The 2-Å Crystal Structure of 6-Oxo Camphor Hydrolase

NEW STRUCTURAL DIVERSITY IN THE CROTONEASE SUPERFAMILY

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6-Oxo camphor hydrolase (OCH) is an enzyme of the crotonase superfamily that catalyzes carbon-carbon bond cleavage in bicyclic β-diketones via a retro-Claisen reaction (Grogan, G., Roberts, G. A., Bougioukou, D., Turner, N. J., and Flitsch, S. L. (2001) J. Biol. Chem. 276, 12565–12572). The native structure of OCH has been solved at 2.0-Å resolution with selenomethionine multiple wave anomalous dispersion and refined to a final Rfree of 19.0. The structure of OCH consists of a dimer of trimers that resembles the "parent" enzyme of the superfamily, enoyl-CoA hydratase. In contrast to enoyl-CoA hydratase, however, two octahedrally coordinated sodium atoms are found at the 3-fold axis of the hexamer of OCH, and the C-terminal helix of OCH does not form a discrete domain. Models of the substrate, 6-oxo camphor, and a proposed enolate intermediate in the putative active site suggest possible mechanistic roles for Glu-244, Asp-154, His-122, His-45, and His-145.

The microbial metabolism of the bicyclic monoterpene camphor 1 has been a topic of great interest for more than 40 years. Much of this work has been stimulated by the adoption of the hydroxylation enzyme cytochrome P450cam from Pseudomonas putida as a structural and mechanistic model for the activity of cytochromes P450 in general (1). In addition, microbial enzymes from P. putida involved in the degradation of camphor, including cytochrome P450cam, have been exploited as biocatalysts for asymmetric oxidation reactions in synthetic chemistry (2). The metabolism of (1R)-(−)-camphor 1 (Fig. 1) by P. putida proceeds via hydroxylation in the 5-exo position to give hydroxycamphor 2, followed by oxidation to diketone 3, which is oxidatively cleaved by an enzymatic Baeyer-Villiger reaction via lactone 4 (3). However, in Rhodococcus sp. NCIMB 9784, an alternative regio-distinct pathway is adopted whereby camphor is hydroxylated in the 6-endo position to give hydroxycamphor 5, followed by oxidation to give a symmetrical diketone 6. 6 is not ring-opened oxidatively, but a carbon-carbon is cleaved, possibly hydrolytically, by a β-diketone hydrolase” enzyme (4) termed 6-oxo camphor hydrolase (OCH),1 to yield the optically active (2R,4S)-α-camphoronic acid 7 (Fig. 2). Our interests in preparative synthetic biocatalysis led us to isolate OCH and to apply it to the asymmetric cleavage of monosubstrate analogues (5). In addition, we cloned and sequenced the gene camK, which encodes OCH (6). Analysis of the primary structure of OCH showed it to have significant homology to the crotonase superfamily of enzymes.

The crotonase superfamily of proteins is a group of low sequence homology enzymes that catalyze a wide range of biochemical reactions (7). The parent enzyme of the superfamily, enoyl-coenzyme A hydratase (ECH) catalyzes the stereospecific hydration of enoyl-coenzyme A, an integral step in the β-oxidation of fatty acids (8). Other members of the superfamily catalyze dehalogenation (9), carbon-carbon bond cleavage (10), and decarboxylation (11) (Fig. 3). In each case, the substrate for enzymatic reaction is an acyl-coenzyme A (CoA) thioester.

Each member of the crotonase superfamily, for which a mechanism has been proposed, shares mechanistic similarities, related in each case to the stabilization of a transition state enolate by a conserved oxanyon hole (12). Gerlt and others (7, 12, 15) have described how the crotonase superfamily serves as a paradigm for the study of enzymatic adaptive evolution, whereby fundamental enzymatic activity such as enoyl-CoA hydration is recruited to more atypical metabolic function such as the dehalogenation of chlorobenzoic acids for the derivation of metabolic energy.

