Refined X-ray Structures of Haloalkane Dehalogenase at pH 6.2 and pH 8.2 and Implications for the Reaction Mechanism

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The crystal structure of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 has been refined at 1.9 Å resolution at two different pH values, the pH of crystallization (pH 6.2) and the pH of optimal activity (pH 8.2), to final *R*-factors of 16.8% and 16.4%, respectively. Both models show good stereochemical quality. Two non-glycine residues have main-chain torsion angles that are located outside the “allowed” regions in a Ramachandran plot. One of them is the nucleophilic residue Asp124, which, together with the two other active site residues His289 and Asp260, is situated in an internal, predominantly hydrophobic cavity. The other residue, Asn148, helps stabilize the conformations of two of these active-site residues, Asp124 and Asp260. Comparison of the models at pH 6.2 and pH 8.2 revealed one major structural difference. At pH 6.2, a salt-bridge is present between the N^{ε2} atom of His289 and the O^{δ1} atom of Asp124, while at pH 8.2, this salt-bridge is absent, indicating that the N^{ε2} atom of the histidine residue is mostly deprotonated at the pH of optimum activity. This is in agreement with the putative reaction mechanism in which the O^{δ1} atom of Asp124 performs a nucleophilic attack on the substrate, resulting in an intermediate ester. This ester is subsequently cleaved by a hydrolytic water molecule. The high-resolution data sets clearly show the exact position of this water molecule. It is in an ideal position for donating a proton to the N^{ε2} atom of His289 and subsequently cleaving the covalently bound intermediate ester, releasing the alcohol product.

Detailed investigation of both refined models showed a number of unusual structural features. Four out of 11 helices contain an internal proline residue other than in the first turn. Two other α -helices have adopted in their central part a 3_10 conformation. A novel four-residue turn between a helix and a strand, the $\alpha\beta_4$ turn, is located at the site of the bend in the central eight-stranded β -sheet of the dehalogenase structure.

Keywords: biodegradation; dehalogenase; X-ray structure; catalytic mechanism; proline-helices

1. Introduction

Hydrolytic dehalogenases play a key role in the detoxification of aliphatic halogenated compounds. They catalyze the release of halide ions from their substrate with water as the sole co-substrate, resulting in the replacement of the halogen substituent by a hydroxyl group (Goldman *et al.*, 1968; Motosugi & Soda, 1983; Janssen *et al.*, 1985).

Xanthobacter autotrophicus GJ10 is a nitrogen-fixing hydrogen bacterium that produces at least two different chloride-releasing enzymes: one, haloalkane dehalogenase is specific for 1-haloalkanes, while the other, haloalkanoate dehalogenase, requires halogenated carboxylic acids as a substrate (Janssen *et al.*, 1985; van der Ploeg *et al.*, 1991). In conjunction with two dehydrogenases, these two enzymes confer to the bacterium the ability to grow on 1,2-dichloroethane as a sole carbon and energy source (Janssen *et al.*, 1989; Fig. 1).

The haloalkane dehalogenase from *X. autotrophicus* GJ10 has a molecular mass of 35 kDa and comprises 310 amino acid residues. The nucleotide

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Figure 1. Catabolic route for 1,2-dichloroethane in *Xanthobacter autotrophicus* GJ10 (adapted from Janssen *et al.*, 1989).

sequence has been reported (Janssen *et al.*, 1989). The enzyme has a preference for 1,2-dichloroethane as substrate, which it converts into 2-chloroethanol and chloride without any requirement for oxygen or cofactors (Janssen *et al.*, 1989). The optimum pH for this reaction is around 8.2. In addition to this compound, the haloalkane dehalogenase is able to hydrolyze terminally chlorinated alkanes with a chain length up to at least four carbon atoms, but also brominated alkanes with chain lengths up to ten carbon atoms can be degraded (Janssen *et al.*, 1989). Some of these aliphatic substrates are produced in large amounts for use as solvents, cleaning agents, pesticides and as intermediates for chemical syntheses. Among them are a number of environmentally harmful compounds such as the chlorinated hydrocarbons methylchloride, ethylchloride and 1,2-dichloroethane (Janssen *et al.*, 1985). They are classified as priority pollutants by the U.S. Environmental Protection Agency (Patterson & Kodukala, 1981). Unfortunately, improper disposal of wastes and accidental or deliberate release of these chlorinated hydrocarbons have caused several environmental pollution incidents, so that contamination of soils, underground waters and surface waters has often occurred (McConnell *et al.*, 1975; Keith & Telliard, 1979; Christian & Moorehead, 1985; Richards & Sieh, 1986).

Enzymes such as haloalkane dehalogenase are an intriguing perspective to be applied as environmental protectants. They offer a simple route for detoxification, because the organic character of the

halogen, which causes the toxicity, is lost (Keuning *et al.*, 1985). However, the practical application of the dehalogenase as an "antipollutant" is not yet very efficient: the affinity of the enzyme towards its substrates is not high ($K_m = 1.1$ mM), and also the turnover number is low ($k_{cat} = 6/s$; Keuning *et al.*, 1985). Therefore protein engineering, in combination with protein crystallography, is being used in an attempt to improve the enzyme with respect to catalytic activity and altered substrate specificity.

Haloalkane dehalogenase has been crystallized and its three-dimensional structure has been elucidated at 2.4 Å resolution (1 Å = 0.1 nm) by Franken *et al.* (1991). Here, we report the refinement to a resolution of 1.9 Å of the model at two different pH values: one at the pH of the crystallization conditions (pH 6.2) and the other at the pH of optimum activity (pH 8.2). These refined structures may serve as a firm basis for further experiments to define the catalytic mechanism and substrate specificity, and may be very useful in view of the practical goal to use the dehalogenase as a future environmental antipollutant.

2. Materials and Methods

(a) Crystallization

Purified haloalkane dehalogenase from *X. autotrophicus* GJ10 (Keuning *et al.*, 1985) was kindly provided by the group of D. Janssen (Laboratory of Biochemistry, University of Groningen). After the gene coding for the enzyme had been cloned into *Escherichia coli* strain HB101 (Janssen *et al.*, 1989), we included an extra purification step on a Mono Q ion-exchange column in order to obtain crystals suitable for X-ray diffraction. The protein was eluted with a linear NaCl gradient (0.0 M to 1 M) in a 50 mM bis-Tris buffer (bis(2-hydroxyethyl)-imino-tris-(hydroxymethyl)-methane), pH 6.4. The fractions around 260 mM NaCl were concentrated and used for crystallization experiments.

