 was lyophilized and resuspended in ^2H_2O (100 μL). The deuteriated MhpC was then added to 100% deuteriated potassium phosphate buffer,[2] pH 8.0 (0.7 mL) and added to solid **2** (10 mg). The resulting enzymatic reaction was quickly transferred to an NMR tube for ^{1}H NMR spectral acquisition.

(4) Conversion of [3,5-$^2H_2$]-Ring Fission Product (2) in ^1H_2O. [3,5-$^2H_2$]-Ring fission product (**2**, 5 mg) was prepared as described above by isotope exchange in 100% deuteriated potassium phosphate buffer,[2] pH 8.0. The sample of lyophilized [3,5-$^2H_2$]**2** was resuspended in 50 mM potassium phosphate buffer, pH 8.0 (5.0 mL), and MhpC (30 units) was added immediately. The remaining procedure is the same as for standard method.

Rate Determination of Deuterium Isotope Exchange in 2. This experiment was carried out an a Bruker 500 MHz NMR spectrometer with the assistance of Dr. D. Turner (University of Southampton). Samples of **2** (5 mg) were placed in two NMR tubes. To the first was added 100% deuteriated potassium phosphate buffer,[2] pH 8.0 (0.7 mL). Immediately upon dissolution of **2** a series of 40 ^{1}H NMR spectra were recorded every 24 s. Integrations for the doublets observed at 6.21 and 6.38 ppm for H-3 and H-5 of **2**, respectively, were measured, and the rates of nonenzymatic ^{2}H exchange at H-3 and H-5 obtained from exponential plots of atom % ^{1}H vs time.

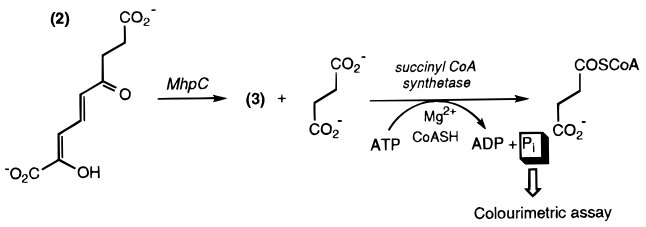
To the second was added 100% deuteriated potassium phosphate buffer,[2] pH 8.0 (0.7 mL) containing 1 unit of MhpC. ^{1}H NMR spectra were recorded as above, and the resulting integrations and rate constants for MhpC-catalyzed ^{2}H exchange measured.

RESULTS

Purification of 2-Hydroxy-6-keto-nona-2,4-diene-1,9-dioic Acid 5,6-Hydrolase (MhpC). After the identification of the phenylpropionate catabolic pathway in *E. coli* (Burlingame & Chapman, 1983), genetic mapping studies revealed that the *mhpA*, *mhpB* and *mhpC* genes were located at minute 8 of the *E. coli* chromosome (Burlingame, 1983). Subcloning of this region of chromosomal DNA has previously yielded two constructs pTB7 and pTB9 containing 9.0 and 5.5 kb of chromosomal DNA, respectively, which when expressed in *E. coli* gave rise to 10−20 fold overproduction of MhpB and MhpC activities (Bugg, 1993). W3110/pTB9 was therefore selected for the purification of both MhpB and MhpC.

Dioxygenase MhpB, purified as previously described (Bugg, 1993), was used to prepare 50−100 mg quantities of the ring fission product 2-hydroxy-6-keto-nona-2,4-diene-1,9-dioic acid (**2**), which by ^{1}H NMR spectroscopic analysis was found to exist in the *trans transoid* conformation (Lam & Bugg, 1994). This material was used as a substrate for MhpC, for which two assays were developed. The assay used routinely to assay MhpC activity was the disappearance of a UV absorbance peak at 394 nm due to the dienolate form of **2**, which gave linear slopes vs time. However, in order to monitor whole enzymatic reaction and verify product

Scheme 4: Succinate Release Assay for MhpC



formation, a new assay was developed. Release of succinate product was coupled to succinyl CoA synthetase, purified from an overexpressing strain of *E. coli* (Buck & Guest, 1989), which catalyzes the ATP-dependent conversion of succinate to succinyl CoA and the concomitant release of inorganic phosphate (Scheme 4). Release of 1−10 nmol of P_i was then detected using a colorimetric assay (Lanzetta et al., 1979). Using succinyl CoA synthetase alone, release of P_i was found to be linear with respect to succinate and coenzyme A. When coupled with MhpC, a linear time course of P_i release was observed over a 10 min period from which rates of succinate release could be calculated.

Previous work had established that MhpB and MhpC activities could be effectively separated on phenyl agarose media (Bugg, 1993). Thus, following a 25−50% ammonium sulfate fractionation, elution from a phenyl agarose column allowed MhpB to be pooled and used for preparation of ring fission product (**2**). The MhpC pool was further purified by Q sepharose anion exchange and S300 gel filtration chromatography, each giving only 2−3-fold purification. FPLC purification by MonoQ anion exchange and phenyl superose hydrophobic interaction columns gave homogeneous enzyme of specific activity 75 units/mg, which appeared as a single band at 29 kDa by SDS−PAGE (see Table 1).

