The Desymmetrization of Bicyclic β-Diketones by an Enzymatic Retro-Claisen Reaction

A NEW REACTION OF THE CROTONASE SUPERFAMILY

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The enzyme 6-oxocamphor hydrolase, which catalyzes the desymmetrization of 6-oxocamphor to yield (2R,4S)-α-campholic acid, has been purified with a factor of 35.7 from a wild type strain of Rhodococcus sp. NCIMB 9784 grown on (1R)-(+) camphor as the sole carbon source. The enzyme has a subunit molecular mass of 28,488 Da by electrospray mass spectrometry and a native molecular mass of ~83,000 Da indicating that the active protein is trimeric. The specific activity was determined to be 357.5 units mg⁻¹, and the K_m was determined to be 0.05 mM for the natural substrate. The N-terminal amino acid sequence was obtained from the purified protein, and using this information, the gene encoding the enzyme was cloned. The translation of the gene was found to bear significant homology to the crotonase superfamily of enzymes. The gene is closely associated with an open reading frame encoding a ferredoxin reductase that may be involved in the initial step in the biodegradation of camphor. A mechanism for 6-oxocamphor hydrolase based on sequence homology and the known mechanism of the crotonase enzymes is proposed.

The desymmetrization of prochiral substrates in organic synthesis remains a powerful technique for the generation of chiral intermediates with, in principle, 100% yield with absolute optical purity. In addition to chemical processes that have been reviewed recently (1), enzyme-catalyzed methods have assumed an important role in desymmetrization (2) owing to the well documented advantages of biocatalysis in general. Such methods have usually exploited the regioselectivity of a hydrolytic enzyme, such as a lipase or nitrilase, to effect transforma-

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Restriction endonucleases were from New England Biolabs. T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were from Roche Molecular Biochemicals. RNase was purchased from Sigma-Aldrich. T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were from Roche Molecular Biochemicals. RNase was purchased from Sigma-Aldrich (Poole, United Kingdom). Protein and DNA size markers were obtained from (Amersham, United Kingdom) Pharmacia Biotech. [γ²³⁵]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Isopropyl-β-D-thiogalactopyranoside was obtained from U.S. Biochemical Corp. All other chemicals were purchased from Sigma-Aldrich. 6-Oxocamphor 3 was synthesized by pyridinium chlorochromate oxidation (10) of 6-endo-hydroxy camphor derived from ethyl acetate extractions of the mother liquor of fermentations of Rhodococcus sp. NCIMB 9784 grown on (1R)-(+) camphor as described below.

Maintenance and Growth of Microorganisms—Escherichia coli XL1 Blue supercompetent cells were obtained from Stratagene (La Jolla, CA) and grown on 1% yeast extract, 1% Tryptone, and 0.5% NaCl at 37 °C. Rhodococcus sp. NCIMB 9784 was obtained from the National Culture of Industrial and Marine Bacteria (Aberdeen, United Kingdom). The bacterium was maintained on nutrient agar slopes at room temperature. Ten 250-ml shake flasks containing 50 ml of basal salts
6-Oxocamphor Hydrolase from Rhodococcus sp.

medium supplemented with 35 mM sodium pyruvate were inoculated from slope and grown on an orbital shaker at 220 rpm at 30 °C for 3 days. This combined inoculum was used to seed 10 liters of basal salts medium in a 12-liter fermentation vessel (BioFlo 1000 fermenter, New Brunswick Scientific) supplemented with 1 g liter⁻¹ (R) (+)-camphor (Aldrich). Following 2 days of growth at 30 °C with an impeller speed of 250 rpm and an air flow of 4 liters min⁻¹, a further 1 g liter⁻¹ was added. After an additional 1 day of growth, the bacteria were harvested by centrifugation to yield a typical wet weight of 4 g liter⁻¹.

