Catalytic Mechanism of a C−C Hydrolase Enzyme: Evidence for a *Gem*-Diol Intermediate, Not an Acyl Enzyme†

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Received October 5, 1999; Revised Manuscript Received November 22, 1999

ABSTRACT: 2-Hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid 5,6-hydrolase (MhpC) from *Escherichia coli* catalyses the hydrolytic cleavage of the extradiol ring fission product on the phenylpropionate catabolic pathway and is a member of the α/β hydrolase family. The catalytic mechanism of this enzyme has previously been shown to proceed via initial ketonization of the dienol substrate (Henderson, I. M. J., and Bugg, T. D. H. (1997) *Biochemistry 36*, 12252−12258), followed by stereospecific fragmentation. Despite the implication of an active site serine residue in the α/β hydrolase family, attempts to verify a putative acyl enzyme intermediate by radiochemical trapping methods using a ^{14}C-labeled substrate yielded a stoichiometry of <1% covalent intermediate, which could be accounted for by nonenzymatic processes. In contrast, incorporation of 5−6% of two atoms of ^{18}O from $H_2{}^{18}O$ into succinic acid was observed using the natural substrate, consistent with the reversible formation of a *gem*-diol intermediate. Furthermore, time-dependent incorporation of ^{18}O from $H_2{}^{18}O$ into the carbonyl group of a nonhydrolysable analogue 4-keto-nona-1,9-dioic acid was observed in the presence of MhpC, consistent with enzyme-catalyzed attack of water at the ketone carbonyl. These results favor a catalytic mechanism involving base-catalyzed attack of water, rather than nucleophilic attack of an active site serine. The implication of this work is that the putative active site serine in this enzyme may have an alternative function, for example, as a base.

Hydrolytic cleavage of a carbon−carbon bond adjacent to a carbonyl is a rare class of reaction in chemistry and biochemistry. Examples in organic chemistry include the haloform reaction and the base-catalyzed C−C cleavage of malonic esters (*1*). There are a small number of enzyme-catalyzed reactions which involve hydrolytic C−C cleavage, including kynureninase from *Pseudomonas fluorescens* (*2*) and dioxo acid hydrolase from beef liver (*3*). In each of these examples, an electron sink is present adjacent to the site of the departing carbanion. In the course of the bacterial meta-cleavage pathways for the degradation of aromatic compounds, ring fission products containing a δ-keto dienol are formed by oxidative cleavage of catechol substrates (*4*). These ring fission products are then substrates for hydrolytic C−C cleavage by a family of hydrolase enzymes, as illustrated in Figure 1. Members of this family of enzymes have been purified from *Pseudomonas putida* pWW0 (*5*), *P. putida* NCIB 10015 (*6*), *Pseudomonas cruciviae* (*7*), *Burkholderia cepacia* LB400 (*8*), and *Escherichia coli* (*9*). They are dimeric proteins of subunit mass 27−32 kDa, requiring no cofactors for activity.

Detailed studies of the reaction catalyzed by 2-hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid 5,6-hydrolase (MhpC)[1] on the phenylpropionate catabolic pathway of *Escherichia coli* have provided some insight into the catalytic mechanism (*9*−*11*). The overall stereochemistry of the reaction has been determined to proceed with retention of regiochemistry at C-5 (i.e., insertion of a hydrogen atom in the H-5E position) (*9*, *10*). Enzyme-catalyzed exchange of ^{2}H at C-5 of the substrate has been observed, consistent with an initial ketonization of the ring fission product (RFP) to give a keto-intermediate (RFP^K), as shown in Figure 2 (*9*). Further evidence for the existence of a discrete keto-intermediate has been obtained by pre-steady-state kinetic mesurements (*11*). The keto- intermediate contains an α,β-unsaturated ketone functional group, which serves as an electron sink for a carbanion arising from C−C cleavage.

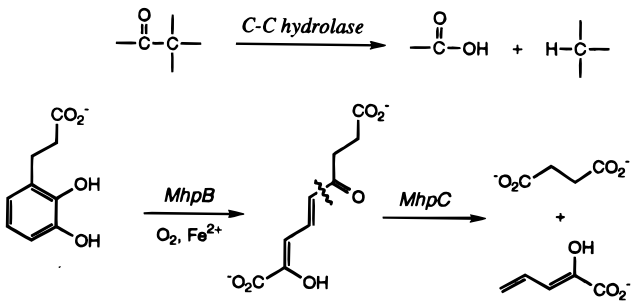

FIGURE 1: Reactions catalyzed by dioxygenase MhpB and hydrolase MhpC.

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† This work was supported by a grant (B04835) from the Biomolecular Sciences committee of BBSRC.

[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; RFP, ring fission product (2-hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid); RFP^k, ketonised ring fission product (2,6-diketo-non-3-ene 1,9-dioic acid); KNDA, 4-keto-nona-1,9-dioic acid.

10.1021/bi9923095 CCC: $19.00
Published on Web 01/19/2000

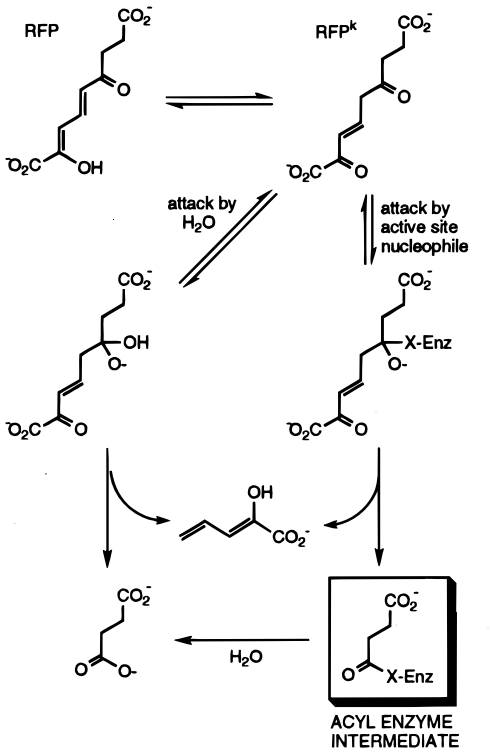

FIGURE 2: Proposed catalytic mechanisms for hydrolase MhpC, via attack of either water or an active site nucleophile upon RFP^k (see refs *9*, *11*).

Mechanistic proposals for the C−C cleavage step of MhpC have involved attack upon the C-6 carbonyl of either water or an active site nucleophile, giving rise in the latter case to a subsequent acyl enzyme intermediate (*9*−*11*). Amino acid sequence alignments imply that this family of C−C hydrolase enzymes are members of the α/*β*-hydrolase family, containing a serine catalytic triad in the order serine-aspartate-histidine as found in the amino acid sequence (*12*). Extensive sequence similarity has been detected between the C−C hydrolases and the sequences of *P. putida* atropinesterase, *Moraxella* sp. lipase 3, *Pseudomonas* sp. KWI-56 esterase V, and *Lactobacillus delbrueckii* proline iminopeptidase (*13*, *14*). A conserved sequence motif Gly-Xaa-Ser-Xaa-Gly was observed at the position of the suspected serine nucleophile, which is also found in the serine proteases (*13*, *14*). There is a large body of biochemical and crystallographic evidence collected for members of the α/*β*-hydrolase family to suggest that the active site serine group acts as a nucleophile (*15*), although acyl enzyme intermediates have only been characterized using unnatural substrates such as *p*-nitrophenyl acetate (*16*).

In the C−C hydrolase family, catalytic activity of *P. putida* XylF was lost upon treatment for 30 min with 0.5 mM phenylmethylsulfonyl fluoride or 1 mM di-isopropyl fluorophosphate (*14*). Furthermore, serine-to-alanine site-directed mutants in this position of *P. putida* XylF or *Comamonas testosteroni* BphD were found to be devoid of catalytic activity (*14*, *17*). Thus, the available biochemical evidence at the start of this work heavily favored a mechanism involving an active site serine nucleophile.