Characterization of Purified MhpC. The purified enzyme was assayed using both the continuous UV assay and the succinate release assay, the former giving a specific activity of 75 units/mg and the latter a value of 30 units/mg. The disparity in these values suggests a certain amount of uncoupling of substrate isomerization and product formation. However, since only a single band was observed by SDS−PAGE at 29 kDa, the comparable activity measured by both assays indicates that there is a single enzyme responsible for the MhpC transformation, rather than separate isomerase and hydrolase enzyme activities. No decrease in specific activity was observed during purification, and no additional cofactors were required for activity, suggesting that no organic cofactors are used by MhpC. No loss of activity was observed upon incubation of MhpC with metal chelators such as EDTA or phenanthroline, indicating that no metal cofactors are required for activity.

A sample of purified MhpC was submitted for N-terminal sequencing, which gave the sequence Ser-Tyr-Gln-Pro-Gln-Thr-Glu-Ala-Ala-Thr-Ser-Arg-Phe-Leu-Asp-Val-Glu. This N-terminal sequence revealed sequence similarity with the inferred amino acid sequences of other C−C hydrolases (Diaz & Timmis, 1995), especially with hydrolase BphD (Hofer et al., 1993), shown in Figure 1. Recent sequencing of the region of DNA encoding the *mhpC* gene has confirmed the identity of the *mhpC* open reading frame, and revealed extensive sequence similarity with other C−C hydrolases (Ferrandez et al., 1997).

The native molecular mass of MhpC was determined by S300 gel filtration versus protein standards to be 62 ± 2 kDa, which suggests that the active form of MhpC is a homodimer. This compares to a native molecular mass of 65 kDa determined for hydrolase XylF, which was also believed to exist as a homodimer (Duggleby & Williams, 1986).

Steady-State Kinetic Analysis. Assays of purified MhpC using the continuous UV assay at a range of substrate concentrations revealed that Michaelis−Menten kinetics were followed. A K_m value of 2.1 μM was measured for substrate **2** from Eadie−Hofstee analysis (data not shown). The enzymatic reaction is apparently irreversible, since **2** is completely consumed upon addition of MhpC, and attempts to carry out the reverse reaction using **3** and succinic acid gave no observable increase in absorbance at 394 nm.

The substrate specificity of purified MhpC was investigated by conversion of a range of 3-substituted catechols by dioxygenase MhpB, which has a relatively broad substrate specificity for the propionate side chain (Spence et al., 1996), to the corresponding dienol ring fission products, and incubation with MhpC. In contrast to MhpB, hydrolase MhpC showed some selectivity for the carboxylate of the side chain, since the methyl ester of the natural substrate was a poor substrate for MhpC. Slow turnover was observed for the ring fission products of 3-methyl catechol or catechol itself. However, the ring fission product of 2,3-dihydroxycinnamic acid was an fairly efficient substrate (see Table 2).

Active Site Groups. Attempts were made to identify possible active site residues using group specific reagents. No significant inactivation was observed using sodium iodoacetate, DTNB, succinic anhydride, or EDC/ethylenediamine using either the UV assay or the succinate release assay. However, 68% inactivation was observed using *p*-hydroxymercuribenzoate, suggesting the existence of a modifiable cysteine residue at or near the active site. Diethylpyrocarbonate, which has been found to inactivate other C−C hydrolases, showed no significant inactivation after 10 min at 5 mM concentration, but gave 96% inactivation at 200 mM concentration (see Table 3). In no cases was it possible to inhibit succinate formation without also inhibiting substrate disappearance at 394 nm.

A pH/rate profile for the MhpC reaction was measured using a range of constant ionic strength buffers (see Figure 2). The results show inflexions at pH 6.5 and 9.5. The dependence on a deprotonated group of $pK_a = 6.5$ suggests the possibility of an active site histidine base, although this is only partially supported by the diethylpyrocarbonate inactivation studies. The inflexion at pH 9.5 suggests a role for an active site acidic group of $pK_a = 9.5$. It is also evident from Figure 2 that MhpC shows higher activity in Tris-HCl buffer, pH 8.0 (k_{cat} = 36 s^{-1}), than potassium phosphate, pH 8.0 (k_{cat} = 12 s^{-1}), in which many of the earlier experiments were carried out.