The diverse biochemical functions of the crotonase superfamily suggest that, from a genomic perspective, it is very difficult to delineate the role of any particular crotonase homologue in any genome from sequence alone. At this time, individual expression and assay of each gene and gene product would be required to begin to understand its contribution to metabolism. Even then, the mechanistic details of crotonase activity will be difficult to determine. Structural studies of enzymes of the crotonase superfamily are thus vital to the current understanding of the biochemical nature of this group. The structures of four members of the crotonase superfamily have been published to date: ECH (13), dienoyl-CoA isomerase (DCI) (14), 4-chlorobenzoyl-CoA dehalogenase (15), and methylmalonyl decarboxylase (MMD) (11). In addition, the structure of an enoyl-CoA hydratase homologue from humans has been published recently, which exhibits the ability to bind RNA (16).

In this paper, we present the crystal structure of OCH, into which we have modeled the native substrate, 6-oxo camphor,

1 The abbreviations used are: OCH, 6-oxo camphor hydrolase; ECH, enoyl-coenzyme A hydratase; DCI, dienoyl-CoA isomerase; MMD, methylmalonyl decarboxylase; MES, 4-morpholineethanesulfonic acid; CBD, 4-chlorobenzoyl-CoA dehalogenase.
**Experimental Procedures**

**Chemicals**—All chemicals were obtained from Sigma unless otherwise specified. 6-Oxo camphor was obtained as detailed in Ref. 6. Plasmid pET-26b(+) was obtained from Novagen Ltd., Madison, WI. Plasmid pGEMT, restriction enzymes, and buffers were purchased from Promega.

**Bacterial Strains, Plasmids, and Culture Conditions**—*Escherichia coli* strains used in this study were BL21(DE3) and B834(DE3). The plasmid vectors used were pGEMT (Promega) and pET-26b(+) (Novagen). *E. coli* strains were cultured in Luria-Bertani broth with 30 μg ml\(^{-1}\) kanamycin at 37 °C. Cultures were routinely grown to an optical density of \(A_{600} = 0.6\) and then induced for expression of OCH by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and then grown for a further 3 h.

**Preparation of Selenomethionine Derivative of OCH**—The methionine auxotroph *E. coli* B834 (DE3) transformed with pGEM3 was cultured in 4 × 500 ml of a liquid culture medium containing 2 mM MgSO\(_4\); 37 mM NH\(_4\)Cl; 44 mM KH\(_2\)PO\(_4\); 85 mM NaH\(_2\)PO\(_4\); 0.09 mM FeSO\(_4\); 22.2 mM glucose; 40 mg each of the 20 common amino acids (except methionine, which was substituted with D/L selenomethionine); 1 mg each of riboflavin, niacinamide, thiamine, and pyridoxine monohydrochloride; 30 μg ml\(^{-1}\) kanamycin. Cultures were grown at 37 °C in an orbital shaker at 200 rpm until an optical density of \(A_{600} = 0.4\) was reached, at which point, 1 mM isopropyl-β-D-thiogalactopyranoside was added as inducer. Cells were harvested after 3 h of further incubation at 37 °C in an orbital shaker at 200 rpm.

**Cryocrystallization**—Crystals of the native and selenomethionine derivative were purified as detailed in Ref. 6.

**Data Collection and Data Processing**—A native data set extending to 2.0 Å was collected on a single crystal of the native enzyme, which had been flash-frozen previously at 120 K in a cryoprotectant solution consisting of 95% (v/v) reservoir and 5% (v/v) ethylene glycol. The data were collected on station 14.2 at the Synchrotron Radiation Facility, Daresbury, United Kingdom, using an ADSC Quantum-4 detector. The data were processed, scaled, and merged using the HKL suite (19). Subsequently, a three-wavelength multi-anomalous dispersion experiment was conducted on beamline BM14 at the European Synchrotron

