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The X-ray Structure of Epoxide Hydrolase from Agrobacterium radiobacter AD1

AN ENZYME TO DETOXIFY HARMFUL EPOXIDES

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Epoxide hydrolases catalyze the cofactor-independent hydrolysis of reactive and toxic epoxides. They play an essential role in the detoxification of various xenobiotics in higher organisms and in the bacterial degradation of several environmental pollutants. The first x-ray structure of one of these, from Agrobacterium radiobacter AD1, has been determined by isomorphous replacement at 2.1-Å resolution. The enzyme shows a two-domain structure with the core having the α/β hydrolase-fold topology. The catalytic residues, Asp107 and His275, are located in a predominantly hydrophobic environment between the two domains. A tunnel connects the back of the active-site cavity with the surface of the enzyme and provides access to the active site for the catalytic water molecule, which in the crystal structure, has been found at hydrogen bond distance to His275. Because of a crystallographic contact, the active site has become accessible for the Gln134 side chain, which occupies a position mimicking a bound substrate. The structure suggests Tyr152/Tyr215 as the residues involved in substrate binding, stabilization of the transition state, and possibly protonation of the epoxide oxygen.

Epoxide hydrolases (EC 3.3.2.3) are a group of functionally related enzymes that catalyze the cofactor-independent hydrolysis of epoxides to their corresponding diols by the addition of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule. Epoxides are very reactive electrophiles and are converted to less toxic, water-soluble compounds by the action of a water molecule.

Until now most research has been focused on mammalian epoxide hydrolases (2, 3), which, together with glutathione S-transferases, are the most important enzymes to convert toxic epoxides to more polar and easily excretable compounds (4). However, much progress has recently also been made in the characterization of bacterial epoxide hydrolases (5, 6, 7). These enzymes show a significant sequence homology with those of mammalian origin. They can be easily obtained in large amounts, and they exhibit enantioselectivity with various industrially important epoxides, which makes them promising biocatalysts for the large scale preparation of enantiopure epoxides and/or their corresponding vicinal diols (8). In particular, extensive studies have been performed on the epoxide hydrolase from Agrobacterium radiobacter AD1, a Gram-negative bacterium that is able to use the environmental pollutant epichlorohydrin as its sole carbon and energy source (5, 6, 8). This epoxide hydrolase is a soluble monomeric globular protein of 35 kDa with a broad substrate range. Epichlorohydrin and epibromohydrin are its best substrates, and the optimum pH range for catalysis is 8.4–9.0. Sequence and secondary structure analysis suggested that this enzyme belongs to the α/β hydrolase-fold family of enzymes (9). Site-specific mutations indicated Asp107, His275, and Asp246 as the catalytic triad residues. The proposed catalytic mechanism involves two steps analogous to haloalkane dehalogenase (10). In the first reaction step, an ester bond is formed between enzyme and substrate by attack of the nucleophilic Asp107 on the primary carbon atom of the substrate; in the second step, this ester bond is hydrolyzed by a water molecule activated by the His275/Asp246 pair. The reaction proceeds via two different transition states, one during the binding and opening of the epoxide ring and the second during the hydrolysis of the ester intermediate. However, several important questions remained unanswered. Until now it has not been possible to identify the residue responsible for the binding and protonation of the epoxide oxygen, nor was the location known of the oxyanion hole that stabilizes the Asp107 oxyanion during the hydrolysis of the ester intermediate. Structural information may also resolve why an Asp246 → Ala mutant still retains some residual activity (6).

Here we report the 2.1-Å resolution x-ray structure of the epoxide hydrolase from A. radiobacter AD1 (Ephy). It is the first epoxide hydrolase for which the structure has been solved. The result of this work can provide a general better understanding about the structural basis of the reaction mechanism for this class of important ubiquitous enzymes.