Evidence from sequence alignments (Hofer et al., 1993; Horn et al., 1991) and site-directed mutagenesis (Diaz & Timmis, 1995; Ahmad et al., 1995) of other members of this family of enzymes has implicated an active site serine residue which may function as a nucleophile in a similar fashion to the serine hydrolase family. In order to probe the existence of an acyl enzyme intermediate, attempts were made to trap the putative intermediate using hydroxylamine treatment

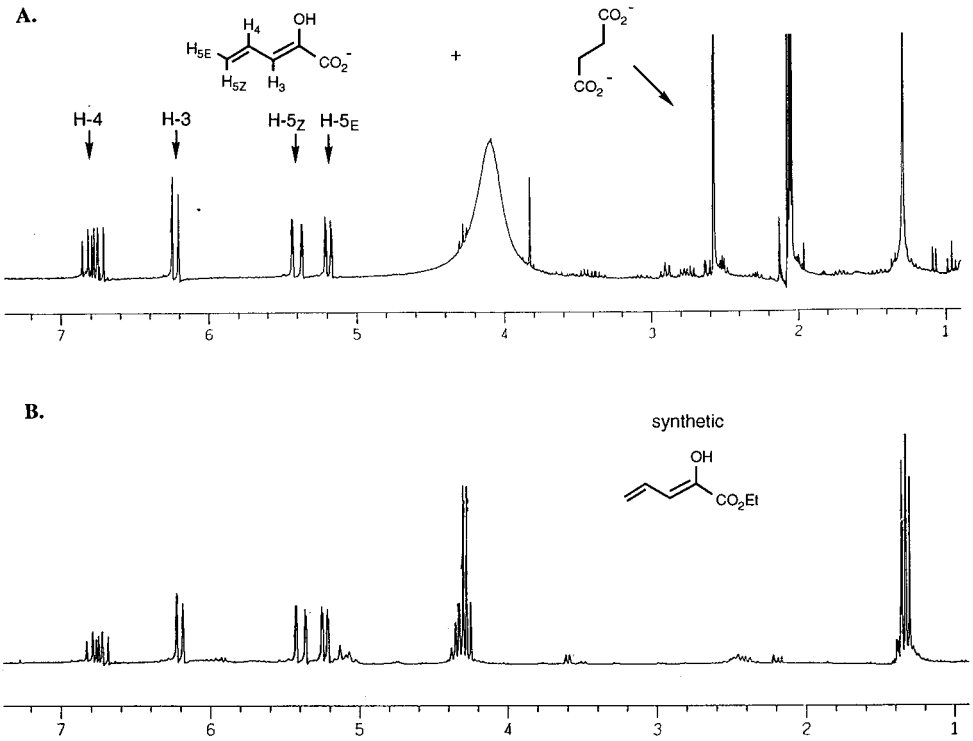
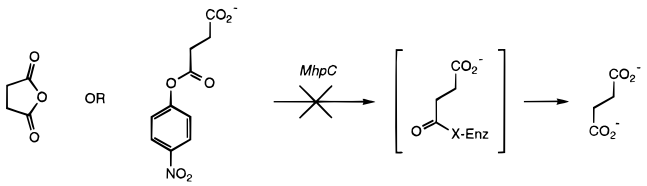


FIGURE 3: ^{1}H NMR spectra of product **3**. (A) Products obtained from MhpC conversion of **2**. (B) Sample of synthetic ethyl 2-hydroxypentadienoate (**5**), prepared as described in Materials and Methods.

followed by a ferric chloride hydroxamate assay (Bernhard et al., 1960). Incubation of authentic succinic anhydride with hydroxylamine resulted in the formation of succinyl hydroxamate, which was detected as the purple Fe(III) complex at 540 nm upon treatment with acidic ferric chloride. However, incubation of 0.2 unit MhpC with substrate **2** and 1 M hydroxylamine gave no detectable succinyl hydroxamate product.

Two alternate substrates for hydrolytic cleavage, namely succinic anhydride and *p*-nitrophenyl succinate, were prepared as described in Materials and Methods. Each of these



compounds, if attacked by the putative active site nucleophile, would generate the succinyl enzyme intermediate, which would be hydrolyzed to succinate. Both were incubated with MhpC, but in neither case was enzyme-catalyzed production of succinic acid observed using the succinate release assay.

Stereochemical Elucidation. Conversion of 5−10 mg quantities of ring fission product by MhpC, as described in Materials and Methods, yielded the products succinic acid and 2-hydroxypentadienoic acid (**3**). Confirmation of the structure of **3** was achieved by preparation of authentic ethyl 2-hydroxypentadienoate (**5**) by reaction of allyl magnesium bromide with diethyl oxalate (see Materials and Methods). The ^{1}H NMR spectra of the enzymatic product **3** and the synthetic material **5** show almost identical vinylic signals (Figure 3); in particular, there is clear separation between the H-5$_Z$ proton at 5.41 ppm and the H-5$_E$ proton at 5.19 ppm. Since one of these two protons is derived from enzymatic protonation, the overall stereochemistry of the reaction should be deduced by carrying out the enzymatic reaction in ^2H_2O. However, relatively large quantities of MhpC were required for this experiment, since exchange of the H-3 and H-5 protons of ring fission product is rapid in ^2H_2O.

