Structure of 6-Oxo Camphor Hydrolase H122A Mutant Bound to Its Natural Product, (2S,4S)-α-Campholinic Acid

MUTANT STRUCTURE SUGGESTS AN ATYPICAL MODE OF TRANSITION STATE BINDING FOR A CROTONASE HOMOLOG*

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The crotonase homolog, 6-oxo camphor hydrolase (OCH), catalyzes the desymmetrization of bicyclic β-diketones to optically active keto acids via an enzymatic retro-Claisen reaction, resulting in the cleavage of a carbon-carbon bond. We have previously reported the structure of OCH (Whittingham, J. L., Turkenburg, J. P., Verma, C. S., Walsh, M. A., and Grogan, G. (2003) J. Biol. Chem. 278, 1744–1750), which suggested the involvement of five residues, His-45, His-122, His-145, Asp-154, and Glu-224, in catalysis. Here we report mutation studies on OCH that reveal that H145A and D154N mutants of OCH have greatly reduced values of $k_{cat}/K_m$, derived from a very large increase in $K_m$ for the native substrate, 6-oxo camphor. In addition, H122A has a greatly reduced value of $k_{cat}$ and its $K_m$ is five times that of the wild-type. The location of the active site is confirmed by the 1.9-A structure of the H122A mutant of OCH complexed with the minor diastereoisomer of (2S,4S)-α-campholinic acid, the natural product of the enzyme. This shows the pendant acetate of the product hydrogen bonded to a His-145/Asp-154 dyad and the endocyclic carbonyl of the cyclopentane ring hydrogen bonded to Trp-40. The results are suggestive of a base-catalyzed mechanism of C-C bond cleavage and provide clues to the origin of prochiral selectivity by the enzyme and to the recruitment of the crotonase fold for alternate modes of transition state stabilization to those described for other crotonase superfamily members.

Enzymatic desymmetrization processes are of great interest to the synthetic organic chemist as they provide quantitative routes to optically active synthetic intermediates from prochiral starting materials (1). We have described the application of the enzyme 6-oxo camphor hydrolase (OCH) to the desymmetrization of bicyclic β-diketones for the preparation of optically active keto acids (2), which may be of use in the preparation of chiral synthons of a range of bioactive compounds. This unusual enzymatic process is the biological equivalent of a retro-Claisen or retro-Dieckmann reaction. The carbon-carbon bond of a β-dicarbonyl species is cleaved to yield a carboxylic acid and a methylene group. The natural substrate for the enzyme is bornane-2, 6-dione, or 6-oxo camphor 1 (Fig. 1), a natural catabolite of the monoterpene camphor when processed by the natural host strain, Rhodococcus sp. NCIMB 9784 (3). We have previously demonstrated (2) that the enzyme yields a 6:1 diastereomeric ratio of the major product, (2R,4S)-campholinic acid 2 and the diastereoisomer 3 of the (2S,4S) configuration. The cleavage of β-dicarbonyl species in nature is mechanistically diverse (4), incorporating, among others, enzymes such as polyvinyl ketone hydrolase (5) from Pseudomonas sp., a serine triad-type hydrolase, and acetylene-cleaving enzyme Dke1 (6), a dioxygenase from Acinetobacter johnsonii. On cloning and sequencing the gene encoding OCH, camK, from Rhodococcus sp. 9784 (7), we were surprised to find that its closest amino acid sequence homologs in the SwissProt data base were enzymes of the crotonase superfamily. Crotonases are a mechanistically diverse group of enzymes that catalyze a variety of chemical reactions and for which a number of x-ray crystal structures have been reported, including enzymes catalyzing asymmetric double bond hydration (8), decarboxylation (9), dehalogenation (10), the isomerization of double bonds in fatty acids (11), and ring closure to form an aromatic ring (12). Each crotonase superfamily member shares common characteristics despite apparent differences in reaction chemistry. The substrate in each homolog described hitherto is an acyl coenzyme A thioester. Each reaction is thought to proceed via an intermediate enolate that appears to be stabilized by a conserved oxanion hole in the enzyme tertiary structure, formed by the peptidic backbone N-Hs of the central residue of a conserved GG(A)G motif for oxyanion stabilization (13). The crotonase superfamily has thus served as a useful paradigm for illustrating the mechanisms of divergent evolution of enzyme action through the recruitment of the crotonase (enoyl-CoA hydratase) fold for alternate reaction chemistry. It was clear from the sequencing results that OCH represented a further diverged member of the crotonase superfamily; not only was the natural substrate not an acyl coenzyme A thioester but the distinctive GG(A)G motif for oxanion stabilization was not present, being replaced instead by an NHP sequence (7).