EXPERIMENTAL PROCEDURES

Crystallization and Heavy Atom Search—The epoxide hydrolase from A. radiobacter AD1 was cloned, overexpressed, and purified as described previously (6). The stock protein solution, containing 5 mg

1 The abbreviations used are: Ephy, epoxide hydrolase from A. radiobacter AD1; DlHA, haloalkane dehalogenase from X. autotrophicus GJ10; Pgl, triacylglycerol lipase from P. glumae; Hpl, human pancreatic lipase; NCS, noncrystallographic symmetry; r.m.s., root mean square.
X-ray Structure of Epoxide Hydrolase

Table I
Data collection and single isomorphous replacement including anomalous scattering (SIRAS) analysis

<table>
<thead>
<tr>
<th>Data set</th>
<th>Native (λ = 1.0 Å)</th>
<th>Hg⁺ (λ = 1.0 Å)</th>
<th>Hg⁺ (λ = 1.5418 Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C2</td>
<td>C2</td>
<td>C2</td>
</tr>
<tr>
<td>a =</td>
<td>146.62 Å</td>
<td>147.15 Å</td>
<td>147.05 Å</td>
</tr>
<tr>
<td>b =</td>
<td>100.20 Å</td>
<td>100.14 Å</td>
<td>100.33 Å</td>
</tr>
<tr>
<td>c =</td>
<td>96.88 Å</td>
<td>97.70 Å</td>
<td>97.34 Å</td>
</tr>
<tr>
<td>β =</td>
<td>100.68°</td>
<td>100.94°</td>
<td>100.51°</td>
</tr>
<tr>
<td>Resolution</td>
<td>2.1 Å</td>
<td>3.5 Å</td>
<td>4.0 Å</td>
</tr>
<tr>
<td>Observations</td>
<td>222,880</td>
<td>65,941</td>
<td>59,453</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>73,445</td>
<td>15,782</td>
<td>10,514</td>
</tr>
<tr>
<td>Completeness (%) overall (final shell)</td>
<td>91.5 (82.0)</td>
<td>93.9 (91.7)</td>
<td>88.3 (91.2)</td>
</tr>
<tr>
<td>Rmerge (final shell)*</td>
<td>0.06 (0.36)</td>
<td>0.13 (0.23)</td>
<td>0.12 (0.25)</td>
</tr>
<tr>
<td>Phasing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavy atom sites</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Phasing power (iso/ano)</td>
<td>1.80/1.87</td>
<td>2.01</td>
<td></td>
</tr>
<tr>
<td>Figure of merit (iso/ano)*</td>
<td>0.35/0.40</td>
<td>0.47</td>
<td>0.43</td>
</tr>
<tr>
<td>Figure of merit overall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCalcb*s</td>
<td>0.49</td>
<td>0.47</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*The Hg derivative is ethyl mercury phosphate (C2H3HgO)2PO4.

Phase Determination and Refinement—The structure of the epoxide hydrolase from A. radioresistens AD1 was solved by the method of single isomorphous replacement supplemented by anomalous scattering, using both the in-house and synchrotron derivative data sets. A major heavy atom site (8.5 σ) for the ethyl mercury phosphate derivative was located in a difference Patterson map (12.0–4.5 Å data). The remaining 21 heavy atom positions were determined using difference Fourier techniques. Heavy atom position search, parameter refinement including anomalous data, and phase calculations were performed with PHASES (13) (Table I). The initial phases calculated at 3.7 Å yielded a figure of merit of 0.47 and were improved by solvent flattening and histogram matching techniques using the program DM (14). The non-crystallographic symmetry (NCS) operators (three orthogonal 2-fold axes) relating the 4 molecules in the asymmetric unit were determined with the help of FINDNCS,2 using the 8 heavy atom sites with the highest occupancies. They were checked by comparing them with the rotation matrices calculated from a self-rotation function (16). An initial mask was built around one molecule in the asymmetric unit with the program MAMA (17); this mask was then used to refine the NCS operators by maximizing the correlation between the electron density maps of the 4 molecules in the asymmetric unit using the program IMP (17). Iterative cycles of density averaging, improvement of the mask, and refinement of the NCS operators, along with solvent flattening and phase extension to 2.6 Å resolution, resulted in a map of interpretable quality.