[Diagram of the partial pathways for degradation of (1R)-(+)-camphor by *P. putida* ATCC 17453 and *Rhodococcus* sp. NCIMB 9784]

[Diagram of carbon-carbon bond cleavage reaction catalyzed by OCH]
Structure of 6-Oxo Camphor Hydrolase

Table I
Data collection, processing, and refinement statistics for OCH structure solution

| Data set          | Native   | SeMet A1 | SeMet A2 | SeMet A3 |
|-------------------|----------|----------|----------|----------|
| Beamline          | 14.2     | 19.5     | 17.6     | 19.0     |
| Wavelength (Å)    | 0.9700   | 0.9797   | 0.9783   | 0.9650   |
| Space group       | P21      | P2       | P2       | P2       |
| Resolution (Å)    | 30.0–2.00 (2.03–2.00) | 40.0–2.40 (2.44–2.40) | 40.0–2.40 (2.44–2.40) | 40.0–2.40 (2.44–2.40) |
| Unique reflections | 95932    | 58684    | 58699    | 59919    |
| Completeness (%)  | 99.7 (97.0) | 99.9 (98.6) | 99.8 (96.9) | 99.9 (98.1) |
| Rwork (%)         | 4.3 (15.7) | 6.9 (19.6) | 5.7 (20.7) | 6.7 (22.2) |
| Multiplicity      | 2.4      | 3.7      | 3.6      | 3.7      |
| I/σI              | 25.8 (7.1) | 17.4 (5.1) | 23.2 (5.0) | 19.5 (5.7) |

Refinement statistics

| Rwork (%) | 14.9     |
| Rfree (%) | 19.0     |
| Mean B factor | 20.5     |
| Root mean square bonds | 0.017 (0.021) |
| Root mean square angles | 1.630 (1.950) |
| Ramachandran | 95.0     |
| Water molecules | 1490 (~248 per monomer) |
| Metal ions | 2       |

RESULTS

Quaternary Structure of OCH—The quaternary structure of OCH is superficially similar to the parent enzyme of the superfamily, enoyl-CoA hydratase (13), dienoyl-CoA isomerase (14), and methylmalonyl decarboxylase (11), being a homogeneous hexamer composed of two stacked trimers (Fig. 4). Homology between the six monomers of the OCH hexamer is high (root mean square deviation = 0.15 to 0.22 Å calculated on Ca). The hexamer is a flattened sphere with approximate overall dimensions of 80 × 76 × 71 Å. An octahedrally coordinated Na⁺ ion is present at the 3-fold axis of each trimer, coordinated via water molecules to residues Asp-168 and Asp-186 from each constituent monomer (Fig. 5). The conclusion that this coordinating species is a sodium ion rather than a water molecule or isoelectronic Mg²⁺ was made because of the very distinctive octahedral coordination geometry and metal/water distances of 2.4–2.5 Å, both of which correlate with sodium ion binding (17, 18). Trimer stabilization appears to be mediated by a mixture of polar and hydrophobic interactions between α-5 (residues 167–174) and α-8 (residues 214–226) of each monomer. Tyr-171 (A) is H-bonded to Glu-192 (B; 2.5 Å); Leu-174 (monomer A) interacts with Leu-225 (monomer B). In addition, Asn-167 (A) is H-bonded to the carbonyl of Val-127 (B; 2.8 Å).

Monomer Structure—The OCH monomer consists of nine α-helices, ranging in length from four to 24 residues, and five well defined β-strands. The secondary structural elements, in common with other crotonase superfamily members, form four turns of a βαβ superhelix, resulting in an inverted prism whereof the top layer of twisted β-sheets, both of the five well defined β-strands, the most N-terminal being antiparallel, the remainder parallel. One face of the inverted prism consists of a layer formed from five near-flat coil regions, which exhibit classical β-sheet bonding only in the central region of the sheet. Of these strands, all are parallel except the most C-terminal, which is antiparallel. The other face of the prism is formed by three α-helices, α-2, (residues 42–57), α-3 (residues 87–106), and α-8 (residues 214–226), the last being the N-terminal region of the large, kinked C-terminal helix (α-8/α-9), the distal region of which (α-9) loops around the core monomer structure. This is in contrast with the constituent monomers of other hexameric members of the crotonase superfamily, wherein the
C-terminal helix of e.g. ECH forms a discrete, second domain (Fig. 6). H9251-9 is also involved in hexamer formation, making contact with one monomer of the adjacent trimer.