**Enzyme Assays**—Activity of 6-oxocamphor hydrolase was monitored using a Hewlett Packard 8453 UV-visible spectrophotometer. The assay was performed as follows: to a 3-ml stirred cuvette containing 2990 μl of 50 mM Tris/HCl buffer, pH 7.0 (henceforth referred to as “buffer”) and the mixture was homogenized twice for 15 min each at 4500 rpm in a Type KDL Dynomill. The resulting homogenate was centrifuged at 35,000 × g for at least six weeks with no discernible loss of activity. The isoelectric point of the purified protein was determined by isoelectric focusing using an Amersham Pharmacia Biotech PhastSystem. The native molecular weight was determined by gel filtration chromatography on a Superox 12 gel filtration column against a range of commercially available standards (Amersham Pharmacia Biotech). The void volume of the column was determined using blue dextran 2000. N-terminal sequencing was performed according to the method of Hayes et al. (11), and 18 residues were unambiguously assigned as Met-Lys-Glu-Leu-Ala-Thr-Pro-Phe-Gln-Glu-Tyr-Ser-Gln-Lys-Tyr-Glu-Asn-Ile. Liquid chromatography-mass spectrometry of pure 6-oxocamphor hydrolase was performed on a Waters/Alliance 2690 HPLC system fitted with a Phenomenex Jupiter C18 300-Å, 250 mm × 2 mm × 5 μm column. The flow rate was 200 μl min⁻¹, and a gradient of 0–95% water/acetonitrile was employed. The liquid chromatography apparatus was fitted to a Waters 2990 UV detector and a Micromass Platform II single quadrupole mass spectrometer utilizing an electrospray ionization source controlled via the VG Mass-Lynx software (VG Biotechnology Ltd., Altrincham, Cheshire, United Kingdom). The source temperature was 140 °C. Capillary voltage was 3.3 kV, and the cone voltage was ramped from 40–90 V over a range of 500–2000 m/z. The instrument was calibrated over this Mᵣ range with horse heart myoglobin (Sigma).

**Nucleic Acid Preparation and Cloning**—Genomic DNA was prepared by a modified version of the method described by Kulakova et al. (12). Approximately 0.5 g of wet cell paste was washed twice in 5 ml of 10 mM EDTA, pH 8.0, and resuspended in 5 ml of 75 mM NaCl, 25 mM EDTA, pH 8.0, 20 mM Tris/HCl, pH 8.0, containing 5 mg/ml lysozyme. The mixture was incubated for 2 h at 37 °C before adding 50 μl of protease K solution (20 mg/ml) and 300 μl of 10% (w/v) SDS. This was incubated for a further 2 h at 55 °C with occasional inversion. The solution was extracted once with phenol (equilibrated with Tris/HCl, pH 8.0) and then twice with chloroform. DNA was precipitated with isopropanol and snowy on a glass rod. After washing with 70% (v/v) ethanol, the DNA was air-dried and dissolved in 0.5 ml of Tris/HCl, pH 8.1, 1 mM EDTA, pH 8.1.

A partially degenerate oligonucleotide (36-mer) designed against the N-terminal sequence (residues 7–18) of the 6-oxocamphor hydrolase activity was synthesized as a hybridization probe: 5′-CCCTTTCCAG-GAGTACWSSCCAGAATCAGAACATC-3′ (where S represents G or C, and W represents A or T). The oligonucleotide mix was radiolabeled by a kinase reaction using T4 polynucleotide kinase and [γ-³²P]ATP under standard conditions (13). Total DNA, digested to completion with different restriction endonucleases and blotted onto a Hybond-N membrane (Amer sham Pharmacia Biotech) was hybridized to the radio labeled oligonucleotide mix at 55 °C for 48 h. The membrane was then washed twice at room temperature with 300 mM NaCl, 30 mM sodium citrate containing 0.1% (w/v) SDS for 15 min and twice at 55 °C with 30 mM sodium citrate containing 0.1% (w/v) SDS for 15 min. Following autoradiography, a single cross-hybridizing band was detected in each lane. EcoRI, SacI, and SmalI digests of genomic DNA were separated on preparative gels; the regions spanning −4.5, 3.6, and 2.0 kb, respectively, were excised; and the DNA was extracted and shotgun-cloned into pUC18 vector. Following transformation into E. coli XL1 Blue, positive clones were isolated by a colony lift procedure (13) using hybridization and washing conditions identical to those used for the Southern blot. Clones were verified using a dot blot procedure (13) prior to sequencing.