The model was traced using the program O (18). Nearly the complete polypeptide chain of one monomer could be interpreted in agreement with the amino acid sequence. By applying the refined NCS operators to the coordinates of the first molecule, coordinates for the other three molecules in the asymmetric unit were generated. The four molecules were then refined using the program X-PLOR (19). During the first runs of the refinement (simulated annealing and individual B-factor refinement), tight NCS restraints were applied (17), but in the final stage of the refinement (conventional positional refinement and individual B-factor refinement), they were gradually released or not even used at all for those residues that clearly showed different conformations in the 4 monomers in the asymmetric unit. The best refinement results were obtained using a flat bulk solvent correction. Special care was taken in the selection of the test set for the Rfree calculation; the test set was selected by dividing the reflections in 102 thin-resolution shells to minimize the correlation between test set and working set reflections that could be caused by the presence of NCS (20). Water molecules were placed according to strict density and distance criteria, starting with the buried and NCS-related ones.

The final model consists of 4 × 282 residues, 610 water molecules (33 of them refined with double positions), and 4 potassium ions. The crystallographic R factor and Rfree are 19.0% and 22.7%, respectively. PROCHECK (21) and WHATCHECK (22) were used to assess the stereochemical quality. The structure was further analyzed using the

program VOIDOO (23), the programs from the CCP4 suite (14), the BIOMOL package, and the program DALI (24). Refinement statistics are given in Table II. The atomic coordinates and the structure factors have been deposited to the Protein Data Bank with the entry code 1ehy.

Modeling of Asp246—As a starting model, the atomic coordinates of the refined structure of the wild type epoxide hydrolase were used in which only the internal solvent molecules were retained. The crystal structure was energy-minimized prior to the modeling using a conjugate gradient routine implemented in X-PLOR (19). To completely remove the possible bias because of the conformation of the protein in the crystal, a slow-cooling molecular dynamics simulation (19) of 25 ps with temperature coupling (25) was performed in which the temperature was slowly reduced from 1000 K to 300 K. The missing loop 138–148 and the loop containing Asp246 were built using the program O (18).

To model a likely conformation of the active Ephy enzyme, we assumed that the acid member of the catalytic triad, Asp246, should lie at interaction distance to the catalytic His275 side chain, as found in many other members of the αβ hydrolase-fold family (9). Haloalkane dehalogenase (PDB accession code 2HAD) (26) and bromoperoxidase A2 (PDB accession code 1BRO) (27) were used as templates to model the new Ephy Asp246 position, analogous to Asp250 of dehalogenase and Asp238 of bromoperoxidase, respectively. Secondly, we assumed that the Gln134 side chain should be removed from the active site, as it blocks the putative substrate binding site. This was done by giving the Pro32-His133-Gln134 loop a similar conformation as the human pancreatic lipase (PDB accession code 1LPB) (28) Pro177, Ala178, Gln179 motif, which has an equivalent topological position. The loop of residues 138–148, which is not observed in the electron density, was built like in the bromoperoxidase structure, connecting the core and the cap domains.

Several cycles of stereochemical regularization were performed using the REFI and LEGO option of O (18). The model was subsequently subjected to energy minimization to tidy up unacceptable close contacts and poor stereochemistry. To overcome the possibility that the energy-minimized structure was trapped in a local minimum, an energy-minimization was applied until convergence was reached, leading to a model with no residues outside the allowed regions in the Ramachandran plot and 29 (20) and a good stereochemical quality (r.m.s. deviation bond lengths = 0.005 Å, r.m.s. deviation bond angles = 1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°). Asp107 and Asp131 have slightly deviating bond angles (1.61°).