The divergence of OCH from the parent crotonase, enoyl-CoA hydratase (ECH) (8), was further emphasized by comparison of the enzyme structures (14). While retaining the overall quaternary arrangement of enoyl-CoA hydratase, the tertiary fold of the poorly sequence-conserved C-terminal helix of OCH appeared to assume a different motif, curling around the $\beta$-α-$\beta$-
superhelix of the N-terminal portion in contrast to forming a second discrete domain as observed in ECH and some other crotonase superfamily structures such as 4-chlorobenzoyl dehalogenase (10). This stark difference in tertiary structure was also observed for methylnaloyl-CoA decarboxylase (9) from Escherichia coli and yeast ∆2-∆2-enoyl-CoA isomerase of the crotonase superfamily (15). At that stage, despite repeated co-crystallization experiments with the natural substrate, we were not able to acquire a structure of OCH with either its natural substrate or product bound. However, a large cavity in the enzyme, completed by the recruitment of the C-terminal helical domain, was identified as a possible active site based on the protrusion of a number of acid-base amino acid residues into its center. Using the known prochiral selectivity of OCH, we were able to model both the substrate 6-oxo camphor and major diastereomeric product (2R,4S)-α-campholic acid into the putative active site and to intimate a putative mechanistic role for the acid-base residues His-45, His-122, His-145, Asp-154, and Glu-244.

In this study, we report the kinetic analysis of mutants H45A, H122A, H145A, D154N, and E244Q of OCH and describe rational use of the low kcat plus low Km mutant H122A for the acquisition of a 1.9-Å crystal structure of OCH H122A bound to the minor diastereoisomer of the reaction product, (2S,4S)-α-campholic acid. The kinetic data, in conjunction with the mutant crystal structure, support a possible mechanistic role for a His-145/Asp-154 dyad in a general base-catalyzed mechanism and also illuminate the important roles of the putative active site and to intimate a putative mechanistic role for the acid-base residues His-45, His-122, His-145, Asp-154, and Glu-244.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were obtained from Sigma unless otherwise specified. 6-oxo camphor was obtained as detailed in Ref. 7. Plasmid pET-26b(+) was obtained from Novagen Ltd. Primers for mutagenesis of camK were obtained from MWG Biotech.

Bacterial Strains, Plasmids, and Culture Conditions—Wild-type and mutant OCH genes were expressed in E. coli strain BL21(DE3). The plasmid pGG3 had been obtained previously by ligating the camK gene from Rhodococcus sp. NCIMB 9784 into pET-26b(+) for expression in E. coli. Each mutant plasmid was purified using an identical protocol as detailed in Ref. 7.

Site-directed Mutagenesis of OCH—The H45A, H122A, H145A, D154N, and E244Q mutants were constructed using the QuikChange® mutagenesis kit (Stratagene) with the pGG3 plasmid as a template. The primers used to create the mutants were 5'-GGTGGGACCTCACA-CGCCAGCCGACAGCTGCTACTG-3' and 5'-CATGTAAGGCAGCTCGTGGCTGCTATCGTACTC3'- and 5'-CATGACG-GGATCTCCGGCGTGGTGTTCACCAGCAGGAAGGG for H45A, 5'-CAAGCGAC-GGTGACCCAACGGCCGATCTCCGGCTG-3' and 5'-CATGACG-GGATCTCCGGCGTGGTGTTCACCAGCAGGAAGGG for H122A, 5'-CACAGTGGCGGACACCCCGCAGACGGCGCCTGACGTCCGGTGCTGCGCGCGCC for H145A, 5'-CCGGCATCGTGCCCGGGAACGGCGCCCACGTGGT-G'-3' and 5'-CACCCAGTGGGCGGTGCTGCTGCGCGCGCCAGATCGGGC for D154N, and 5'-GGTGGGACCTCACA-CGCCAGCCGACAGCTGCTACTG-3' for E244Q. The following parameters were used during thermal cycling: 1 cycle of 99 °C for 30 s, followed by 20 cycles of 99 °C for 30 s, 55 °C for 60 s, and 68 °C for 840 s. Mutant plasmids were purified using standard methods and sequenced to confirm the presence of the substituted bases.