In contrast, previous studies in our laboratory had failed to provide confirmatory evidence in favor of an acyl enzyme intermediate in the MhpC-catalyzed reaction. Attempts to trap the putative acyl enzyme intermediate using hydroxylamine failed to give any hydroxamic acid product (*9*). Synthesis of *p*-nitrophenyl succinate as an alternative substrate for pre-steady-state kinetics revealed that this compound was not a substrate for MhpC (*9*). A major kinetic difference between MhpC and the serine proteases was also observed: the serine proteases are inactive at pH < 5, accumulating the acyl enzyme intermediate at their active sites (*15*, *16*); whereas MhpC was found to be fairly active at low pH, showing k_{cat} values of 3.9 and 1.0 s^{-1} at pH 5.0 and 4.0, respectively, and showed no initial burst of product release under pre-steady-state kinetic analysis (*11*).

The mechanistic relationship of the MhpC family of C−C hydrolases with the "classical" serine proteases is, therefore, an unanswered problem, the key question being whether an acyl enzyme intermediate exists in the catalytic mechanism. To resolve this question, we decided to carry out trapping experiments using a ^{14}C-labeled substrate. This paper describes the synthesis and assay of a ^{14}C-labeled substrate, and experiments using ^{18}O labeling and a nonhydrolysable substrate analogue to investigate an alternative mechanism, namely, base-catalyzed attack of water.

MATERIALS AND METHODS

General. NMR spectra were recorded on a Bruker AC300 Fourier Transform Spectrometer (300 MHz). Mass spectra and GC−MS were recorded on a VG 70−250 mass spectrometer. HPLC analysis was carried out on a Waters Associates Chromatograph using a Bio-Rad HPX-87H Organic Acids column. Scintillation counting was carried out on a Beckman LS6500 multipurpose scintillation counter, using Optiphase HiSafe 3 scintillation fluid.

[2-^{14}C]-malonic acid was purchased from Dupont NEN. Isotopically enriched water (^{18}O, 95−98 atom %) was purchased from Promochem Ltd. 2,3-Dibenzyloxybenzaldehyde was prepared by benzylation of 2,3-dihydroxybenzaldehyde, using the method of Comber et al. (*18*). 4-Keto-nona-1,9-dioic acid (KNDA) was prepared by treatment of 2-(2-cyanoethyl)-cyclohexanone with hydrogen peroxide, according to the method of Chiusoli et al. (*19*), in 30% yield: δ_H (300 MHz, D_2O) 2.68 (2H, t, J = 8 Hz), 2.43 (4H, t, J = 8 Hz), 2.22 (2H, t, J = 8 Hz), 1.4−1.5 (4H, m) ppm; δ_C (75 MHz, D_2O) 217.90, 181.27, 180.02, 44.19, 39.46, 36.05, 30.34, 26.29, 25.22 ppm; *m*/*z* (ES -ve ion) 237/239 $(M + Cl)^-$. Propiohydroxamate was prepared from methyl propionate using the method of Fishbein et al. *(20)* (mp 86−90 °C, lit (*21*) 92−93 °C). All other chemicals and biochemicals were purchased from Sigma-Aldrich.

Escherichia coli MhpC and MhpB were prepared from *E. coli* W3110/pTB9 as previously described (*11*). 2-Hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid (RFP) was prepared enzymatically as previously described (*9*).

Enzyme Kinetics. MhpC was assayed by monitoring the decrease in absorbance at 317 or 394 nm, as previously described (*11*), upon addition of enzyme to a solution of RFP in the appropriate buffer. Time-dependent inhibition of MhpC was assayed by preincubation of samples of MhpC (1 unit) with KNDA (10 mM final concentration) in 50 mM sodium citrate pH 5.0, followed by 10-fold dilution into 50 mM sodium citrate pH 5.0 containing ring fission product (50 μg), and monitoring of the decrease in absorbance at 317 nm.

*[2-^{14}C]-3-(2,3-Dibenzyloxyphenyl)-propenoic acid (**1**).* A solution of [2-^{14}C]-malonic acid (250 μCi) in pyridine (2 mL) was added to 2,3-dibenzyloxybenzaldehyde (0.30 g), followed by the addition of a solution of unlabeled malonic acid (98 mg) in pyridine (5 mL). The mixture was heated to 50 °C to dissolve the reagents, then piperidine (0.3 mL) was added. The mixture was heated to 80 °C for 1 h, then heated at reflux for 3 h. The reaction mixture was cooled, then poured into cold water (20 mL), and acidified (concd HCl) to pH 1. The precipitate was filtered, washed with water, and dried. Recrystallization from aqueous ethanol yielded **1** as a white crystalline solid (245 mg, 72%). A specific activity of 0.19 μCi/mole was determined by scintillation counting (total 129 μCi, radiochemical yield 52%). R_f (1:1 EtOAc/petroleum ether) 0.48; mp 190−192 °C; IR (Nujol mull) 3000−2600, 1692, 1632, 1573 cm^{-1}; δ_H (300 MHz, d$_6$-DMSO) 7.91 (2H, d, J = 16.0 Hz, ArC*H*═C), 7.40 (2H, dd, J = 8.0, 1.5 Hz), 7.32 (4H, m), 7.24 (4H, m), 7.13 (1H, t, J = 5.5 Hz), 7.01 (2H, d, J = 5.5 Hz), 6.35 (1H, d, J = 16.0 Hz, C═C*H*CO$_2$H), 5.10 (2H, s), 4.95 (2H, s) ppm; δ_C (75 MHz, d$_6$-DMSO) 168.1, 151.9, 146.7, 136.6, 136.3, 128.9, 128.3, 128.2, 128.0, 127.7, 127.2, 124.1, 119.9, 119.1, 115.2, 75.0, 70.5 ppm; *m*/*z* (FAB) 361 (MH$^+$, 14%), 91 (100%).

*2,3-Dibenzyloxy-1-([1-^{14}C]-ethenyl)-benzene (**2**).* To a solution of the ^{14}C-labeled acid **1** (169 mg, 89 μCi) in quinoline (5 mL) was added copper (I) oxide (42 mg) under a nitrogen atmosphere. The reaction mixture was heated at 180 °C for 4 h. After the reaction mixture was cooled, the reaction mixture was diluted with diethyl ether (5 mL), filtered, and the precipitate was washed with ether. The combined ether fractions were washed with 2 M HCl (10 mL), 2 M NaOH (10 mL), water (3 × 10 mL), brine (10 mL), then dried ($MgSO_4$) and evaporated under reduced pressure. The resulting oil was purified by silica column chromatography (petroleum ether → 9:1 petroleum ether/EtOAc) to give **2** as an orange oil (80 mg, 50%, 43 μCi). R_f (10% EtOAc/petroleum ether) 0.48; IR (liquid film) 1574, 1496 cm^{-1}; δ_H (300 MHz, CDCl$_3$) 7.5−7.3 (10H, m), 7.19 (1H, dd, J = 8.0, 1.5 Hz), 7.10 (1H, dd, J = 17.5, 11.0 Hz, ArC*H*═C), 7.05 (1H, t, J = 8.0 Hz), 6.94 (1H, dd, J = 8.0, 1.5 Hz), 5.77 (1H, dd, J = 17.5, 1.5 Hz, C═C*H*$_2$), 5.29 (1H, dd, J = 11.0, 1.5 Hz, C═C*H*$_2$), 5.16 (2H, s), 5.03 (2H, s) ppm; δ_C (75 MHz, CDCl$_3$) 152.4, 146.2, 137.8, 137.2, 132.6, 131.5, 129.6, 128.7, 128.6, 128.5, 128.1, 127.7, 124.3, 118.4, 115.3, 113.8, 75.4, 71.1 ppm; *m*/*z* (EI) 316 (M$^+$, 9%), 225 (20%), 91 (100%); HRMS (*m*/*z*):[M + H]$^+$ calcd for $C_{22}H_{20}O_2$, 316.1463; found, 316.1447.