Although the position of the modeled loop 132–148 is only one of the possible conformations it can assume, we are confident that the rebuilding of the loop containing Asp246, in a fashion common to many αβ hydrolase-fold enzymes, gives a plausible picture of the catalytic site of the fully active epoxide hydrolase.

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>Refinement statistics and stereochemical quality of the final model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>25–2.1</td>
</tr>
<tr>
<td>R factor* (R_free)</td>
<td>0.190 (0.227)</td>
</tr>
<tr>
<td>No. of residues in the asymmetric unit</td>
<td>4 × 282</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>610</td>
</tr>
<tr>
<td>No. of potassium ions</td>
<td>4</td>
</tr>
<tr>
<td>Average B-factor (Å²)</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>28.3</td>
</tr>
<tr>
<td>Main chain</td>
<td>27.2</td>
</tr>
<tr>
<td>Side chain</td>
<td>28.7</td>
</tr>
<tr>
<td>Water molecules</td>
<td>33.5</td>
</tr>
<tr>
<td>Potassium ions</td>
<td>33.3</td>
</tr>
<tr>
<td>r.m.s. deviation from ideality</td>
<td></td>
</tr>
<tr>
<td>bond lengths (Å)</td>
<td>0.008</td>
</tr>
<tr>
<td>bond angles (°)</td>
<td>1.338</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td></td>
</tr>
<tr>
<td>Residues in most favored regions (%)</td>
<td>89.7</td>
</tr>
<tr>
<td>Residues in additionally allowed regions (%)</td>
<td>9.3</td>
</tr>
<tr>
<td>Residues in generously allowed regions (%)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* R factor = Σo(|F_o| − |F_c|)/Σo|F_o|, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

RESULTS

Quality of the Model—Epoxide hydrolase from A. radiobacter AD1 (Ephy) crystallizes in the monoclinic space group C2, with 4 molecules in the asymmetric unit. A superimposition of the Ca atoms of the four molecules gave an average r.m.s. difference of 0.24 Å for molecules B, C, and D and a higher r.m.s. difference of 0.40 Å if molecule A is included. All results discussed below apply to all 4 molecules (A, B, C, and D) unless stated otherwise. The final model consists of 4 × 282 residues, 610 water molecules (33 of them refined with double positions), and 4 potassium ions, originating from the crystallization buffer, one for each molecule in the asymmetric unit. In each monomer (294 residues), the first N-terminal residue (Met) is not visible nor is there interpretable electron density for the loop 138–148. The final crystallographic R factor and R_free values are 19.0% and 22.7%, respectively. The r.m.s. deviations from ideal geometry are 0.008 Å for bond lengths and 1.338° for bond angles. No residues are in the disallowed regions of the Ramachandran plot (29). Pro39 was found in a cis conformation.

Overall Structure—The Ephy monomer has a nearly globular shape with approximate dimensions of 48 × 47 × 47 Å². It consists of two domains: domain I (or “core” domain), which shows the typical features of the αβ hydrolase-fold topology (9), and the mainly α-helical domain II (or “cap” domain), which lies on top of domain I (Figs. 1 and 2). The core domain comprises amino acids 1–137 and 219–294, and it consists of a central eight-stranded β-sheet with seven parallel strands (only the second strand is antiparallel). The β-sheet is flanked on both sides by α-helices, two on one side and four on the other. Helix α7 is a one-turn 310 helix. Domain II, containing α-helices α4 to α8, forms a large excursion between β-strands β6 and β7 of the core domain. It has a double-layered structure with helices α7 and α8 located between the core domain and the plane formed by α4, α5, and α6.

