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Primary Structure and Catalytic Mechanism of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1*

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Rick Rink‡, Marko Fennema‡, Minke Smids‡, Uwe Dehmel‡§, and Dick B. Janssen‡¶

From the ‡Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

The epoxide hydrolase gene from *Agrobacterium radiobacter* AD1, a bacterium that is able to grow on epichlorohydrin as the sole carbon source, was cloned by means of the polymerase chain reaction with two degenerate primers based on the N-terminal and C-terminal sequences of the enzyme. The epoxide hydrolase gene coded for a protein of 294 amino acids with a molecular mass of 34 kDa. An identical epoxide hydrolase gene was cloned from chromosomal DNA of the closely related strain *A. radiobacter* CFZ11. The recombinant epoxide hydrolase was expressed up to 40% of the total cellular protein content in *Escherichia coli* BL21(DE3) and the purified enzyme had a k_{cat} of 21 s^{-1} with epichlorohydrin. Amino acid sequence similarity of the epoxide hydrolase with eukaryotic epoxide hydrolases, haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, and bromoperoxidase A2 from *Streptomyces aureofaciens* indicated that it belonged to the α/β-hydrolase fold family. This conclusion was supported by secondary structure predictions and analysis of the secondary structure with circular dichroism spectroscopy. The catalytic triad residues of epoxide hydrolase are proposed to be Asp^{107}, His^{275}, and Asp^{246}. Replacement of these residues to Ala/Glu, Arg/Gln, and Ala, respectively, resulted in a dramatic loss of activity for epichlorohydrin. The reaction mechanism of epoxide hydrolase proceeds via a covalently bound ester intermediate, as was shown by single turnover experiments with the His^{275} → Arg mutant of epoxide hydrolase in which the ester intermediate could be trapped.

Epoxide hydrolases can hydrolyze epoxides to their corresponding diols by addition of a water molecule. There is a strong interest in these enzymes since they play a key role in the detoxification of xenobiotic compounds and have great potential in enantioselective chemistry. Most research has focused on mammalian epoxide hydrolases since these enzymes are of toxicological relevance. The epoxide hydrolase genes that have been cloned so far are of mammalian, insect, and plant origin (1–9), and they can be distinguished into a class of microsomal enzymes and a class of soluble enzymes based on cellular localization and biochemical properties like substrate specificity. The mammalian epoxide hydrolases are believed to belong to the α/β-hydrolase fold family (10–12) since they show a low but significant sequence similarity with haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (13) of which the three-dimensional structure has been solved by x-ray crystallography (14). Sequence similarities are low within this α/β-hydrolase fold family and are restricted to specific areas within the topology, such as the preserved positions of the catalytic triad residues.

Agrobacterium radiobacter AD1 is a Gram-negative bacterium that was isolated for its ability to use epichlorohydrin as the sole carbon and energy source (15). Epichlorohydrin is hydrolyzed by an epoxide hydrolase to 3-chloro-1,2-propanediol, which is then converted to glycidol by a haloalcohol dehalogenase. Glycidol is further converted to glycerol which then enters the central metabolic pathway (16). The epoxide hydrolase that catalyzes the cofactor-independent hydrolysis of epichlorohydrin was purified to homogeneity and found to be a monomeric globular protein with a molecular mass of 35 kDa. Its similarity to haloalkane dehalogenase was suggested but could not be proven by biochemical analysis (15). The enzyme has a broad substrate range and epichlorohydrin and epibromohydrin were found to be the best substrates.

Not much is known about structure and sequence of microbial epoxide hydrolases. To obtain insight in the structure and mechanism of microbial epoxide hydrolases, we decided to characterize the epoxide hydrolase gene from *A. radiobacter* AD1. Due to its small size compared with microsomal and soluble epoxide hydrolase and its bacterial origin, this enzyme has potential in structural and biocatalytic studies. Based on the sequence and secondary structure analysis, the epoxide hydrolase is predicted to be an α/β-hydrolase folded enzyme. The residues Asp^{107}, His^{275}, and Asp^{246} were identified as the catalytic triad residues by sequence analysis and site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Acros chimica, Merck, or Sigma. Super taq polymerase was purchased from Sphaero Q. Restriction enzymes and other molecular biology enzymes were from Boehringer Mannheim. Oligonucleotide construction and amino acid analysis were done by Eurosequence BV, Groningen. Sequencing was done with the T7 sequencing kit from Pharmacia.

Strains and Growth Conditions—*A. radiobacter* AD1, formerly *Pseudomonas* sp. strain AD1 (15), is able to grow on epichlorohydrin and was maintained at 30 °C on sealed MMY plates (17) with 5 μl of epichlorohydrin added to a piece of filter paper in the lid of the Petri dish. *Agrobacterium* strain CFZ11 was isolated on 1,3-dichloro-2-propanol. Its properties are similar to those of strain AD1.[1] For the isolation of chromosomal DNA, *A. radiobacter* AD1 was cultivated in closed flasks containing one-fifth of the total volume of MMY medium with 5 mM 1,3-dichloro-2-propanol as the growth substrate. For the prepara-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y12804.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands. Tel.: 31-50-363-4208; Fax: 31-50-363-4165; E-mail: d.b.janssen@chem.rug.nl.

§ Present address: National Research Center for Biotechnology, Division of Cell Biology and Immunology, Mascheroder Weg 1, 38124 Braunschweig, Germany.

[1] A. J. van den Wijngaard and D. B. Janssen, unpublished data.

tion of chromosomal DNA, strain CFZ11 was cultivated in NB medium. All strains of *Escherichia coli* were cultivated in liquid LB medium with, when needed, ampicillin added to a final concentration of 100 μg/ml (18). *E. coli* JM101 was used for the production of single-stranded DNA for sequencing purposes (19). *E. coli* BW313 was used for the production of uracil containing single-stranded DNA for Kunkel mutagenesis (20). *E. coli* BL21(DE3) was used for the high-level expression of epoxide hydrolase (21).