The enzyme was crystallized from ammonium sulfate by vapor diffusion in hanging drops (Rozeboom *et al.*, 1988). The best crystals were grown from a 60% saturated (at 0°C) $(\text{NH}_4)_2\text{SO}_4$ solution in a 100 mM bis-Tris buffer solution of pH 6.2. Within 3 weeks colorless, lozenge-shaped crystals with an average size of 0.40 mm × 0.40 mm × 0.15 mm were obtained, which diffract to a maximum resolution of 1.9 Å. They belong to space group $P2_12_12$ with unit cell dimensions $a = 94.80$ Å, $b = 72.80$ Å, $c = 41.40$ Å. They contain 1 molecule/asymmetric unit.

Crystals at pH 8.2, the pH of optimum activity for the enzyme, were obtained by soaking native crystals in mother liquor at pH 8.2. This procedure did not have any dramatic effect on the crystals.

(b) Data collection and processing

In order to improve the initial multiple isomorphous replacement (MIR†) model of the haloalkane dehalogenase structure at 2.4 Å, a new data set to a maximum resolution of 1.9 Å was collected at room temperature from a single crystal on a FAST area detector (Enraf

† Abbreviations used: MIR, multiple isomorphous replacement; r.m.s., root-mean-square.

Table 1
Data collection and processing

	pH 6.2	pH 8.2
Max. resolution (Å)	1.89	1.90
Number of crystals	1	1
Total exposure time (h)	48	48
Total number of observations	45,547	46,954
Number of unique reflections	18,923	19,853
Number of discarded observations	1685	1005
R_{merge} (%)†	4.40	5.13
Completeness to 1.90 Å (%)	85.8	85.2
Completeness in last shell (1.92 to 1.89 Å: (%))	54.9	
(1.92 to 1.90 Å: (%))	69.0	73.8

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_{\text{refl}} |I(hkl, j) - \bar{I}(hkl)|}{\sum_{hkl} \sum_{\text{refl}} |I(hkl, j)|} \times 100\%.$$

Nonius, Delft, The Netherlands). The detector was equipped with a CAD4 κ -goniostat with graphite monochromatized $\text{CuK}\alpha$ radiation from an Elliot GX21 rotating anode generator as X-ray source. Data collection and reduction was carried out using the MADNES system (Messerschmidt & Pflugrath, 1987). Profile fitting of the data was done according to Kabsch (1988). The intensities were collected in 0.15° frames using 3 different crystal settings of 90° for the crystal. The resulting pH 6.2 data set contained a total of 45,547 observed reflections, of which 19,517 reflections were unique. The intensities that deviated too much (multiply measured reflections not within 3 σ from the mean intensity) or were negative, were omitted (1685 reflections). Data were scaled (Hamilton *et al.*, 1965) and merged together using programs of the Groningen BIOMOL crystallographic software package. The resulting 18,923 unique observations were then merged together with the previously collected 2.4 Å pH 6.2 data set (Franken *et al.*, 1991), resulting in a final native data set with 20,296 unique reflections, which is 85.8% complete in the resolution range ∞ to 1.89 Å. The final results are listed in Table 1.

A similar procedure was used for the crystal soaked in mother liquor at pH 8.2. Statistics of the data collection of the structure at this pH are also included in Table 1.

(c) Refinement

The least-squares restrained refinement program TNT (Tronrud *et al.*, 1987) was used to refine the model with the 2.4 Å structure of the dehalogenase as a starting model (Franken *et al.*, 1991). The weighting procedure was varied: at first the weighting on structure factors was favored, later on increased weight on ideal geometry was used. The stereochemical parameters of the protein were defined by the amino acid dictionary provided by the authors of the TNT package. Bond distances, bond angles, planarity of groups, van der Waals distances and non-bonded contacts were restrained. Since some side-chain torsion angles have a broad distribution because of low energy barriers between energetically preferred rotamers (Janin *et al.*, 1978), the torsion angles were not included in the refinement by setting their weighting factor to zero. The torsion angles in peptide bonds and the planar rings in side-chains, which are more restricted in the structure, were covered by other stereochemical terms. The isotropic temperature factors were restrained on the differences of temperature factors between

connected atoms. Occupancies were set to unity and were also not refined.

During all stages of the refinement, the model was checked and manually rebuilt on an Evans and Sutherland PS390 picture system using the programs FRODO and O (Jones, 1978; Jones *et al.*, 1991). During the refinement, clear density was seen for the complete amino acid chain in a $(2mF_o - DF_c) \exp(i\alpha_c)$ electron density map (Read, 1986), with the exception of residue Asn14 and the C-terminal residue, which were somewhat disordered, and could therefore not readily be assigned. Solvent molecules were introduced in the model once the R -factor dropped below 23%. Peaks were included at sites where the density in a σ_A weighted (Read, 1986) $(F_o - F_c) \exp(i\alpha_c)$ Fourier map was at least 3.3 σ , and only if the peaks were within hydrogen-bonding distance from an H-bond donor or acceptor in the model. For the refinement of the pH 8.2 model, the procedure used was analogous to that used for the pH 6.2 structure.

The atomic co-ordinates of the final models at pH 6.2 and pH 8.2 have been deposited with the Brookhaven Protein Data Bank (accession numbers 2HAD (pH 6.2) and 1EDE (pH 8.2); Bernstein *et al.*, 1977).

(d) Assignment of secondary structure elements

Secondary structural elements were assigned on the basis of the main-chain hydrogen-bonding pattern and dihedral angles. Main-chain hydrogen bonds are considered to exist if the donor-acceptor distance is less than 3.50 Å and the associated angles are greater than 120° (Baker & Hubbard, 1984), with the assumption that a hydrogen bond between 3.20 Å and 3.50 Å is defined as a "possible" hydrogen bond. Amino acids with such a possible hydrogen bond are included in the secondary structural elements. Furthermore, end residues are considered to be part of a helix or a strand, only if their dihedral angles are located in or near the proper region of the Ramachandran plot (Ramakrishnan & Ramachandran, 1965).