*2,3-Dihydroxy-1-([1-^{14}C]-ethyl)-benzene (**3**).* To a degassed solution of ^{14}C-styrene **2** (80 mg, 43 μCi) in THF (5 mL) was added 10% palladium/charcoal (16 mg). The reaction vessel was purged with nitrogen, then purged with hydrogen, and left under an atmosphere of hydrogen for 4 h at room temperature. The reaction mixture was filtered through Celite, then concentrated in vacuo to give a brown oil. The oil was dissolved in anhydrous aetonitrile (2 mL), and dried sodium iodide (150 mg) was added. Freshly distilled trimethylsilyl chloride (0.15 mL) was added dropwise, and the reaction was heated at reflux for 9 h. The reaction mixture was cooled, then diluted with water (4 mL), and the product was extracted into diethyl ether (3 × 5 mL). The ether fractions were washed with 10% sodium thiosulfate (2 × 10 mL), water (3 × 10 mL), and brine (10 mL), then dried ($MgSO_4$) and evaporated under reduced pressure to give a brown oil. Purification by silica column chromatography (15% EtOAc/petroleum ether) gave **3** as a red/brown oil (26 mg, 76%, 32 μCi). The final specific activity was determined to be 0.18 μCi/mole. The sample was found to darken upon storage under nitrogen and was re-purified prior to reuse. R_f (15% EtOAc/petroleum ether) 0.23; IR (liquid film) 3406, 1594 cm^{-1}; δ_H (300 MHz, CDCl$_3$) 6.75 (3H, s, Ar*H*), 4.7 (2H, br s, O*H*), 2.65 (2H, q, J = 7.5 Hz, ArC*H*$_2$), 1.25 (3H, t, J = 7.5 Hz, C*H*$_3$) ppm; δ_C (75 MHz, CDCl$_3$) 143.0, 142.0, 129.0, 121.2, 120.2, 113.0, 23.0, 14.2 ppm; *m*/*z* (EI) 138 (M$^+$, 48%), 123 (100%).

*Preparation of ^{14}C-labeled Ring Fission Product (**4**).* ^{14}C-Labeled ring fission product **4** (0.4 μmol, 160 000 cpm) was prepared by treatment of ^{14}C-ethyl catechol (**3**) (100 μL of 3 mg/mL solution) with MhpB (0.5 units) in 50 mM potassium phosphate pH 8.0, giving a bright yellow solution. For trapping experiments at pH 8.0, this solution was used immediately. For trapping experiments at pH 4.0, the solution was acidified to pH 1, and **4** was extracted into ethyl acetate (3 × 5 mL). The organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a brown oil, which was resuspended in 50 mM sodium citrate buffer pH 4.0.

Rapid-quench Trapping Experiments at pH 8.0. The rapid-quench apparatus consisted of 3 × 1 mL plastic syringes connected via a central plunger. The output from syringes 1 and 2 was mixed via a three-way joint, leading via a 20 cm length piece of tubing to a further three-way joint, which mixes with the eluent from syringe 3. Syringe 1 was loaded with MhpC (29 units, 1 mL), which had been dialyzed overnight into the appropriate buffer (50 mM potassium phosphate, pH 8.0, or 50 mM sodium citrate, pH 4.0). Syringe 2 was loaded with ^{14}C-labeled ring fission product, prepared as described above. Syringe 3 was filled with 8 M urea. The three syringes were emptied at 1 mL/s (mixing time 100 ms), and the quenched eluent was collected. The mixture was loaded onto a G50 sephadex gel filtration column (30 × 2 cm), which was eluted with 50 mM sodium citrate pH 4.0. Fractions were analyzed for radioactivity and residual MhpC activity. MhpC-containing fractions were pooled, and the ^{14}C stoichiometry determined by scintillation counting and Bradford protein assay (*22*).

5% Trichloroacetic Acid (TCA) Precipitation Experiments. MhpC (30 units, 1 mg) was dialyzed overnight into either 50 mM potassium phosphate, pH 8.0, or 50 mM sodium citrate, pH 4.0. This was added to a solution of ^{14}C-labeled ring fission product (in either 50 mM potassium phosphate, pH 8.0, or 50 mM sodium citrate, pH 4.0). Enzyme and substrate were incubated for a set period of time at 20 °C, at which point 100 μL of ice-cold 100% TCA solution (final concentration 5%) was added, to precipitate the protein. The samples were left on ice for 30 min, then centrifuged at 13 000 rpm for 5 min in a microcentrifuge. The supernatant was removed, and the pellet was washed with 5% TCA (8 times). A sample of wash was counted in order to check removal of soluble ^{14}C label. The final protein pellet was resuspended in 50 mM potassium phosphate, pH 8.0, and counted for ^{14}C label. Controls using bovine serum albumen or lysozyme were carried out in the same way.

Chemical Treatment of Putative Acyl Enzyme Intermediates. Treatment with NaOH. Selected protein pellets from the 5% TCA trapping experiments were dissolved in water

(5 mL), adjusted to pH 11 by addition of 2 M NaOH, and left overnight at room temperature. The solutions were acidified to pH 1, and labeled products were extracted into diethyl ether (3 × 10 mL). The ether extracts were dried (Na_2SO_4) and evaporated at reduced pressure. The samples were then analyzed on a Bio-Rad Organic Acids HPLC column, eluting in 5 mM H_2SO_4 at 0.6 mL/min. Fractions corresponding to ^{14}C-propionic acid (t_R = 21 min) were collected and counted for ^{14}C label. *Treatment with Hydroxylamine.* Selected protein pellets were dissolved in 50 mM sodium citrate, pH 4.0, containing 250 mM hydroxylamine (2 mL) and were left overnight at room temperature. The protein was reprecipitated by addition of 100 μL of 100% TCA solution, followed by centrifugation. The supernatant was then freeze-dried. The resulting solid was resuspended in 5 mM H_2SO_4 and analyzed on a Bio-Rad Organic Acids HPLC column, as above. Fractions corresponding to ^{14}C-propiohydroxamate (t_R = 32 min) were collected and counted for ^{14}C label.

Chemical Treatment of ^{14}C-labeled Ring Fission Product. Samples of ^{14}C-labeled ring fission product (160 000 cpm) were treated either with 50 mM sodium citrate, pH 4.0, containing 250 mM hydroxylamine, or with an aqueous sodium hydroxide solution at pH 11, overnight at room temperature. Samples were freeze-dried, then resuspended in 5 mM H_2SO_4 and analyzed by Organic Acids HPLC as above. Samples of ^{14}C-labeled ring fission product (160 000 cpm) were also treated with MhpC (20 units) in 50 mM potassium phosphate, pH 8.0, in order to generate sodium ^{14}C-propionate. Incubations were acidified to pH 1, extracted into ethyl acetate (5 mL). The organic phase was dried (Na_2SO_4) and evaporated at reduced pressure to give ^{14}C-propionic acid. Samples of ^{14}C-propionic acid were treated with 50 mM sodium citrate, pH 4.0, containing 250 mM hydroxylamine overnight at room temperature. Samples were freeze-dried, then resuspended in 5 mM H_2SO_4 and analyzed by Organic Acids HPLC as above.

Base-catalyzed Cleavage of ^{14}C-3-Ethyl Catechol. ^{14}C-Labeled ring fission product (20 000 cpm), prepared as above, was dissolved in 2 M NaOH (1 mL), and incubated at 25 °C; 100 μL aliquots were removed at the intervals from 0 to 48 h and immediately quenched with 2 M sulfuric acid (60 μL) and frozen at −20 °C. The collected samples were made up to 300 μL volume in 5 mM sulfuric acid and spiked with a 50% solution of propionic acid (1 μL). The samples were analyzed on a Bio-Rad HPX-87H Organic Acids column, eluting with 5 mM sulfuric acid at 0.6 mL/min. The peak corresponding to propionic acid (t_R = 20 min) was collected, and a sample counted for ^{14}C.