Cloning and Sequencing of the Epoxide Hydrolase Gene—The epoxide hydrolase gene was initially cloned by means of the polymerase chain reaction, using degenerate primers that were designed on the amino acid sequence of the N- and C-terminal amino acid sequences of the protein (15). The N-terminal sequence was determined again and found to be TIRRPEDFKHYEVQLPDVKIHYVREGAGPTLLL. An ATG start codon within a *Nco*I restriction site was present in the forward primer: 5′-CGGGTACC**ATG**GCAATTCGACGTCCAGAYGAXTTXAAYCAXTAXGA-3′ (Y = A/G; X = C/T; start codon shown in bold; *Nco*I site underlined). A stop codon and a *Nco*I restriction site were incorporated in the reverse primer: 5′-CGGGATCCATGG**CTA**GCGYAAZGCZGTXTTRAT-3′ (Z = G/A/T/C; *r* = T/G/A; stop codon shown in bold; *Nco*I site underlined).

Total DNA of *A. radiobacter* AD1 was isolated from cells that were cultivated on 1,3-dichloro-2-propanol, using standard procedures (22). DNA amplification by the polymerase chain reaction was done with 1 μg of total DNA on a Perkin Elmer PCR[2] apparatus using the standard amplification protocol described by Innis and Gelfand (23), with the exception that primer annealing was first done at 37 °C (3 rounds) and then at 40 °C (25 rounds). The amplified DNA was digested with *Nco*I and ligated into the *Nco*I site of the expression vector pGEF+ (plasmid pGELAF+ (24) without the *Nco*I fragment containing the *dhlA* gene), resulting in a translational fusion. The ligation mixture was transformed to *E. coli* BL21(DE3) cells by means of electroporation and a colony displaying epoxide hydrolase activity (see under "Enzyme Assays") was selected. Plasmid DNA was isolated (18) and the cloned fragment was sequenced by the dideoxy method (25). The construct pEH20 was used for further study.

Total DNA from strain CFZ11 was isolated by standard methods (22). For Southern blot analysis the DNA was digested with different restriction enzymes. After agarose gel electrophoresis and capillary transfer onto positively charged nylon membrane (Boehringer Mannheim) the DNA was hybridized with digoxygenin-labeled DNA of the epoxide hydrolase gene from the construct pEH2O. For detection of the hybridizing fragments the standard protocol of Boehringer Mannheim was followed. A hybridizing 2.3-kilobase *Bam*HI/*Hin*dIII fragment was cloned into pWKS130 (26). This clone was finally sequenced using the Tag DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer) and the ABI 373 automated sequencer (Applied Biosystems Division of Perkin Elmer). Potential promoter sites were searched by using the promoter prediction program Promoter Prediction by Neural Network NNPP (27).

Expression and Purification of Epoxide Hydrolase—Both wild type and mutant epoxide hydrolase were expressed in *E. coli* BL21(DE3). Plasmid DNA was transformed by electroporation to *E. coli* cells, which were then plated out on LB plates containing ampicillin and incubated overnight at 30 °C. A preculture was started by inoculating 100 ml of LB containing ampicillin with the transformants from a plate to a starting OD_{600} of 0.1 and was incubated at 30 °C until an OD_{600} of 1 to 2 was reached. The preculture was diluted in 1 liter of LB, containing ampicillin, and the culture was incubated overnight at 20 °C. The cells were centrifuged, washed, and resuspended in 50 ml of TEMAG buffer (10 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, and 10% glycerol, pH 7.5). Cells were broken by continuous sonication and the extract was centrifuged (200,000 × *g*, 90 min, 4 °C). The supernatant was applied on a 30-ml DE52 anion exchange column and elution was carried out with a gradient of 0 to 1 M ammonium sulfate in TEMAG. The collected fractions that displayed epichlorohydrin activity and had the highest protein content were pooled and dialyzed against PEMAG buffer (5 mM potassium phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, and 10% glycerol, pH 6.8). A 30-ml hydroxylapatite column was used for further purification, using a gradient of 5 to 100 mM of phosphate in PEMAG. The fractions that contained purified epoxide hydrolase were pooled and dialyzed against TEMAG buffer. The enzyme was stored at 4 °C or −20 °C. Glycerol (10% v/v) was used for its stabilizing effect on epoxide hydrolase activity upon storage.

Construction of Epoxide Hydrolase Mutants—Site-directed mutagenesis was done as described by Kunkel (20) with uracil-containing single-stranded plasmid pEH20 as template. Asp^{107} was mutated to Glu with primer D107E (5′-CGTTGGCCAT**G(C/A)A**TTCGCGGCCAT-3′, Glu codon in bold, *Eco*RI restriction site underlined), and to Ala with primer D107A (5′-GGCCAT**GCG**TTCGCG-3′, Ala codon in bold). Confirmation of a mutated codon was obtained by PCR with a control primer (5′-CGTTGGCCATGCG-3′, mutation underlined) and a downstream primer (5′-TGGCAGCAGCCAACTCAGCT-3′). His^{275} was mutated to Arg and Gln with primer H275RQ (5′-GACGATTGAAGACTGCGGT**(A/C)(AG)G**TTCTTGATGGTC-3′, mutated codon shown in bold, removed *Pvu*I restriction site underlined). Asp^{246} was mutated to Ala with primer D246A (5′-TTGGGA**GCT**ACTTGC-3′, Ala codon shown in bold). Confirmation of a mutated codon was obtained by PCR with a control primer (5′-GGGCACGCAAGTAG-3′, mutation underlined) and an upstream primer (5′-TAATACGACTCACTATAGGG-3′). The Kunkel mixture was transformed to *E. coli* BL21(DE3) cells by electroporation. Recombinants were screened for epoxide hydrolase activity and plasmid DNA was isolated. Mutations were confirmed either by restriction analysis or by PCR with primers that could only amplify DNA with the desired mutation. Finally, the mutations were checked by dideoxy sequencing (25).

Enzyme Assays—Epoxide hydrolase activities in whole cells and column fractions were determined by a microtiter plate assay, based on a chromogenic reaction of an epoxide with 4-nitrobenzylpyridine (28). Small amounts of cells or 10-μl samples of column fractions were added to a 96-well microtiter plate together with 100 μl of TE buffer (50 mM Tris-SO_4, 1 mM EDTA, pH 9.0) containing 10 mM epichlorohydrin. After incubation for 1–10 min at room temperature, 50 μl of reagent A (100 mM 4-nitrobenzylpyridine in 80% ethylene glycol and 20% acetone (v/v)) was added. The microtiter plate was tightly sealed with silicone rubber and was incubated for 10 min at 80 °C. After cooling to room temperature, 50 μl of reagent B (50% triethylamine and 50% acetone (v/v)) was added. A blue color appeared when epichlorohydrin was not degraded, else the mixture stayed colorless.