Conversion of **2** was first attempted in 90% ^2H_2O/10% ^1H_2O using 15 units of MhpC, which revealed deuteriated product **3** containing significantly reduced intensity for the H-5$_E$ proton (Figure 4B). It was subsequently found that MhpC could be lyophilized and resuspended in ^2H_2O without significant loss of activity, allowing conversion of **2** to be carried out in 100% ^2H_2O. This was carried out using 30 units of MhpC and the reaction continuously monitored by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the deuteriated product revealed the complete disappearance of the H-5$_E$ signal (Figure 4C), establishing that it is the H-5$_E$ proton which is derived from enzyme-catalyzed protonation. This result was further confirmed using [3,5-^{2}H]**2** prepared by complete exchange of H-3 and H-5 in ^2H_2O as described in Materials and Methods (see Scheme 5). Conversion of [3,5-^{2}H]**2** in ^1H_2O followed by ^{1}H NMR spectroscopic analysis of the product revealed that the H-5$_E$ proton had been inserted as expected (see Figure 4D).

Deuterium Isotope Exchange Analysis. Inspection of the ^{1}H NMR spectra in Figure 4 revealed that, as well as complete insertion of the H-5$_E$ proton, there is also exchange of the H-3 and H-5$_Z$ protons taking place. During the conversion of protio-**2** in ^2H_2O there is 38% ^{2}H exchange at H-3 and 68% ^{2}H exchange at H-5$_Z$, whereas during the conversion of [3,5-^{2}H]**2** in ^1H_2O, there is 2% ^{1}H exchange at H-3 and 30% ^{1}H exchange at H-5$_Z$ (see Figure 5). The possibility that isotope exchange might be occurring after enzymatic conversion was tested by incubation of partially

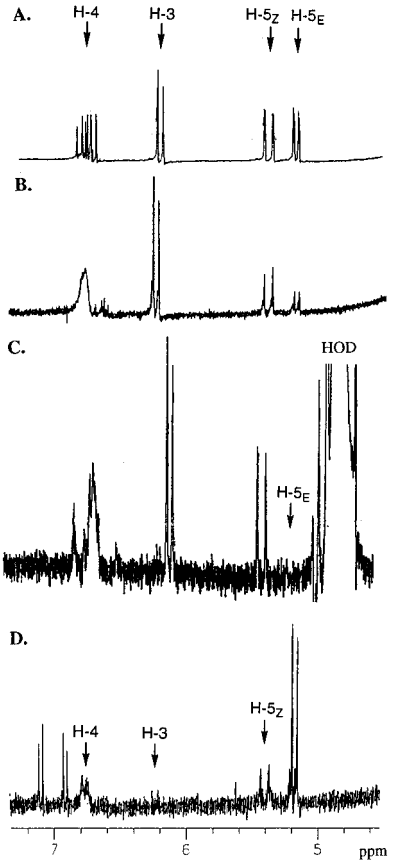
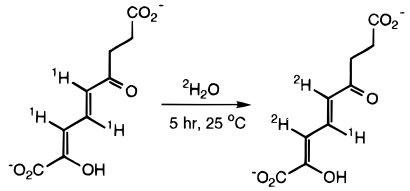


FIGURE 4: ^{1}H NMR spectra (270 MHz) of deuteriated products (**3**) obtained from MhpC conversions. (A) Product of conversion of **2** in ^1H_2O (solvent: d_6-acetone). (B) Product of conversion of **2** in 90% ^2H_2O/10% ^1H_2O (solvent: d_6-acetone). (C) Product of conversion of **2** in 100% ^2H_2O (solvent: ^2H_2O). The position of the absent H-5$_E$ doublet is indicated. (D) Product of conversion of [3,5-$^2H_2$]**2** in ^1H_2O (solvent: d_6-acetone). The pair of doublets at 6.9 and 7.1 ppm are due to an unknown impurity.

Scheme 5: Preparation of [3,5-$^2H_2$]-Labeled Ring Fission Product (**2**)



deuteriated product **3** for 1 h after MhpC conversion in ^2H_2O, at which point no reduction in intensity was observed for any of the ^{1}H NMR signals of **3**. Since the enzyme appears to be regiospecific in the insertion of the H-5$_E$ proton, the observed isotope exchange can only be explained by isotope exchange taking place in substrate **2** prior to enzymatic conversion. Further analysis of this isotope exchange data is significant, since it provides indirect evidence for MhpC-catalyzed production of keto-intermediate **4**.