In order to define a reverse turn, we used the same criteria as described by Venkatachalan (1968) and Crawford *et al.* (1973): four consecutive amino acids for which the distance between $\text{C}\alpha^1$ and $\text{C}\alpha^4$ is less than 5.70 Å; the (ϕ_2, ψ_2) and (ϕ_3, ψ_3) torsion angles are within 15° of the values given by Venkatachalan, except that 1 of the 4 may be 30° off (i.e. for type I turns (−60°, −30°, −90°, 0°), for type II (−60°, 120°, 80°, 0°) and for

Figure 2. Real-space R -factor for the pH 6.2 (continuous line) and the pH 8.2 (broken line) dehalogenase model, as calculated by the program O (Jones *et al.*, 1991).

type III ($-60^\circ, -30^\circ; -60^\circ, -30^\circ$) as well as the sign-reversed torsion angles for type I', II' and III'); and there exists a hydrogen bond between the main-chain oxygen atom of the first residue and the backbone nitrogen atom of the last residue of the turn. A near reverse turn fulfils 2 of the 3 criteria and an open turn only 1 (Crawford *et al.*, 1973). A *cis*-proline turn (or type VI_b) is defined as a reverse turn in which a proline residue occupies the third position in the turn and adopts β -strand (ϕ, ψ) values.

3. Results & Discussion

(a) Quality of the model

The final 1.9 Å structures of haloalkane dehalogenase at pH 6.2 and 8.2 presented here contain all 310 amino acid residues. The main-chain and most of the side-chains could unambiguously be assigned, except for the C-terminal Glu310. The electron density for this residue was very weak in both models, indicating some disorder. Weak density was also found for residue Asn14 in the pH 6.2 model. This is reflected in the real-space R -factor (Jones *et al.*, 1991) for this amino acid, which in the pH 6.2 data set exceeds 70% (Fig. 2). In the pH 8.2 model with much clearer electron density for this region, the real-space R -factor for residue Asn14 is 27%. This better density is probably due to stronger hydrogen bonds for the side-chain atoms of this residue to its main-chain carbonyl atom ($N^{\epsilon 2}$ Asn14-O Asn14: 2.71 Å) and to the $N^{\epsilon 2}$ atom of

Figure 3. R -factor as a function of resolution for the model at pH 6.2 (thick line) and pH 8.2 (thin line). The theoretical curves (Luzzati, 1952) for mean positional errors of 0.10, 0.15 and 0.20 Å are shown.

residue Gln167 of a symmetry-related molecule in the crystal at pH 8.2 (see Table 7: $O^{\delta 1}$ Asn14- $N^{\epsilon 2}$ Gln167: 2.96 Å at pH 8.2). The overall real-space R -factor for all atoms in the two models is 22.7% for the pH 6.2 dehalogenase structure and 25.2% for the pH 8.2 model, respectively. For the pH 6.2 structure, a total of 258 solvent molecules are included; the pH 8.2 structure contains 205 water molecules. The refinement of the two models of the enzyme at pH 6.2 and at pH 8.2 resulted in final crystallographic R -factors of 16.8% and 16.4%, respectively. The r.m.s. (root-mean-square) shift of the main-chain atoms of the refined pH 6.2 model is 1.15 Å, compared to the initial MIR model at 2.4 Å (Franken *et al.*, 1991). The co-ordinate error estimated from a σ_A plot (Read, 1986) is 0.15 Å and 0.13 Å for the pH 6.2 and pH 8.2 model, respectively. The overall co-ordinate error in the atomic positions can also be derived by plotting the R -factor as a function of resolution (Luzzati, 1952), although this tends to overestimate the error. Compared to the theoretical curves for constant error, the estimated average error for the pH 6.2 structure is 0.20 Å. A similar result is obtained with the refined dehalogenase structure at pH 8.2, as can be seen in Figure 3. In Table 2 some details of the quality of the two final models are given. Figure 4(a) and (b) show a representative part of the final electron density map for the two structures at the two different pH values, which gives a good

Table 2
Quality of the final model

	pH 6.2	pH 8.2	Target σ	Number of parameters
r.m.s. deviations from ideality for:				
bond lengths (Å)	0.009	0.008	0.020	2552
bond angles ($^\circ$)	2.740	2.608	3.000	3448
planarity	0.010	0.009	0.020	369
trigonal planes	0.008	0.007	0.020	68
bad contacts (Å)	0.044	0.056	0.100	
Number of bad contacts	22	28		
Average B -factor ($\text{\AA}^2$)	16.37	14.71		
r.m.s. B -factor of connected atoms	1.647	1.664		2552
Final R -factor (%)†	16.84	16.38		

† R -factor = $\Sigma||F_o| - |F_c|| / \Sigma|F_o|$.

Figure 4. Representative part of the final $(2mF_0 - DF_c)$ exp $(i\alpha_c)$ electron density map (Read, 1986) at 1.33σ contour level. (a) The model at pH 6.2: the surroundings of Asn148, a residue with energetically unfavorable (ϕ, ψ) angles, are shown. (b) The model at pH 8.2: the surroundings of the catalytic triad.

Figure 5. Ramachandran plot of the main-chain (ϕ, ψ) conformational angles of haloalkane dehalogenase at pH 6.2. The glycine residues are shown as open circles and the non-glycine residues as filled circles. Continuous lines define the areas of preferred (ϕ, ψ) combinations and broken lines indicate the sterically allowed areas in conformational space when the constraints on the van der Waals contacts are relaxed and a value of $\tau(N, C^{\alpha}, C)$ -bond angles to 115° is allowed (Ramachandran *et al.*, 1963). Non-glycine residues with energetically unfavorable (ϕ, ψ) values are labeled. The (ϕ, ψ) plot of the pH 8.2 model is virtually the same.

indication of the quality of the map for the whole structure.

The following description of the structure of haloalkane dehalogenase is based on the refined model at pH 6.2. Comparison with the enzyme model at the pH of optimum activity, pH 8.2, revealed almost no differences. Apart from some flexible side-chains on the surface of the protein, the two structures superimpose very well (r.m.s. difference = $0.28 \text{ \AA}$ for all atoms), except for a small, but significant, movement of the Asp124 side-chain located in the interior of the structure, a residue vital for the reaction mechanism (see below).