Active Site—The proposed active-site residues (6) Asp107 and His275 are located in a predominantly hydrophobic internal cavity between domains I and II. The core domain contributes to the lining of the cavity with residues Gly37, Trp38, Pro39,
X-ray Structure of Epoxide Hydrolase

FIG. 2. Secondary structure topology diagram and location of the catalytic triad residues, Asp\textsuperscript{107}, Asp\textsuperscript{246}, and His\textsuperscript{275}. The dashed line represents the missing loop 138–148. Short \( \beta \)-strands are located at the N terminus (\( \alpha' \)), between \( \beta 3 \) and \( \alpha 1 \) (\( \alpha'1 \)), between \( \beta 4 \) and \( \alpha 2 \) (\( \alpha'2 \)), and between \( \alpha 3 \) and \( \beta 6 \) (\( \alpha'3 \)). The last \( \alpha \)-helix shows a conspicuous bend at residue 281 (\( \alpha'11-\alpha'11 \)) because of the presence of Pro\textsuperscript{285} in the center of the helix.

Glutamic acid, His\textsuperscript{106}, Asp\textsuperscript{107}, Phe\textsuperscript{108}, Ile\textsuperscript{133}, Phe\textsuperscript{137}, Ile\textsuperscript{219}, His\textsuperscript{275}, Phe\textsuperscript{276}, and Val\textsuperscript{279}. The cap domain supplies Tyr\textsuperscript{152}, Trp\textsuperscript{183}, and Tyr\textsuperscript{215} (Fig. 3). Asp\textsuperscript{107} is situated at the very sharp “nucleophile elbow” between the central strand \( \beta 5 \) and helix \( \alpha 3 \). At this topological position, all \( \alpha/\beta \) hydrolase-fold enzymes present the nucleophile, which can either be Ser, Cys, or Asp (9). The \( (\phi, \psi) \) angles of Asp\textsuperscript{107} are slightly unfavorable (\( \phi = 57\degree, \psi = -124\degree \)), but its conformation is stabilized by a network of hydrogen bonds involving residues of the sharp turn, as has been found in other \( \alpha/\beta \) hydrolase enzymes (9). In addition, the main chain nitrogen atom of Asp\textsuperscript{107} interacts via a hydrogen bond with the backbone oxygen atom of Asp\textsuperscript{131}, the other residue with slightly deviating backbone torsion angles (\( \phi = 31\degree, \psi = 69\degree \)). Furthermore, the side chain of Asp\textsuperscript{107} is stabilized by a hydrogen bond of its O\textsuperscript{\textsubscript{\beta}} atom with the backbone amide groups of Trp\textsuperscript{38} and Phe\textsuperscript{108} and by a salt bridge between the O\textsuperscript{\textsubscript{\beta}} and the N\textsuperscript{\textsubscript{\alpha}} atom of the His\textsuperscript{275} side chain.

An –20 Å long tunnel, filled with water molecules, is located between \( \alpha \)-helices \( \alpha 1 \), \( \alpha 10 \), the loop connecting \( \alpha \)-helix \( \alpha 1 \) and \( \beta \)-strand \( \beta 3 \) of the core domain, and \( \alpha 7 \) of the cap domain (Fig. 4). This tunnel leads to the back of the active-site cavity, and it is perfectly suited to replenish the hydrolytic water molecule at hydrogen bond distance to the N\textsuperscript{2} atom of the His\textsuperscript{275} side chain (Fig. 3) after the reaction. In our structure, the active site cavity is exposed to the solvent from the front part too, where the missing loop is located. Because of the position of the hydrolytic water molecule in the back of the active site, it is likely that the substrate enters the active-site cavity from the front part.