Epoxide hydrolase activities were determined quantitatively by following substrate depletion using gas chromatography of ethereal extracts (15) or by following substrate depletion and diol production by gas chromatography of reaction mixtures quenched in acetone. A suitable amount of epoxide hydrolase was incubated in TE buffer with 5 mM substrate. At various time points, 100 μl of sample was added to 1 ml of ice-cold acetone containing 1-nonanol as the internal standard. Protein and salts were removed by centrifugation (15 min, 4000 × *g*) and the extract was analyzed by GC using a 0.2 mm × 25-m CP-Wax57-CB column (Chrompack, Middelburg, The Netherlands) and a flame-ionization detector. 1 Unit of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of diol/min. Protein determination was carried out with Coomassie Brilliant Blue with bovine serum albumin as a standard or by measuring the absorbance of purified enzyme at 280 nm. One OD_{280} unit corresponded with 0.42 mg of epoxide hydrolase/ml as was determined by the biuret method (29), amino acid analysis, and by dissolving a known amount of freeze-dried epoxide hydrolase in water.

The specific activities of the mutant enzymes and wild type enzyme were determined in concentrated cell free extracts. A pellet of cells that was washed with TEM buffer (50 mM Tris-SO_4, mM EDTA, and 5 mM β-mercaptoethanol, pH 7.5), was resuspended in one pellet volume of buffer and disrupted by sonication. The suspension was centrifuged for 90 min (200,000 × *g*, 4 °C). The cell-free extract contained protein concentrations of 80–150 mg/ml and the epoxide hydrolase content was 30–40% of the total protein content, as confirmed by SDS-polyacrylamide gel electrophoresis and density scanning. 200 μl of protein extract or an adequate dilution thereof was added to 1.5 ml of TE buffer (Tris-SO_4, 5 mM EDTA, pH 9.0) with 5 mM epichlorohydrin and incubated at 30 °C. Samples of 200 μl were taken in time and quenched in 1.8 ml of ice-cold acetone with 1-nonanol as the internal standard and analyzed by GC.

Circular Dichroism (CD)—Far-UV CD-spectra were recorded on a AVIV circular dichroism spectrometer (62A DS) by measuring the change in ellipticity in millidegrees. Enzyme was dialyzed against a 5 mM phosphate buffer, pH 6.8, and spectra were recorded in a 1-mm cuvette at 25 °C. The CD-spectra were corrected for buffer absorbance. Secondary structure elements were extracted from the spectra by using the programs CONTIN (30), SELCON (31), and K2D (32).

[2] The abbreviations used are: PCR, polymerase chain reaction; EchA, epichlorohydrin epoxide hydrolase from *A. radiobacter* AD1; DhlA, haloalkane dehalogenase from *X. autotrophicus* GJ10 (13); BpA2, bromoperoxidase A2 from *S. aureofaciens* (43); DehH1, fluoroacetate dehalogenase from *Moraxella* sp. strain B (41).

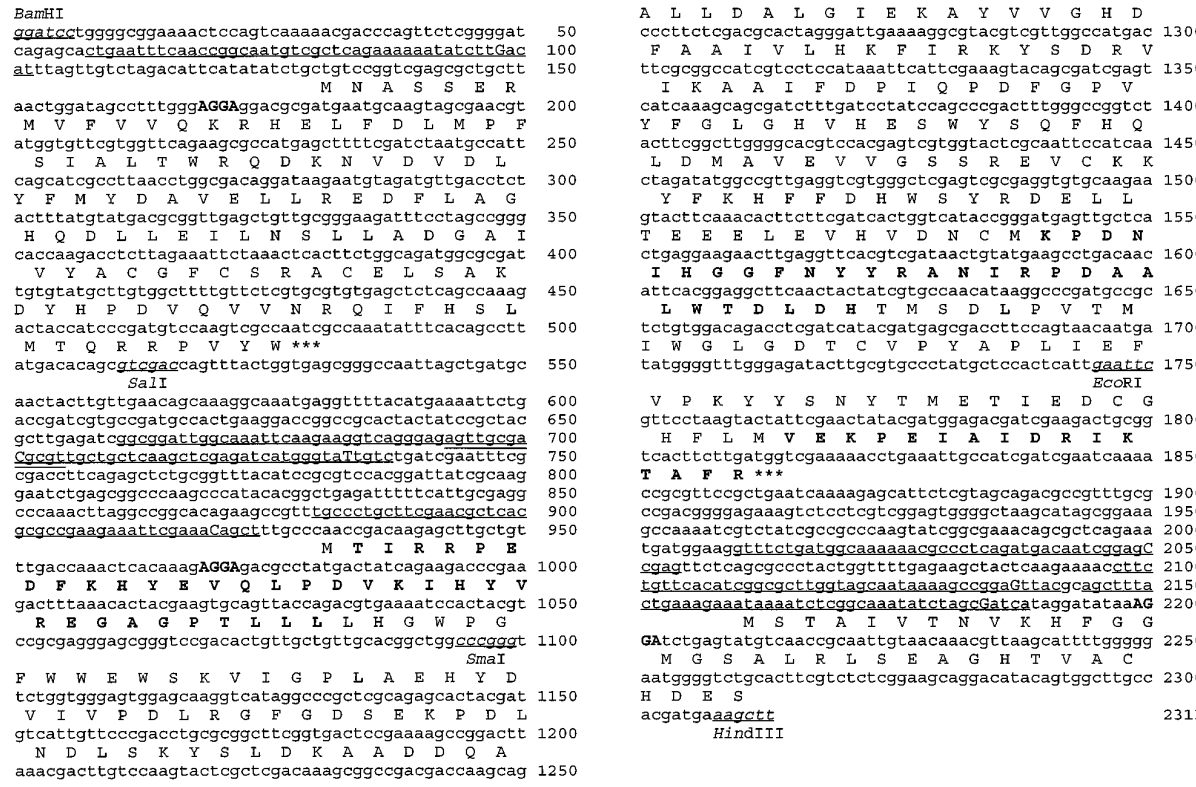

FIG. 1. **Cloned 2.3-kilobase fragment of chromosomal DNA from *A. radiobacter* CFZ11.** Amino acid sequence corresponding to the three largest open reading frames are indicated in single letter code. The amino acids of epoxide hydrolase that are confirmed by peptide sequencing are shown in *bold* (15). Ribosome-binding sites are shown in *bold capitals*. Putative promoter sequences are *underlined* and the predicted point of transcription is shown as a *capital letter*. Restriction sites that were confirmed by Southern blot analysis are indicated.

