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Histidine 289 Is Essential for Hydrolysis of the Alkyl-enzyme Intermediate of Haloalkane Dehalogenase*

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Haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 catalyzes the hydrolytic cleavage of carbon–halogen bonds in a broad range of halogenated aliphatic compounds. Previous work has shown that Asp^{124}, which is located close to the internal substrate-binding cavity, carries out a nucleophilic attack on the C-α of the alkylhalide, displacing the halogen. The resulting alkyl-enzyme intermediate is subsequently hydrolyzed. In order to study the role of His^{289} in the hydrolysis of the intermediate, a His^{289} → Gln mutant was constructed by site-directed mutagenesis. The purified mutant enzyme was not catalytically active with haloalkanes, but a halide burst stoichiometric to the amount of enzyme was observed with 1,2-dibromoethane. Using ion spray mass spectrometry, accumulation of the covalent alkyl-enzyme and binding of the alkyl moiety of the substrate to an Asp^{124}-containing tryptic peptide were shown. Fluorescence-quenching experiments indicated that halide ions are strongly bound by the alkyl-enzyme but not by the substrate-free enzyme. The results show that His^{289} is the base catalyst for the dealkylation of the covalent intermediate, but that it is not essential for the initial nucleophilic attack of Asp^{124} on the C-1 atom of the haloalkane. Furthermore, the halide ion that is released in the first step probably leaves the active site only after hydrolysis of the alkyl-enzyme.

The first step in the degradation of 1,2-dichloroethane by bacteria is hydrolytic conversion to 2-chloroethanol catalyzed by haloalkane dehalogenase. The enzyme from *Xanthobacter autotrophicus* hydrolyzes a broad range of haloalkanes to the corresponding alcohols, without a requirement for cofactors or oxygen. The sequence of the gene (*dhlA*) that encodes the 310-amino acid enzyme is known (1). Furthermore, the x-ray structure was determined (2, 3). The protein is composed of a main domain, which is formed by an eight-stranded β-sheet and α-helices, and a cap domain, which is formed by helices and loops located on top of the main domain. On the basis of the topology of the main domain, haloalkane dehalogenase was classified as a member of the α/β hydrolase fold enzymes (4). This group of hydrolytic proteins have a conserved arrangement of active site residues in the main domain, forming a catalytic triad.

Two tryptophans that line an internal cavity of 37 Å^3, which is the substrate binding site, bind the halogen atom of the substrate and halide ions (5, 6). Substrate and halide binding can be followed by quenching of tryptophan fluorescence. On the basis of x-ray crystallographic studies (5, 6) and mass spectrometric analysis of ^{18}O incorporation from $H_2{}^{18}O$ (7), Asp^{124} was proposed to act as a nucleophile, causing displacement of the halide ion and formation of an alkyl-enzyme intermediate. The aspartate is located on the nucleophile elbow, a conserved structural element present in the main domain of α/β hydrolase fold enzymes (4). The alkyl-enzyme ester bond of the covalent intermediate was proposed to be hydrolyzed by water. A water molecule could be activated by His^{289}, which is also at a conserved position in the α/β hydrolase fold structure and may act as a proton acceptor (Fig. 1). The "charge relay" residue that activates His^{289} is Asp^{260}, which is also present at a conserved position in these enzymes.

In this study, we analyze the properties of a His^{289} → Gln mutant and show that the histidine is essential for hydrolysis of the covalent intermediate but not for its formation. Mutation of His^{289} and alkylation of Asp^{124} were also found to influence the affinity of the enzyme for halide ions and the rate of substrate-independent exchange of the carboxylate oxygens of Asp^{124} with solvent water.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Merck or Sigma. Restriction enzymes and other molecular biology enzymes were from Boehringer Mannheim. α-Chymotrypsin and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were obtained from Worthington Biochemical Corp., NJ. All DNA primers were purchased from Eurosequence BV, Groningen. DNA sequencing was done with the T7 sequencing kit from Pharmacia Biotech Inc. $H_2{}^{18}O$ (97%) was obtained from Isotec Inc., Miamisburg, OH.

Construction of the Mutant H289Q—Mutants were constructed by two consecutive PCR[1] reactions. Wild-type *dhlA* DNA was amplified with the mutant primer 5′-GCGGACGCTGGCCAGTTCGTACA (Gln codon underlined) and the antisense primer located downstream of the *dhlA* gene 5′-ATAGAATTCATGGATCCTCAGTTTTCGTACCGGCACCGG (*Bam*HI site underlined). The PCR product was elongated by a subsequent PCR reaction with primer 5′-AACCCCTCGAGATAGCGGACCC (*Xho*I site underlined) and the above downstream primer. The new PCR product was used as mutant DNA and exchanged with the corresponding fragment of a derivative of the haloalkane dehalogenase expression vector pPJ123 (8), which contained an additional *Xho*I site in the *dhlA* gene,[2] and finally recloned in the much better DhlA expression vector pELA (8). Sequences were confirmed by T7 DNA polymerase dideoxy sequencing (9).