**DNA Sequencing and Analysis**—Double-stranded DNA sequencing (14) of plasmid DNA prepared from positive clones was carried out with an automated DNA sequencer (ABI PRISM 377, PerkinElmer Life Sciences). All sequencing was carried out on both strands. Computer-assisted sequence analysis was performed using the DNAStrider and MacVector software packages. Data base homology searches (SwissProt release 39 protein data base) were carried out using the NCBI BLAST server. The nucleotide sequence data reported in this paper has been deposited at EMBL and GenBank™ with the accession number AF323755.

**RESULTS**

**Purification of 6-Oxocamphor Hydrolase**—Table I shows that the purification of 6-oxocamphor hydrolase from crude cell extract proceeds with a yield of 5% and a factor of 35.7. SDS-polyacrylamide gel electrophoresis of the purified protein (Fig. 2) sug- gested a denatured molecular mass of ~35,000 Da but electrospray mass spectrometry confirmed a smaller subunit mass of 28,488 Da. Analysis of the native protein by gel filtration chromatography revealed an apparent native molecular mass of 83,000 Da (average of two determinations). This suggests the protein exists in solution as a trimer (assuming the molecule is not highly elongated, which would give it a much larger than expected Stokes radius). An isoelectric point of 8.5 was recorded for the enzyme.

1 The abbreviations used are: kbp, kilobase pair; bp, base pair; ORF, open reading frame; ECH, enoyl-CoA hydratase; 4CBD, 4-chlorobenzoyl-CoA dehydrogenase.
6-Oxocamphor hydrolase was observed to have the same pH as penta-2,4-dione hydrolase and a comparable native molecular mass, the latter being a monomer of 75,000 Da (14). The pH optimum of the enzyme was ascertained to be 7.0, and the enzyme displayed 25% higher activity in 50 mM phosphate buffer than 50 mM Tris/HCl at the same pH.

**Kinetic Properties**—The specific activity of 6-oxocamphor hydrolase was determined to be 357.5 units mg⁻¹, the $K_m$ to be 0.05 mM, and the $K_m$ to be 167 s⁻¹ for 6-oxocamphor. The high specific activity of the enzyme is reflected in an inability to detect any 6-oxocamphor in fermentation extractions of *Rhodococcus* sp. NCIMB 9784, which yield a high proportion of coccus; lane 2, 6-oxocamphor hydrolase.

Effect of Metal Ions, Salt, and Inhibitors—1 mM Cu²⁺ was found to inhibit 6-oxocamphor hydrolase activity (72% relative activity), whereas 1 mM Zn²⁺ (104%) had no significant effect. In common with penta-2,4-dione hydrolase (15), 6-oxocamphor hydrolase was greatly inhibited by Hg²⁺ ions (1 mM gave only 2% relative activity). High salt concentrations were shown to strongly inhibit penta-2,4-dione hydrolase, with no observable activity at 1 M NaCl (15). No such inhibition was observed with 6-oxocamphor hydrolase. Gel filtration studies indicated no significant alteration in the apparent molecular size of 6-oxocamphor hydrolase at this concentration of NaCl.

The effect of EDTA and various inhibitors of both thiol nucleophile-dependent hydrolases and the serine hydrolase inhibitor phenylmethylsulfonyl fluoride were tested. 6-oxocamphor hydrolase was inhibited to some degree by thiol active reagents, such as 1 mM N-ethylmaleimide (68% relative activity), but most notably 1 mM hydroxymercurobenzoate (14%); 1 mM EDTA had a slight activating effect (119%). Phenylmethylsulfonyl fluoride (1 mM) had almost no effect on activity.

**Gene Cloning**—The gene encoding the 6-oxocamphor hydrolase was cloned by hybridization with a mixture of oligonucleotides designed against the N-terminal sequence of the purified protein activity. Three overlapping fragments of DNA (2.0 kbp SmaI, 3.6 kbp SacI, and 4.3 kbp EcoRI) were isolated by this procedure (Fig. 3) and cloned into pUC18 (clones S2.0, Sa3.6, and E4.3, respectively). Sequence analysis of the clones revealed several potential open reading frames (ORFs) (Fig. 4).