Homology Search and Secondary Structure Predictions—The BLAST program (33) was used to screen protein and DNA data bases for proteins that shared sequence similarity. Multiple sequence alignments were made in ClustalW v1.6 (34). Secondary structure predictions were carried out with the programs SopM (35), ssp (36), Sspred (37), and Predict-Protein (38) that were offered as services on the World Wide Web. The results were compared and similar predictions were taken as a consensus prediction.

Single Turnover Experiments—Single turnover experiments were performed at 30 °C with wild type enzyme and the $His^{275} \rightarrow Arg$ mutant on a rapid quench-flow apparatus (RQF-63) from Kintek Instruments using 1 mM purified enzyme and 0.5 mM epichlorohydrin in TEMAG buffer, pH 7.5. All concentrations given represent values after one-to-one mixing of protein and substrate. The enzyme was concentrated with an Amicon ultrafiltration cell using a PM10 filter. After mixing, the reaction mixture with a total volume of 100 μl was quenched with 117 μl of acetone, and directly injected into 800 μl of ice-cold acetone with 1-nonanol as the internal standard. The concentrations of epichlorohydrin and 3-chloro-1,2-propanediol were determined by GC. The experiment was repeated with 0.6 mM $His^{275} \rightarrow Arg$ mutant of epoxide hydrolase, using 0.6 or 0.3 mM epichlorohydrin, all in TEM buffer, pH 7.5.

RESULTS

Cloning and Sequencing of the Epoxide Hydrolase Gene—The epichlorohydrin epoxide hydrolase *echA* gene was originally cloned by amplifying the gene by PCR by using two degenerate primers that were designed on basis of the N- and C-terminal amino acid sequence. The amplified gene was fused into the start codon of the expression vector pGEF+, resulting in the construct pEH20. Upon cloning, the second amino acid was changed from a threonine to an alanine residue, but it did not have a noticeable effect on the activity or stability of the enzyme. The cloning procedure was repeated twice for separate polymerase chain reactions to identify possible errors that could be introduced during amplification of the gene. All three clones were entirely sequenced. When the three constructs were compared with each other, two silent mutations (G231 → A, and C618 → T) were detected in the *echA* gene of the construct pEH2O. Since the amino acid sequence of epoxide hydrolase was not effected, plasmid pEH20 was used for further study.

To eliminate the possibility that the sequenced epoxide hydrolase gene differs from the actual sequence due to PCR errors, we decided to clone the gene directly from chromosomal DNA. Attempts to clone the epoxide hydrolase gene from chromosomal DNA of *A. radiobacter* AD1 failed. However, using *A. radiobacter* CFZ11, we were able to clone the epoxide hydrolase gene based on homology with the PCR clone from strain AD1. An open reading frame was found on a 2.3-kilobase *Bam*HI/*Hin*dIII fragment that was identical to the one in pEH20, that was cloned from strain AD1 (Fig. 1). The *echA* gene coded for a polypeptide of 294 amino acids and had a G + C content of 52%, which was very close to an overall G + C content of 51%. The epoxide hydrolase has a calculated molecular mass of 34,064 kDa, which fitted the experimentally determined mass of 35 kDa (15). The N-terminal sequence and the two peptides that were determined by Edman degradation were fully conserved in the translated amino acid sequence of the *echA* gene (15). The internal and C-terminal peptide were also flanked by methionine residues at the sites where the protein was originally cleaved (Fig. 1). Southern blot analysis with an *echA* probe on double digested chromosomal DNA of strain AD1 with combinations of *Bam*HI, *Sal*I, *Sma*I, *Eco*RI, and *Hin*dIII, resulted in hybridization signals of equal size as can be deduced from Fig. 1. This indicates that strains AD1 and CFZ11 contain identical stretches of DNA.

A perfect ribosome-binding site was found 7 base pairs upstream of the *echA* gene and also a possible promoter sequence was predicted by the program NNPP with a score of 0.33 on a

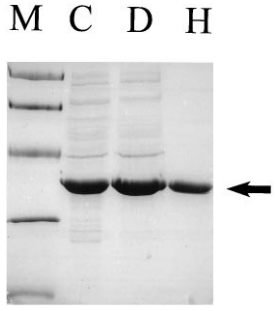

FIG. 2. **SDS-polyacrylamide gel of pooled fractions during the purification of epoxide hydrolase.** *C*, crude extract; *D*, pooled DE52 fractions; *H*, pooled hydroxylapatite fractions; *M*, marker with protein masses of 94, 67, 43, 30, and 20 kDa. Epoxide hydrolase is marked by an *arrow*.

TABLE I
Specific activities of purified recombinant epoxide hydrolase measured at a substrate concentration of 5 mM

Substrate	Specific activity
	units/mg protein
Epichlorohydrin	38
Epibromohydrin	38
Epifluorohydrin	14
Glycidol	4
Ethylene oxide	8
Propylene oxide	5.5
1,2-Epoxybutane	11.5
1,2-Epoxyhexane	7.5
1,2-Epoxyoctane[a]	25
Isoprene monoxide	14
Styrene oxide	7.5

[a] Substrate concentration, 3 mM.

scale of 0 to 1. Further upstream of the *echA* gene, two other promoter sites are predicted with scores of 0.90 and 0.92. Upstream of the *echA* gene an open reading frame was found coding for 116 amino acids with a ribosome-binding site and two potential promoter sequences (scores 0.93 and 0.77). The hypothetical protein showed 24% sequence similarity with a hypothetical protein of *E. coli* (YCHN_ECOLI; SwissProt entry code) of 117 amino acids of which the function is not described. Another open reading frame of 315 base pairs, ranging from base 539 to 854, lacked a ribosome-binding site and had no significant sequence similarity with sequences in the DNA and protein libraries. Downstream of the *echA* gene the beginning of an open reading frame was found that coded for 34 amino acids which had 41% sequence similarity with the N terminus of haloalcohol dehalogenase HheA of *Corynebacterium* sp. strain N-1074 (39). Upstream of this open reading frame lies a perfect ribosome-binding site and three putative promoter sequences (scores 0.90, 0.93, and 0.96).