Expression and Purification of Haloalkane Dehalogenase—Wild-type and mutant H289Q haloalkane dehalogenase were purified from *Escherichia coli* BL21(DE3) as described by Schanstra *et al.* (8). Cells with pELA constructs expressing haloalkane dehalogenase were grown at 30 °C in LB medium containing 50 μg/ml ampicillin to an A_{600} of about 1. Cultivation was continued at 17 °C, and IPTG (0.4 mM) was added

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[1] The abbreviations used are: PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

[2] F. Pries, G. H. Krooshof, and D. B. Janssen, unpublished results.

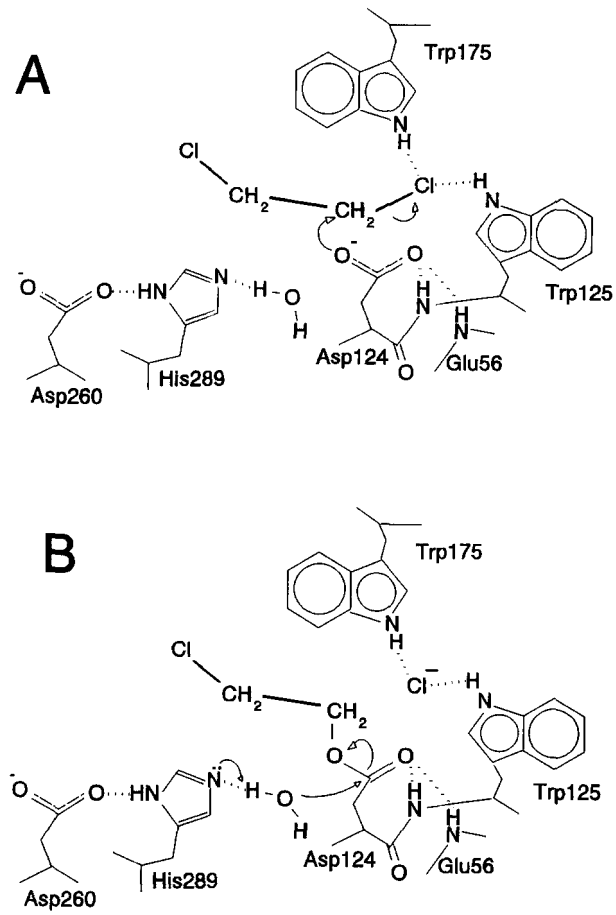

FIG. 1. **Proposed catalytic mechanism of haloalkane dehalogenase.** *A*, nucleophilic attack of Asp^{124} leading to formation of the covalent alkyl-enzyme intermediate. *B*, His^{289} catalyzed hydrolysis of the intermediate. The oxygen atom of the water molecule is incorporated in the carboxylate group of Asp^{124}. Main chain amide protons serve as oxyanion hole. See Introduction for details.

after 1 h as an inducer for DhlA expression. Cells were harvested after 16 h (A_{600} 2), washed with TEMAG buffer (25 mM Tris·SO_4, pH 7.5, containing 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM sodium azide, and 10% (v/v) glycerol), resuspended in TEMAG buffer, and sonicated for 10 s/ml cell suspension. Cell debris and other nonsoluble material were removed by centrifugation at 120,000 × *g* for 60 min. This was followed by DEAE-cellulose chromatography and hydroxylapatite chromatography to yield pure enzyme (8). All enzyme samples used were more than 99% pure as determined by SDS-polyacrylamide gel electrophoresis. Stocks of the enzyme were stored in TEMAG buffer. For incubations with $H_2{}^{18}O$, the enzyme was concentrated by ultrafiltration over an Amicon PM10 filter to 10–20 mg/ml. Stoichiometric bromide release from 1,2-dibromoethane was tested with enzyme concentrated to 42 mg/ml.

Assays—Measurements of haloalkane dehalogenase activity with different substrates were performed by following halide liberation with a colorimetric assay as described previously (1). The assay is done in 1 N nitric acid and thus results in denaturation of the enzyme. Bromide release from 1,2-dibromoethane by a high concentration of DhlA was tested in incubation mixtures containing 1.2 mM enzyme, 3 mM 1,2-dibromoethane, and 50 mM Tris·SO_4, pH 8.2, in a total volume of 500 μl. Halide liberation was measured colorimetrically after 30 s and 5 min of incubation at 30 °C. Calculations were corrected for the absorbance caused by enzyme, using a control incubation containing no substrate. Protein concentrations were determined with Coomassie Brilliant Blue or by measuring the absorbance at 280 nm.

The N-terminal amino acid sequence was determined by Eurosequence BV, Groningen, using automatic Edman degradation (10) with an Applied Biosystems model 477A sequencer. In the wild-type and mutant dehalogenases produced by pELA-based expression vectors, the N-terminal sequence of the enzyme is MVNAIR instead of MINAIR, which reduces the molecular weight of native DhlA to 34,990.

Fluorescence Measurements—Fluorescence spectra and fluorescence quenching by halide or substrate were determined with an Aminco SPF500-C spectrofluorometer at 25 °C as described previously (5). All incubations were done using 1 μM dehalogenase in TEMAG buffer with an excitation wavelength of 290 nm. Emission spectra were recorded from 300 to 400 nm.

