Crystal Structure of the (R)-Specific Enoyl-CoA Hydratase from Aeromonas caviae Involved in Polyhydroxyalkanoate Biosynthesis*

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The (R)-specific enoyl coenzyme A hydratase (R-hydratase) from Aeromonas caviae catalyzes the addition of a water molecule to trans-2-enoyl coenzyme A (CoA), with a chain-length of 4–6 carbons, to produce the corresponding (R)-3-hydroxyacyl-CoA. It forms a dimer of identical subunits with a molecular weight of about 14,000 and is involved in polyhydroxyalkanoate (PHA) biosynthesis. The crystal structure of the enzyme has been determined at 1.5-Å resolution. The structure of the monomer consists of a five-stranded antiparallel β-sheet and a central α-helix, folded into a so-called “hot dog” fold, with an overhanging segment. This overhang contains the conserved residues including the hydratase 2 motif residues. In dimeric form, two β-sheets are associated to form an extended 10-stranded β-sheet, and the overhangs obscure the putative active sites at the subunit interface. The active site is located deep within the substrate-binding tunnel, where Asp31 and His16 form a catalytic dyad. These residues are catalytically important as confirmed by site-directed mutagenesis and are possibly responsible for the activation of a water molecule and the protonation of a substrate molecule, respectively. Residues such as Leu165 and Val139 are situated at the bottom of the substrate-binding tunnel, defining the preference of the enzyme for the chain length of the substrate. These results provide target residues for protein engineering, which will enhance the usefulness of this enzyme in the production of novel PHA polymers. In addition, this study provides the first structural information of the (R)-hydratase family and may facilitate further functional studies for members of the family.

Metabolism of various fatty acids via β-oxidation includes a hydration step in which 2-enoyl coenzyme A (CoA) intermediates are converted to 3-hydroxyacyl-CoAs. Two stereoisomers of the product are used in different metabolic pathways, and, accordingly, enzymes with different stereospecificities are involved in each pathway. The enzyme responsible for the production of the S-isomer is the classical enoyl-CoA hydratase (crotonase) and is involved in degradation of fatty acids in mitochondria, peroxisomes, and bacterial cells. The rat mitochondrial enzyme has been studied extensively. The rat enzyme has a specific activity for CoA thioesters of straight chain fatty acids with a broad range of chain lengths (C14–C16) (1). It is a hexamer of identical subunits composed of 261 residues in the mature form (2). Extensive studies (3–5), including crystallographic analyses (6–8), have revealed that two amino acid residues, Glu144 and Glu146, are important for the catalytic reaction that proceeds through an acid-base mechanism. However, the enzyme responsible for the production of the R-isomer, (R)-specific enoyl-CoA hydratase, hydratase 2, or β-hydratase (hereafter referred to as (R)-hydratase), has been identified recently in mammals, yeast, and some polyhydroxyalkanoate (PHA)-producing bacteria. In mammals, this enzyme occurs as a domain of the peroxisomal multifunctional enzyme type 2 (MFE-2) and is involved in the degradation of very long chain and 2-methyl-branched fatty acids and the biosynthesis of bile acids (9–13). The yeast enzyme is also localized in the peroxisome as a part of MFE-2 but is involved in the degradation of straight chain fatty acids (14). These eucaryotic enzymes are similar in size, composed of about 300 amino acid residues, and are highly homologous (15). Bacterial (R)-hydratases have been found to date in Aeromonas caviae (16, 17), Pseudomonas aeruginosa (18), Rhodopseudomonas palustris (19), and Methylobacterium rhodesianum (20), all of which are known to accumulate PHA granules in their cells. These enzymes have been characterized at the molecular level except for the case of M. rhodesianum. However, the A. caviae enzyme is the only bacterial one whose physiological role has been identified to date.