A Ramachandran (ϕ, ψ) plot (Ramakrishnan & Ramachandran, 1965) of the pH 6.2 structure is visualized in Figure 5. A similar plot of the main-chain dihedral angles was obtained for the pH 8.2 model (data not shown). Only two non-glycine residues are clearly outside the "allowed" regions. These are Asp124 and Asn148, each of them having a well-defined density (Fig. 4(a) and (b)). When residues in an enzyme have deviating main-chain torsion angles, they usually have a special structural or functional purpose (Morris *et al.*, 1992). Asp124 is the nucleophilic residue in the proposed reaction mechanism, as will be discussed below. This amino acid is located in an extremely sharp hairpin-like turn between the central strand of the β -sheet in the structure and its subsequent helix, a feature typical for all α/β hydrolase fold enzymes (Ollis *et al.*, 1992). This sharp turn is stabilized by several hydrogen

bonds to and from backbone atoms and this might compensate for the unfavorable (ϕ, ψ) angles for this residue of $+49^\circ$ and -135° , respectively. The same is observed for the (ϕ, ψ) values of the nucleophilic active-site residues of the other members of the α/β hydrolase fold family. Asp124 is situated at the N-terminal end of the helix and its backbone oxygen makes hydrogen bonds with the amide atoms of residues Gly127 and Phe128 (O124-N127, 2.68 Å; O124-N128, 2.95 Å). Similarly, the Gln123 backbone oxygen atom is hydrogen-bonded to the backbone nitrogen atom of Gly126 (O123-N126, 2.82 Å). In addition, the main-chain nitrogen atom of Asp124 is fixed in its position by a hydrogen bond to the backbone oxygen atom of Asn148 (N124-O148, 2.89 Å), the other residue with deviating main-chain torsion angles ($\phi = -9^\circ$, $\psi = +94^\circ$). Furthermore, the side-chain N^{δ2} atom of Asn148 makes a hydrogen bond with the side-chain O^{δ2} atom of Asp260, another residue involved in catalysis (N^{δ2}-O^{δ2}, 2.79 Å). The side-chain of this charged active-site residue is pointing into the interior of the protein and this hydrogen bond is one of the four bonds that help stabilize its conformation.

The (ϕ, ψ) combinations of three other non-glycine residues, Gln17, Asp114 and Asn117, fall in the left-handed α -helical region around ($60^\circ, 40^\circ$). They are associated with chain reversals in the polypeptide chain and will be discussed later. The structure contains two *cis*-proline residues (Pro57 and Pro168).

The χ^1 torsion angles in the model were not restrained during the refinement. Nevertheless, the observed χ^1 angles are in good agreement with the preferred conformers $g^- (+60^\circ)$, $t (+180^\circ)$ or $g^+ (-60^\circ)$ (Finkelstein & Ptitsyn, 1977; Janin *et al.*, 1978), with the g^- position less favorable than the two other positions, g^+ and t . The average g^- , t and g^+ angles are, respectively, $+64.9(\pm 11.3)^\circ$, $+181.4(\pm 15.1)^\circ$ and $-63.6(\pm 19.7)^\circ$. Morris *et al.* (1992) have found that there is a good correlation between the resolution limit, the final *R*-factor, the width of the distribution around these idealized χ^1 torsion angles and the quality of the refined model. They calculated χ^1 to be, respectively, $+64.1(\pm 15.7)^\circ$ (*gauche*⁻), $+183(\pm 16.8)^\circ$ (*trans*) and $-66.7(\pm 15.0)^\circ$ (*gauche*⁺) for well-refined structures at high resolution. As can be seen, we have found very similar values for the average unrestrained χ^1 angles in the refined model of haloalkane dehalogenase at pH 6.2. A similar result for the distribution of the χ^1 torsion angles has been obtained for the refined structure of the enzyme at pH 8.2.

(b) Overall structure

Haloalkane dehalogenase is a globular enzyme with overall dimensions of about $51 \text{ Å} \times 54 \text{ Å} \times 46 \text{ Å}$ (Franken *et al.*, 1991; Fig. 6(a)). The protein has all the features typical for α/β hydrolase fold enzymes (Ollis *et al.*, 1992). Despite the lack of sequence

similarity, the dehalogenase has a similar eight-stranded β -sheet, of which the second strand is antiparallel, the catalytic triad residues have the same sequence order and the active site is structurally similar, with the 'nucleophile elbow', a very sharp strand-nucleophile-helix motif placing the nucleophilic residue on top of the turn, having (ϕ, ψ) angles in the unfavorable region of the Ramachandran-plot being the most prominent feature (Ollis *et al.*, 1992).

Figure 6(b) gives a C α backbone drawing of the refined structure. Two domains can be distinguished. Residues 1 to 155 and 230 to 310 form the first domain which consists of an eight-stranded β -pleated sheet with seven parallel strands, forming a hydrophobic core, surrounded by six α -helices (2 on one side of the sheet, 4 on the other side) and a small two-stranded antiparallel β -sheet. The second domain (residues 156 to 229) lies like a cap on top of the first domain. It is composed of five helices connected by loops (Franken *et al.*, 1991). This domain forms a large intersection between the sixth β -strand and the fourth helix of domain I. Between these two domains, an internal cavity of approximately 37 Å^3 size is located (Fig. 6(b)) which is predominantly lined with hydrophobic residues, except for Asp124, His289 and Asp260. These residues form the catalytic triad. This cavity can be reached from the solvent through a tunnel. The side-chains of two lysine residues (Lys259 and Lys261) serve as a lid at the beginning of the entrance.

Using the criteria described in Materials and Methods, we have defined 11 helices (comprising 43.2% of the residues), 10 β -strands (15.8%) and 14 turns (17.4%) in the structure by manually inspecting hydrogen bonds and torsion angles as calculated by the programs HBONDS and PHIPSI from the Groningen BIOMOL program system for protein crystallography.

(i) β -Strands

The hydrogen-bonding pattern of the β -strands can be seen in Figure 7. The total of ten β -strands are all located in domain I, forming two separate β -sheets. The small one consists of two short antiparallel β -strands of three residues each (Fig. 7(a)).