All displayed the typical codon usage pattern found in *Rhodococcus* sp., with a strong bias toward GC-rich codons. The deduced polypeptide translation of one such ORF (*camK*) matches the N-terminal sequence obtained from the isolated 6-oxocamphor hydrolase activity. *camK* encodes a protein of 257 amino acids, and the predicted ATG start codon is positioned 5 bp 3’ of a purine-rich region that may act as a ribosome binding site (Fig. 5). Furthermore, the calculated mass of the polypeptide (28,482 Da) is very close to the experimentally determined mass of the purified protein by electrospray mass spectrometry (28,485 Da). Comparison of the translated sequence with the SwissProt data base revealed significant homology to the crotonase superfamily of enzymes from several sources. The best alignments were obtained with crotonase (enol-CoA hydratase) from Clostridium acetobutyllicum (16) and *E. coli* (17), revealing 45 and 42% homology, respectively (data not shown). Significantly, close homology was also observed with 2-ketocyclohexanecarboxyl coenzyme A hydrolase from Rhodopseudomonas palustris (18) and 4-chlorobenzoyl-CoA dehalogenase from a Pseudomonas sp. (19). A sequence comparison of the translated sequence against representative members of the crotonase superfamily is given in Fig. 6.

The 3’ end of the gene encoding 6-oxocamphor hydrolase (*camK*) has an overlap of 1 nucleotide, encompassing the TGA stop codon and a predicted GTG start codon of another open reading frame (ORF1). ORF1 appears to be translationally coupled to *camK* and encodes a protein of 167 amino acids. A BLAST search of the SwissProt data base indicates homology to maoC gene from Klebsiella aerogenes (20), which belongs to the aldehyde dehydrogenase family and to the short-chain dehydrogenase/reductase family of enzymes (e.g. 17β-estradiol dehydrogenase from rat).

Downstream of ORF1 is an ORF encoding a polypeptide of 408 amino acids that displays similarity to nonspecific lipid transfer proteins from various species (e.g. 49% homology to chicken protein). The function of this open reading frame in relation to camphor metabolism is not known.

Upstream of *camK*, an ORF encoding a polypeptide of 206 amino acids was digested to completion with either EcoRI (lane 1), PstI (lane 2), SacI (lane 3), or Smal (lane 4), resolved on an agarose gel, blotted, and probed.

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amino acids (ORF3) was identified on the opposite strand. This divergently transcribed open reading frame has a potential ribosome binding site located 5 bp 5' of the proposed ATG start codon. Sequence analysis of the translated product revealed significant homology to a number of transcriptional repressor proteins of the TetR/AcrR family from various microorganisms.
crobial sources (e.g. 47% homology to the acred operon repressor from E. coli (24)). A potential DNA-binding motif based on sequence homologies was identified (38SVRDLGGEALQPGSVYAHI) that may form a helix-turn-helix motif. 

Further upstream of the proposed transcriptional regulator is ORF4, transcribed in the same direction as camK. Two alternative potential ATG start codons in the same reading frame were identified at positions 492 and 498 in the nucleotide sequence (Fig. 5). Because both ATG codons are positioned just downstream (5 and 6 bp, respectively) of a potential ribosome binding site, it is not clear which one represents the start of the ORF. For clarity, we have assigned the more 5' ATG as being the start codon. The ORF encodes a polypeptide of 396 amino acids. Sequence analysis of the translated product revealed convincing homology to a number of ferredoxin reductase proteins in the database. Conservation of sequence was particularly obvious in the regions involved in adenine nucleotide binding (data not shown). The best homology was found with the ferredoxin (rhodocoxin) reductase (ThcB) involved in the biodegradation of thiocarbamate from Rhodococcus sp. NI86/21 (46% homology) and the putidaredoxin reductase involved in the hydroxylation of camphor by P. putida (22) (46% homology).