**Active Site Geometry**—Although the location of the OCH active site has yet to be established from a ligand-bound structural complex, we have identified a hydrophobic pocket near the center of the inverted prism apex, which bears many characteristics suggestive of a putative active site. This pocket is wholly enclosed within one monomer, in common with MMD (11), but in contrast to ECH, DCI, and CBD, for which the active sites are observed at the trimer interface. Entrance to the pocket from the solvent appears to be gated by Phe-79. The pocket is bound on one side by helices H9251-2, H9251-3, and H9251-9 and is largely hydrophobic, but the protrusion of five polar residues, Glu-244, Asp-154, His-145, His-122, and His-45, into the cavity is noteworthy.

The distinctive (G/A)G(G/A) turn motif in the majority of other crotonase sequences (7) e.g. ECH (residues 140–142) is absent in OCH, being replaced by an Asn-121 HP motif (residues 121–123). His-122 adopts unfavorable \( \psi \) and \( \psi \) angles of \( 42.8^\circ \) and \( 58.1^\circ \). The dihedral angles for most other amino acid residues lie within permitted regions of the Ramachandran plot. The other notable exception is His-145, which adopts \( \psi \) and \( \psi \) angles of \( 97.6^\circ \) and \( 105.1^\circ \), respectively.

**Modeling**—We have so far been unable to obtain a crystal structure of OCH bound with either the natural substrate, 6-oxo camphor (this would be unlikely as turnover is very rapid), or the product, \( (2R,4S)\)-campholinic acid. However, the location of a putative active site, in conjunction with known crotonase mechanisms and the stereochemical constraints of the reaction, have allowed us to model both substrate and putative intermediate into the proposed active site region (Fig. 7).

We have suggested previously (5) that cleavage of the carbon-carbon bond may proceed via nucleophilic attack at one prochiral carbonyl in 6-oxo camphor by a water molecule activated by an acidic residue in the enzyme active site, analogous to the hydrolase mechanism of water addition observed in ECH (27). Such attack would have to occur from the left hand side of the molecule as drawn in Fig. 8 (pro-S) to result in the observed product stereochemistry (S) at the acetate-substituted center. When the substrate is modeled into the active site (Fig. 7, top), the geminal dimethyl group of the substrate is shown to be oriented toward a hydrophobic region of the active site making interactions with dominant hydrophobic residues...
such as Phe-82 (5.1 Å), Ile-93 (4.0 Å), Trp-90 (3.9 Å), and Leu-84 (3.8 Å). In this orientation, either the prominent Glu-244 or Asp-154 residue is well placed for the activation of a water molecule for attack at the (pro-\(S\)) carbonyl side of the substrate. The modeled orientation also allows for the (pro-\(R\)) carbonyl of the substrate to be stabilized by hydrogen bonding to His-122. Ring opening of the substrate may proceed via the enolate intermediate 8 shown in Fig. 8. In other crotonase homologues, intermediate enolates, thought too active to be kinetically competent (7), are thought to be stabilized by an oxyanion hole formed between two peptidic N-H groups from residue positions that are conserved throughout the previously determined superfamily structures (e.g. Ala-98 and Gly-141 in ECH). In OCH, the replacement of the GG\(^{141}\)G motif with the N\(^{121}\)HP motif suggests that this oxyanion hole is absent or constructed differently. Any proposed enolate must be stabilized by some other factor. Modeling of the enolate intermediate (Fig. 7, bottom) suggests that the enolate may be H-bonded to His-45 (2.6 Å), the carboxylate moiety of the intermediate being H-bonded both to His-122 (2.7 Å) and His-145 (2.9 Å), which are both observed to be held in unusual conformations (see above).