Incorporation of $H_2{}^{18}O$ into MhpC Reaction Products. Samples of 2-hydroxy-6-keto-nona-2,4-diene-1,9-dioic acid (5 μg, 23 nmole) were applied to microcentrifuge tubes by evaporation from ethanol solution. To each sample was added 110 μL of 95% $H_2{}^{18}O$ and 10 μL of 1 M potassium phosphate buffer (pH 8.0) prepared in $H_2{}^{18}O$. The samples were incubated for time intervals of 0, 120, and 300 s. Aliquots of MhpC (2 units), which had been freeze-dried and resuspended in $H_2{}^{18}O$, were added and left for 60 s to ensure complete conversion (reaction was visibly completed in 1−2 s as judged by the disappearance of the yellow substrate). The mixture was then acidified to pH 1 by addition of 10 μL of 2 M HCl, and the sample immediately frozen on dry ice. The samples were freeze-dried, and the resulting solids taken up in methanol (200 μL). The solutions were then treated with an ethereal solution of diazomethane. After 10 min, excess diazomethane was destroyed by dropwise addition of acetic acid. Samples were then centrifuged (13 000 rpm), and the supernatant evaporated by a stream of nitrogen gas.

Samples were then analyzed by GC−MS using chemical ionization conditions and selected ion monitoring. For each sample, a series of peaks were obtained for $[M+1]^+$, $[M+3]^+$, and $[M+5]^+$ ion currents for dimethyl succinate (M_r 146), retention time of 8.36 min on a BPX5 25 m column with an internal diameter of 0.22 mm. The following temperature gradient was applied: 35 °C for 3 min; heated to 340 °C at 20 °C/min; 340 °C for 3 min. The observed $[M+5]^+$ intensities were corrected for the natural background isotope pattern for dimethyl succinate ($C_6H_{11}O_4$) by subtraction of 1.032% of the $[M+3]^+$ intensities. The total percentage incorporation was calculated, and the corrected $[M+5]^+$ intensities expressed as a percentage of this figure.

^{18}O Incorporation into KNDA. MhpC (5.0 mg) was freeze-dried and resuspended in 230 μL $H_2{}^{18}O$ (>98 atom % ^{18}O), containing ^{18}O-labeled potassium phosphate buffer, pH 7.0 (50 mM), and KNDA (0.5 mg). A control incubation was also set up, containing no MhpC. At time intervals of 0.5, 15, 30, and 60 min, 50 μL aliquots were withdrawn and treated with a solution of sodium borohydride (1 mg) in ethanol (0.5 mL). Samples were then acidified by addition of formic acid (2 drops) and freeze-dried. The resulting solids were treated with an ethereal solution of diazomethane for 10 min, then excess diazomethane was destroyed by dropwise addition of acetic acid. Solutions were evaporated, then resuspended in hexane and analyzed by GC−MS (electron impact ionization). GC was carried out on a BP1 25 m column with an internal diameter of 0.22 mm. The following temperature gradient was applied: 35 °C for 5 min; heated to 300 °C at 20 °C/min; 300 °C for 5 min. Peaks corresponding to the seven-membered lactone and five-membered lactone were observed at 1230 and 1246 s, respectively. The five-membered lactone (*m*/*z* 169 (30%), 150 (20%), 115 (30%), 85 (100%), 55 (25%)) showed a major fragment ion at *m*/*z* 85, which showed clear incorporation of ^{18}O vs time. Selected ion monitoring of this peak at *m*/*z* 85 and 87 was used to determine the percentage ^{18}O incorporation.

RESULTS

Synthesis of a ^{14}C-labeled Substrate. To investigate the possibility of an acyl enzyme intermediate in the MhpC catalytic mechanism, synthesis of a ^{14}C-labeled substrate that would react with MhpC to give a ^{14}C-labeled acyl enzyme intermediate was undertaken. Extensive work on the serine protease α-chymotrypsin has demonstrated that the acyl enzyme intermediate of this and other serine hydrolases can be quantitatively trapped under acidic conditions (*23*, *24*). Indeed, the X-ray crystal structure of an acyl enzyme intermediate of α-chymotrypsin has been determined (*25*). Previous stopped flow UV analysis of the MhpC-catalyzed reaction had revealed that turnover of the MhpC-catalyzed reaction occurs under acidic conditions, but that under these conditions, the release of the acyl equivalent succinate is rate-limiting (*11*). We, therefore, reasoned that trapping

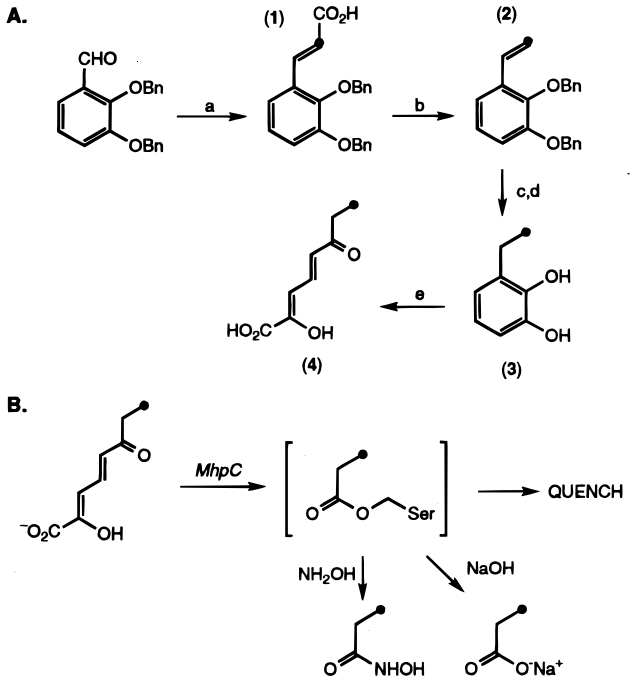

FIGURE 3: (A) Synthetic route for ^{14}C-labeled 3-ethyl catechol (**3**). Reagents and yields: a, [2-^{14}C]-malonic acid, piperidine, pyridine, 72%; b, Cu_2O, quinoline, 180 °C, 50%; c, H_2, Pd/C, THF; d, Me_3-SiCl, NaI, MeCN, 76% overall; e, MhpB, 50 mM potassium phosphate pH 8.0. B. Scheme for trapping of putative acyl enzyme intermediate by ^{14}C-labeled substrate.

studies using a ^{14}C-labeled substrate under acidic conditions should give a good yield (>50%) of a trapped acyl enzyme intermediate, if present.

To discourage the hydrolysis of such an acyl enzyme intermediate, we decided to use a substrate analogue containing an ethyl side chain in place of the natural propionate side chain. It has been found previously that substrates containing alkyl side chains are converted at lower catalytic efficiency by MhpC (*9*). 3-Ethyl catechol is known to be an efficient substrate for MhpB-catalyzed extradiol ring cleavage (K_m = 185 μM, k_{cat} = 34 s^{-1}) (*26*). The ethyl side chain ring fission product was not stable toward isolation, but could be converted in situ by addition of hydrolase MhpC, with a turnover number of 9 s^{-1} at pH 8.0 (K_m = 7.5 μM). The synthetic route to ^{14}C-labeled 3-ethyl catechol is shown in Figure 3A. Knoevenagel condensation of 2,3-dibenzyloxybenzaldehyde with [2-^{14}C]-malonic acid gave the 2,3-disubstituted cinnamic acid (**1**), which was decarboxylated to give the ^{14}C-labeled styrene **2**. Hydrogenation of the alkene side chain and debenzylation gave ^{14}C-3-ethyl catechol (**3**) with a specific activity of 0.19 μCi/μmole.

Radiochemical Trapping Experiments. ^{14}C-Labeled ring fission product **4** was generated in situ using dioxygenase MhpB and was used for a series of trapping experiments with samples of purified hydrolase MhpC, under a variety of mixing and quenching conditions, as shown in Table 1. Mixtures from rapid quench experiments were applied to a Sephadex G-75 gel filtration column, and the eluted sample of protein was analyzed for radioactivity by scintillation counting. Trapping was also carried out by precipitation of samples with 5% trichloroacetic acid, followed by repeated washing (5 or 6 times) with further 5% trichloroacetic acid until washings contained no ^{14}C label, followed by scintillation counting of the precipitate. The results are shown in Table 1.