Incubations with $H_2{}^{18}O$—Incubations of dehalogenase with $H_2{}^{18}O$ were carried out in 100 μl of incubation mixtures containing 0.1 mM dehalogenase, 50 mM Tris·SO_4, pH 8.2, and 48 or 83% $H_2{}^{18}O$ for the wild-type and H289Q DhlA, respectively. After 2.5 min or 3 h at 30 °C, 14 μl of 2 M ammonium acetate/ammonium carbonate, pH 8.0, and 5 μl of 1 mg/ml trypsin, freshly dissolved in 0.2 M of the same buffer, were added. Digestion was carried out for 4 h at 37 °C. Controls were performed similarly but with 1,2-dibromoethane and 1-bromopropane omitted.

HPLC Isolation of the Peptide Fragments—Trypsin cleavage was carried out with 5–20 mg/ml haloalkane dehalogenase and 0.05–0.2 mg/ml trypsin in 0.2 M ammonium acetate/ammonium bicarbonate buffer, pH 8. After 4 h at 37 °C, the mixture was separated by reversed phase HPLC on a Nucleosil 10C18 column, using a linear gradient of 0–67% acetonitrile in 0.1% trifluoroacetic acid as the eluant. Eluting peptides were either subjected directly to mass spectrometric analysis or collected, lyophilized, and used for further experiments. The alkylated mutant enzyme was significantly less sensitive to trypsin digestion, and therefore a 4-fold higher trypsin concentration and an incubation time of 20 h were used.

For determining incorporation of ^{18}O from $[^{18}O]H_2O$ in Asp^{124}, trypsin cleavage was done as described above, and fragments were isolated by HPLC using 0–67% acetonitrile in 0.1% ammoniumacetate, pH 6, as the solvent instead of trifluoroacetic acid. The 24-amino acid peptide containing the nucleophilic aspartate (Asn^{117}–Arg^{140}) was subsequently digested by chymotrypsin, and a pentapeptide (Val^{121}–Trp^{125}) containing Asp^{124} was isolated by HPLC as described before (7). Control experiments and previous work (7) showed that no significant exchange of carboxylate oxygens with solvent water oxygen occurred after trypsin digestion of the dehalogenase. The C-terminal carboxylate of the 24-mer is cleaved off during chemotrypsin digestion.

Ion Spray Mass Spectrometry—For mass spectrometry, lyophilized peptides were dissolved at approximately 100 nmol/ml in a solution of 0.02% trifluoroacetic acid in 80% (v/v) methanol in water and analyzed by pneumatically assisted electrospray ionization on a Nermag R 3010 quadrupole instrument as described previously (11, 12). Data acquisition and data reduction took place by means of the standard Nermag SIDAR software. Each peptide solution was examined by full scan mass spectra recorded in 1-atomic mass unit steps in order to confirm the identity of the peptide and to determine the incorporation of ^{18}O.

Whole proteins were also analyzed by ion spray mass spectrometry (13). Proteins were dissolved in a solution of 1% trifluoroacetic acid in 80% (v/v) methanol in water. Multiply charged ion mass spectra were recorded in 1-atomic mass unit steps.

Alkylation of Asp^{124}-containing peptides was determined by direct introduction of the HPLC column eluate into the mass spectrometer using a Nucleosil 10C18 reversed phase column for separation and a 0–67% gradient of acetonitrile in 0.1% trifluoroacetic acid for elution.

Gas Chromatography Analysis—The presence of 2-bromoethanol and 1-bromopropane in dialyzed enzyme solutions was measured by gas chromatography. The samples were extracted with diethylether containing 1-bromohexane as the internal standard. Analysis and fitting of the data were performed as described by Schanstra *et al.* (8), with the modification that for 1-bromopropane the temperature program of the GC started with 3-min isothermal operation at 30 instead of 45 °C.

RESULTS

Activity of the H289Q Haloalkane Dehalogenase—Mutant H289Q haloalkane dehalogenase was constructed by PCR mutagenesis, expressed in *E. coli* BL21(DE3) and purified. Using incubations containing 0.8 mg/ml of enzyme (22 μM) and 5 mM substrate at pH 8.2, no catalytic activity was found with 1,2-dibromoethane and dibromomethane (detection limit 0.006 units/mg protein). The wild-type enzyme has an activity with 1,2-dibromoethane of 4 units/mg of protein under these conditions. Thus, the activity of the mutant was reduced more than 660-fold.

The possibility that bromide was released stoichiometrically to enzyme by a large amount of mutant DhlA was checked with 1,2-dibromoethane. Incubation of 1.2 mM H289Q DhlA with 3