The A. caviae (R)-hydratase is essentially involved in the biosynthesis of an energy storage material of PHA, functioning as a monomer-supplying enzyme (16, 17). It is encoded as a phaJ gene in the PHA biosynthesis operon, including the phaC and phaP genes coding for PHA synthase (16) and granule-associated protein (21), respectively. The hydratase acts on the β-oxidation intermediates trans-2-enoyl-CoAs to produce (R)-3-hydroxyacyl-CoAs, which are subsequently polymerized to form PHA by PHA synthase. The hydratase, and the synthase
as well, preferably act on the substrates with a chain length of 4–6 carbon atoms, accounting for the fact that the produced PHA is a copolymer of (R)-3-hydroxybutyric (C4) and (R)-3-hydroxyhexanoic (C6) acids (22). Unlike the eucaryotic enzymes, this bacterial (R)-hydratase is a monofunctional enzyme forming a homodimer of identical subunits with a molecular weight of 13,954. The mature polypeptide chain contains 133 amino acid residues (17). Interestingly, despite the lack of a physiological relationship with the eucaryotic enzyme, the A. caviae enzyme has significant sequence homology with the C-terminal region of the eucaryotic enzyme (e.g., a 38.4% identity with the yeast enzyme for a limited region of 73 amino acid residues (16), indicating that they are derived from a common ancestor.

The reaction mechanism of the (R)-hydratase has not been well characterized, in contrast to the rat crotonase. Recently, a highly conserved amino acid sequence, referred to as the hydratase 2 motif, has been identified by comparing the hydratase 2 domains of peroxisomal MFE-2s and several fungal and bacterial proteins (15). On the basis of the sequence comparison, pK measurement, and mutagenesis, it has been proposed that in the human hydratase 2, Glu466 and Asp510 play a critical role in the catalytic reaction through an acid-base mechanism that is similar to that for the crotonase (15). However, this cannot be applied to the case of the A. caviae enzyme. Although Asp510 of the human enzyme, one of the hydratase 2 motif residues, corresponds to Asp31 of the A. caviae enzyme, there is no counterpart of Glu466 in the A. caviae enzyme, because of the lack of a region corresponding to the N-terminal half (residues from 319 to 483) of the human enzyme where Glu466 is located. Therefore, if the catalytic reaction of the A. caviae hydratase also proceeds via an acid-base mechanism, which residue partners with Asp31 to form the catalytic dyad? A preference for the chain length of the CoA thioester substrate is another remarkable difference between bacterial and peroxisomal enzymes. Because no structure of either bacterial or peroxisomal enzymes has been reported, it is not clear whether or not the difference in substrate specificity is linked to the large difference in the polypeptide chain length of these proteins.

To elucidate the mechanisms of the catalytic reaction and recognition of the chain length of substrates, a three-dimensional structure of the enzyme is indispensable. Here we report the first crystal structure of (R)-hydratase. The structure of A. caviae (R)-hydratase has a so-called “hot dog” fold as a main frame, with an overhanging segment that contains conserved residues including the hydratase 2 motif residues. The catalytic residues Asp31 and His36 are on the overhanging segment and are located at the dimer interface. The substrate-binding site is tunnel-shaped, which accounts for the preference for a substrate with chain length of four to six. The structure will provide invaluable information for the application of this enzyme to the production of PHAs that have attracted considerable attention because of their potential as renewable and biodegradable plastics (23–27). It will also provide a plausible model for the human enzyme, the deficiency of which causes a peroxisomal disorder (28–30).

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Crystallization, and Data Collection**—Recombinant (R)-hydratase was overexpressed in *Escherichia coli* and purified according to the procedure described by Fukui et al. (17). Because the sample was found to be degraded perhaps due to contaminated proteins, further purification by gel filtration was performed with a Superdex 70pg column (Amersham Biosciences). Crystals of the enzyme were obtained by sitting-drop vapor-diffusion using a mother liquor containing 20% polyethylene glycol 4000, 5% 2-propanol, and 20 mM HEPES (pH 7.0), as described previously (31). The crystallization experiments were set up at 25 °C. Large crystals were grown in 2–3 weeks to maximum dimensions of 0.5 × 0.1 × 0.04 mm and were stabilized in a solution containing 28% polyethylene glycol 4000, 5% 2-propanol, and 20 mM MES (pH 6.0). These crystals belong to space group C2, with cell parameters *a* = 111.5 Å, *b* = 59.3 Å, *c* = 47.3 Å and *β* = 112.9°, and contain two subunits per asymmetric unit. They diffracted CuKα radiation up to a resolution of 2 Å. The intensity data (Native 1) were processed to a resolution of 2.5 Å with an Rmerge of 7.8% using PROC-ESS (Rigaku). Higher resolution data (Native 2; up to 1.5 Å resolution) from a flash-cooled crystal were collected using synchrotron radiation at SPring-8, Harima, Japan (32), and were processed with an Rmerge of 5.5% using MOSFLM (33). On flash-cooling, the cell parameters of the crystal changed slightly to *a* = 110.0 Å, *b* = 57.8 Å, *c* = 47.0 Å, and *β* = 112.7°. The statistics of the data collection are summarized in Table I.