Turnover of **4** at pH 8.0 by MhpC proceeds with a turnover number of 9 s^{-1}, hence rapid quench experiments were carried out using a mixing time of 100 ms. Mixing at pH 8.0 followed by trapping at pH 5.0 or 4.0 (expts 1, 2) gave no enzyme-bound ^{14}C label. Trapping via denaturation with 8 M urea (expt 3), or TCA precipitation (expt 4), gave a small amount of enzyme-bound ^{14}C label, both yielding a stoichiometry of 0.09 mol ^{14}C/mole enzyme subunit. However, a control experiment using bovine serum albumen in place of MhpC (expt 5) also gave a similar stoichiometry of 0.07 mol ^{14}C label/mole enzyme subunit after gel filtration, under identical conditions.

To increase the likelihood of trapping the putative intermediate, the mixing of enzyme and substrate was carried out at pH 4.0, under which conditions earlier pre-steady-state kinetic experiments using the natural substrate have shown that conversion of the succinyl−enzyme intermediate to enzyme + succinate is the rate-determining step (*11*). Under these conditions, the substrate **4** is processed very slowly by MhpC (k_{cat} = 0.2 min^{-1}). Using the 5% TCA precipitation method, at quench times of 1−10 min, stoichiometries of 0.05−0.11 mol ^{14}C/mole enzyme subunit were obtained (expts 6−10). Control experiments using the protein lysozyme in place of MhpC, under identical conditions, gave stoichiometries of 0.06−0.08 mol ^{14}C/mole enzyme subunit (expts 11−13). Quenching the MhpC reaction using 8 M urea under these conditions gave a very low stoichiometry of 0.01 mol ^{14}C/mole enzyme subunit (expt 14).

In view of the low level of enzyme-bound label observed, and the results of control experiments, it was necessary to carry out further experiments to establish whether the observed enzyme-bound ^{14}C label was due to the trapping of an acyl enzyme intermediate, or due to nonspecific labeling arising from hydrophobic or ionic aggregation. If the trapped label were due to an acyl enzyme intermediate, then treatment with sodium hydroxide or hydroxylamine would generate sodium ^{14}C-propionate or ^{14}C-propionic hydroxamate, respectively, which could be identified by HPLC analysis (see Figure 3B).

Accordingly, samples of precipitated, ^{14}C-labeled MhpC were treated with either 2 M sodium hydroxide or 0.25 M hydroxylamine for 16 h. Samples were then analyzed by Organic Acids HPLC, and peaks corresponding to authentic propionic acid (t_R = 21 min) and propionic hydroxamate (t_R = 32 min) were collected and counted. The results are shown in Table 2. ^{14}C Label was detected at low levels for both propionic acid (8−12% of applied label, 0.4−1.3% overall yield) and propionic hydroxamate (6−9% of applied label, 0.3−1.0% overall yield). The low proportion of counts observed indicated that much of the original enzyme-bound ^{14}C label was indeed due to nonspecific labeling (the major ^{14}C-labeled species identified by HPLC was in fact unreacted ^{14}C-3-ethyl catechol (**3**), which accounted for 10−20% of applied label). Thus, at best, only 12% of observed ^{14}C label could be due to a propionyl−enzyme species, corresponding to an overall yield of approximately 1%.

To account for the observed trapped ^{14}C label, further control experiments were carried out in which ^{14}C-ring fission product was treated in the absence of MhpC with either 2 M sodium hydroxide or 0.25 M hydroxylamine. HPLC

Table 1: Stoichiometry of ^{14}C Labelling of MhpC by ^{14}C-labeled Substrate **4** ($^{14}C-RFP$)[a]

expt	mixing conditions	quench	mixing time	stoichiometry (mole ^{14}C per mole enz subunit)
1	MhpC + $^{14}C-RFP$, 50 mM phosphate pH 8.0	50 mM citrate pH 5.0	100 ms	0.00
2	As 1	50 mM citrate pH 4.0	100 ms	0.00
3	As 1	8 M urea	100 ms	0.09
4	As 1	5% trichloroacetic acid	4 s	0.09
5	BSA + $^{14}C-RFP$, 50 mM phosphate pH 8.0	5% trichloroacetic acid	4 s	0.07
6	MhpC + $^{14}C-RFP$, 50 mM citrate pH 4.0	5% trichloroacetic acid	1 min	0.05
7	As 6	5% trichloroacetic acid	2 min	0.08
8	As 6	5% trichloroacetic acid	5 min	0.07
9	As 6	5% trichloroacetic acid	7 min	0.05
10	As 6	5% trichloroacetic acid	10 min	0.11
11	Lysozyme + $^{14}C-RFP$, 50 mM citrate pH 4.0	5% trichloroacetic acid	5 min	0.06
12	As 11	5% trichloroacetic acid	7 min	0.08
13	As 11	5% trichloroacetic acid	10 min	0.08
14	As 6	8 M urea	7 min	0.01

[a] Experiments carried out as described in Materials and Methods. Each incubation contained 30 units MhpC (1 mg, 35 nmol) and 0.4 μmol **4**, specific activity 0.19 μCi/μmol. Experiment 5 contained 1 mg BSA; experiments 11−13 contained 1 mg lysozyme. BSA, bovine serum albumen.

Table 2: HPLC Analysis for ^{14}C-Propionic Acid and ^{14}C-Propionic Hydroxamate, Obtained from Chemical Treatment of Putative Enzyme-linked Intermediates, ^{14}C-labeled **4** ($^{14}C-RFP$), and ^{14}C-Propionic Acid[a]

		^{14}C-propionic acid[b]		^{14}C-propionic hydroxamate[b]	
reagents	total ^{14}C[a] (cpm)	(cpm)	% yield	(cpm)	% yield
ENZ-INT (ppt from Expt 9) + 2 M NaOH	2046	246	[12%]		
ENZ-INT (ppt from Expt 10) + 2 M NaOH	4428	333	[8%]		
ENZ-INT (ppt from Expt 10) + 0.25 M NH_2OH, pH 4.0	1508	47	[3.1%]	129	[8.6%]
ENZ-INT (ppt from Expt 10) + 0.25 M NH_2OH, pH 4.0	1379	51	[3.4%]	91	[6.6%]
$^{14}C-RFP$ + 2 M NaOH	30654	989	[3.2%]		
$^{14}C-RFP$ + 0.25 M NH_2OH, pH 4.0	72425	456	[0.6%]	979	[1.4%]
^{14}C-propionic acid + 0.25 M NH_2OH, pH 4.0	400	150	[37.5%]	250	[62.5%]

[a] Total amount of ^{14}C-label applied to the HPLC column. [b] Analysis was carried out on a Bio-Rad HPX-87H Organic Acids column, eluted with 5 MM H_2SO_4 at 0.6 mL/min. Retention times: propionic acid, 21 min; propionic hydroxamate, 32 min.

analysis (see Table 2) revealed small amounts of ^{14}C-propionic acid (3.2% yield) and ^{14}C-propionic hydroxamate (1.4% yield), respectively, indicating that there are background nonenzymatic reactions with the ^{14}C-ring fission product that can quantitatively account for the ^{14}C-propionic acid and ^{14}C-propionic hydroxamate observed above. In addition, treatment of ^{14}C-propionic acid with hydroxylamine at pH 4.0 gave a 62% yield of ^{14}C-propionic hydroxamate.

In conclusion, these radiochemical trapping experiments gave a low stoichiometry of trapped ^{14}C label, which can be quantitatively attributed either to nonspecific aggregation or to background nonenzymatic processes. Earlier pre-steady-state kinetic analysis of the MhpC-catalyzed reaction have shown that at pH 4 the breakdown of the enzyme−product complex is rate-determining (*11*), hence the putative acyl enzyme intermediate should accumulate under these conditions, yet <1% stoichiometry was observed under the same conditions using a sensitive radiochemical method. These results, therefore, do not support the existence of a covalent acyl enzyme intermediate in the MhpC catalytic cycle.