Asp\textsuperscript{246} has been proposed to be the acidic member of the catalytic triad, responsible for assisting His\textsuperscript{275} in activating the water molecule that hydrolizes the ester intermediate formed at Asp\textsuperscript{107} (6). Asp\textsuperscript{246} is located in a turn between \( \beta 7 \) and helix \( \alpha 10 \), in a position topologically conserved within the \( \alpha/\beta \) hydrolase-fold family (Fig. 2) (9). However, in our crystal structure Asp\textsuperscript{246} is not at interacting distance from His\textsuperscript{275}. Instead the loop containing this residue is pulled away from the active site, and the Asp\textsuperscript{246} side chain is pointing into the solvent. This is probably the result of crystal packing forces because helix \( \alpha 10 \) of molecule A, which follows the loop containing Asp\textsuperscript{246}, is involved in an intermolecular contact with helix \( \alpha 10 \) of molecule B. A similar contact exists between molecules C and D, which even involves an intermolecular disulfide bridge between Cys(C)\textsuperscript{248} and Cys(D)\textsuperscript{248}. The absence of this disulfide bond between molecules A and B results in a slightly different conformation of the loop containing Cys\textsuperscript{248} in molecule A compared with the other three molecules in the asymmetric unit. As a consequence, the difference between the Ca positions of molecules A, B, C, and D (average r.m.s. difference of 0.40 Å) is higher than for the B, C, and D molecules only (r.m.s. difference of 0.24 Å). The conformational plasticity of the region between residues 244 and 257 is also reflected by very high B-factors (between 28 Å\textsuperscript{2} and 84 Å\textsuperscript{2}) and a not easily interpretable electron density map, often poorly defined or showing multiple conformations even for the backbone. The space vacated by Asp\textsuperscript{246} makes it possible for the side chain of Glu\textsuperscript{134} to move into the active site, occupying the site where the substrate is likely to be bound (Fig. 3). Its position is stabilized by a hydrogen bonding network involving the hydroxyl groups of Tyr\textsuperscript{152} and Tyr\textsuperscript{215} and the carbonyl oxygen of Glu\textsuperscript{134} of Asp\textsuperscript{107}. The folding of Ephy strongly resembles that of bromoperoxidase A2 from Streptomyces aureofaciens (27) (BpaA2, PDB accession code 1BRO; r.m.s. deviation –1.7 Å for 193 Ca atoms) and haloalkane dehalogenase from Xanthobacter autotrophicus (10, 26, 30) (DhaA, PDB accession code 2HAD; r.m.s. deviation –2.0 Å for 204 Ca atoms) and a number of other members of the \( \alpha/\beta \) hydrolase-fold family (9). The matching is best for the central \( \beta \)-sheet and for helices \( \alpha 2 \) and \( \alpha 3 \) (Fig. 5), but all other structural elements are equivalent as well, especially in the regions close to the catalytic residues. The \( \alpha \)-helices in the cap domain superimpose less well, showing a different relative orientation. These helices contribute several residues important for the interaction with substrates.

Despite a low sequence homology (33% homology, 20% identity), the structural similarity of epoxide hydrolase and haloalkane dehalogenase is particularly interesting. These enzymes have both an Asp-His-Asp catalytic triad. Asp\textsuperscript{107} and His\textsuperscript{275} of Ephy superimpose very well on Asp\textsuperscript{124} and His\textsuperscript{289} of DhaA; their side chains are in the same relative position and make similar hydrogen bonds. In dehalogenase the halogen atom of the substrate is bound between the indole ring N-atoms of Trp\textsuperscript{255} and Trp\textsuperscript{275}. In epoxide hydrolase, Phe\textsuperscript{108} and Trp\textsuperscript{183} occupy the equivalent positions, suggesting that they may be involved in substrate binding (6). However, a stabilizing structural role for Phe\textsuperscript{108} is also conceivable, as it has a “T-shaped” interaction with the Tyr\textsuperscript{215} side chain (31). In dehalogenase,
the $O_{d2}$ atom of Asp$^{124}$, which in the putative transition state will become negatively charged, is stabilized by interaction with the main chain nitrogen atoms of residues 125 and 56. Asp$^{107}$ $O_{d1}$ in Ephy has similar interactions with the amide nitrogen atoms of residues 108 and 38, suggesting Phe 108 and Trp38 as part of the oxyanion hole.