For the preparation of heavy atom derivatives, the (R)-hydratase crystals were transferred to a synthetic mother liquor containing 28% polyethylene glycol 4000, 5% 2-propanol, and 100 mM MES (pH 6.0). Three isomorphous heavy atom derivatives were prepared with 1 mM HgCl2, 3 mM KI3IrCl6, or 3 mM K2PtCl4. All sets of derivative data were collected using CuKα radiation at room temperature and processed with PROCESS (Table I).

**Structure Determination and Refinement**—Initial phase calculation was conducted with the Native 1 and derivative data sets. Two mercury sites were identified by calculating the difference-Patterson function using PHASES (34). Two iridium atom sites and one platinum site were identified using the difference-Fourier technique. Heavy atom parameters were refined at 3.5 Å resolution in PHASES, including the anomalous dispersion signals of mercury. Following solvent-flattening density modification and phase extension to 3.0 Å resolution, the electron density map was clearly interpretable. A model was built interactively using XTALVIEW (35) and TURBO-FRODO (36). Because the encoded first Met residue does not exist in the mature enzyme due to a post-translational modification (17), the sequence number was started with the encoded second residue Ser as the first. This model was positioned in a unit cell by molecular replacement using REPLACE (37) calculated against the Native 2 data set, and then refined with a simulated annealing protocol using X-PLOR (38) and CNS (39). The final model includes residues 2–133 and 1–133 for chains A and B, respectively, one 2-propanol, and 347 water molecules. Fig. 1 shows a representative (2mFo − DFo) electron density map of the protein. The stereochemistry was verified using PROCHECK (40). The atomic coordinates have been deposited in the Protein Data Bank (accession code 1IQ8).

**Mutagenesis, Activity Assay, and CD Spectra Measurement**—Site-directed mutagenesis was performed by means of the PCR method (41) and was confirmed by DNA sequencing. The PCR primers containing mutational sites were designed in inverted tail-to-tail directions and used for the amplification reaction with the expression vector for the (R)-hydratase (pETNB3) as a template. The oligonucleotide sequences of the primers used are as follows: 5′-CTCTCGGAGGCCTTCAAC-CCTGTCG-3′ for the D31A mutation; 5′-CTCTCGGAGGACTTACAACC-CCTGTCGGCTTGACCACCCGGCC-3′ for the H36A mutation; 5′-CGGCTCAGAAGGCGGCTTCTCCGACGCACGC-3′ for the H63A and H64A mutations; and 5′-CTCTCGGAGGCGGCTTGAGGCGGCACGCA-3′ and 5′-GCTGCG- GAGCGACATGGCGGACTTGGG-3′ for the S62A mutation (the changed codons causing the mutations are indicated as boldface letters). The products were self-ligated and transformed into *E. coli* BL21 (DE3). The expression, purification, and activity assay for the mutant enzymes were carried out in the manner described previously (17) for

![FIG. 1. A representative 2Fo - DFo electron density map at 1.5-Å resolution (contoured at 1σ).](http://www.jbc.org/)

*Author contributions: K. Shibata, S. Uetani, M. Inoue, K. Shibata, S. Uetani, M. Inoue, T. Kishi, A. Moriyama, K. Hasegawa, and H. Shiomi performed the experiments and wrote the paper. T. Kishi, A. Moriyama, and H. Shiomi designed the study and supervised the research.*
the wild-type enzyme. The structural conformations of the mutants were evaluated by far-UV CD spectroscopy using a Jasco J720 spectropolarimeter in a range of 200–250 nm with a 1-mm path length quartz cuvette at 20 °C.