Substrate Specificity—We have recently carried out initial studies on the substrate specificity of 6-oxocamphor hydrolase (23) (refer to Fig. 7). Acyclic diketones such as pentane-2,5-dione (a substrate for the β-diketone hydrolase from Pseudomonas vesicularis var. pavaloyticus (15)) and 3,3-dimethylpenta-2,5-dione were not substrates. 2,2-Disubstituted cyclohexa-1,3-diones were the only monocyclic diketones transformed, although the transformation of two of these, 2-methyl-2-propylcyclohexa-1,3-dione and 2-methyl-2-butylcyclohexa-1,3-dione, resulted in racemic keto acid products (which were converted to their methyl esters for analysis). Transformations of the bicyclic diketone substrates bicyclo[2.2.1]heptane 2,6-dione and bicyclo[2.2.2]octane-2,6-dione were, however, shown to yield (S)-keto acid products of 84% and 95% enantiomeric excess (methyl ester 11 and methyl ester 12, respectively) (Fig. 7). It is noteworthy that all substrates accepted by the enzyme are nonenolizable either due to quaternary substitution between the carbonyl...
groups (e.g., 3, 5, 6) or due to ring-strain-related restrictions imposed by Bredt’s rule (3, 9, 10).

**DISCUSSION**

The recent completion of the *E. coli* genome (24) has revealed seven genes encoding paralogues of enoyl-CoA hydratase, three with unknown function (17). This suggests that there remain activities of the crotonase superfamily yet to be described. In this paper, we present evidence that 6-oxocamphor hydrolase, which catalyzes the asymmetric hydrolysis of β-diketones, represents a new addition to the spectrum of activities catalyzed by the crotonase superfamily.

The activity of crotonase, or enoyl-CoA hydratase (ECH), has been the subject of intensive study over many years owing to its central role in the β-oxidation pathway. The essential activity of ECH in this regard has been the stereospecific reversible hydration of enoyl-CoA molecules of varying fatty acid length to yield β-hydroxy thioesters (25, 26, 30). The catalytic mechanism of ECH is dependent on stabilization of an enolate anion by hydrogen bonding to an oxyanion hole created by two peptidic NH groups in the active site of the enzyme, Ala-98 and Gly-141 (Fig. 8). In recent years, the comparison of genetic sequence information for a wide range of enzymatic activities has revealed that there exists a superfamily of crotonase-like proteins, each member of which catalyzes a reaction that is dependent on the same general stabilization of an enolate anion (17). Activities assigned to the crotonase family include double-bond isomerization (27), aromatic ring closure (28), 1,3-dioxo cleavage (18), dehalogenation (19), and decarboxylation (29), in addition to double bond hydration (Fig. 9). Although the overall amino acid sequence is well conserved between these enzymes, crucial active site residues have been shown to be present in some members of the family and absent in others, suggesting a nonconserved mechanism of enol stabilization and water transfer. In enoyl-CoA hydratase from rat mitochondria, two glutamate residues at the active site are responsible for acid/base catalysis of double bond hydration: Glu-144 facilitates attack of nucleophilic water to the carbonyl, and Glu-164 donates a proton to the α-carbon in the final step to yield the hydrated product (Fig. 8).

Homology between ECH and 6-oxocamphor hydrolase is conserved throughout most of the length of the polypeptide chain. Identity is most pronounced in the central region of the protein, which constitutes the spiral domain of ECH, most especially in the A3 B3 aβ-strand region 129PVIAAVNG, although the 140GGG turn that is present in both ECH, 2-ketocyclohexanecarboxyl-CoA hydrolase (27); 2KCH, 2-ketocyclohexanecarboxyl-CoA hydrolase from *Rhodospseudomonas palustris* (18); 4CBD, 4-chlorobenzoyl-CoA dehalogenase from a *Pseudomonas* sp. (19). Regions of similarity are highlighted in the boxes.

**Fig. 6.** Amino acid sequence alignment of 6-oxocamphor hydrolase (6-OCH) with other members of the crotonase superfamily of enzymes. ECH, enoyl-CoA hydratase of rat mitochondria (27); 2KCH, 2-ketocyclohexanecarboxyl-CoA hydrolase from *Rhodospseudomonas palustris* (18); 4CBD, 4-chlorobenzoyl-CoA dehalogenase from a *Pseudomonas* sp. (19). Regions of similarity are highlighted in the boxes.

**Fig. 7.** Hydrolysis of nonenolizable β-diketones by 6-oxocamphor hydrolase from *Rhodococcus* sp. NCIMB 9784.
first trimerization domain of ECH, T1 (Lys-185 through Thr-217), conservation between the second trimerization domain, T2 (Lys-234 through His-290) of ECH, 4CBD, and 6-oxocamphor hydrolase is more poorly conserved. Nevertheless, all three enzymes exist as trimers at one level of quaternary substructure.