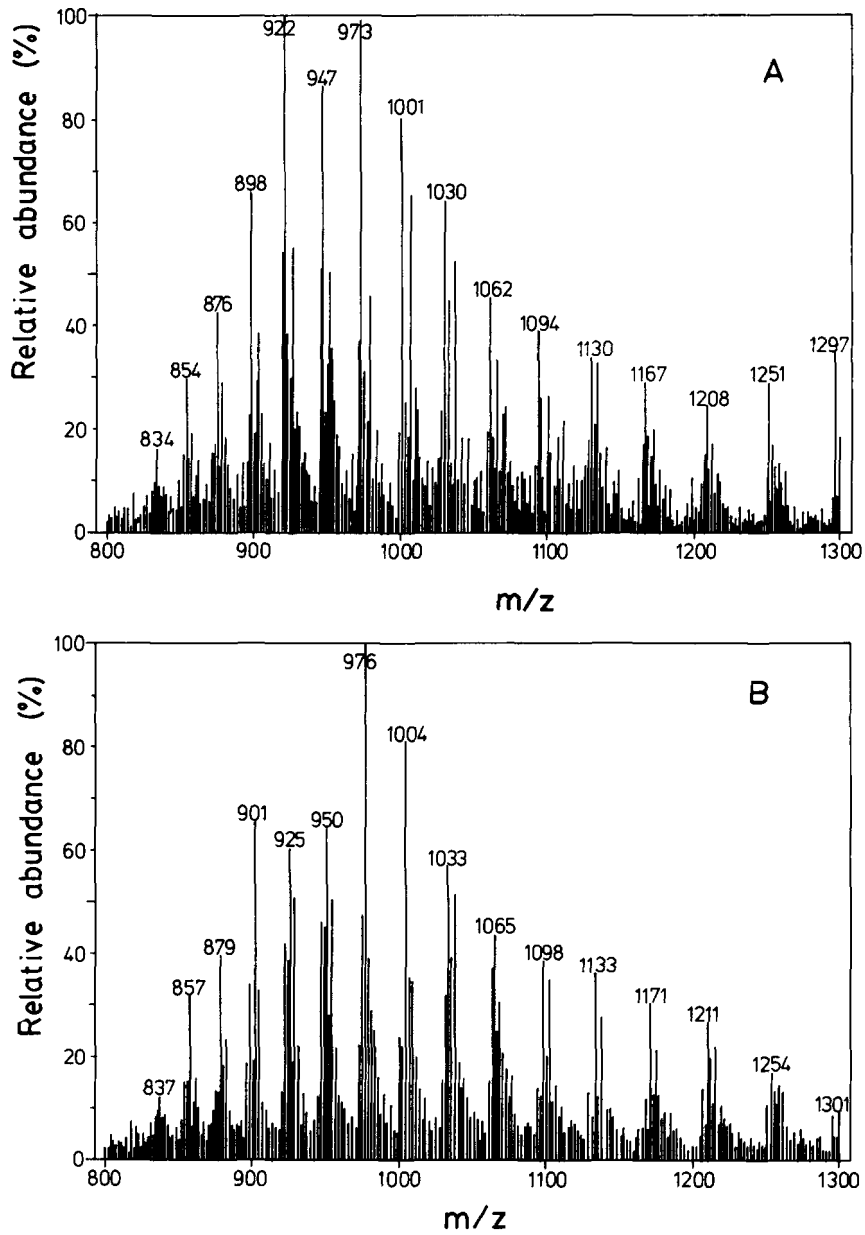

FIG. 2. **Partial ion spray mass spectra of the H289Q haloalkane dehalogenase incubated without (*A*) and with (*B*) 1,2-dibromoethane as substrate.** Both spectra were obtained with an injection of 20 μl of a 10 nmol/ml protein solution. Multiply protonated protein ion series in *A* are observed from *m/z* 834 (42 charges) through *m/z* 1944 (18 charges) and in *B* from *m/z* 837 (42 charges) through 1951 (18 charges).

mM 1,2-dibromoethane led to the release of 1.1 ± 0.2 mM bromide after 30 s and 1.02 ± 0.2 mM bromide after 5 min, as determined by colorimetric analysis. No formation of 2-bromoethanol could be detected by gas chromatography (detection limit, 0.1 mM), while the concentration of 1,2-dibromoethane had decreased by 31%. The data indicate that after the first stoichiometric release of bromide, the enzyme activity was completely blocked and that the covalent intermediate accumulated.

Identification of the Covalent Alkyl-Enzyme—To determine whether a covalent alkyl-enzyme did indeed accumulate during incubation of H289Q DhlA with bromoalkane, whole enzyme and a 24-mer peptide containing Asp^{124} were analyzed by ion spray mass spectrometry.

Analysis of H289Q enzyme, which was not incubated with substrate and dialyzed against water, showed the presence of multiply protonated ions with the number of positive charges varying from 18 to 42 (Fig. 2*A*). N-terminal sequencing of the enzyme showed that 80% of the mutant H289Q haloalkane dehalogenase molecules had lost the N-terminal methionine. The molecular mass was calculated from the observed *m/z* values of multiply charged ions (13). The experimentally determined molecular mass was 34,990 (±2), which is identical to the molecular mass predicted from the amino acid sequence of H289Q DhlA. The mass of H289Q DhlA that had reacted with 1,2-dibromoethane was determined in the same way (Fig. 2*B*). A value of 35,100 (±2) was found, which is 110 mass units higher than the mass of the unreacted mutant protein. This is very close to the calculated mass increment upon incorporation of the bromoethyl group, which is 107.