RESULTS AND DISCUSSION

The crystal structure of the (R)-hydratase was solved by isomorphous replacement with anomalous scattering using the mercury, iridium, and platinum derivatives. The final model was refined to 1.5-Å resolution with crystallographic and free (5% of the total reflections) R-factors of 20.3 and 23.1%, respectively (Table I). The asymmetric unit contained two polypeptide chains, referred to as A and B, which together formed a functional dimeric molecule. Because the first encoded residue, histidine, is post-translationally deleted (17) and not present in the mature protein, the encoded second residue serine represents residue 1 in our numbering system. Chain B consists of all of the 133 amino acid residues, whereas chain A contains 132 residues, because Ser1 was not well defined in the electron density map. Both chains are effectively identical with a root-mean-square (r.m.s.) deviation between the 130 Ca atoms of each monomer (residues 4–133) of 0.59 Å. However, small conformational differences occur in the region from Asn33 to Arg49 and its adjacent region from Leu65 to Ile75 (r.m.s. deviation of 1.28 Å for 28 Ca atoms). As a result, the segment from Pro39 to Ala42 forms a 3 10-helix in chain B, whereas it is an α-helix in chain A. This helix in chain B has more contact with the central helix H4, the interaction with the hot dog main body, other than the central helix H4, is then followed by a long extended loop of about 10 residues, and finally the central helix H4, with four turns, comes to be connected to strand S2. Helix H4 has a very interesting feature. It is a complex of three helices that are fused continuously along a common axis. The first and the last portions, Gly54–Leu57 and Phe61–Gln68, form α-helices, whereas the middle portion, Leu57–Phe61, forms a 3 10-helix. Helix H4 is wrapped around by the largely wound β-sheet like a hot dog, where the β-sheet and the central helix H4 are a “bun” and a “sausage,” respectively.

The hot dog fold has been originally observed for the β-hydroxydecanoyl thioester dehydrase from E. coli (42) and later for the 4-hydroxybenzoyl-CoA thioesterase from Pseudomonas sp. CBS-3 as well (43). Two hot dog repeats in a single peptide, called the “double hot dog” fold, have also been reported for the medium chain length acyl-CoA thioesterase II from E. coli (44). Although they have a fold similar to the core structure, (R)-hydratase has a feature that distinguishes it from other hot dog fold enzymes. The most notable difference is an overhanging segment situated between the first strand S1 and the central helix H4. This region in other hot dog fold enzymes is a relatively short loop of about 15 residues, whereas in (R)-hydratase, it is a large fragment of about 35 residues. This overhang in (R)-hydratase is rather compact and has little interaction with the hot dog main body, other than the central helix H4. The B-factor values for the main chain atoms in the region from Asn33 to Arg49 (from helix H2 to the loop after helix H3; see Fig. 2b) are relatively high compared with those in the hot dog main frame (the average B-factors for the Ca atoms of chains A and B are 18.01 and 17.03 Å², respectively, whereas those in the region from Asn33 to Arg49 of both chains are 23.65 and 23.24 Å², respectively), indicating the intrinsic lability of

### Table I. Data statistics

| Data set    | Native 1 | Native 2 | HgCl₂ | K₂IrCl₆ | K₃PtCl₄ |
|-------------|----------|----------|-------|---------|---------|
| Wavelength  | 1.5418   | 1.0000   | 1.5418| 1.5418  | 1.5418  |
| Resolution (Å) | 2.5     | 1.5     | 3.0   | 3.5     | 3.6     |
| Total observations | 30,245  | 198,695  | 17,069| 10,235  | 10,949  |
| Unique reflections | 9,461   | 52,985   | 5,614 | 3,554   | 3,299   |
| Completeness (%) | 93.0    | 99.4     | 95.6  | 96.3    | 95.5    |
| Rmerge (%) | 7.2      | 5.2      | 10.2  | 10.3    | 11.0    |
| Rfree (%) | 20.6     | 14.6     | 19.9  | 19.9    | 19.9    |
| Phasing power | 1.58    | 1.14     | 1.28  |         |         |
| Mean figure of merit |         |         | 0.50  |         |         |

* Rmerge = Σ|Fnative|−|Fobs|/|Fnative|, where Fnative and Fobs are structural factors of the native and derivative data, respectively.

### The Monomer Structure

The (R)-hydratase monomer consists of a five-stranded antiparallel β-sheet and four helices (Fig. 2, a and b). The order of strands in the β-sheet is 1-3-4-5-2, and the length of each strand ranges between 7 and 11 residues. All the helices (H1–H4) are connected between the first strand, S1, and the second strand, S2. Strand S1 is followed by the first α-helix H1 having three turns. Two contiguous short helices with one turn for each follow helix H1. In chain A, both helices are in the α-helical conformation, whereas in chain B, helices H2 and H3 are in the α- and 3 10-helical conformations, respectively, as described above. Helix H3 is then followed by a long extended loop of about 10 residues, and finally the central helix H4, with four turns, comes to be connected to strand S2. Helix H4 has a very interesting feature. It is a complex of three helices that are fused continuously along a common axis. The first and the last portions, Gly54–Leu57 and Phe61–Gln68, form α-helices, whereas the middle portion, Leu57–Phe61, forms a 3 10-helix. Helix H4 is wrapped around by the largely wound β-sheet like a hot dog, where the β-sheet and the central helix H4 are a “bun” and a “sausage,” respectively.
this region. This is consistent with the observed conformational differences between chains A and B as described above. The residues in this overhang are highly conserved among the \((R)\)-hydratases, indicating the importance of this overhang. In fact, the catalytically important residues Asp\(^{31}\) and His\(^{36}\) are involved in this segment (see below). Thus, this overhang is both structurally and functionally characteristic to the \((R)\)-hydratase.