Purification and Assay of OCH Mutants—All five mutant genes were expressed and purified using an identical protocol as detailed in Ref. 7. Levels of soluble OCH mutant proteins were obtained with comparable yield and solubility to the wild-type. The activity of the OCH mutants was evaluated as described previously (7) by measuring the decrease in absorption at 294 nm over time, indicating the rate of cleavage of the 6-oxo camphor substrate by enzyme. It was possible to construct Michaelis-Menten plots for each mutant by measuring the initial rate of catalysis over a range of substrate concentrations. Lineweaver-Burk plots were used to obtain the Km and kcat for the natural substrate for each mutant enzyme.

Crystallographic Analysis of the H122A Mutant—Crystals of the H122A mutant of OCH were grown by the vapor diffusion hanging drop technique. A 10 mg/ml protein solution containing 50 mM Tris-HCl, pH 7.1, 1 mM dithiothreitol, and 20 μM phenylmethylsulfonyl fluoride was mixed in a 50:50 ratio with reservoir to form the hanging drops. The reservoir solution consisted of 0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 5.6, 0.2 M calcium acetate, and 26% (v/v) polyethylene glycol monomethyl ether, 2,000 Da. Prior to data collection, crystals were transferred to a saturated solution of 6-oxo camphor in reservoir and soaked for 30 min. The H122A mutant crystallized in space group P21, with cell dimensions a = 83.27 Å, b = 132.00 Å, c = 135.43 Å, β = 94.11°.

Data Collection and Data Processing—A data set extending to 1.9-Å resolution was collected on a single crystal of the H122A mutant enzyme that had been flash frozen at 120 K using the soak solution, which acted as a cryoprotectant because of the presence of polyethylene glycol monomethyl ether, 2,000 Da. The data were collected on beamline ID14-EH3 at the European Synchrotron Radiation Facility, Grenoble, France, using a MAR165 CCD detector. The data were processed, scaled, and merged using the HKL suite (16). Data collection and processing statistics are given in Table I.

Structure Solution and Refinement—The structure was initially phased by molecular replacement using the CCP4 program AMORE (18), starting with a model from the 2-Å resolution structure of OCH (Protein Data Bank entry 180u). The OCH hexamer was used as a search model with all water atoms removed. The resulting H122A model contained 12 monomers in the asymmetric unit corresponding to 2 hexamers. Following molecular replacement, initial refinement was performed using the CCP4 program REFMACS5 (19). Rigid body refinement was performed for 10 cycles. The model was then subjected to positional refinement and initial maximum likelihood weighted 2 Fobs - Fcalc and Fobs - Fcalc maps were calculated. The model was adjusted to fit the electron density maps using the XTALVIEW molecular graphics program (20). Electron density was present in both maps corresponding to the bound product of catalysis, (2S,4S)-α-campholic acid. The CCP4 (17) program SKETCHER was used to make the product molecule model; a library file was created for use in REFMAC5 (19) using the CCP4 (17) program LIGCHECK. This was built into each of the 12 sites in the asymmetric unit. Further positional refinement was performed with non-crystallographic symmetry restraints applied between all 12 sites.

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FIG. 1. Cleavage of 6-oxo camphor catalyzed by 6-oxo camphor hydrolase.
The crystal structure of the H122A mutant complexed with (2S,4S)-\(\alpha\)-camphorlic acid was determined to 1.9-Å resolution. The structure (Fig. 2) revealed that the location of the active site had been correctly identified in our previous publication (14), bound on one side by helices \(\alpha\)-2, \(\alpha\)-3, and \(\alpha\)-9, the last of these being the C-terminal helix. The quality of the final model was analyzed using the CCP4 (17) program PROCHECK (22). The Ramachandran plot shows that 95.3% of residues occupy the most favored regions, with a further 4.2% occupying additional allowed regions. Only residue His-145 adopts dihedral angles significantly outside the allowed regions of the Ramachandran plot. His-145 is located within a type I \(\beta\)-turn immediately preceding a \(\beta\) strand and helix \(\alpha\)-5, and the electron density of this residue is unambiguous.