The nucleophilic aspartate (Asp^{124}) of DhlA is present in a 24-amino acid tryptic peptide that can be isolated by reversed phase HPLC (7). To show that this peptide is alkylated during reaction of H289Q DhlA with 1,2-dibromoethane, the enzyme was incubated with substrate, digested with trypsin and analyzed by HPLC/mass-spectrometry (Fig. 3). The peak corresponding to the 24-amino acid peptide (mass, 2601 Da) was detected in trypsin-cleaved H289Q enzyme that was not exposed to a bromoalkane but was no longer present in enzyme reacted with 1,2-dibromoethane or 1-bromopropane (Table I and Fig. 3). Instead, peaks with longer retention times were detected after trypsin cleavage of enzyme reacted with the alkylbromides. Mass spectrometry showed the presence of frag-

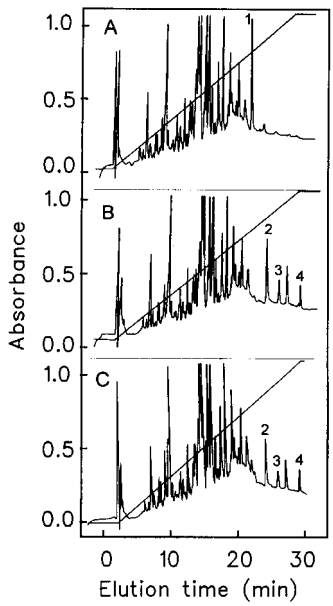

FIG. 3. **Reversed-phase HPLC elution profiles of proteolytic fragments.** *A*, trypsin digest of haloalkane dehalogenase prepared from the unreacted mutant protein. *Peak 1*, 24-mer Asn^{117}–Arg^{140}, as determined by N-terminal amino acid sequencing and mass spectrometry. *B*, trypsin digest of H289Q DhlA after reaction with 1-bromopropane. The mass of *peaks 2–4* was determined and corresponded to propyl derivatives (see Table I). *C*, trypsin digest of H289Q DhlA after reaction with 1,2-dibromoethane. The alkylated peaks are indicated, and their masses and identities are given in Table I.

ments of 2643, 3140, and 4210 Da (accuracy ± 1 Da) in enzyme reacted with 1-bromopropane. This corresponds to the 24-mer Asn^{117}–Arg^{140}, the 28-mer Leu^{113}–Arg^{140}, and the 37-mer Asn^{104}–Arg^{140}, each with a propyl group attached. Analysis of fragments obtained by trypsin cleavage of H289Q enzyme incubated with 1,2-dibromoethane revealed the presence of the same peptides as obtained from the 1-bromopropane-incubated protein but with a molecular mass that was 65 Da higher, which corresponds to the difference between the propyl and bromoethyl groups.

These results confirm that incubation of H289Q DhlA with substrate leads to accumulation of an alkyl-enzyme with the alkyl group covalently attached to Asp^{124}.

Fluorescence Quenching Experiments—Fluorescence measurements were used to investigate halide and substrate binding to H289Q dehalogenase. The binding of halides to wild-type haloalkane dehalogenase was previously determined by quenching of fluorescence of the two tryptophan residues that form the halide binding site (5). At pH 8, wild-type enzyme bound chloride (5) and bromide ions (Table 3) with K_d values of 57 and 5.2 mM, respectively. Dissociation constants for chloride could not be determined with mutant H289Q DhlA at pH 8 or pH 6 since quenching was very low at concentrations up to 800 mM. At pH 8, significant quenching of fluorescence of H289Q DhlA was also not detected with 200 mM bromide, a concentration above which it became an aspecific collisional quencher. Some quenching was observed with bromide at pH 6. The degree of quenching was only 15%, as compared with 30% with the wild-type, and the K_d was 4.2 ± 0.7 mM (Table II). This indicates that binding of bromide and chloride to the H289Q mutant was very poor as compared with the wild-type enzyme.

Strong quenching of fluorescence was observed after incubation of H289Q DhlA with low concentrations of 1,2-dibromoethane and 1-bromopropane, indicating that Trp^{125} and Trp^{175} could still bind substrate and/or bromide after alkylation (Fig. 4). Quenching with 1,2-dibromoethane was somewhat stronger than with 1-bromopropane. Dialysis of enzyme incubated with 1,2-dibromoethane or 1-bromopropane against TEMAG buffer or water did not abolish quenching of fluorescence, indicating that the quenching is caused either by covalent modification or by very tight binding.

Theoretically, this quenching of fluorescence of bromoalkane-exposed and dialyzed H289Q enzyme could be caused by a bromide ion that did not leave the active site cavity after cleavage of the carbon-bromine bond by self quenching of the two tryptophans caused by the covalently attached alkyl group or by a second substrate molecule that becomes bound to the free tryptophans of the alkyl-enzyme intermediate after release of the halide. To determine whether a bromopropane molecule was present in the active site, 0.93 mM H289Q DhlA was incubated with 5 mM 1-bromopropane for 10 min at 30 °C and pH 8.2 and then dialyzed for 48 h against TEMAG. The mixture was extracted with diethylether. No bromopropane could be detected by GC analysis (detection limit, 0.01 mM). Furthermore, titration of H289Q DhlA with 1-bromopropane indicated that the quenching of fluorescence was maximal after addition of an equimolar amount of 1-bromopropane (Fig. 5). These data suggest that the persistence of quenching upon dialysis is caused by a tightly bound bromide ion or by the covalently attached alkyl group, and not by excess substrate bound to the enzyme.