Expression and Characterization of Epoxide Hydrolase—The *echA* gene in pEH20 is under control of a T7 promoter and epoxide hydrolase was expressed constitutively in a soluble and active form up to 40% of the total cellular protein content in *E. coli* BL21(DE3). For purification of the enzyme, cells were harvested at an OD_{600} of 4–5 and typically 100 mg of more than 98% pure protein could be obtained from a 1-liter culture with a purification factor of 2.5 (Fig. 2). The protein could be stored for at least 3 months at 4 °C or at −20 °C without significant loss of activity.

The specific activities of purified epoxide hydrolase for some substrates are listed in Table I. Epichlorohydrin and epibromohydrin are the best substrates. Short and long chain 1,2-epoxyalkanes are good substrates for epoxide hydrolase, and since styrene oxide is also degraded, the active site pocket must be sufficiently large to harbor these substrates. Isoprene monoxide (2-methyl-2-vinyloxirane) was also converted, indicating that branching at the second carbon atom of the epoxide ring is possible. No activity was found for *cis*-2,3-epoxybutane and since both isomers of stilbene oxide were also not degraded (15), it is essential that the epoxide ring is located at the primary carbon atom.

A substrate depletion curve of epoxide hydrolase with epichlorohydrin as the substrate, followed a straight line to the detection limit of 50 μM, indicating that the K_m value for epichlorohydrin was below 50 μM. Since the K_m value for epichlorohydrin is very small, the specific activity of 38 units/mg of protein^{-1} at a substrate concentration of 5 mM can considered to be the V_{max}, corresponding to a k_{cat} of 21 s^{-1}.

Sequence Similarity with α/β-Hydrolase Fold Enzymes—A sequence similarity search with the amino acid sequence of epichlorohydrin epoxide hydrolase (EchA) was performed in various protein and DNA data bases. A selection of the most similar proteins is shown in a ClustalW alignment in order of their sequence similarity to EchA (Fig. 3). All epoxide hydrolase sequences that were present in the data banks were scored in the search. The soluble epoxide hydrolases from mammalian and plant origin (1–3, 7, 8) were found to be more similar to EchA than the microsomal epoxide hydrolases from mammalian and insect origin (4–6, 9), which are membrane-associated enzymes. Sequence similarity was also found with putative hydrolases from *Caenorhabditis elegans* (40) and *Stigmatella aurantiaca* (41), but they were omitted from the alignment because the proteins have not been studied. The highest similarity was found between EchA and the fluoroacetic acid dehalogenase (DehH1) from *Moraxella* sp. strain B. Based on sequence similarity with haloalkane dehalogenase (DhlA) from *X. autotrophicus* GJ10 (13), DehH1 is believed to be an α/β-hydrolase fold enzyme (42). Other hydrolases that had significant sequence similarity with EchA are two 2-hydroxymuconic semialdehyde hydrolases (SwissProt entry codes: DMPD_PSEPU and XYLF_PSEPU), 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase (TODF_PSEPU), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BPHD_PSES1), magnesium-chelatase 30-kDa subunit (BCHO_RHOCA), and dehydrolipoamide *S*-acetyltransferase (ACOC_ALCEU).

It has been proposed that the soluble and microsomal epoxide hydrolases of mammalian origin belong to the α/β-hydrolase fold family (10–12, 43). The sequence similarity of EchA with bromoperoxidase A2 (BpA2) from *Streptomyces aureofaciens* (44) and DhlA, of which the three-dimensional structures are known, indicates that EchA also belongs to this class of hydrolases. The overall sequence similarity of EchA with homologous proteins is low but significant (between 13 and 23%), and is mainly located in the N-terminal region. Low sequence similarities are common between the members of the α/β-hydrolase fold family (45). Two N-terminally located motifs in EchA, *i.e.* HG*X* and GarG*X*S (*X* = any amino acid, ar = aromatic residue) that are often found in α/β-hydrolase fold enzymes, were also found in other epoxide hydrolases (10, 12). Both motifs are located on loops that excurse from the β-sheet and are in proximity of the cap domain. The part of the alignment that corresponds to the cap domain region of DhlA and BpA2 shows no sequence similarity at all, which is in agreement with a role in determining the substrate specificity.

The sequence alignment of Fig. 3 suggests catalytic residues for EchA, which are in the same position in the alignment as the identified catalytic residues of BpA2 (44), DhlA (14), soluble epoxide hydrolase (46), and microsomal epoxide hydrolase (47). The nucleophile (Nu), an aspartic acid or a serine in the case of BpA2, is conserved among the depicted hydrolases in the nucleophile elbow sequence Sm-*X*-Nu-*X*-Sm-Sm (Sm = small res-

FIG. 3. **Sequence alignment of EchA with other hydrolases.** The sequences were aligned using the multiple alignment program ClustalW and are shown in order of sequence similarity to the epoxide hydrolase cloned in this study. Conserved amino acids are marked with #, four or more conserved residues are marked with +. The nucleophile is marked by *N*, the catalytic histidine by *H*, and the catalytic acid residue by *A*. Below the sequence alignment, the predicted secondary structure elements of EchA, and the determined secondary structures of DhlA (14) and BpA2 (44) are shown. β-Strands are shown as *arrows* (numbered as for DhlA) and α-helices are shown as *waves*. Sequences: EchA, epoxide hydrolase from *A. radiobacter* strain AD1; DehH1, fluoroacetic acid dehalogenase from *Moraxella* sp. strain B (42); sEHs, soluble epoxide hydrolase from potato (7); sEHh, soluble epoxide hydrolase from human (1); BpA2, bromoperoxidase A2 from *S. aereofaciens* (44); DhlA, haloalkane dehalogenase from *X. autotrophicus* GJ10 (13); and mEHh, microsomal epoxide hydrolase from human (4). The other epoxide hydrolase sequences were omitted from the alignment due to high sequence similarity (over 80%) to the depicted epoxide hydrolases (2, 3, 5, 6, 8, 9).