**DISCUSSION**

The structure of 6-oxo camphor hydrolase suggests that OCH is more removed from a possible progenitor of the crotonase superfamily of proteins than members described previously. Although the hexameric global structure is superficially similar to ECH, for example, the presence of sodium ions at the 3-fold axis of each constituent trimer of OCH was not observed in ECH, even though a similar coordination environment was observed in the latter protein. A nickel ion was reported to be coordinated by equivalent histidines along the 3-fold trimer axis of MMD, but this was thought to be an artifact of nickel/His tag-dependent purification (11). In the case of OCH, sodium may arise from the purification or crystallization buffer. There does not appear to be an absolute dependence on any metal ion for OCH activity as evidenced by previous studies, however. Indeed, an increase in NaCl concentration was not observed to affect enzymatic activity (6).

Significant divergence from other members of the crotonase superfamily is also observed in the structure of the constituent monomer of OCH. Comparison of residues 13–192 of the ECH monomer and residues 31–210 of OCH (Fig. 6) reveals that the N-terminal catalytic domain of ECH is, for the most part,
structurally conserved in OCH (root mean square deviation value of 4.82 Å). The C-terminal helix is positioned very differently, however, not forming a discrete domain as in ECH, but rather looping around the monomer structure of OCH to form the wall of a hydrophobic pocket, which may constitute the active site. The active site of OCH appears to have been recruited from a hydrophobic well bound by helices in the interior of the monomer, which already exists partially in ECH. In the case of OCH, this active site has been completed by recruitment of the C-terminal trimerization domain helix as a further wall of the active site seemingly at no cost to trimer stabilization, which is satisfied by alternative regions in OCH than ECH. It may be that trimer interactions centered around a sodium ion have evolved as a mechanism of stabilization to offset any loss of stabilization as a result of the recruitment of the C-terminal helix in ECH. It is also possible that the recruited C-terminal helix is positioned to provide the primary catalytic residue (Glu-244) for water activation leading to carbon-carbon bond cleavage. The C-terminal divergence among crotonase homologs is reflected additionally in the sequence alignment of a series of members of the crotonase superfamily (Fig. 9), illustrating comparatively poor conservation of sequence in this region.

The sequence alignments of selected regions of OCH, ECH, MMD, CBD, and DCI (Fig. 9) are also revealing in respect to conserved catalytic residues throughout crotonase homologs. In conjunction with sequence comparison and structural studies, site-directed mutagenesis experiments have revealed that although catalytic residues may be conserved between superfamily members, they may make no catalytic contribution. This appears to be the case with Glu-124 in OCH (equivalent to catalytic Glu-44 in ECH), which is H-bonded to His-122 and therefore presumably cannot have a primary catalytic role. Asp-154 (OCH), which protrudes into the putative active site, is near homologous to the catalytically active Asp-145 in CBD. The presence of Glu-244 in the putative active site is particularly noteworthy, as such an extremely C-terminal residue in the active site of a crotonase homolog would be unprecedented, this area usually being involved in trimerization.

Another striking divergence of the primary structure of OCH from that of other crotonase homologs is the absence of the (G/A)G(G/A) motif, which, in other members, provides the conserved glycine for formation of the oxyanion hole for enolate stabilization. In OCH, (G/A)G(G/A) appears to be replaced by an N^{121}HP motif, His-122 being held in an unusual conformation, perhaps suggestive of a mechanistic role for this residue. In other homologues, the oxyanion hole is formed by peptidic (Glu-244) for water activation leading to carbon-carbon bond cleavage. The C-terminal divergence among crotonase homologs is reflected additionally in the sequence alignment of a series of members of the crotonase superfamily (Fig. 9), illustrating comparatively poor conservation of sequence in this region.

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