The observed hydroxide ion-catalyzed cleavage of the ^{14}C-ring fission product was an unexpected finding, and it provides chemical precedent for a cleavage mechanism involving base-catalyzed attack of water onto the keto-intermediate. To investigate further this reaction, samples of ^{14}C-ring fission product were incubated with 2 M sodium hydroxide for varying lengths of time, followed by acidification and analysis by Organic Acids HPLC. The reaction yielded 710 cpm ^{14}C-propionic acid after 1 h, 1180 cpm after 2 h, rising slowly to 1840 cpm after 24 h, indicating a slow, nonlinear but time-dependent reaction. The nonlinearity of the reaction is probably due to the chemical instability of RFP at high pH. The initial rate of production of ^{14}C-propionic acid over the first hour can be calculated as 4.9×10^{-9} M^{-1} s^{-1}. Since the catalytic efficiency of the MhpC-catalyzed reaction is 1.7×10^7 $M^{-1}s^{-1}$ for its natural substrate[9] and 1.1×10^6 $M^{-1}s^{-1}$ for **4**, the enzyme accelerates the rate of nonenzymatic C−C cleavage by a factor of $>10^{14}$.

$H_2{}^{18}O$ Incorporation Experiments. The alternative mechanism for C−C cleavage involves base-catalyzed attack of water upon RFP^K (see Figure 2), which would generate a *gem*-diol intermediate, fragmenting with C−C cleavage to give succinic acid directly (*9*). This type of intermediate is reminiscent of the amide hydrate intermediate believed to exist in the catalytic mechanism of the aspartic proteases (*27*). Evidence for the existence of an amide hydrate intermediate in the reaction catalyzed by the HIV protease has been obtained by exchange with $H_2{}^{18}O$, i.e., the reversible formation of an amide hydrate intermediate, and the occasional cleavage of a $C-{}^{16}O$ bond in the reverse reaction, leading to incorporation of 5−10% ^{18}O from $H_2{}^{18}O$ (*27*).

This type of ^{18}O exchange experiment could also be applied to the MhpC-catalyzed reaction in which case a small incorporation of two atoms of ^{18}O would be observed in the product succinic acid, as illustrated in Figure 5. In fact, studies involving enzymatic processing of 2,3-dihydroxyphenylpropionic acid by MhpB/MhpC in $H_2{}^{18}O$ (50 atom % ^{18}O) have already been carried out in our laboratory, to analyze the mechanism of dioxygenase MhpB (*28*). In those experiments, analysis of the succinic acid product as either its dimethyl ester or di-trimethylsilyl ester had shown, in addition to the expected M+2 peak, a small M+4 peak at

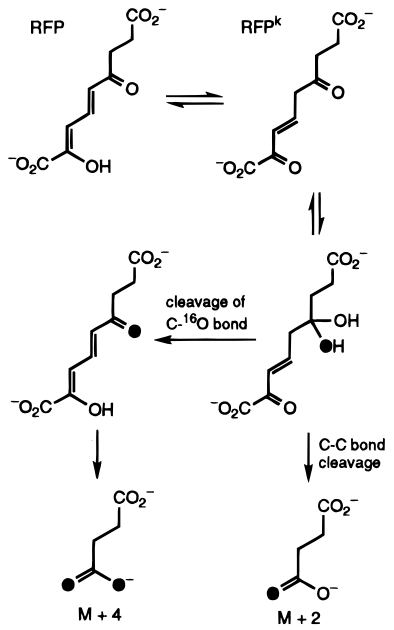

FIGURE 4: Enzymatic processing of RFP by MhpC in the presence of $H_2{}^{18}O$ to generate singly (M+2) and doubly (M+4) labeled succinic acid products.

the level of approximately 10% incorporation. In our previous paper, we had assigned this peak to a small amount of nonenzymatic exchange at the C-6 ketone of RFP, prior to enzymatic conversion by MhpC (*23*). We, therefore, resolved to repeat these experiments, to distinguish between nonenzymatic exchange in the carbonyl vs MhpC-mediated exchange.

Accordingly, samples of ring fission product were suspended in ^{18}O-containing buffer (at 95 atom % ^{18}O) for varying lengths of time, prior to enzymatic conversion by addition of MhpC (resuspended in ^{18}O-containing buffer). Enzymatic conversion could be observed visually, due to the yellow color of RFP at pH 8, and proceeded in 1−2 s upon addition of enzyme. Samples were then frozen, lyophilised, derivatized using diazomethane, and analyzed by GC−MS, using secondary ion monitoring to determine accurate peak height intensities. The results shown in Table 3 have been adjusted to subtract background peak height due to natural abundance isotopic peaks (^{13}C, ^{2}H, etc.) of the parent molecular ion. In each case, after subtraction of natural abundance peaks, there remained a small additional M+4 peak corresponding to an incorporation of 5−6%, confirming our earlier observation (*28*). There was no significant variation in percentage incorporation vs preincubation time, indicating that nonenzymatic exchange at the C-6 ketone does not occur on this time scale.

Although the level of M+4 incorporation is relatively small, it is a reproducible result, and is significantly above background M+4 levels. The low level of ^{18}O exchange is not surprising, as exchange requires a mis-processing of the *gem*-diol intermediate, i.e., cleavage of a C−^{16}O bond, rather than a C−^{18}O bond, or its release from the enzyme active site and dehydration in solution (see Figure 4). It is documented that additional ^{18}O exchange is not observed in the serine proteases, which proceed via an acyl enzyme intermediate, but is observed in the aspartyl proteases, which are believed to proceed via an amide hydrate intermediate (*29*). These data thus provide the first tentative evidence in favor of a reversibly formed *gem*-diol intermediate in the catalytic mechanism.

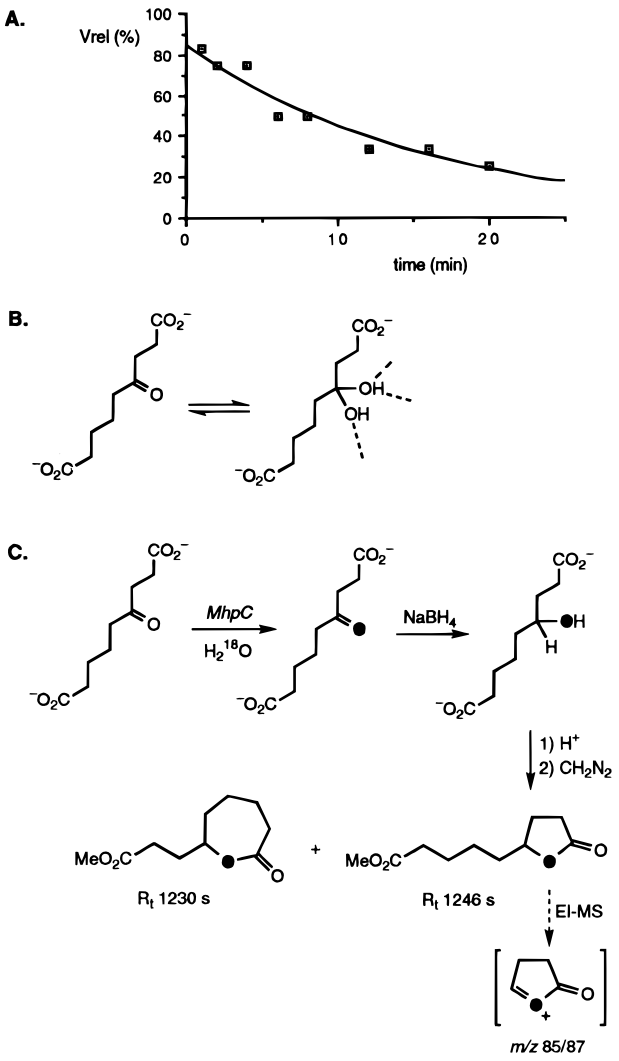

FIGURE 5: (A) Time-dependent inhibition of MhpC by KNDA. (B) Proposed scheme for time-dependent inhibition via hydration of the C-4 ketone carbonyl at the MhpC active site. (C) Investigation of MhpC-catalyzed exchange of ^{18}O from $H_2{}^{18}O$ into the C-4 ketone carbonyl of KNDA. The GC retention times of the five-membered and seven-membered lactone products are shown, together with the structure of the observed *m/z* 85/87 fragment ion.