It is notable that of the two active site glutamate residues in ECH, only Glu-144, which facilitates attack of water on the 3-carbon of the enoyl-CoA substrate, is conserved in 6-oxocamphor hydrolase (Glu-124). This residue is absent in other members of the superfamily, including those of which the activity most closely resembles that of 6-oxocamphor hydrolase, e.g. 2-KCH (18) and 4-chlorobenzoyl-CoA dehalogenase, in which Asp-145 has been identified as the crucial catalytic residue (19). Glu-144 is conserved between ECH and methylmalonyl-CoA decarboxylase (29), but in this case, it has been shown that this glutamate residue cannot be catalytic. Hence, it cannot be assumed with certainty that the homologous glutamate in 6-oxocamphor hydrolase is catalytic. The other catalytic residue of ECH, Glu-164, is not conserved in 6-oxocamphor hydrolase. There are several candidate residues with the required acid/base character capable of forming an acid-base couple with Glu-124 in 6-oxocamphor hydrolase, including Glu-136 (conserved with rat ECH and 2-KCH), Asp-142, and perhaps Asp-154, which is one residue distant from the active Asp-145 of 4CBD. Despite the absence of structural studies or site directed mutation experiments to determine the actual active site residue in 6-oxocamphor hydrolase responsible for water activation, it is nevertheless still possible to tentatively postulate a mechanism for asymmetric β-diketone hydrolysis based on the results of sequence comparison.

The activation of water in the active site of 6-oxocamphor hydrolase, possibly by Glu-124, in concert with another residue would facilitate nucleophilic attack of water at the pro-S carbonyl, yielding the (S)-enantiomer of keto acid 4 (Fig. 10). It is possible that tautomerization of the keto form is not an enzyme-catalyzed process because the same ratio of diastereomers (predominantly cis-) is also observed in acid-catalyzed hydrolysis of 3 to 4.

The desymmetrization of bicyclic β-diketones, and indeed the hydrolysis of 2,2-dialkylcyclohexanones, constitutes a novel reaction in the crotonase superfamily. The previously reported activity of a crotonase homologue that bears the closest resemblance is that of 2-KCH, which hydrolyzes a β-dicarbonyl species. Importantly, however, 6-oxocamphor hydrolase is the first crotonase homologue not to have an activity dependent on coenzyme A for substrate activation. Indeed, of those residues shown to be responsible for coenzyme A binding in ECH (e.g. Lys-92, Lys-101, and Lys-282, involved in forming salt bridges to the phosphates of ADP) and 4CBD (Arg-24 and Arg-87), none is conserved in 6-oxocamphor hydrolase.

Interestingly, all the compounds which act as substrates for 6-oxocamphor hydrolase have nonenolizable 1,3-diketones. This factor may explain why the retro-Claisen reaction mediated by 6-oxocamphor hydrolase is effected using a crotonase type mechanism. In contrast, similar reactions on enolizable diketones, such as fumarylacetoacetate (mediated by fumary-
lactoacetate hydrolase (9)), utilize a serine hydrolytic triad type mechanism, in which the energy barrier to hydrolysis may be greater. It remains to be seen whether other activities, such as cyclohexane-1,3-dione hydrolase (31) or polyvinylketone hydrolase (which is active against pentane-1,4-dione (15)), are of the fumarylacetoacetate hydrolase or crotonase type.

Sequence analysis of the clone encoding the 6-oxocamphor hydrolase activity revealed several proximal genes encoding proteins that may be involved in camphor metabolism. ORF1 is immediately downstream and apparently translationally coupled to camK, and it encodes a small protein that displays homology to dehydrogenase enzymes. This open reading frame awaits elucidation by site directed mutagenesis and detailed kinetic analysis of mutant activity. The wild type enzyme is already effective at performing the desymmetrization of bicyclic β-diketones with a high degree of enantioselectivity and hence constitutes an important addition to the array of biocatalysts that might be employed in the synthesis of fine chemical intermediates.

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