The major β -sheet (Fig. 7(b)), which is the core of the first domain, is an eight-stranded β -pleated sheet with seven parallel and one antiparallel strands (Franken *et al.*, 1991). The topological arrangement of the strands in this sheet, one of the characteristics of the α/β hydrolase fold enzymes (Ollis *et al.*, 1992), is shown in Figure 8. Except for the N-terminal part of which the first three strands are not interrupted by helices, the sheet follows the typical β - α - β connectivity (Sternberg & Thornton, 1977) with all cross-over connections being right-handed (Richardson, 1976). As can be seen in Figure 8, strand 4 interrupts the topological connectivity between strand 2 and 3 and is, *via* two turns, followed by a small β -strand (β_b) and a helix before it reaches the next β -strand. Strand 6 and the helix

Figure 6. (a) Stereo drawing, created with the program RIBBON (Priestle, 1988) of the overall structure of haloalkane dehalogenase, highlighting α -helices and β -strands and coils, represented by helical ribbons, arrows and ropes, respectively. (b) Stereo plot of the C^α backbone of dehalogenase. The cap domain in orange and the 2 β -sheets of domain I in blue are represented by thicker lines. The catalytic triad residues are in red. Dots represent the internal active-site cavity located between the 2 domains and the cavity with the hydrolytic water molecule, which is, together with the 3 other water molecules occupying these cavities, colored yellow.

The eight strands are left-hand twisted in such a way that the sheet covers almost one half of a cylinder (Ollis *et al.*, 1992). Perpendicular to this, the sheet is also bent by about 35° at β -strand 5 (Fig. 9).

(ii) *Helices*

residues) shows also a typical α -helical geometry, but is connected to a 3_{10} helix at the N terminus through an elongated turn. This turn is stabilized by a water molecule, forming hydrogen bonds with the main-chain atoms of this internal turn (Fig. 10). This kind of stabilization of an internal turn in a helix by a water molecule has been noticed more often in proteins (Sundaralingam & Sekharudu, 1989). Also the N-terminal part of helix H_1 contains such a water molecule to maintain the α -helical hydrogen-bonding pattern. Surprisingly, four helices in the enzyme have a distorted hydrogen-bonding pattern caused by the occurrence of a proline residue in or just after the center of the helix. This remarkable feature is observed also in the refined model at pH 8.2. Four out of 11 helices (approx. 36%) with an internal proline residue other than in the first turn, is an unusually high occurrence in globular proteins. Barlow & Thornton (1988) found only ten helices with an internal proline residue out of 291 helices examined in 57 proteins (approx. 3%) and these proline residues were mostly conserved, indicating a special structural function. No conservation of helix proline residues is found within the known α/β hydrolase fold enzymes; thus, if there is any special function, it must be within the dehalogenase structure itself. The proline residue (Pro68) in helix H_1 (14 residues) results in a kink in this helix. Except for the loss of a hydrogen bond, since the proline backbone amide group cannot donate a proton, and the usual loss of the hydrogen bond between the main-chain nitrogen atom of the $(i+1)$ th residue (Val69 in the dehalogenase structure) and the oxygen of the $(i-3)$ th amino acid residue (Lys65), thereby disturbing the α -helical hydrogen-bonding pattern (Barlow & Thornton, 1988), this proline residue causes hardly any disruption of the α -helical (ϕ, ψ) angles of the residues involved. Helix H_1 is located on the outside of the enzyme. Pro68 is situated on the solvent-exposed side and causes the helix to bend around the hydrophobic core. Helix H_3 (12 residues) is the least solvent-accessible helix in the protein (Table 6). It connects β -strands 5 and 6 of the main β -sheet and is located at the base of the bend in this sheet. The proline (Pro134) is situated in the last turn of the helix and causes a strongly kinked conformation in which a water molecule is inserted to repair the hydrogen bond between the NH group of the $(i+1)$ th residue (Met135) and the oxygen atom of the $(i-3)$ th residue (Leu131). Two residues, Phe222 and Pro223 of helix H_8 (11 residues) line the internal active-site cavity. The incorporation of a proline residue (Pro223) in this helix does not result in a kink in the helix conformation, since the backbone amide group of the proline residue, despite the lack of a hydrogen bond, can come closer to the backbone atoms of residue $(i-4)$ than normally observed in proline-kinked helices. This is accomplished by the backbone oxygen atom of the $(i-4)$ th residue, Val219, being pulled outside the helix conformation by a tight hydrogen bond to the $O^{\delta 2}$ atom of Glu56, a residue outside the helix.

Figure 8. Topological arrangement of the secondary structure elements in the refined structure of haloalkane dehalogenase. The numbers represent start and end residues of the helices and the β -strands. The location of the catalytic triad residues are given by an asterisk (*). Compared to the topology of the enzyme at 2.4 Å resolution shown by Franken *et al.* (1991), a small antiparallel β -sheet (residues 3 to 5 and 90 to 92) has been added.

(O219-O⁴² Glu56, 2.42 Å). The fourth helix, helix H₁₀ (11 residues), contains two proline residues: one, Pro265, at the second position of the first turn, which according to MacArthur & Thornton (1991) is the most favored location of a proline residue in an

α -helix, not disturbing the α -helical bonding pattern; and the other, Pro270, in the middle. Helix H₁₀ is a surface helix and, as in the case of helix H₁, Pro270 is exposed to the solvent and it causes the helix to kink around the hydrophobic core.

Figure 9. Stereo drawing, created with the program MOLSCRIPT (Kraulis, 1991), of the main β -sheet together with the nucleophile elbow, characteristic for the α/β hydrolase fold enzymes. The sheet is bent by about 35° in the middle.

Figure 10. Schematic view of the hydrogen-bonding pattern of the helices in the structure of dehalogenase. Continuous and broken lines represent hydrogen bonds shorter than 3.20 Å and between 3.20 and 3.50 Å, respectively. Broken lines with an open circle indicate that the hydrogen-bonding pattern is maintained *via* a water molecule.