There are two processes which might be responsible for isotope exchange in **2**: (A) nonenzymatic exchange into H-3 and H-5 of **2**, which occurs on a time scale of 5−10 min in ^2H_2O, prior to conversion by MhpC; and/or (B) enzymatic formation and release of keto-intermediate **4** into solution, followed by nonenzymatic enolization to regenerate **2** (see Figure 5). Process B would be expected to give rise to exchange at H-5 only, whereas process A would give

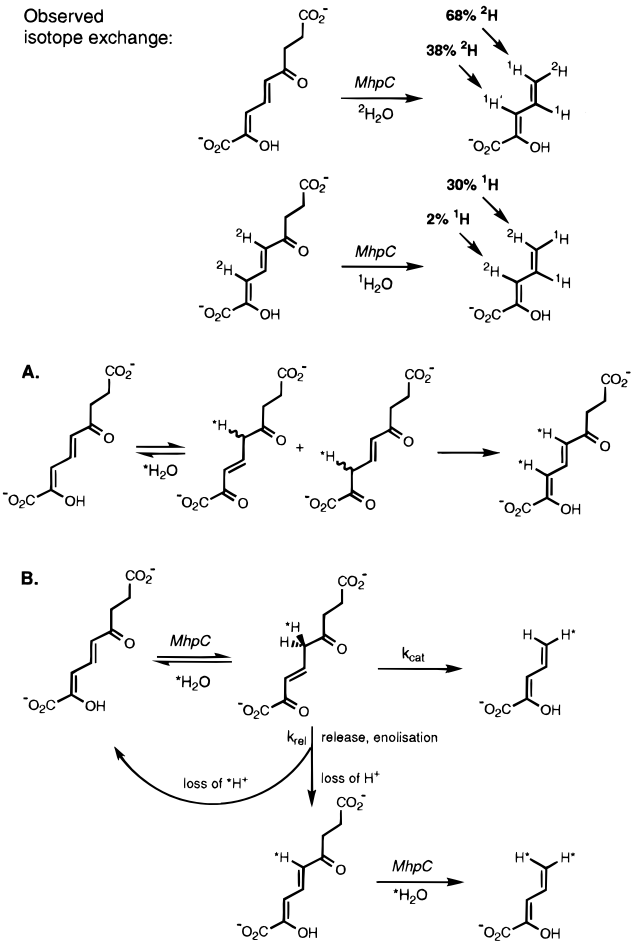


FIGURE 5: Isotopic distributions observed in deuteriated products (**3**) and processes A (nonenzymatic) and B (enzymatic) which could give rise to isotope exchange at H-3 and H-5.

exchange at both H-3 and H-5, the relative rates depending on the rates of α- vs γ-protonation of dienol **2**. If the observed isotope exchange was due only to process A, then the same ratio of exchange at H-3 and H-5$_Z$ would be expected in the two experiments, which is not observed.

Process A would be expected to occur approximately 6 times slower in the [^{2}H]**2**/^1H_2O experiment, where the removal of H* would be favored by a kinetic isotope effect (see Figure 5). The amounts of isotope exchange observed in the [^{2}H]**2**/^1H_2O experiment are indeed smaller than for the [^{1}H]**2**/^2H_2O experiment, consistent with the expected isotope effect; however, it is noticeable that in the [^{2}H]**2**/^1H_2O experiment there is a much higher ratio of exchange at H-5$_Z$ (30%) to H-3 (2%), consistent with process B. For process B, the rate of isotope exchange would also be reduced in the $^2H \rightarrow {}^1H$ direction by a kinetic isotope effect, but exchange would still be observed if $k_{rel} \approx k_{cat}$. The observation that significant isotope exchange is observed in the [^{2}H]**2**/^1H_2O experiment selectively at H-5$_Z$ is consistent with process B and hence with MhpC-catalyzed production of the keto-intermediate **4**.

In order to seek more direct evidence for MhpC-catalyzed ^{2}H exchange at H-5 of the substrate **2**, incubations of **2** in ^2H_2O in the presence or absence of a modest amount of MhpC (sufficient to catalyze isotope exchange but insufficient to convert **2** rapidly) were monitored directly by ^{1}H NMR spectroscopy, recording spectra every 0.4 min over a 10 min period. Isotope exchange at H-3 and H-5 was