To determine whether halide ions remained bound to the enzyme after hydrolysis of the C–Br bond during formation of the covalent adduct, 0.4 mM enzyme was incubated with 0.4 mM 1-bromopropane for 10 min at 30 °C. Subsequently, enzyme and buffer were separated by centrifugation on a Centricon-10 microconcentrator membrane (cutoff, 10,000). Halide assays revealed the presence of 0.15 mM bromide in the dialysate. Exact determination of the amount of bromide bound to enzyme was not possible due to the high background caused by the protein, but it was estimated that 0.8 ± 0.2 mM bromide was present in the enzyme fraction that contained 1.6 mM dehalogenase. Thus, halide ions were not completely released from the alkylated enzyme into the surrounding medium.

To determine the binding strength of alkylated enzyme and halide, H289Q enzyme incubated as above with 1-bromopropane was extensively dialyzed against TEMAG buffer, pH 7.5. Colorimetric analysis indicated that the dialyzed enzyme contained less than 10% (on a molar basis) bromide. Fluorescence quenching persisted, however. Both at pH 6 and pH 8, the addition of bromide to the diluted dialyzed protein (1 μM) caused an increase in fluorescence with typical ligand binding profiles and K_d values that were much lower than those of wild-type or free H289Q mutant DhlA (Table II). These observations are in agreement with quenching of fluorescence of alkylated enzyme being caused by the alkyl group of the bound substrate and/or self quenching of the two tryptophan residues. No excimer fluorescence emission peak at higher wavelength (350–450 nm) was detected with the bromide-free dialyzed alkylated enzyme. Apparently, the alkylated enzyme binds bromide ions much more tightly than the substrate-free mutant or wild-type enzymes (Table II), and the degree of quenching is lower with a bromide ion present in the active site than in the bromide-free alkylated H289Q dehalogenase (Fig. 4).

Exchange of Carboxylate Oxygens of Asp^{124} with Solvent Water—Based on sequence homology, eukaryotic epoxide hydrolase was proposed to have an overall structure and reaction mechanism similar to haloalkane dehalogenase. Lacourciere and Armstrong (14) have incubated epoxide hydrolase under single turnover conditions with substrate and $H_2{}^{18}O$, dialyzed the enzyme, and reincubated it with substrate in the absence of $H_2{}^{18}O$. This led to incorporation of ^{18}O in the second product.

TABLE I
Mass of peptide fragments of H289Q DhlA

Enzyme	Peak no.	*m/z*	*z*	Experimental molecular mass	Theoretical molecular mass	Fragment
H289Q DhlA (unreacted)	1	1301	2	2600	2601	Asn^{117}–Arg^{140}
	1	868	3	2601		
H289Q DhlA + 1-Bromopropane	2	1322	2	2642	2643	Asn^{117}–Arg^{140} + propyl
	2	882	3	2643		
	3	1571	2	3140	3141	Leu^{113}–Arg^{140} + propyl
	3	1048	3	3141		
	4	1403	3	4209	4211	Asn^{104}–Arg^{140} + propyl
	4	1054	4	4212		
H289Q DhlA 1,2-dibromoethane	2	1355	2	2708	2708	Asn^{117}–Arg^{140} + bromoethyl
	2	903.5	3	2707.5		
	3	1603	2	3204	3206	Leu^{113}–Arg^{140} + bromoethyl
	3	1609	3	3204		
	4	1426	3	4275	4276	Asn^{104}–Arg^{140} + bromoethyl
	4	1070	4	4276		

TABLE II
Fluorescence quenching of dehalogenases by bromide

Enzyme	pH	f_a[a]	K_d[b]
			mM
Wild-type	6	0.29 ± 0.08	0.25 ± 0.08
Wild-type	8	0.34 ± 0.04	4.2 ± 0.7
H289Q mutant	6	0.19 ± 0.09	3.9 ± 1.7
H289Q-propyl	6		0.066 ± 0.015
H289Q-propyl	8		0.011 ± 0.002

[a] f_a, fractional accessibility.
[b] K_d values were calculated using the modified Stern-Volmer equation as described previously (5).

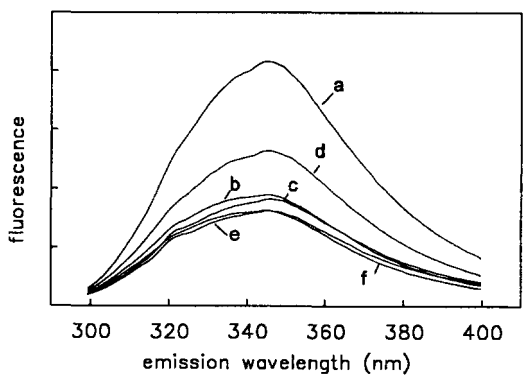

FIG. 4. **Fluorescence spectra of mutant H289Q haloalkane dehalogenase and quenching by 1,2-dibromoethane or 1-bromopropane.** In all measurements, 1 μM mutant enzyme was used in TEMAG buffer. *a*, fluorescence spectrum of free H289Q DhlA; *b*, fluorescence in the presence of 0.5 mM 1-bromopropane; *c*, as *b*, after 48 h of dialysis against TEMAG buffer; *d*, as c, after addition of 40 mM KBr; *e*, fluorescence spectrum of H289Q DhlA in the presence of 0.5 mM 1,2-dibromoethane; *f*, as *d*, after dialysis.