Another remarkable feature, noticed in both refined structures at pH 6.2 and pH 8.2, is found in helices H₇ (9 residues) and H₉ (17 residues). Helix H₉ has an α -helical conformation, but has a tendency to be replaced by a 3_{10} configuration in the center. This helix is a straight, solvent-exposed helix with a large "hydrophobic moment" (Eisenberg *et al.*, 1982). By incorporating a tightening of a turn in the middle by forming hydrogen bonds between the main-chain oxygen atoms of residues Ser237 and Thr238 and the backbone amides of residues Ala240 and Ile241, respectively, hydrophobic aromatic residues of the second half of the helix, like Phe243 and Trp244, remain positioned at the hydrophobic side of the helix. Similarly, helix H₇ has also a 3_{10} conformation in the middle and an α -helical configuration at both ends. This helix is also not curved, and is located at the outside of the enzyme.

(iii) Turns

In total, 14 turns were assigned in the structure of the dehalogenase, and divided into ten different types. They are summarized in Table 3. Of the 13 reverse turns identified on the basis of the 4-1 hydrogen-bonding pattern, only one is a type I turn, two are type II and another is a clear 3_{10} (or type III) reverse turn (Crawford *et al.*, 1973; Venkatachalan, 1968). There are six near reverse turns of respectively, type I (2 turns), type II (1), type III (2) and type II' (1) and one open turn of type III. All type II turns, whether they are reverse or near reverse, contain a glycine residue in the third position (Venkatachalan, 1968), since a residue with a side-chain at this position would experience sterical hindrance from the carbonyl oxygen atom of the central peptide in this turn. The type II' turn has, for the same reason, a glycine residue in the second position of the turn, because a side-chain would bump into the backbone amide of the central peptide. The remaining two reverse turns are *cis*-proline turns (or type VI_b) (Lewis *et al.*, 1973; Chou & Fasman, 1977), with the proline residue in the third position of the turn having (ϕ, ψ) combinations in the β -strand region of the Ramachandran plot.

Since haloalkane dehalogenase is a member of the α/β hydrolase fold enzymes, one of the characteristics of this fold is the "nucleophile elbow" located at Asp124 (Ollis *et al.*, 1992): a sharp γ -like turn that connects β -strand 5 with helix H₃. The sharp nature of this turn results in Asp124 having unfavorable (ϕ, ψ) angles and forces the side-chain of this residue to point into the active-site cavity.

Besides these defined turns, there are several other chain reversals in the dehalogenase structure. Three residues associated with these turns have main-chain dihedral angles in or near the left-handed helical region in the Ramachandran plot. They are Gln17, Asp114 and Asn117. Gln17 is located in a long connection between the first 3_{10} turn (residues 9 to 12) and β -strand 1.

Figure 11. Schematic view of the $\alpha\beta_4$ turn (residues Asp114 to Asn117) in haloalkane dehalogenase, compared to the $\alpha\beta_3$ loop in the parallel α/β -barrel folding motif (Scheerlinck *et al.*, 1992).

Residues Asp114 to Asn117 form the turn in the helix-turn-strand motif between helix H_3 and β -strand 5. The two central amino acid residues of this turn have (ϕ, ψ) values in the β -region and the two outer residues have main-chain torsion angles located in the left-handed α -helical region. This turn does not belong to any known classical turn, but some features show some resemblance to the $\alpha\beta_3$ loop found in the parallel α/β -barrel folding motif (Edwards *et al.*, 1987), in which the first (residue i) of the three amino acid residues of the turn adopts a positive ϕ -angle, which results in a tight reversal of the main-chain, hydrophobic residues occur at positions $(i-4)$, $(i-1)$, $(i+1)$ and $(i+4)$ and hydrogen bonds are formed between the backbone amide groups of residues i and $(i+1)$, and the backbone oxygen atoms of residues $(i-3)$ and $(i-4)$, respectively (Scheerlinck *et al.*, 1992: Fig. 11). In the dehalogenase structure, residues Ile110 (residue $i-4$), Leu113 ($i-1$), Leu115 ($i+1$) and Ile118 ($i+4$) occur at topologically similar positions as in the $\alpha\beta_3$ loop and the first residue of this motif has a positive ϕ -value. Also in dehalogenase, the main-chain nitrogen atoms of residues Asp114 (residue i) and Leu115 ($i+1$) are hydrogen-bonded to the

main-chain oxygen atoms of Glu111 ($i-3$) and Ile110 ($i-4$), respectively (N114-O111, 2.89 Å; N115-O110, 3.25 Å). However, this (Asp114-Asn117)-turn consists of four amino acid residues and thus has one residue more (Asn117) than in the α/β -barrel, with dihedral angles in the left-handed α -helical region, which causes a second tight reversal of the main-chain. To the best of our knowledge, this kind of special motif has not yet been reported previously, therefore, we like to refer to it as an $\alpha\beta_4$ turn (Fig. 11). Furthermore, a salt-bridge is formed between the first residue of this turn with a residue just before β -strand 6, the first strand after the bend in the sheet (Asp114 O^{δ1}-Arg140 N^{δ1}, 2.96 Å). Similarly, there exists also a hydrogen bond between the last residue of this turn with the backbone oxygen atom of a residue before β -strand 3, the last strand before the bend (Asn117 N^{δ2}-Glu47 O, 2.89 Å). This "crossed" hydrogen-bonding pattern can help stabilize the bend of 35° in the β -sheet.

(iv) Ionic interactions and hydrogen bonds

Salt-bridges can contribute considerably to the stabilization of the native conformation of proteins (Perutz, 1978; Walker *et al.*, 1980). The 24 salt-bridges that are found in the haloalkane dehalogenase structure at pH 6.2 are listed in Table 4. At the pH of optimum activity, pH 8.2, three of them are lost. Two salt-bridges have disappeared because of a slight movement of the side-chain of Arg186 located at the surface of the enzyme. They have been replaced at pH 8.2 by a hydrogen bond between the side-chain of this arginine and a residue of a symmetry-related molecule in the crystal (Asn43, see Table 7). The third and most important difference in salt-bridges between the two structures of different pH values is the loss of the ionic interaction between the two active-site residues, His289 and Asp124, indicating that the N^{ε2} atom of the histidine residue is mostly deprotonated at pH 8.2 (see below).