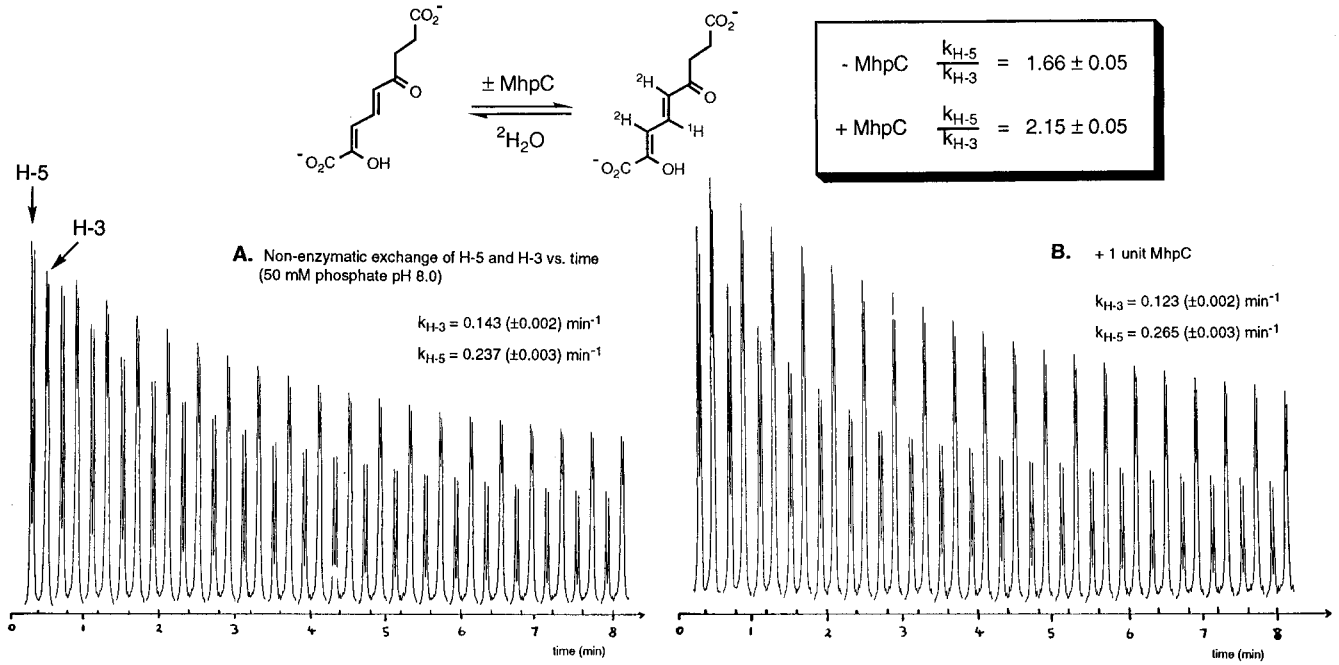


FIGURE 6: ^{1}H NMR spectral (500 MHz) analysis of H-3 and H-5 peaks of substrate **2** under ^2H_2O exchange conditions. Left-hand signal of each pair is H-5 (6.38 ppm, J = 16 Hz), right-hand signal of each pair is H-3 (6.21 ppm, J = 11 Hz). Signals from 20 consecutive spectra recorded every 0.4 min plotted vs time. (A) Nonenzymatic exchange in deuteriated potassium phosphate buffer (pH 8.0). (B) Deuterium exchange in the same buffer in the presence of 1 unit MhpC. Rate constants for isotopic exchange determined by exponential plots of peak intensity vs time.

monitored directly by integration of the signals at 6.21 and 6.38 ppm, respectively (see Figure 6). Plotting values of integration versus time gave exponential plots from which rate constants could be accurately calculated for ^{2}H exchange at H-3 and H-5 with and without MhpC.

In the absence of MhpC, values of 0.143 ± 0.002 min^{-1} and 0.237 ± 0.003 min^{-1} were measured for ^{2}H exchange at H-3 and H-5, respectively, showing that at pH 8.0 there is a 1.7-fold preference for γ-protonation of dienol **2** (k_{H-5}/k_{H-3} = 1.66 ± 0.05). In the presence of 1 unit of MhpC, the rate constants were 0.123 ± 0.002 min^{-1} for H-3 and 0.265 ± 0.003 min^{-1} for H-5, giving a ratio of k_{H-5}/k_{H-3} = 2.15 ± 0.05. The rate of exchange at H-3 is slightly slower than in the first experiment, but the observed value of k_{H-5} has increased in the presence of MhpC. The lower k_{H-3} can be rationalized by the presence of 1.4% ^1H_2O in the second experiment, which would slightly reduce the apparent rate of nonenzymatic ^{2}H exchange, but would not affect the ratio k_{H-5}/k_{H-3}.[3]

The observation that the ratio k_{H-5}/k_{H-3} is increased from 1.66 ± 0.05 to 2.15 ± 0.05 in the presence of MhpC implies that the enzyme is able to catalyze isotope exchange at H-5 of **2**. This preferential isotope exchange is consistent with release of keto-intermediate **4**, even though **4** was not detected in significant amounts during this ^{1}H NMR experiment.

Kinetic Isotope Effects. The existence of kinetic isotope effects in the MhpC reaction was examined using steady-state kinetics. Values of v_{max} were measured for substrate **2** in 100% ^2H_2O using lyophilized enzyme as mentioned above, and for [3,5-^{2}H]**2** prepared as mentioned above. The results shown in Table 4 indicate that there is a measurable D_2O solvent isotope effect on v_{max} of 1.42 for both [^{1}H]**2** and [3,5-^{2}H]**2**. Attempts to investigate a possible secondary isotope effect on v_{max} using [3,5-^{2}H]**2** gave isotope effects of 1.1 ± 0.1 in both H_2O and D_2O, suggesting that there may be a small secondary isotope effect. A K_m value of 4.4 μM was measured for [3,5-^{2}H]**2** in ^2H_2O, indicating a further solvent isotope effect on K_m; however, we were unable to obtain consistent K_m values for **2** in ^2H_2O or [3,5-^{2}H]**2** in ^1H_2O, perhaps due to the simultaneous isotope exchange processes taking place. The observed solvent isotope effect is difficult to interpret in isolation but would be consistent with either a rate-determining proton transfer or base-catalyzed attack of water.