Modeling of Asp$^{246}$—As mentioned above, the third catalytic residue, Asp$^{246}$, is pulled out of the active site. However, the positions of Asp$^{260}$ in haloalkane dehalogenase and Asp$^{228}$ in bromoperoxidase A2 give a reliable suggestion where Asp$^{246}$ should be located in the active conformation of Ephy. Superposition of the $\beta$-sheets of DhlA and BpA2 on that of Ephy brings the $O_{d2}$ atom of Asp$^{260}$ of dehalogenase and Asp$^{228}$ of bromoperoxidase in coincidence with the water molecule in Ephy, which is hydrogen-bonded to N$\delta1$ of His$^{275}$ and O$\delta2$ of Asp$^{131}$ (Fig. 6). This information was used to model a likely conformation of the “active” Ephy enzyme, with DhlA, BpA2, and human pancreatic lipase (Hpl) structures as templates for reconstructing the Ephy loops containing Asp$^{246}$ and Gln$^{134}$ (See “Experimental Procedures”).

The final model shows an intact and empty active site cavity, capable of accommodating substrates. It is lined with Gly$^{37}$, Trp$^{38}$, Pro$^{39}$, Glu$^{44}$, His$^{106}$, Asp$^{107}$, Phe$^{108}$, Ile$^{133}$, Phe$^{137}$, Tyr$^{152}$, Trp$^{183}$, Tyr$^{215}$, Ile$^{219}$, Cys$^{246}$, His$^{275}$, Phe$^{276}$, and Val$^{279}$ (Fig. 7). Asp$^{246}$ is hydrogen-bonded to the His$^{275}$ N$\epsilon2$, and it now occupies the position where the acidic member of the catalytic triad is normally found in $\alpha/\beta$ hydrolase-fold enzymes. The rest of the active site has undergone only minor changes in the relative positions of the atoms. The hydrolytic water molecule is still present at hydrogen bond distance to the His$^{275}$ N$\epsilon2$ atom. The Tyr$^{152}$ and Tyr$^{215}$ hydroxyl groups, which in the crystal structure were hydrogen-bonded to the Gln$^{134}$ side chain, still point in the same direction, enabling them to donate the proton needed for opening of the epoxide ring (Fig. 8).

**DISCUSSION**

**Active Site and Substrate Binding**—Epoxide hydrolase has a two-domain structure (Fig. 1). The core domain displays an $\alpha/\beta$ hydrolase-fold topology, which provides the scaffolding for the catalytic triad residues Asp$^{107}$, His$^{275}$, and Asp$^{246}$, whereas the $\alpha$-helical cap domain contributes several residues important for the interaction with substrates. The active site is located in a cavity between the two domains, which contains Asp$^{107}$ and His$^{275}$. At the optimum pH for catalysis (8.4–9.0) His$^{275}$ is most likely deprotonated and therefore able to activate the water molecule located nearby. In
the crystal structure, this water molecule is in contact with the solvent via a narrow tunnel between the core and cap domains (Fig. 4). This tunnel resembles the active site back entrance in bromoperoxidase A2, which was proposed to provide access to the active site for small molecules participating in the reaction, like peroxide and halide, or to expel water molecules from the active site during substrate binding (27). In epoxide hydrolase, it seems more likely that the tunnel serves to replenish the hydrolytic water molecule after the reaction has been completed.

The third member of the catalytic triad, Asp<sup>246</sup>, assists His<sup>275</sup> in activating the hydrolytic water molecule (6). To our surprise it is not at hydrogen bonding distance from the N<sub>d1</sub> atom of His<sup>275</sup>, but it has moved away into the solvent region. This is most probably a consequence of crystal packing forces: the Asp<sup>246</sup> → Ala mutation strongly decreases the activity of the enzyme (~0.5% of the wild type activity) (6), and such a dramatic effect on activity is difficult to rationalize for the position of the residue as observed in our crystals. The exposed position of Asp<sup>246</sup> has made it possible for the Gln<sup>134</sup> side chain to move into the active site and block it (Fig. 3). Because a Gln<sup>134</sup> → Ala mutant has an activity comparable with that of wild-type enzyme, it is unlikely that Gln<sup>134</sup> is normally present in the active site. However, human microsomal epoxide hydrolase has been reported to be inhibited by amides (32). The A. radiobacter epoxide hydrolase shows competitive inhibition by amides as well, especially by compounds like phenylacetamide (30 μM). Therefore, we conclude that the Gln<sup>134</sup> side chain may act as such an inhibitor, mimicking the binding mode of epoxide substrates. Thus, the combination of crystal contacts of helix α10 (residues 252 to 261) and the affinity of the active site for amide compounds have probably led to the observed exposed position of Asp<sup>246</sup>. Nevertheless, the high structural similarity (Fig. 5) between the core domains of epoxide hydrolase, haloalkane dehalogenase, bromoperoxidase A2, and human pancreatic lipase allowed us to use the latter three enzyme structures as templates to remodel the loops containing Asp<sup>246</sup> and Gln<sup>134</sup>. The result is a plausible model of the active site in the fully active enzyme (Fig. 7).