idue), as described by Ollis *et al.* (45). The sequence similarity indicates that Asp^{107} may be the nucleophile in EchA. The histidine residue of the catalytic triad is completely conserved among the hydrolases and is located close to the C terminus at position 275 in EchA. The sequence around the acid residue of the catalytic triad is not conserved in the α/β-hydrolase fold family (45), but Asp^{246} of EchA is clearly aligned with the catalytically active aspartic acids of BpA2, DhlA, and soluble epoxide hydrolase (Fig. 3). Residue Glu^{404} of human microsomal epoxide hydrolase, that was proposed to be the third member of the catalytic triad (11), and Glu^{401} of juvenile hormone epoxide hydrolase from insect (9) also align with Asp^{246}. No acid residue is present in DehH1 at a corresponding position. The spacing between the conserved histidine and the aspartic acid (26–34 residues) is similar for all other enzymes. Based on sequence similarity, we propose that the catalytic triad of epoxide hydrolase consists of the residues Asp^{107}, His^{275}, and Asp^{246}.

Secondary Structure of Epoxide Hydrolase—Since the three-dimensional structures of BpA2 and DhlA are known, we studied the secondary structure elements of epoxide hydrolase in more detail by circular dichroism spectroscopy and secondary structure prediction. The experimental secondary structure elements of the main domains of BpA2 and DhlA are conserved in the sequence alignment (Fig. 3). This suggests that the secondary structure elements of DhlA and BpA2 can be extrapolated to EchA. The secondary structure predictions on the amino acid sequence of EchA led to almost similar results as the alignment. All the β-strands of EchA were predicted to be at the same position in the alignment as the β-strands of DhlA and BpA2, only β-strand 6 was not present. The same holds for some of the predicted α-helices. When secondary structure predictions were done with the amino acid sequences of DhlA and BpA2, the β-strands were predicted very well (not shown). This indicates that the residues that form the β-sheet highly favor β-stranded structure. The secondary structure predictions were most consistent in the N-terminal part of EchA, which is also the region with the highest sequence similarity. The secondary structure of the cap domain region of EchA was predicted to be predominantly α-helical, although β-stranded structures were also predicted with some programs. The cap domains of DhlA and BpA2 are completely α-helical, but they differ in their tertiary structure and the sequence similarity is too low to predict the location of secondary structure elements in EchA.

Circular dichroism spectroscopy (CD) was performed on purified epoxide hydrolase to obtain more structural information. A CD spectrum of wild type epoxide hydrolase and purified DhlA was recorded in the far-UV region (Fig. 4). The recorded spectra are much alike and are typical for proteins with α-helical and β-stranded structure (48). With the programs CONTIN, SELCON, and K2D, the ratios of the secondary structure elements of EchA, α-helix/β-strand/other, were determined to be 31/28/41, 32/22/46, and 37/12/51, respectively. Clearly, all programs predict an α/β structure for EchA. Predictions of the secondary structure elements of DhlA were made as a control

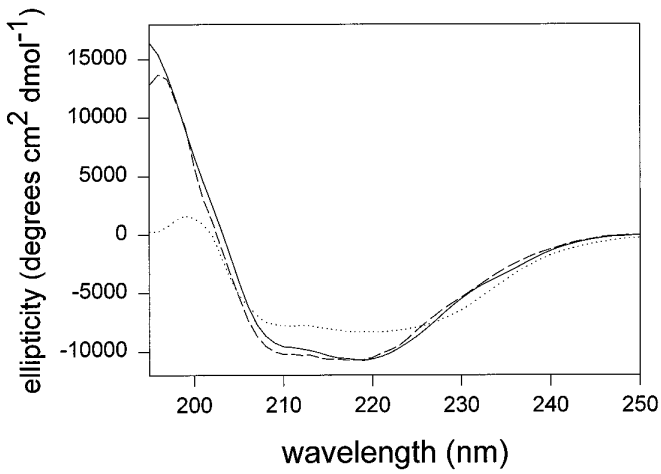

FIG. 4. **Circular dichroism spectra of haloalkane dehalogenase, epoxide hydrolase, and the His^{275} → Arg mutant of epoxide hydrolase.** The spectra were recorded in 5 mM phosphate buffer, pH 6.8, in the far-UV region between 195 and 250 nm at 25 °C. The *solid line* represents epoxide hydrolase (6.5 μM), the *dashed line* the His^{275} → Arg mutant of EchA (9.5 μM), and the *dotted line* the haloalkane dehalogenase (19 μM).

with the same programs and resulted in ratios of 22/18/60, 32/22/46, and 33/13/54, respectively. The programs SELCON and K2D gave almost identical predictions for EchA and DhlA, indicating that both enzymes have similar structures. The ratio of DhlA, 43/14/43, that was determined by x-ray crystallography (14), compares relatively well with the predictions made by SELCON and K2D. The β-strand content was especially predicted very well by K2D.

Characterization of Epoxide Hydrolase Mutants—In the sequence alignment of Fig. 3, the residues Asp^{107}, His^{275}, and Asp^{246} of epoxide hydrolase were pointed out as the catalytic residues. To test if these residues were catalytically active, Asp^{107} was mutated to Ala and Glu, His^{275} was mutated to Arg and Gln, and Asp^{246} was mutated to Ala. The epoxide hydrolase mutants were all expressed at 20 °C as soluble protein, and in quantities similar to wild type enzyme. The activities of all mutant enzymes measured in a cell-free extract with epichlorohydrin were drastically reduced compared with wild type enzyme, indicating that all three residues are involved in catalysis. The Asp^{246} → Ala mutant still had some activity for epichlorohydrin (Table II).