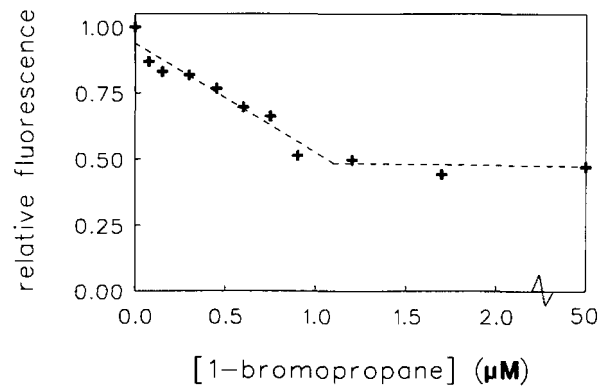

FIG. 5. **Complete quenching of fluorescence of H289Q mutant haloalkane dehalogenase by a stoichiometric amount of 1-bromopropane.** The enzyme concentration used in each measurement was 1 μM, and the fluorescence was measured after addition of varying concentrations of 1-bromopropane. Fluorescence was measured at λ_{max} = 346 nm and is given relative to the unquenched fluorescence of the H289Q mutant enzyme.

Similar experiments performed by us with haloalkane dehalogenase failed since there was too much substrate-independent exchange of the Asp^{124} carboxylate oxygens of the intact wild-type enzyme with solvent water during dialysis (data not shown). The role of His^{289} in this exchange reaction was investigated by comparing the rates of ^{18}O incorporation in the absence of substrate between wild-type DhlA and H289Q DhlA. The exchange was stopped by trypsin digestion and only occurred in intact enzyme. Pentapeptides (Val^{121}–Trp^{125}) containing Asp^{124} were subsequently isolated by HPLC and analyzed by ion spray mass spectroscopy to identify exchange of the carboxylate oxygens that had occurred in the presence of $H_2{}^{18}O$ (Table III).

No exchange of the oxygens of the nucleophilic Asp^{124} of mutant H289Q haloalkane dehalogenase was detected after 3 h of incubation in the presence of $H_2{}^{18}O$, as the mass of the pentapeptide was predominantly 646. In wild-type enzyme, however, incorporation of approximately 30% ^{18}O occurred after 3 h of incubation, indicating a rate of exchange exceeding 0.3 h^{-1}. ^{18}O incorporation was hardly observed when wild-type enzyme was incubated for only 2.5 min (Table III). Apparently, His^{289} is involved in the substrate-independent exchange of the carbonyl oxygens of Asp^{124} in the wild-type enzyme. The exchange in the wild-type dehalogenase in the absence of substrate occurs at a low rate compared with the reaction rate and rate of incorporation of ^{18}O from solvent of wild-type haloalkane dehalogenase in the presence of substrate. The exchange can be caused by a His^{289}-bound water molecule that is observed in the x-ray structure and thus appears to be highly nucleophilic.

DISCUSSION

We have investigated the role of histidine 289 in the hydrolysis of bromoalkanes by haloalkane dehalogenase. The properties of the H289Q mutant show that His^{289} is essential for the hydrolysis of the covalent alkyl-enzyme intermediate, which is formed by the nucleophilic attack by Asp^{124} and not for its formation. Using mass spectrometry, we showed that the intermediate accumulated when the mutant was incubated with 1-bromopropane or 1,2-dibromoethane. We could not detect any catalytic activity of the mutant, which indicates that the first order rate constant for hydrolysis of the covalently alkylated enzyme must be below 3.5×10^{-3} s^{-1}. For the wild-type en-

TABLE III

Partial mass spectra obtained by ion spray mass spectrometry of the pentapeptide VVQDW of wild-type and H289Q mutant haloalkane dehalogenase

Values are expressed as relative abundance with respect to the MH^+ ion isotopomer at m/z 646.

m/z	Predicted[a]	WT enzyme in $H_2{}^{16}O$		Wild-type enzyme in $H_2{}^{18}O$				H289Q enzyme in $H_2{}^{18}O$ (3-h incubation)		Wild-type enzyme expected	
				2.5-min incubation		3-h incubation					
		Measured	Corrected[b]	Measured	Corrected[b]	Measured	Corrected	Measured	Corrected	48% expected[c]	30% expected[c]
646	100	100	100	100	100	100	100	100	100	100	100
647	37.3	44.1	6.8	39.2	1.9	36.6	0	41.6	4.3		
648	8.3	13.3	2.5	15.9	6.9	86.1	78.0	15.4	5.5	185	87
649	1.2	4.9	2.2	3.8	0	27.8	0	8.7	5.1		
650		1.4	0.4	1.1	0.5	34.5	29.1	3.2	0.9	85	19
651		1.4	1.3	0.3	0	9.6	0	3.5	2.8		

[a] Predicted isotope pattern of the $C_{30}H_{44}N_7O_9^+$ ion, based on the natural isotope abundances of ^{13}C, ^{15}N, ^{17}O, ^{18}O, and ^2H.
[b] Corrected for natural isotope contributions (22).
[c] Relative abundancies of m/z 646, 650, and 652 expected from 48 and 30% ^{18}O incorporation.

zyme, this rate constant must be higher than the k_{cat}, which is 2.3 s^{-1}.