The H122A mutant forms a hexamer, which can be described as a dimer of trimers, identical to that of the wild-type enzyme (14). The \(\alpha\)-carbons of the mutant hexamer superimpose on those of the wild-type hexamer with a root mean square displacement of 0.38 Å and a maximum displacement of 2.50 Å, calculated using the CCP4 program LSQKAB (23). The differences between the two structures can be attributed to the presence of the bound product molecule in the active site (Fig. 3). There are three regions of change with respect to the wild-type structure upon binding of the product molecule, defined by Glu-76 \(\rightarrow\) Asn-83, Pro-118 \(\rightarrow\) Glu-124, and Glu-141 \(\rightarrow\) Ile-150. The first region contains a \(\beta\) strand and helix \(\alpha\)-3. The region contains two phenylalanines, one of which, Phe-79, appears to move to close off the entrance to the active site as suggested previously (14). The second phenylalanine, Phe-82, facilitates this movement by forming a stacking interaction between the plane of its aromatic ring and the pro-(\(R\)) face of the cyclopentane ring of the product, suggesting a mechanism whereby the entrance to the active site is closed during catalysis by direct interaction with the substrate. The second region is positioned after helix \(\alpha\)-3 and strand 6 and contains a type IV \(\beta\)-turn. The mutated residue Ala-122 is contained within this region, and the movement may reflect its role in catalysis, but it may also be an artifact of the mutation. The final region contains a type I \(\beta\)-turn followed by a \(\alpha\) helix. This region contains His-145, which hydrogen bonds to the N-H group of the carboxylate oxygens of the pendant acetate at C-4 of the bound product.

The density of the bound ligand in each of the 12 subunits in the model is unambiguous, as illustrated in Fig. 2. The ligand observed is not the major product of carbon-carbon bond cleavage observed with the wild-type enzyme, which exhibits the cis-stereochemistry of the 2-methyl and 4-acetate groups as confirmed by x-ray crystallography of the enzymatic product generated \textit{in vitro} (2), but rather the (2S,4S)-diastereomer of the \textit{trans} conformation. The structure provides useful information on important active site interactions between the product molecule and the active site. In addition, the product is bound in a closed conformation, with the structure giving the impression of the substrate molecule just after carbon-carbon bond cleavage, and retains the three-dimensional character of the substrate.

In addition to those already described, further interactions are apparent between active site residue side chains and the product (Fig. 4). The endocyclic carbonyl of the product is hydrogen bonded at a distance of 2.56 Å to the N-H group of Trp-40. The carboxylate oxygen of the pendant acetate at C4 that hydrogen bonds to His-145 also hydrogen bonds to Glu-244 at a distance of 2.50 Å. The other carboxylate oxygen of the acetate hydrogen bonds with Asp-154 at a distance of 2.67 Å. It is possible that His-145 and Asp-154 form a catalytic dyad. There may also be an indirect interaction between this carboxylate oxygen and His-45 via an active site water that is hydrogen bonded to both the carboxylate oxygen at a distance of 2.76 Å and His-45 at a distance of 2.72 Å. Another active site water hydrogen bonds the first water at a distance of 2.72 Å and also