A secondary structure-based sequence alignment shows that \((R)\)-hydratase from \(A.\) \textit{caviae} has some homology with the \((R)\)-hydroyxacyl thiol ester dehydrase from \(E.\) \textit{coli} (Fig. 3). A total of 12 identical residues are localized in S1, H4, S3, and S5 (the \((R)\)-hydratase notation). This suggests that \((R)\)-hydratase and dehydrase may have been derived from a common ancestor protein. This is not surprising because the functions of both enzymes are very similar; both catalyze the addition/elimination of a water molecule to/from 2-enoyl/(\(R\))-3-hydroxyacyl thioesters (CoA and acyl-carrier protein thioesters for \((R)\)-hydratase and dehydrase, respectively). In the case of \((R)\)-hydratase, the overhang, including the functionally important residues, may have been inserted in the course of molecular evolution.

\textit{The Dimer Structure}—In the dimeric form (Fig. 2, c and d), two monomers make extensive contacts, burying 1426.02 Å\(^2\) (about 20\%) of the surface area of the monomer, 68\% of which
is apolar (values from www.biochem.ucl.ac.uk/bsm/PP/server/). Helices H1 and H4 and strand S2 are associated in an antiparallel direction so that the two β-sheets form a 10-stranded β-sheet as a whole. The extended 10-stranded β-sheet wraps around the two central helices, with the overhangs covering the gaps that occur at the interface of the two subunits. Helices H1 and H4 are associated with their counterparts across the molecular dyad, facing side by side in an antiparallel direction. These paired helices are then packed so that the axes of the helices run almost perpendicular to each other. Thus, the two H4 helices are sandwiched between the two H1 helices and the 10-stranded β-sheet. This results in the helix H4 being almost entirely buried in the molecule, with the C terminus of the helix partially exposed to the solvent. In fact, helix H4, which is composed of 14 residues (Gly54 – Ser29), is dominantly hydrophobic with the exception of only three polar residues (Ser 59, Ser62 and His63) which are important (see below). On the other hand, helix H1 is half-exposed to solvent. It contains 11 residues (Ala19 – Ser20), including two polar ones (Glu21 and Ser29). The exposed face of the helix are lined alanine residues (Ala19, Ala20, Ala23, Ala24, and Ala27) in addition to Glu21. Ser29 is buried in the protein, hydrogen-bonded to the carbonyl oxygen of Phe25 and the amide nitrogen of Asp31. The dimeric molecule has an ellipsoidal shape, with dimensions of $-56 \times 46 \times 35 \text{ Å}$.

The Hydratase 2 Motif and Other Homologous Proteins—Only a few examples (17 – 19) of bacterial (R)-hydratases have been described thus far. They are similar in polypeptide chain length (133 – 174 residues), substrate specificities (for short chain length enoyl-CoA), and amino acid sequence (about 45%). Eucaryotic enzymes (around 300 residues), on the other hand, are about twice as large as the bacterial enzymes and have a substrate specificity for very long chain length, 2-branched, and C27 bile acid intermediate 2-enoyl-CoAs. Qin et al. (15) recently identified the highly conserved amino acid sequence, referred to as the hydratase 2 motif ([Phe25]-[Ser29]), by comparing the hydratase 2 domains of MFE-2s and several fungal and bacterial proteins. The sequence alignment shows that the bacterial (R)-hydratases also have this motif, with a deviation in which the glycine residue is replaced by Glu30 for the A. caviae (R)-hydratase and the yeast, rat, human, and A. caviae enzymes are shown only for the regions equivalent to the A. caviae enzyme.