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of the epoxide ring by hydrogen bonding and protonating the epoxide oxygen. In agreement with this hypothesis, mutagenesis studies of these tyrosines have shown that only a double Tyr-Phe mutant is completely inactive, suggesting that both Tyr152 and Tyr215 are able to provide the proton needed for the opening of the epoxide ring. Because Tyr215 is absolutely conserved within the epoxide hydrolase family and Tyr152 is mostly conserved in the soluble epoxide hydrolases, it is likely that the Tyr activation is a general property of this class of enzymes (Fig. 8). In the past, one of three lyses, Lys174, Lys176, and Lys177, was proposed to be involved in the protonation (6), but the crystal structure of Ephy unambiguously shows that these three lysine residues are located far from the active site, exposed to the solvent on top of the cap domain.

**Position and Function of Asp131—**The Asp246 ← Ala mutation resulted in a strong reduction of enzymatic activity. Nevertheless, this mutant still has some residual activity (~0.5% of wild type activity), indicating the importance of Asp131 in catalysis but not its essentiality (6). The three-dimensional structure of Ephy shows the presence of another aspartic acid, Asp131, which may act as a backup of Asp246 as a hydrogen bond philic Asp124 (10, 26). In epoxide hydrolase, a similar hydrogen bonding pattern is present between the Oε2 atom of the nucleophile Asp107 and the amide nitrogen atoms of Trp38 and Phe108. Thus these peptide nitrogen atoms are in an optimal position to stabilize the negative charge that develops on the Oε2 atom of the nucleophile during the hydrolysis of the ester intermediate. In addition, the negative charge on Asp107 Oε2 may be further stabilized by the α-helix dipole of helix α3 (36).

A second role of the tetrapeptide motif may be in stabilizing the position of the putative hydrolytic water molecule (Fig. 6).

Indeed, in the crystal structure this water molecule is at interacting distance to the backbone oxygen atom of Trp38.

These essential structural functions may explain the importance of the HGXP motif for enzymatic activity within the epoxide hydrolase family, as already demonstrated by mutation of His to Ala in the rat microsomal epoxide hydrolase (37).

**Conclusions—**The x-ray structure reveals for the first time the fold of an epoxide hydrolase and provides novel, detailed information on the residues involved in the enzymatic mechanism. It localizes the catalytic residues, the hydrolytic water molecule, and the position of the oxyanion hole, and it proposes a possible backup for the acidic member of the catalytic triad. Most importantly, it unambiguously identifies the previously unanticipated Tyr152/Tyr215 as the acidic group responsible for binding and possibly protonation of the transition state of the formation of the ester intermediate. The residues important for catalysis are conserved within the epoxide hydrolase family. Therefore all these structural features are likely to be shared by other epoxide hydrolases and allow us to gain a better understanding of the behavior and mechanism of this class of biologically and biotechnologically important enzymes. At present we are investigating the structural basis of the enzymatic enantioselectivity by mutation analysis and by docking the substrates in the modeled active site.

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**REFERENCES**

X-ray Structure of Epoxide Hydrolase