His^{289} of haloalkane dehalogenase is part of a catalytic triad, which is also present in other α/β hydrolase fold enzymes. The catalytic amino acids are present along the primary sequence in the order of nucleophile, aspartate (or glutamate), histidine. Histidine is also completely conserved in the catalytic triad of serine proteases of the chymotrypsin class and the subtilisin class, where the three residues are located along the sequence in the order of histidine, aspartate, nucleophile and aspartate, histidine, nucleophile, respectively. In the serine proteases, the histidine is needed both for formation and hydrolysis of the acyl-enzyme, since it activates the nucleophile by base catalysis and is also able to donate a proton to the serine leaving group when the ester is hydrolyzed. The role of His^{289} in DhlA is probably to increase the nucleophilicity of a water molecule that is close to the carbonyl carbon of Asp^{124} in the covalent intermediate in the x-ray structure (3). The proton abstracted by the imidazole N-ϵ may be transferred to the alcohol that is released during hydrolysis of the intermediate.

Although the position of the histidine residue in the β-sheet thus is conserved in other α/β hydrolase fold enzymes (4), its role in catalysis is very different between members of this group. In dienelactone hydrolase from *Pseudomonas* B13, the equivalent His^{202} is only involved in maintaining the nucleophilicity of Cys^{123} and not hydrolysis of the covalently bound intermediate (15). In lipase from *Geotrichum candidum* and acetylcholinesterase from *Torpedo californica*, the histidine is suggested to act as it does in serine proteases, *i.e.* it is involved both in formation and in hydrolysis of the covalent acyl-enzyme intermediate (16–18). For wheat carboxypeptidase A, the catalytic histidine was also suggested to act as an acceptor of a proton from the nucleophilic serine (19). Thus, the role of the general base histidine varies in the different α/β hydrolase fold enzymes. It is noteworthy that hydrolysis of the ester intermediate by DhlA occurs by nucleophilic attack of this water molecule on the carbonyl carbon of Asp^{124} (7) and not on the carbon from which the first leaving group is displaced, as in the α/β hydrolase fold enzymes that have a serine as the nucleophile. In the latter enzymes, the carbonyl function essential for hydrolysis of the substrate and the ester intermediate is provided by the substrate rather than by the enzyme.

The role of His^{289} in hydrolysis of the alkyl-enzyme intermediate is based on the enhancement of the nucleophilicity of a water molecule. In the x-ray structure, the presence of a water nearby the carboxylate carbon of Asp^{124} was indeed observed (5). His^{289} not only facilitates the cleavage of the alkyl-enzyme but also causes an unusually rapid exchange of the carboxylate oxygens of Asp^{124} with oxygen from water. This exchange explains why we never observed transfer of ^{18}O via enzyme to the second product when DhlA was incubated with substrate in $H_2{}^{18}O$, dialyzed, and subsequently incubated in unlabeled water with a second substrate. Such a transfer was recently observed with microsomal epoxide hydrolase (14). This enzyme has sequence similarity to DhlA, suggesting that the enzymes are mechanistically similar (7, 14, 20). The nucleophilicity of the water molecule toward Asp in epoxide hydrolase thus could be lower than in haloalkane dehalogenase. Epoxide hydrolases also have a conserved histidine residue that aligns with His^{289} of haloalkane dehalogenase (14) and site-directed mutagenesis of the corresponding His^{431} of rat microsomal epoxide hydrolase has indeed shown that this residue is essential for activity (21).

Formation and trapping of the covalent intermediate formed from DhlA and bromoalkanes appeared to cause quenching of tryptophan fluorescence, which did not disappear when the enzyme was dialyzed against buffer to remove excess substrate and free bromide, indicating self-quenching of tryptophan fluorescence of the alkyl-enzyme. The addition of bromide to dialyzed alkyl-enzyme caused a slight increase in fluorescence, which can be explained by bromide binding to the alkylated protein and a less efficient quenching of tryptophan in the bromide-bound enzyme than in the bromide-free alkylated H289Q DhlA.

The dissociation constant of bromide with the alkylated mutant enzyme at pH 8 was determined to be more than 1000-fold lower than the dissociation constant of bromide with the nonalkylated wild-type enzyme. Furthermore, the nonalkylated H289Q enzyme had a very low affinity for bromide, even at pH 6. These observations indicate that protonation of His^{289} is required for proper halide binding to the free wild-type enzyme in order to compensate for the repulsive negative charge of Asp^{124} if the carboxylate of this residue is not esterified. The strong binding of bromide to the alkylated enzyme also indicates that the halide ion, which is the first reaction product, will not leave the active site until the alkyl-enzyme is hydrolyzed. Probably, halide release occurs after the alcohol has left the active site cavity since the alcohol was never observed in x-ray crystallography experiments of enzyme soaked in substrate, whereas halide was always present (6).

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