Table 4: Steady-State Kinetic Isotope Effects Observed for the MhpC-Catalyzed Reaction[a]

substrate	v_{max} (nmol min^{-1})	$k(H_2O)/k(D_2O)$	k_H/k_D
[^{1}H]**2** in ^1H_2O	87.9 ± 4		
[^{1}H]**2** in ^2H_2O	61.9 ± 3	1.42 ± 0.1	
[3,5-$^2H_2$]**2** in ^1H_2O	79.5 ± 4		1.11 ± 0.1
[3,5-$^2H_2$]**2** in ^2H_2O	56.1 ± 2	1.42 ± 0.1	1.10 ± 0.1

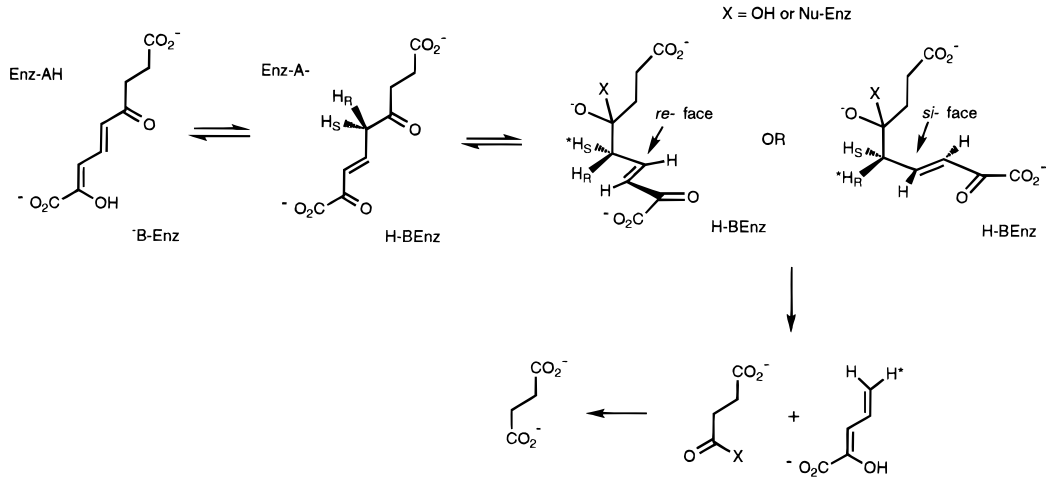
[a] Assays carried out using continuous UV assay as described in Materials and Methods.

DISCUSSION

C−C hydrolase MhpC has been purified to near homogeneity, revealing that the purified enzyme is a 29 kDa homodimer. The purified enzyme possesses comparable activity using the succinate release assay or monitoring substrate consumption at 394 nm, indicating that a single polypeptide is responsible for the entire MhpC reaction, as

[3] Since the MhpC enzyme was added in 10 μL of ^1H_2O, the second experiment contained 1.4% ^1H_2O (or 2.8% HOD). During the nonenzymatic ketonization reaction (Figure 5, process A), incorporation of ^{1}H into the keto intermediate would be favored over ^{2}H by a solvent kinetic isotope effect and hence would reduce the apparent rate of ^{2}H exchange. These effects could explain quantitatively the reduced k_{H-3} observed in the second experiment, but would not affect the ratio k_{H-5}/k_{H-3}.

Scheme 6: Proposed Mechanistic Scheme for the MhpC-Catalyzed Reaction, Illustrating the Two Possible Stereochemical Courses[a]



[a] The hydrogen atom incorporated from solvent is indicated by H*

opposed to the possibility of separate isomerase and hydrolase activities. The observation that none of the group specific reagents tested were able to diminish succinate release without also diminishing substrate consumption suggests that a single active site is responsible for the whole transformation. The enzyme is highly specific for the propionate side chain, and pH/rate studies suggest the involvement of an active site base of pK_a = 6.5 and an active site acid of pK_a = 9.5.

We have shown formally the addition of the elements of water across the C-5/C-6 carbon−carbon bond: the present study has shown the regiospecific incorporation of ^1H from ^1H_2O or ^2H from ^2H_2O into the product **3**, while separate studies into the mechanism of the preceding dioxygenase MhpB have shown that one atom of ^{18}O is incorporated by MhpC from $H_2{}^{18}O$ into the succinate carboxylate (Sanvoisin et al., 1995).

The stereochemistry of the reaction has been shown to proceed via overall replacement of a succinyl group by a proton from solvent water with retention of regiochemistry. A mechanistic interpretation of these results is shown in Scheme 6. Since there are two steps in the mechanism which define the overall stereochemistry, the observed stereochemical outcome is consistent either with protonation at the *proR* position at C-5 of the proposed keto-intermediate **4**, followed by fragmentation onto the *si* face of the C-3/C-4 double bond, or protonation at the *proS* position at C-5 of keto-intermediate **4**, followed by fragmentation onto the *re* face of the C-3/C-4 double bond.