In the (R)-hydratase structure, the residues corresponding to the hydratase 2 motif are located in the region from the C-terminal half of helix H1 to the short helix H3 (25) and the water molecule and may be functionally important (see below). On the other hand, helix H1 is half-exposed to solvent. It contains 11 residues (Ala19 – Ser20), including two polar ones (Glu21 and Ser29). On the exposed face of the helix are lined alanine residues (Ala19, Ala20, Ala23, Ala24, and Ala27) in addition to Glu21. Ser29 is buried in the protein, hydrogen-bonded to the carbonyl oxygen of Phe25 and the amide nitrogen of Asp31. The dimeric molecule has an ellipsoidal shape, with dimensions of $-56 \times 46 \times 35 \text{ Å}$.

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FIG. 4. a, close view of the putative active site of (R)-hydratase. The main chain is represented as a ribbon drawing. For clarity, helices H2 and H3 are represented as a coil model. The side chains of Asp31, His36, Phe25, Phe83, Pro86, Ser62, Leu65, Pro70, Ser74, Tyr76, and Val130 are shown as a ball-and-stick model. Carbon, oxygen, nitrogen, and sulfur atoms are indicated in black, red, blue, and yellow, respectively. Water molecules are represented by orange balls. b, a docking model of crotonyl-CoA in the active site of the (R)-hydratase. The model was built manually on a SGI graphics workstation using TURBO-FRODO (36), with reference to the binding mode of the suicide inhibitor in the E. coli dehydrase (42). Possible specific interactions between the carboxyl group of the thioester bond of crotonyl-CoA and the amide group of Gly44, and between the phosphate groups of crotonyl-CoA and the side chains of Asp105, Arg106, and Lys131 were considered. Carbon atoms of crotonyl-CoA are indicated in gray. Only crotonyl and pantetheine moieties are shown as a ball-and-stick model with a different radius from those of residues. A water molecule that is possibly activated by Asp31 is shown above the C3 atom of crotonyl-CoA.

Glu10 adopts a left-handed helical conformation (μ, ϕ) = (71.8° and 16.3°) (chain A) and (74.3° and 13.0°) (chain B), which accounts for the high preference for the glycine residue at this position in other hydratase 2 motif enzymes. Due to the conformation of Glu10, the side chain of the following catalytic residue Asp31 can be properly oriented to the catalytic site cavity. Note that the side chain of Glu10 is hydrogen-bonded to Lys55, which may contribute to stabilizing the energetically less favorable conformation. Another notable residue is Ser39. This residue, situated at the C terminus of the helix H1, is buried in the protein, hydrogen-bonded to the carbonyl oxygen of Phe25 and the amide nitrogen of Asp31. This interaction may also stabilize the conformation of the peptide Glu10–Asp31.

Other homologous proteins include the β-subunits of fattyacid synthases and hypothetical proteins from various sources, and collectively, they are classified into a family in which a conserved domain referred to as the MaoC-dehydratase domain in the Pfam data base (CD ID: pfam 01575) (45) is shared. Therefore, the (R)-hydratase structure described here provides the first and representative example for this conserved domain.

Active Site and Implications for the Catalytic Mechanism—Attempts to prepare crystals of the enzyme with substrates or competitive inhibitors have, thus far, been unsuccessful. It is possible, however, to speculate on the location of the active site of the (R)-hydratase on the basis of the structural similarity with the E. coli β-hydroxycetanoyl thiol ester dehydrase, for which the active site has been identified (42).

Two active sites related by the molecular dyad are formed at the interface of the two monomers. Each active site is located deep within the substrate-binding tunnel, which is contributed by both of the monomers. Although the environment of the tunnel is dominantly hydrophobic, three charged or polar residues, Asp31, His36, and Ser82, are localized at the bottom of the tunnel, making a complex hydrogen bond network with five water molecules (Fig. 4a). An inspection of the active site structure suggests that Asp31 and His36 are the most promising candidates for the catalytic residues.

To examine the importance of these residues, as well as Ser82, for catalysis, four mutants, D31A, H36N, H36A, and S62A, were prepared and assayed. For the former three mutants, the Vmax values were reduced by 10-2-fold compared with the wild-type enzyme, from 6.2 × 105 units mg−1 to 9.7 × 102, 5.7 × 102, and 9.4 × 102 units mg−1, respectively. The Km values were largely unchanged (23, 11, and 16 μM for the three mutants, respectively, compared with 29 μM for the wild-type enzyme). These data indicate that Asp31 and His36 are essential for catalysis. It should be noted that the CD spectra for these mutants showed profiles similar to the wild-type enzyme (data not shown), indicating that the observed reduced activities for these mutants are not due to conformational changes of the proteins. The mutant S62A, on the other hand, showed Vmax and Km values of 9.6 × 105 units mg−1 and 27 μM, respectively, and thus retained significant activity, suggesting that Ser82 is less important for catalysis. An observation of the hydrogen bond between Asp31 and Ser82 may suggest that Ser82 plays a significant role in maintaining the orientation of the side chain of Asp31 to be favorable for catalysis and in increasing the overall efficiency of the catalysis.