Single turnover experiments were performed with wild type enzyme and the His^{275} → Arg mutant, to test if epichlorohydrin is converted via a covalently bound ester intermediate. Enzyme (1 mM) was incubated with 0.5 mM epichlorohydrin and the concentrations of epichlorohydrin and 3-chloro-1,2-propanediol were measured after different incubation times (Fig. 5). With wild type enzyme the reaction was complete within 100 ms and product was formed as soon as the reaction started. No significant difference between the rates of substrate disappearance and product formation was observed, which would otherwise be an indication for a covalently bound ester intermediate.

The single turnover experiment with the His^{275} → Arg mutant clearly showed that the concentration of epichlorohydrin decreased significantly in the first 10 s while no product was formed (Fig. 5*B*). This implicates that the reaction mechanism of epoxide hydrolase proceeds via a covalently bound ester intermediate. After 1 min of reaction time, some product could be detected (detection limit 50 μM) and after 10 min epichlorohydrin was almost completely converted to 3-chloro-1,2-propanediol. Although epichlorohydrin was covalently trapped in the His^{275} → Arg mutant, the enzyme was still able to hydrolyze the covalently bound ester intermediate with a rate constant of less than 0.001 s^{-1}, which is below the detection limit of the steady state rate measurements (Table II).

TABLE II
Specific activities of wild type and mutant enzyme for 5 mM epichlorohydrin measured in cell free extract

Enzyme	Specific activity
	unit/mg protein
Wild type	12
Asp^{107} → Ala	<0.002
Asp^{107} → Glu	<0.002
His^{275} → Arg	<0.002
His^{275} → Gln	<0.002
Asp^{246} → Ala	0.050

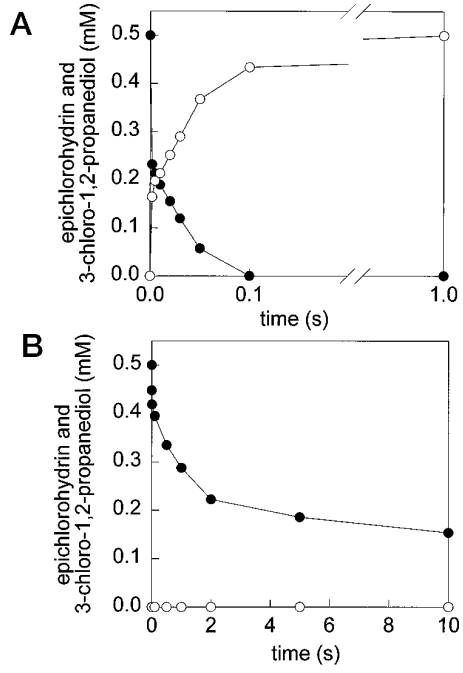

FIG. 5. **Single turnover reaction of epoxide hydrolase with epichlorohydrin.** The concentrations of epichlorohydrin (●) and 3-chloro-1,2-propanediol (○) were determined after different incubation times. *A*, single turnover experiment with wild type epoxide hydrolase (1 mM) and epichlorohydrin (0.5 mM). *B*, single turnover experiment with the His^{275} → Arg mutant of epoxide hydrolase (1 mM) and epichlorohydrin (0.5 mM).

Substrate depletion was relatively fast in the first 2 s after which conversion proceeded at a slower rate. This is in agreement with a slow rate of ester hydrolysis. Repetition of this experiment with another batch of purified enzyme and various enzyme/substrate ratios gave reproducible results and indicated that 70% of the His^{275} → Arg enzymes is probably not participating in the reaction, which may be related to enzyme heterogeneity. The results could not be explained by preference of the mutant enzyme for one of the enantiomers of epichlorohydrin since both were hydrolyzed at the same rate (data not shown). A circular dichroism spectrum of the His^{275} → Arg mutant showed no significant structural distortions, since the spectrum is similar to that of wild type epoxide hydrolase (Fig. 4).

DISCUSSION

The epichlorohydrin epoxide hydrolase (*echA*) gene of *A. radiobacter* AD1 was cloned and expressed in *E. coli* BL21(DE3). The identity was confirmed by the high activity for epichlorohydrin and fragments of amino acid sequence. Epichlorohydrin epoxide hydrolase (EchA) was found to be more similar to soluble epoxide hydrolase than to microsomal epoxide hydro-

lase. No other bacterial epoxide hydrolase gene has been cloned, but a DNA fragment of 112 base pairs, located upstream of the haloalcohol dehalogenase gene *hheB* in *Corynebacterium* sp. strain N-1074, codes for a C terminus of 37 amino acids and has 90% sequence identity with the C-terminal sequence of EchA (39). Downstream of the *echA* gene of *A. radiobacter* CFZ11/AD1, we found a segment of an open reading frame encoding for 34 amino acids that had 41% sequence similarity with haloalcohol dehalogenase HheA of *Corynebacterium* sp. strain N-1074 (39). Since both bacteria have a similar degradation route of epichlorohydrin, it is very likely that the open reading frame found in strain CFZ11 codes for the N terminus of a haloalcohol dehalogenase.

Epoxide hydrolase appears to be an α/β-hydrolase folded enzyme. One of the characteristics of this family of enzymes is that the sequence similarity is mainly found in some parts of the N-terminal region and around the catalytic triad residues (10, 12, 42, 49). This also holds for the *echA* encoded enzyme. Despite the low sequence similarity, the GarG*X*S and the HG*X*P sequences were strictly conserved. In DhlA, the sequence HGEP forms the oxyanion hole in which the backbone amide hydrogen of Glu^{56}, which is located on a sharp *cis*-proline turn, stabilizes the oxyanion that is formed on the side chain carbonyl oxygen of the nucleophilic Asp^{124} during the hydrolysis of the ester intermediate. His^{54} forms a hydrogen bond ($N_{\delta 1}$-O) with the backbone carbonyl of Gly^{55}, causing a sharp turn (14). Mutation of His^{148} to Asn in this motif in rat microsomal epoxide hydrolase led to a significant decrease in activity (47). This indicates that a sharp turn in the oxyanion hole is essential for enzyme activity and that therefore this motif is well conserved. The function of the GarG*X*S motif in DhlA is not described, but it is part of a large loop with three β-turns, and it is located close to the oxyanion hole and the nucleophilic Asp^{124}. Since this motif is well conserved, it may have a function in positioning of the oxyanion hole in regard to the nucleophile. In DhlA, the side chain of the aromatic residue Phe^{85} of the motif GFGDS is in a tilted-T arrangement with His^{54} of the oxyanion hole motif, in which the positively charged His ring stands perpendicular on the slightly negative charged surface of Phe^{85}. No hydrogen bonds or salt bridges were found between these motifs, but a His-Phe interaction can have a considerable stabilizing effect (50, 51).