We have previously established that upon large scale isolation of ring fission product **2**, only the dienol form is seen by ^1H NMR spectroscopy (Lam & Bugg, 1994), suggesting that the dienol tautomer is thermodynamically more stable than the keto tautomer (**4**). The isotope exchange data observed at H-5_Z in the deuteriated products (**3**) arising from the ^2H_2O conversion of **2** and the ^1H_2O conversion of [3,5-$^2H_2$]**2** by MhpC imply that this enzyme is able to generate and release significant amounts of the keto-intermediate (**4**) which re-enolises in solution giving rise to isotope exchange at H-5 of **2**. Furthermore, we have demonstrated directly that MhpC is able to catalyze ^2H exchange at H-5 of **2** in ^2H_2O. Our results suggest that the initial ketonization step is fast compared with the subsequent C−C fragmentation step and that the rate of release of intermediate **4** is comparable with the rate of subsequent processing, implying that this enzyme is quite leaky with respect to the keto-intermediate (**4**). Experiments described in the following paper in this issue provide presteady-state kinetic data to confirm this hypothesis (Henderson & Bugg, 1997).

The subsequent fragmentation step could in theory take place upon attack of either water or an active site nucleophile at C-6 of **4**. Sequence alignments and site-directed mutagenesis of other C−C hydrolases have implicated an active site serine residue which could form a covalent acyl enzyme intermediate (Diaz & Timmis, 1995; Ahmad et al., 1995). Attempts to obtain evidence for this acyl enzyme intermediate in the MhpC reaction using hydroxylamine trapping and alternate substrates have in our hands proved unsuccessful. However, this could be due to inaccessibility to solvent reagents. The observed ^2H_2O solvent isotope effect could be due to the rate-determining attack of water.

These studies confirm that enzymatic cleavage of the carbon−carbon bond requires the presence of an internal electron sink, in the form of the unsaturated ketone moiety of the keto-intermediate. The following paper in this describes detailed presteady state kinetic measurements to support the proposed mechanism.

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REFERENCES

Ahmad, D., Fraser, J., Sylvestre, M., Larose, A., Khan, A., Bergeron, J., Juteau, J. M., & Sondossi, M. (1995) *Gene 156*, 69−74.

Baddar, F. G., & El-Assal, L. S. (1950) *J. Chem. Soc.* 3606−3608.

Bayly, R. C., & Berardino, D. D. (1978) *J. Bacteriol. 134*, 30−37.

Bernhard, S. A., Coles, W. C., & Norwell, J. F. (1960) *J. Am. Chem. Soc. 82*, 3043−3050.

Blakley, E. R., & Simpson, F. J. (1963) *Can. J. Microbiol. 10*, 175−185.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Buck, D., & Guest, J. R. (1989) *Biochem. J. 260*, 737−747.
Bugg, T. D. H. (1993) *Biochim. Biophys. Acta 1202*, 258−264.
Burlingame, R. (1983) Ph.D. Thesis, University of Minnesota.
Burlingame, R., & Chapman, P. J. (1983) *J. Bacteriol. 155*, 113−121.
Diaz, E., & Timmis, K. N. (1995) *J. Biol. Chem. 270*, 6403−6411.
Duggleby, C. J., & Williams, P. A. (1986) *J. Gen. Microbiol. 132*, 717−726.
Ferrandez, A., Garcia, J. L., & Diaz, E., (1997) *J. Bacteriol. 179*, 2573−2581.
Henderson, I. M. J., & Bugg, T. D. H. (1997) *Biochemistry 36*, 12252−12258.
Hofer, B., Eltis, L. D., Dowling, D. N., & Timmis, K. N. (1993) *Gene 130*, 47−55.
Horn, J. M., Harayama, S., & Timmis, K. N. (1991) *Mol. Microbiol. 5*, 2459−2474.
Lam, W. W. Y., & Bugg, T. D. H. (1994) *J. Chem. Soc., Chem. Commun.* 1163−1164.
Lanzetta, P. A., Alvarez, L. J., Reihach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.
Omori, T., Sugimura, K., Ishigooka, H., & Minoda, Y. (1986) *Agric. Biol. Chem. 50*, 931−937 .
Rambaud, M., Bakasse, M., Duguay, G., & Villieras, J. (1988) *Synthesis* 564−566.
Sanvoisin, J., Langley, G. J., & Bugg, T. D. H. (1995) *J. Am. Chem. Soc. 117*, 7836−7837.
Spence, E. L., Kawamukai, M., Sanvoisin, J., Braven, H., & Bugg, T. D. H. (1996) *J. Bacteriol. 178*, 5249−5256.
Whitman, C. P., Aird, B. A., Gillespie, W. R., & Stolowich, N. J. (1991) *J. Am. Chem. Soc. 113*, 3154−3162.
Whitman, C. P., Hajipour, G., Watson, R. J., Johnson, W. H., Jr., Bembenek, M. E., & Stolowich, N. J. (1992) *J. Am. Chem. Soc. 114*, 10104−10110.

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