### Table I

| H122A data collection, refinement, and final model statistics | ID14-EH3 |
|-------------------------------------------------------------|---------|
| **Data collection statistics**                               |         |
| Beamline                                                    | P2_1    |
| Wavelength (Å)                                              | 20–1.9  |
| Space group                                                 | 271694  |
| Resolution (Å)                                              | 19032   |
| Unique reflections                                          | 100 (100)|
| Completeness (%)                                            | 6.0 (28.2)|
| R_{cryst} (%)                                               | 3.8 (3.7)|
| Multiplicity                                                | 22.2 (4.9)|
| **Refinement statistics**                                   |         |
| R_{cryst} (%)                                               | 16.4    |
| R_{free} (%)                                                | 19.6    |
| Root mean square bond lengths (Å)                           | 0.017   |
| Root mean square bond angles (degree)                       | 1.662   |
| Ramachandran                                                | 95.3    |
| Average B main chain (Å²)                                   | 19.4    |
| Average B side chain (Å²)                                   | 22.5    |
| Average B waters (Å²)                                       | 26.5    |
| Average B ligand (Å²)                                       | 23.8    |
| **Model composition asymmetric unit**                       |         |
| Residues                                                    | 2984    |
| Water molecules                                             | 1929    |
| Ligand molecules                                            | 12      |
the backbone carbonyls of Asp-154 and Gly-97 at distances of 2.66 and 2.90 Å, respectively. Finally, the geminal dimethyl group of the product is situated in a hydrophobic cleft formed by Leu-84, Ile-150, and Phe-82, making hydrophobic contacts at distances of 4.04, 4.12, and 3.59 Å, respectively. All three residues are in regions of change one and three with respect to the wild-type structure described above and appear to move closer upon ligand binding.

Superimposition of the ligand-bound mutant and wild-type structure also reveal water molecules in the native structure in place of the endocyclic carbonyl of the product and the oxygen atoms of the pendant C4 acetate. This is perhaps suggestive of water activation by either Asp-154 or His-145 in a general base-catalyzed mechanism as discussed below.

### DISCUSSION

A variety of mechanisms have evolved in nature for the cleavage of β-diketone molecules between the two carbonyl groups. Pentane 2,4-dione (acetylacetone) is cleaved by a serine hydrolase-type enzyme from *Pseudomonas* to yield acetate and acetone (5) and can also be cleaved oxidatively by the enzyme Dke1 from *Acinetobacter johnsonii* to yield methylglyoxal and acetate (6). Mechanistic investigations of these enzymes are complicated by the existence of the substrate in both diketo and enolate forms in solution. In contrast, all the substrates shown to be cleaved by 6-oxo camphor hydrolase thus far are non-enolizable, either as a result of quaternary substitution at the carbon center between the carbonyl groups or because of restrictions imposed by Bredt’s rule in the bicyclic systems. From

### TABLE II

| Enzyme       | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|--------------|-----------|-------|---------------|
| Wild-type OCH| 642.86    | 0.04  | 1.61 × 10^2   |
| H45A         | 1.14      | 0.01  | 9.50 × 10^1   |
| H122A        | 0.36      | 0.20  | 1.80 × 10^3   |
| H145A        | 0.28      | 3.33  | 8.40 × 10^3   |
| D154N        | 1.42      | 4.01  | 3.55 × 10^2   |
| E244Q        | 20.40     | 0.08  | 2.55 × 10^4   |
the results described above, it appears that in the case of these non-enolizable ketones, further enzyme chemistry using amino acids recruited from the crotonase fold has been adopted for β-diketone cleavage, and indeed a previously unreported mechanism of carbon-carbon bond cleavage appears to operate.

Although OCH is unique among the crotonase superfamily in that it does not act on an acyl-CoA thioester, the retro-Dieckmann reaction catalyzed is reminiscent of the carbon-carbon bond cleavage catalyzed by the crotonase homolog BadI from *Rhodopseudomonas palustris* (24). BadI catalyzes the cleavage of 2-ketocyclohexanecarboxyl-CoA (Fig. 5); a recent abstract (25) proposes a possible general base-catalyzed mechanism of hydrolysis via the activation of a water molecule for nucleophilic attack at the endocyclic carbonyl of the substrate. The data presented here also provide persuasive evidence of a general base-catalyzed mechanism for OCH activity, perhaps by activation of a water molecule for nucleophilic attack at the pro-(S) face of the substrate (Fig. 6). A water molecule that is hydrogen bonded to His-145 in the wild-type structure superimposes with one of the carboxylate oxygens of the product, also H-bonded to the same residue. This may suggest that His-145 activates that water molecule for nucleophilic attack. This histidine appears to form a dyad with Asp-154, as suggested by the product-bound crystal structure. The role of aspartate 154 may be to orientate the correct histidine tautomer for reaction, as suggested for ribonuclease A (26) where mutation of the aspartate of the Asp/His dyad to asparagine was also observed to significantly decrease the catalytic efficiency of the enzyme, although in the case of OCH the decrease in catalytic efficiency is much more pronounced. Attack of the water molecule at the carbonyl would result first in the formation of a tetrahedral oxyanion, 4, which would subsequently rearrange to yield enolate 5. The stabilization of kinetically unstable enolates is a feature of catalysis by crotonase homologs, although in this case the structure of the enolate 5 is very different from the enolates ligated to coenzyme A in other crotonases and would be stabilized, if necessary, by a different structure than the conserved oxyanion hole in the other enzymes. Indeed, the proximity of Trp-40 to the endocyclic carbonyl of the product derived from the enolate oxygen suggests that this interaction may prove sufficient for the stabilization of an enolate that would soon be protonated and tautomerized in solution. It may be that a further oxyanion hole would be required for the stabilization of the initial proposed tetrahedral oxyanion. As the product has retained the three-dimensional character of the substrate, we might speculate that stabilization might be