Interaction of MhpC with a Noncleavable Substrate Analogue. 4-Keto-nona-1,9-dioic acid (KNDA) contains a 1,9-dicarboxylic acid as found in RFP, and contains the ketone at C-4 (C-6 of RFP) where nucleophilic attack occurs, but lacks the α,β-unsaturated ketone functional group required as an electron sink for C−C bond cleavage. This analogue might, therefore, be susceptible to carbonyl addition at the MhpC active site, but not C−C cleavage. The analogue was synthesized by a literature route (*19*) and was assayed against MhpC.

Addition of KNDA to assays of MhpC at 1 mM concentration gave only a small (10%) decrease in rate, implying that it binds fairly weakly to the free enzyme. However, preincubation of KNDA with MhpC was found to cause time-dependent inhibition of enzyme activity (see Figure 5A), implying that a more tightly binding enzyme−KNDA adduct is formed in a time-dependent fashion. Incubation of KNDA with MhpC followed by dialysis gave full recovery of

Table 3: Incorporation of Two Atoms of ^{18}O from $H_2{}^{18}O$ into Succinic Acid by MhpC-Catalyzed Reaction[a]

preincubationtime (sec)	*m/z* 147 (%)	*m/z* 149 (%)	*m/z* 151[a] (%)	total % ^{18}O incorp	% incorp of 2 atoms ^{18}O[b]
0	27.6	68.3	4.1	72.4	5.7
0	28.7	68.1	3.2	71.3	4.5
120	29.3	68.8	1.9	70.7	2.7
300	18.8	76.7	4.5	81.2	5.5
300	19.0	76.3	4.6	80.9	5.7

[a] Experiments were carried out as described in Materials and Methods, using 5 μg RFP (23 nmol) and 2 units MhpC. GC−MS analysis (under NH_3 chemical ionization) of dimethyl succinate obtained via derivatization of succinic acid product, after pre-incubation in 50 mM potassium phosphate, pH 8.0, prior to MhpC treatment. Peak areas for M+1 (*m/z* 147), M+3 (*m/z* 149), and M+5 (*m/z* 151) determined by selected ion monitoring of GC−MS data. [b] The *m/z* 151 intensity has been corrected for the presence of natural abundance isotope peaks due to the preceding peaks. [c] Expressed as a percentage of the total observed ^{18}O incorporation.

Table 4: MhpC-Catalyzed Incorporation of ^{18}O from $H_2{}^{18}O$ into KNDA[a]

	− MhpC		+ MhpC	
time[b] (min)	*m/z* 85 (%)	*m/z* 87 (%)	*m/z* 85 (%)	*m/z* 87 (%)
0.5	>99	<1	94.3	5.7
15	95.7	4.3	91.4	8.6
30	87.9	12.1	75.3	24.7
60	81.1	18.9	38.3	61.7

[a] Experiments were carried out as described in Materials and Methods, using 0.5 mg KNDA (2.5 μmol) and 22 mg/mL MhpC (0.76 μM). KNDA was derivatized as shown in Figure 5C to give a five-membered lactone product, which was analyzed by GC−MS. The data shown is for a *m/z* 85 fragment ion observed under electron impact ionization. Accurate peak areas were determined by selected ion monitoring of the GC−MS data. Mean value from duplicate experiment is quoted (deviation <10%). [a] Incubation time prior to derivatization.

enzyme activity, verifying that the observed inhibition was not irreversible. It, therefore, appears that KNDA acts as a slow-binding inhibitor for MhpC. In view of the evidence presented above, it is reasonable to suppose that, upon binding of KNDA to the MhpC active site, the C-4 ketone is attacked by water to form a *gem*-diol intermediate, which cannot undergo C−C cleavage but would be bound more tightly by the MhpC active site (see Figure 5B). This hypothesis would predict that MhpC might catalyze the exchange of ^{18}O-labeled water into the C-4 position.

To test this hypothesis, samples of KNDA were incubated with or without MhpC in ^{18}O-containing buffer (98 atom % ^{18}O). Aliquots were treated with sodium borohydride, then acidified, and methylated using diazomethane, to give a mixture of five-membered and seven-membered lactone products (see Figure 5C). Under electron impact conditions, the five-membered lactone gave a major fragment ion at *m/z* 85 containing the five-membered ring. Analysis of the derivatization products by GC−MS revealed a time-dependent incorporation of ^{18}O label into the five-membered lactone product arising from the incubation containing MhpC (see Table 4). The sample incubated for 60 min with MhpC showed 62% incorporation of one atom of ^{18}O, whereas the sample incubated in the absence of MhpC for the same length of time showed only 19% incorporation. Further experiments confirmed that the level of ^{18}O incorporation was also dependent on the MhpC concentration (data not shown).

These results imply that MhpC is able to catalyze the exchange of ^{18}O at the C-4 ketone of KNDA, consistent with the reversible formation of a *gem*-diol intermediate. Nucleophilic attack of an active site nucleophile at the C-4 ketone would not lead to ^{18}O exchange. The observed rate of ^{18}O incorporation is approximately 0.15 μmoles ^{18}O−KNDA per μmole MhpC per minute, which is comparable to the first-order rate constant for onset of time-dependent inhibition of MhpC by KNDA (k_{obs} = 0.16 min^{-1}). It is, therefore, reasonable to propose that the observed ^{18}O incorporation is caused by the same process which is responsible for time-dependent inhibition. In view of the structural similarity of KNDA to the MhpC substrate, the observed results provide further experimental evidence in favor of a *gem*-diol intermediate in the MhpC-catalyzed reaction.

DISCUSSION

At the outset of this work, there were convincing indications from sequence alignments and site-directed mutagenesis studies that the active site of the MhpC family of C−C hydrolases contained a reactive serine residue, which could participate as a nucleophile in a fashion similar to the catalytic mechanism of the serine proteases (*14*, *17*). However, the radiochemical trapping studies described above have yielded consistently low stoichiometries of covalently bound material, even under conditions most likely to accumulate such an intermediate. An upper limit of approximately 1% acyl enzyme intermediate can be inferred from these studies, under conditions where previous kinetic studies have shown that product release should be rate-limiting. It is, therefore, very hard to reconcile these data with the existence of an acyl enzyme intermediate for this enzyme.

In contrast, studies of the MhpC-catalyzed reaction in $H_2{}^{18}O$ have shown that there is 5−6% incorporation of a second atom of ^{18}O, consistent with the existence of a *gem*-diol reaction intermediate. Further evidence for the formation of a *gem*-diol intermediate has been obtained from the slow-binding inhibition of MhpC by KNDA, a noncleavable substrate analogue, and the MhpC-catalyzed exchange of ^{18}O into the ketone carbonyl of this analogue.

We, therefore, conclude that the reaction mechanism of this family of C−C hydrolases is most likely to proceed via base-catalyzed attack of water upon RFP^K to generate a *gem*-diol intermediate.[2] This type of intermediate is also thought to be formed during the catalytic mechanism of the aspartic proteases, along similar lines of evidence (*27*). We note that the aspartic proteases are most active at low pH (typically pH_{opt} is 3−4), thus cleavage of a *gem*-diol intermediate is

[2] As pointed out by a referee, the catalytic mechanism of ribulose 1,5-bisphosphate carboxylase/oxygenase also involves the formation of a *gem*-diol intermediate, followed by C−C cleavage. For a recent review, see Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., and Lorimer, G. H. (1998) *Chem. Rev. 98*, 549−561.