Asp31 and His36 are conserved in other bacterial and eucaryotic (R)-hydratases (Fig. 3), which also indicate the importance of these residues for enzyme function. In the case of the human enzyme, on the other hand, it has been proposed that Glu366 and Asp510 play critical roles in the hydratase reaction, acting in a similar manner to the two catalytic glutamic acid residues of the rat crotonase (15). Whereas Asp510 of the human enzyme corresponds to Asp31 of the A. caviae enzyme, there is no counterpart of Glu366 in the A. caviae enzyme due to the lack of a corresponding region to the N-terminal half of the human enzyme, where the Glu366 residue is located. Unfortu-
nately, the investigators in Ref. 15 failed to examine the importance of His$^{36}$ in the human enzyme, which is equivalent to His$^{515}$ of the A. caviae enzyme, because the H515A mutant could not be correctly folded. Thus, the possibility that His$^{515}$ of the human enzyme has a catalytic role cannot be ruled out. For the A. caviae enzyme, the catalytic importance of His$^{36}$ has been demonstrated, as described above.

On the basis of the crystal structure analysis and mutagenesis experiments, and with the aid of a docking model of crot- onyl-CoA to the substrate-binding tunnel (Fig. 4b), we propose that the Asp$^{31}$ and His$^{36}$ residues play critical roles in the catalytic reaction of the enzyme as follows. Asp$^{31}$ may activate a water molecule by abstracting a proton from a water molecule. The activated water molecule would then attack the car- bon atom C3 of crot- onyl-CoA, and cooperatively His$^{36}$ may donate a proton to the C2 carbon atom of the substrate, thus completing one cycle of the catalytic reaction. Note that the docking model also suggests the importance of Gly$^{54}$. The am- ide group of Gly$^{54}$ may hydrogen-bond to the carbonyl group of the thioester bond of the substrate, thus keeping the substrate in an orientation in which the activated water molecule is restricted to attacking the C3 atom of the substrate from the re-face, which is related to the C2–C3 double bond, so as to form the R-isomer of the product. The amide group of Gly$^{54}$ may also function as the oxanion hole, which, in addition to the dipole of the central α-helix H4, stabilizes the reaction intermediate. This interaction between the substrate and the protein re- quires a glycine residue at position 54 exclusively, otherwise steric hindrance between the carbonyl group of the substrate and the side chain of the residue would have disruptive influ- ences.

The proposed reaction mechanism is very similar to that of β-hydroxydecanoyl thiol ester dehydrase from E. coli, which utilizes His$^{70}$ and Asp$^{84}$ as catalytic residues (42). However, there is a large difference between the two proteins with re- spect to the location of the two active site residues. In the case of the dehydrase, the active site residues Asp and His are located on different subunits, whereas in the case of (R)-hydratase, they are on the same subunit. The Ca atoms of the histidine and aspartic acid residues from both proteins are located spatially in different positions. Interestingly, even the location of the Ca atoms of the catalytic residues are different, the architecture of the active site is very similar, that is, the catalytically significant Nε1, Oγ1, and amide nitrogen atoms of the His, Asp, and Gly residues, respectively, can be well super- imposed. Note that Ser$^{62}$ in the A. caviae enzyme, which is located on the central helix, is topologically equivalent to Asp$^{84}$ of the E. coli dehydrase and that the Oγ1 atom of the Ser residue and the Oγ2 atom of the Asp residue come close in the same superposition.