![Fig. 5. Reaction catalyzed by the crotonase homolog BadI, 2-ketocyclohexanecarboxyl hydrolase.](image)

![Fig. 6. Proposed steps in the reaction coordinate of OCH-catalyzed cleavage of 6-oxo camphor 1, based on structural observations.](image)
achieved by interaction with Asp-154, which could explain the high $K_a$ observed when this is mutated to asparagine. We are currently synthesizing non-cleavable analogues of 1 and putative transition state mimics in an attempt to illuminate significant interactions in the wild-type enzyme.

OCH is of interest as a catalyst for preparative biotransformations because of the high degree of prochiral selectivity exhibited by the enzyme. The structure of the H122A mutant is helpful in revealing some of the molecular determinants of that selectivity. Attack of the proposed nucleophile predominantly on the pro-(S) face of the substrate will determine the absolute configuration of the carbon center at C4 of the product. This selectivity appears to be mediated by a number of complex interactions, including specific hydrogen bonding interactions of the substrate pro-(R) carbonyl group with Trp-40 and His-145. In addition, the flattened cyclopentane ring of the product that forms appears to be stacked against Phe-82. The absolute stereochemistry at C2 of the product would be determined by protonation of the enolate 5, but at this stage it is difficult to assign the role of a proton donor to any of the active site amino acid residues. Indeed, we have already suggested that this proton may not be enzyme-derived, as abiotic hydrolysis of the substrate in dilute hydrochloric acid results in the same conformation as that seen in the enzymatic reaction (2). The stereochemistry at the 2-position thus appears to be thermodynamically controlled, and the (2S,4S) product bound by the active site would tautomerize to the 6:1 mixture of (2R,4S):(2S,4S) when released from the confines of the active site. Nevertheless, the identification of the molecular determinants of prochiral facial selectivity in this mutant may prove valuable in experiments designed to rationally engineer OCH to deliver the opposite enantiomer of the chiral keto acid products.

The crotonase superfamily provides a fascinating model for the study of the divergent evolution of enzyme reaction chemistry, incorporating many proteins that have apparently recruited the parent enoyl-CoA hydratase fold for the catalysis of a wide range of reactions. As such, OCH represents a further divergence from other crotonase homologs in that the proposed intermediate stabilized along the reaction coordinate is not the enolate derived from an acyl-CoA thioester and that the conserved oxyanion hole in known crotonase homolog structures is absent (14). Gerlt and Babbitt (27) have proposed new bases for classification of proteins into superfamilies based not only on the tertiary fold but also reaction chemistry. In this system, members of the same superfamily would either (a) catalyze the same chemical reaction or (b) catalyze reactions that share a “common mechanistic attribute,” such as the enolate stabilized by a conserved oxyanion hole in crotonase homologs. The product-bound structure of OCH suggests that stabilization of either the proposed tetrahedral transition state or transient enolate requires a different oxyanion hole than that of the rest of the crotonase superfamily. Although sequence and structure would certainly place OCH in a crotonase “superfamily” (27), the mechanistic attributes of the OCH-catalyzed reaction suggest that OCH is the first member of a new family of enzymes that, as yet, has few close relatives.

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