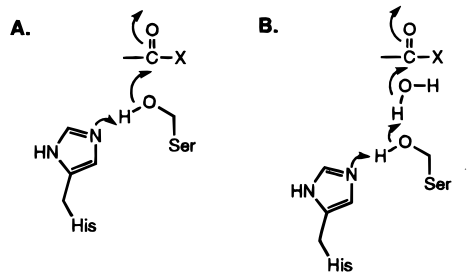

FIGURE 6: Scheme illustrating the involvement of an active site serine (A) as a nucleophile or (B) as a base.

quite feasible at low pH, whereas the acyl enzyme intermediate of the serine protease catalytic mechanism is not hydrolyzed at all at low pH. We have earlier found that MhpC is active at low pH (*11*), behavior which is entirely consistent with formation of a *gem*-diol intermediate, but not with an acyl enzyme intermediate. We have also previously observed a solvent kinetic isotope effect ($k(H_2O)/k(D_2O)$) of 1.4 for the MhpC-catalyzed reaction (*9*).

These conclusions raise two important questions: what is the role of the previously identified serine residue, and what are the implications for the α/β-hydrolase family as a whole? From earlier studies, there certainly appears to be an essential active site serine residue in the C−C hydrolases (*14*, *17*), but it appears not to act as a nucleophile. We speculate that it might act as the base for deprotonation of water, or perhaps even as a proton donor. We note that there is some precedent for the participation of a serine residue as a proton donor in horse liver alcohol dehydrogenase (*30*), and in *E. coli* enolpyruvyl-UDPGlcNAc reductase (*31*). The active site residues involved in substrate enol-keto tautomerisation also remain to be determined for the C−C hydrolases. Both *E. coli* MhpC (*32*) and *B. cepacia* LB400 BphD (*8*) have recently been crystallized, thus it is hoped that X-ray crystallography will provide an insight into the role of the active site serine residue.

Given the structural similarity between the C−C hydrolases and the α/β-hydrolase family (*13*, *14*), these findings may have implications for other members of this family of lipases and esterases. There is a considerable body of biochemical and crystallographic evidence implying that the active site serine in these enzymes acts as a nucleophile (*15*); however, acyl enzyme intermediates have only been detected using unnatural substrates such as *p*-nitrophenyl acetate (*16*). A number of lipases are known to be catalytically active at low pH, behavior which is not consistent with the "classical" serine catalytic triad, but which is also observed for MhpC. Furthermore, there are at least two members of the α/β-hydrolase family which catalyze nonhydrolytic reactions, the cofactor-free haloperoxidase from *Streptomyces aureofaciens* (*33*) and the hydroxynitrile lyase from *Hevea brasiliensis* (*34*), where nucleophilic mechanisms have been proposed for a serine triad identified by X-ray crystallography, in which a general base mechanism would be a realistic alternative. It would require only a subtle repositioning of the active site serine residue of the α/β hydrolases, relative to the incipient carbonyl, to change from a nucleophilic mechanism to a base-catalyzed mechanism (see Figure 6). It is worth recalling that the hydroxide ion is not only a powerful nucleophile in chemical reactions, it is also a strong base.

ACKNOWLEDGMENT

We would like to thank Julie Herniman for assistance with GC−MS analysis.

REFERENCES

1. Gutsche, C. D. (1967) in *The Chemistry of Carbonyl Compounds*, pp 60−61, Prentice-Hall, New York, NY.
2. Philips, R. S., and Dua, R. K. (1991) *J. Am. Chem. Soc. 113*, 7385−7388.
3. Hsiang, H. H., Sim, S. S., Mahuran, D. J., and Schmidt Jr., D. E. (1972) *Biochemistry 11*, 2098−2102.
4. Bugg, T. D. H., and Winfield, C. J. (1998) *Nat. Prod. Reports 15*, 513−530.
5. Duggleby, C. J., and Williams, P. A. (1986) *J. Gen. Microbiol. 132*, 717−726.
6. Bayly, R., and Bernardino, D. D. (1978) *J. Bacteriol. 134*, 30−37.
7. Omori, T., Sugimura, K., Ishigooka, H., and Minoda, Y. (1986) *Agric. Biol. Chem. 50*, 931−937.
8. Seah, S. Y. K., Terracina, G., Bolin, J. T., Riebel, P., Snieckus, V., and Eltis, L. D. (1998) *J. Biol. Chem. 273*, 22943−22949.
9. Lam, W. W. Y., and Bugg, T. D. H. (1997) *Biochemistry 36*, 12242−12251.
10. Lam, W. W. Y., and Bugg, T. D. H. (1994) *J. Chem. Soc., Chem. Commun.*, 1163−1164.
11. Henderson, I. M. J., and Bugg, T. D. H. (1997) *Biochemistry 36*, 12252−12258.
12. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) *Protein. Eng. 5*, 197−211.
13. Hofer, B., Eltis, L. D., Dowling, D. N., and Timmis, K. N. (1993) *Gene 130*, 47−55.
14. Diaz, E., and Timmis, K. N. (1995) *J. Biol. Chem. 270*, 6403−6411.
15. For a recent review of the α/β-hydrolase family, see: Schrag, J. D., and Cygler, M. (1997) *Methods Enzymol. 284*, 85−107.
16. Chapus, C., Semeriva, M., Bovier-Lapierre, C., and Desnuelle, P. (1976) *Biochemistry 15*, 4980−4987.
17. Ahmad, D., Fraser, J., Sylvestre, M., Larose, A., Khan, A., Bergeron, J., Juteau, J. M., and Sondossi, M. (1995) *Gene 156*, 69−74.
18. Comber, M. F., and Sargent, M. V. (1985) *Aust. J. Chem. 38*, 1481−1489.
19. Chiusoli, G. P., Minisci, F., and Quilico, A. (1957) *Gazz. Chim. Ital. 87*, 100−108.
20. Fishbein, W. N., Daly, J., and Streeter, C. L. (1969) *Anal. Biochem. 28*, 13−24.
21. Sosnovsky, G., and Krogh, J. A. (1980) *Synthesis* 654−656.
22. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
23. Balls, A. K., and Wood, H. N. (1956) *J. Biol. Chem. 219*, 245−256.
24. Bender, M. L., Schonbaum, G. R., and Zerner, B. (1965) *J. Am. Chem. Soc. 34*, 49−76.
25. Henderson, R. (1970) *J. Mol. Biol. 54*, 341−354.
26. Spence, E. L., Kawamukai, M., Sanvoisin, J., Braven, H., and Bugg, T. D. H. (1996) *J. Bacteriol. 178*, 5249−5256.
27. Hyland, L. J., Tomaszek, T. A., Roberts, G. D., Carr, S. A., Magaard, V. W., Bryan, H. L., Fakhoury, S. A., Moore, M. L., Minnich, M. D., Culp, J. S., DesJarlais, R. L., and Meek, T. D. (1991) *Biochemistry 30*, 8441−8453.
28. Sanvoisin, J., Langley, G. J., and Bugg, T. D. H. (1995) *J. Am. Chem. Soc. 117*, 7836−7837.
29. Antonov, V. K., Ginodman, L. M., Rumsh, L. D., Kapitannikov, Y. V., Barshevskaya, T. N., Yavashev, L. P., Gurova, A. G., and Volkova, L. I. (1981) *Eur. J. Biochem. 117*, 195−200.

30. Bränden, C. I., Jörnvall, H., Eklund, H., and Furugren, B. (1975) *The Enzymes* (Boyer, P. D., Ed.) Vol. 11, pp 130−190, Academic Press, New York.
31. Benson, T. E., Walsh, C. T., and Hogle, J. M. (1997) *Biochemistry 36*, 806−814.
32. Robertson, T. A., Bugg, T. D. H., and Wood, S., unpublished work.
33. Hofmann, B., Tolzer, S., Pelletier, I., Altenbuchner, J., van Pee, K. H., and Hecht, H. J. (1998) *J. Mol. Biol. 279*, 889−900.
34. Wagner, U. G., Hasslacher, M., Giriengl, H., Schwab, H., and Kratky, C. (1996) *Structure 4*, 811−822.

BI9923095