Most of the 73 charged residues are located at the outside of the protein. The seven charged amino

Table 3
Assignment of turns

Segment	Residues	Type	C ^{α1} ...C ^{α4} (Å)	$\phi^2, \psi^2/\phi^3, \psi^3(^{\circ})$	O-1...N-4 (Å)	Location
9-12	Asp-Gln-Arg-Phe	Near 3_{10} turn	5.84	-61, -15/-67, -23	3.03	β_1 - β_1
28-31	Leu-Pro-Gly-Tyr	Reverse turn II	5.42	-64, +124/+98, -12	3.15	β_1 - β_2
43-46	Asn-Ser-Asp-Ala	Near reverse turn I	5.25	-66, -11/-110, +15	3.05	β_2 - β_3
55-58	Gly-Glu-Pro-Thr	Near <i>cis</i> -proline turn (VI _b)	5.84	-112, +57	3.12	β_3 -H ₁
82-85	Phe-Phe-Gly-Phe	Near reverse turn II	5.69	-58, +136/+100, -6	3.09	β_4 - β_6
85-88	Phe-Gly-Lys-Ser	Near reverse turn II'	5.93	+54, -133/-97, +3	3.14	β_4 - β_6
93-96	Asp-Glu-Glu-Asp	3_{10} turn	5.69	-60, -33/-59, -26	3.13	β_5 -H ₂
123-125	Gln-Asp-Trp	Nucleophile elbow		(+49, -135)		H ₂ - β_5
138-141	Pro-Ser-Arg-Phe	Near 3_{10} turn	5.62	-60, -32/-77, -9	3.14	H ₃ - β_6
154-157	Asp-Pro-Val-Thr	Open turn III	5.79	-61, -31/-72, -45	3.41	β_6 -H ₄
166-169	Thr-Gln-Pro-Ala	Open <i>cis</i> -proline turn (VI _b)	6.91	-79, +167	4.56	H ₄ -H ₅
213-216	Thr-Ser-Tyr-Gln	Reverse turn I	5.11	-64, -20/-91, -4	2.96	H ₇ -H ₈
275-278	Ile-Asn-Gly-Cys	Reverse turn II	5.45	-60, +130/+72, +10	3.06	H ₁₀ - β_8
284-287	Ile-Ala-Asp-Ala	Near reverse turn I	5.65	-62, -35/-91, -4	3.49	β_8 -H ₁₁

Classification of turns was performed according to the criteria described in Materials and Methods.

Table 4

Ionic interactions in haloalkane dehalogenase at pH 6.2 and pH 8.2 (< 3.50 Å)

Residue 1	Residue 2	Distance (Å)		
		pH 6.2	pH 8.2	Domains
Arg6 N ^c	O ^{δ1} Asp89	2.68	2.70	I-I
N ⁷²	O ^{δ2}	3.12	3.17	
Asp27 O ^{δ1}	N ^c Arg112	2.38	2.52	I-I
O ^{δ1}	N ⁷²	3.05	3.09	
O ^{δ2}	N ⁷²	3.22	3.48	
His37 N ^{δ1}	O ^{δ2} Asp81	2.74	2.70	I-I
Asp40 O ^{δ1}	N ^c Arg76	2.92	2.82	I-I
O ^{δ2}	N ⁷²	2.82	2.71	
Asp48 O ^{δ1}	N ^c Lys142	2.88	3.11	I-I
Arg64 N ⁷¹	O ^{c2} Glu293	2.85	2.75	I-I
N ⁷²	O ^{c1}	2.86	2.81	
N ⁷²	O ^{c2} Glu202	2.87	2.95	I-II†
Glu94 O ^{c1}	N ^c Lys224	2.69	2.75	I-II†
O ^{c2}	N ^c Arg220	2.70	2.94	I-II†
Asp114 O ^{δ1}	N ⁷¹ Arg140	2.96	2.85	I-I
Asp124 O^{δ1}	N^{c2} His289	2.71	3.33	I-I
Asp137 O ^{δ2}	N ^c Arg140	2.98	3.11	I-I
Asp170 O ^{δ2}	N ^c Lys261	2.94	2.80	II-I†
Asp178 O ^{δ2}	N ⁷¹ Arg193	3.03	3.10	II-II
Asp184 O ^{δ2}	N ^c Arg186	2.90	—	II-II
	N ⁷²	3.09	—	
Asp260 O^{δ2}	N^{δ1} His289	2.82	3.06	I-I
Glu296 O ^{c2}	N ^c Arg300	2.58	2.55	I-I
	N ⁷²	2.77	2.64	

Salt-bridges between the catalytic triad residues are indicated by bold characters.

† Salt-bridges formed between the 2 domains.

Table 5 (continued)