The α/β-hydrolase fold is a conserved topology with a main domain that consists of a central β-sheet that is alternated with α-helices that cover both sides. In the structures of haloalkane dehalogenase and bromoperoxidase A2, the α-helical cap domain follows β-strand 6 and covers the active site like a cap. The positions of the secondary structure elements of haloalkane dehalogenase and bromoperoxidase A2 compared with the secondary structure elements that were predicted for epoxide hydrolase strongly suggest a specific topology for epoxide hydrolase (Fig. 3). The cap domain of epoxide hydrolase therefore seems to be located between β-strands 6 and 7 (residues 132–209), since this is the only part of the alignment that shows little similarity in both sequence and secondary structure predictions. In α/β-hydrolase fold enzymes the β-sheet forms a scaffold for the catalytic triad residues, that are located on loops excursing from the β-sheet, and since these residues are preserved in the topology, they are also conserved in the sequence. Mutation of the residues Asp^{107}, His^{275}, and Asp^{246} of epoxide hydrolase, resulted in a dramatic drop of enzyme activity, which indicates that these residues are involved in catalysis.

The $Asp^{246} \rightarrow$ Ala mutant still had some residual activity for epichlorohydrin, indicating that this residue is involved in enzymatic activity but not essential. Mutation of the acid residue

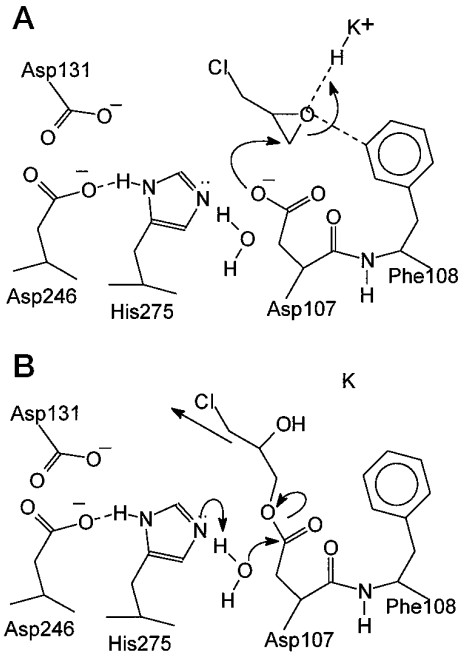

FIG. 6. **Proposed reaction mechanism of epoxide hydrolase.** *A*, nucleophilic attack of Asp^{107} on the C_α of the epoxide ring, leading to the formation of a covalent ester intermediate. *B*, proton abstraction from a water molecule by the His^{275}/Asp^{246} pair and hydrolysis of the intermediate.

in haloalkane dehalogenase,[3] 2-hydroxymuconic semialdehyde hydrolase (49), and soluble epoxide hydrolase (46) resulted in an inactivated enzyme. Probably, Asp^{246} has some backup in EchA. In triacylglycerol lipase from *Pseudomonas glumae*, a partially redundant catalytic aspartate was reported (52). In human pancreatic lipase an alternative catalytic triad was found in which the catalytic aspartate was shifted from β-strand 7 to β-strand 6 at position 176 (53). In the crystal structure of DhlA, Asn^{148} is the analog of Asp^{176} of human pancreatic lipase and is located directly after β-strand 6 where it forms a hydrogen bond with the nucleophilic Asp^{124} (14). In the sequence alignment, Asp^{131} of EchA is aligned with Asn^{148} of haloalkane dehalogenase (Fig. 3). So, Asp^{131} of epoxide hydrolase is probably positioned close to the nucleophilic Asp^{107} and the catalytic His^{275}. The presence of another aspartate may be sufficient to retain some activity in EchA when Asp^{246} is replaced by alanine. The same argument can also explain why no acid residue could be found in Fig. 3 for DehH1. Residue Asp^{129} of DehH1, which is aligned with Asp^{131} of EchA and Asn^{148} of DhlA, could well be part of an alternative catalytic triad, as present in human pancreatic lipase (53).

Based on these results, we propose a reaction mechanism for EchA in which the catalytic Asp^{107} performs a nucleophilic attack on the primary carbon atom of epichlorohydrin, leading to a covalently bound ester intermediate (Fig. 6). It was shown earlier using ^{18}O-labeled water that hydrolysis takes place at the primary carbon atom of the epoxide ring (15). His^{275}, assisted by Asp^{246}, abstracts a proton from a water molecule that hydrolyzes the ester at the carbonyl function of Asp^{107}. Phe^{108} of epoxide hydrolase, which is located next to the nucleophile Asp^{107}, is probably interacting with the epoxide ring. In haloalkane dehalogenase, Trp^{125} is involved in halogen and halide binding (54). The eukaryotic epoxide hydrolases all have a tryptophan at this position, but a positively charged edge of phenylalanine is also capable of binding the electronegative oxygen atom of the epoxide ring (55, 56). A phenylalanine

[3] G. H. Krooshof, unpublished data.

residue next to the nucleophilic serine was also found in 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase and two 2-hydroxymuconic semialdehyde hydrolases that had sequence similarity with EchA.

Beetham *et al.* (11) postulated that a proton donating group (K-H) should be present in the cap domain of soluble epoxide hydrolase and protonates the leaving group, an oxyanion that is formed upon opening of the epoxide ring. They mentioned Lys^{406} as a possible candidate, since this residue is conserved among soluble epoxide hydrolases and since chemical modification resulted in inactivation of the enzyme. An alignment of EchA with all soluble epoxide hydrolases indicates that this is the only lysine that is conserved in the cap domain (data not shown). The alignment points out Lys^{173} of EchA as a possible candidate, but also the two nearby positioned Lys residues 174 and 177 could perform the role as proton donor. The role of Lys^{173}, Lys^{174}, and Lys^{177} is currently studied further by site-directed mutagenesis and fluorescence spectroscopy.

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