**Substrate-binding Site**—The substrate-binding site is a tunnel-shaped pocket 15 Å long and 6 Å wide. The mouth of the tunnel is formed by Phe$^{47}$ and residues from the region imme- diately after strand S2 (Phe$^{83}$–Pro$^{86}$) and the N-terminal re- gion of strand S2’ (Ile$^{75}$–Leu$^{77}$) (Fig. 4a). Of these residues, Phe$^{47}$, Phe$^{83}$, Pro$^{86}$, Ile$^{75}$, and Tyr$^{76}$ are conserved in bacterial (R)-hydratases (Fig. 3), indicating that they are important for interactions with the substrate or the formation of the mouth of the tunnel. The docking model (Fig. 4b) indicates that the 3’-phosphate ADP moiety of the substrate may be bound at the surface of the enzyme and that the pantetheine and acyl chain moieties are bound inside the tunnel. Arg$^{103}$, Arg$^{104}$, and Lys$^{105}$ are located around the mouth which may be involved in the binding of the phosphate groups of the 3’-phosphate ADP moiety. The tunnel can be divided into two parts at the cata- lytic site. They correspond to the regions for binding of the pantetheine and acyl chain moieties. The pantetheine-binding site is contributed by the two subunits, whereas the acyl chain- binding site is formed by a single subunit. The acyl chain- binding site is surrounded by the side chain atoms of Ser$^{89}$, Leu$^{95}$, Pro$^{90}$, Ser$^{94}$, Tyr$^{76}$, and Val$^{130}$, in addition to the main chain atoms of these residues and Gly$^{96}$ and Ile$^{78}$. These resi- dues define the size of the acyl chain-binding pocket, which is sufficient to accommodate a substrate with a carbon chain length of four to six, but which is insufficient to accommodate a longer one. To accommodate substrates with much larger chains, the tunnel may need to be burrowed further into the hydrophobic core of the protein. Thus these residues can be targets for site-directed mutagenesis to modify the preference for the chain length of the substrate, which will generate mon- mers with various structures and enable novel PHAs to be designed.

Compared with the bacterial enzyme, the eucaryotic en- zymes have a larger molecular size of about 300 residues and prefer substrates with a longer carbon chain. For example, the yeast enzyme can act on 2-decenoyl-CoA (14), and the human enzyme on 2-methylhexadecanoyl-CoA and 3a,7a,12a-trihy- droxy-5β-cholest-24-enoyl-CoA (11). It does not appear that the acyl chain-binding site housed in the hot dog structure is able to provide sufficient room for these large substrates. Thus it is likely that the architecture of the acyl chain-binding site in the eucaryotic enzyme is different from that in the bacterial one, that is, the N-terminal portion of the eucaryotic enzyme may contribute to the large part of it. Furthermore, it should be noted that residues corresponding to Ile$^{75}$, Tyr$^{76}$, and Leu$^{77}$ of the A. caviae enzyme around the mouth are not conserved between the eucaryotic and bacterial enzymes, in contrast to residues Phe$^{47}$, Phe$^{83}$, and Pro$^{86}$ which are highly conserved in both groups. This suggests that the N-terminal region of strand S2 might not be involved in interactions with substrates. This further indicates that in the eucaryotic enzyme, the C-terminal portions corresponding to the bacterial enzyme do not associate to form a homodimer with a 10-stranded β-sheet. If this is true, the N-terminal portion might form another hot dog fold and associate with the C-terminal hot dog moiety, that is the enzyme would have a double hot dog fold, as is observed in the E. coli medium chain acyl-CoA thioesterase II (44).

In conclusion, the data herein reveal that (R)-hydratase is a member of the hot dog fold enzyme superfamily. Furthermore, the functionally important overhanging segment, including the hydratase 2 motif residues, is inserted into the hot dog core structure. The structure identifies the key catalytic residues Asp$^{31}$ and His$^{36}$, which were further confirmed by mutagene- sis. It is proposed that Asp$^{31}$ may function as an activator of a water molecule which attacks a substrate molecule and His$^{36}$ as a proton donor to the substrate. The acyl chain-binding pocket is circumscribed by residues such as Leu$^{65}$ and Val$^{130}$, which define the selectivity for the chain length of substrates. The issue of whether the overhang, which has relatively high B-factor values, changes its conformation on binding of sub- strates is an intriguing one. Crystallographic studies of the enzyme-substrate complex are currently in progress to address this issue as well as the detailed catalytic reaction mechanism. In addition, future studies will include the modification of the specificity for the carbon chain length of substrates by target- ing the residues involved in the acyl chain-binding site, in an attempt to obtain enzymes with a specificity useful for the production of PHA polymers with desired structures. Finally, this study provides the first insight into the structure of the (R)-hydratase family with a signature of the hydratase 2 motif, which will facilitate further functional studies of this class of enzymes.
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Crystal Structure of the (R)-Specific Enoyl-CoA Hydratase from Aeromonas caviae Involved in Polyhydroxyalkanoate Biosynthesis
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