Residue 1	Residue 2	Distance (Å)		
		pH 6.2	pH 8.2	Domains
Asn14 N ^{δ2}	Asn14 O	—	2.71	I-I
Asn23 O ^{δ1}	Ser21 O	3.33	3.39	I-I
Tyr31 O ⁿ	Phe101 O	2.49	2.63	I-I
Asn43 O ^{δ1}	Asp45 N	3.01	2.99	I-I
His54 N ^{δ1}	Gly55 O	2.94	2.94	I-I
N ^{c2}	Phe82 N	3.07	3.04	I-I
Glu56 O ^{c1}	Val219 O	3.49	3.49	I-II
O ^{c2}		2.43	2.48	
Trp59 N ^{c1}	Ala205 O	3.00	2.99	I-II
Tyr63 O ⁿ	Leu53 O	2.60	2.49	I-I
	Val121 O	3.36	—	I-I
Arg64 N ⁷¹	Tyr61 O	3.09	3.21	I-I
Lys65 N ^c	Glu293 O	3.01	2.84	I-I
Ser73 O ⁷	Phe70 O	2.78	2.80	I-I
Asp81 O ^{δ1}	Ser60 N	2.95	2.95	I-I
O ^{δ2}	Lys87 N	2.92	2.75	I-I
Ser88 O ⁷	His37 N	2.89	2.83	I-I
	Asp81 O	2.71	2.72	I-I
Asp89 O ^{δ2}	Arg35 N	2.91	2.84	I-I
Lys90 N ^c	Gly86 O	2.70	2.80	I-I
	Ser88 O	3.05	2.93	I-I
Tyr97 O ⁿ	Pro91 N	3.13	3.11	I-I
Thr98 O ⁷¹	Phe101 N	2.96	3.03	I-I
Arg103 N ^c	Thr132 O	3.44	—	I-I
N ⁷²		2.87	2.99	
Asn117 N ^{δ2}	Glu47 O	2.89	3.19	I-I
Gln123 O ^{c1}	His289 O	2.90	3.10	I-I
Asp124 O ^{δ2}	Glu56 N	2.88	2.93	I-I
	Trp125 N	2.72	2.78	I-I
Trp125 N ^{c1}	Phe222 O	3.29	3.38	I-II
Thr132 O ⁷¹	Leu129 O	2.71	2.67	I-I
Arg140 N ⁷¹	Leu115 O	2.96	2.75	I-I
Asn148 O ^{δ1}	Asn148 N	2.64	2.61	I-I
	Gly257 N	2.82	2.90	I-I
N ^{δ2}	Gly257 O	3.22	3.28	I-I
Asp154 O ^{δ1}	Asp154 N	2.93	2.95	I-I
O ^{δ2}	Val156 N	3.09	—	I-II
	Thr157 N	3.01	2.90	I-II
Thr166 O ⁷¹	Ala163 O	3.37	3.14	II-II
Thr173 O ⁷¹	Val165 O	3.33	3.25	II-II
Thr181 O ⁷¹	Tyr177 O	2.92	3.00	II-II
Asp188 O ^{δ1}	Gln189 N	3.18	3.07	II-II
Thr199 O ⁷¹	Glu202 N	3.15	3.31	II-II
Glu202 O ^{c1}	Thr199 N	2.88	2.92	II-II
Ser204 O ⁷	Ala201 O	2.75	3.31	II-II
Asp212 O ^{δ1}	Ser214 N	2.88	2.50	II-II
Gln216 N ^{c2}	Leu185 O	3.49	3.32	II-II
Arg220 N ⁷¹	Thr213 O	3.06	3.01	II-II
	Glu94 O	2.95	—	II-I
Lys221 N ^c	Tyr97 O	2.60	2.90	II-I
Arg229 N ^c	Val226 O	2.85	2.92	II-II
N ⁷¹	Thr153 O	2.84	2.85	II-I
N ⁷²	Phe161 O	3.10	2.91	II-II
	Phe161 O	2.82	2.77	II-II
	Val226 O	3.40	3.39	II-II
Asp230 O ^{δ1}	Cys233 N	2.83	2.75	I-I
Ser237 O ⁷	Cys233 O	2.57	2.64	I-I
Thr238 O ⁷¹	Ile234 O	2.69	2.63	I-I
Trp244 N ^{c1}	Leu131 O	2.88	2.91	I-I
Asn249 O ^{δ1}	Asp247 O	—	3.37	I-I
Gln251 O ^{c1}	Thr309 O	—	3.29	I-I
N ^{c2}		—	3.07	
Asp260 O ^{δ1}	Leu262 N	2.91	2.86	I-I
O ^{δ2}	Leu263 N	3.02	3.00	I-I
Asn276 N ^{δ2}	Trp248 O	2.97	2.94	I-I
Gln292 N ^{c2}	Val291 O	3.38	—	I-I
	N	—	3.34	
	Gln292 N	2.68	2.66	I-I
Thr309 O ⁷¹	Glu308 O	2.82	2.47	I-I

Table 5

Hydrogen bonds in haloalkane dehalogenase at pH 6.2 and pH 8.2 (< 3.50 Å)

Residue 1	Residue 2	Distance (Å)		Domains
		pH 6.2	pH 8.2	
A <i>Side-chain to side-chain hydrogen bonds</i>				
Thr7 O ⁷¹	Lys90 N ^c	2.62	2.60	I-I
Ser60 O ⁷	Tyr18 O ⁿ	2.93	2.80	I-I
	Asp81 O ^{δ1}	2.66	2.77	I-I
Arg103 N ^c	Thr132 O ⁷¹	3.00	3.08	I-I
Thr119 O ⁷¹	Arg143 N ^c	2.89	2.90	I-I
	N ⁷²	3.02	3.15	
Gln23 O ^{c1}	His289 N ^{c2}	—	3.43	I-I
Asp137 O ^{δ1}	Ser139 O ⁷	3.48	3.44	I-I
Asn148 N ^{δ2}	Asp260 O ^{δ2}	2.87	2.79	I-I
Thr153 O ⁷¹	Thr157 O ⁷¹	—	3.47	I-II
Asp178 O ^{δ2}	Trp194 N ^{c1}	2.92	2.83	II-II
Tyr206 O ⁿ	Glu293 O ^{c2}	2.50	2.51	II-I
Asp212 O ^{δ1}	Ser214 O ⁷	2.50	2.52	II-II
Gln231 N ^{c2}	Asp235 O ^{δ1}	2.81	2.77	I-I
Thr238 O ⁷¹	Thr153 O ⁷¹	3.42	3.09	I-I
Trp248 N ^{c1}	Thr252 O ⁷¹	3.01	3.11	I-I
Glu283 O ^{c2}	Tyr269 O ⁿ	2.52	2.53	I-I
Gln292 O ^{c1}	Tyr63 O ⁿ	3.34	3.35	I-I
B <i>Side-chain to main-chain hydrogen bonds</i>				
Thr7 O ⁷¹	Pro209 O	2.72	2.74	I-II
Arg11 N ^c	Ala208 O	2.94	2.82	I-II
	N ⁷²	2.99	2.78	I-II
Ser13 O ⁷	Gln10 O	3.25	3.25	I-I

Figure 12. Variation of the atomic temperature factors of the dehalogenase structure at pH 6.2 along the polypeptide chain, averaged for main-chain atoms (thick line) and for side-chain atoms (thin line). The regions of the helices and the β -strands are indicated at the top of the Figure.

acid residues in the pH 6.2 model situated inside the enzyme, like the catalytic triad residues, which are positioned in the cavity between the two domains of the enzyme, are ion-paired or are involved in hydrogen bonds. Only four of these 24 salt-bridges are formed between the two domains and all residues involved are solvent-accessible. Table 5 gives a list

of all the hydrogen bonds made by side-chain atoms in the dehalogenase structure at both pH values.

(v) Thermal motion

The atomic B -factors are a measure of the mobility of atoms. Figure 12 shows the average B -factor as a function of residue number. The average B -factor for all 2479 protein atoms in the dehalogenase structure is $14.4 \text{ \AA}^2$. The mean B -value for the main-chain atoms is $13.1 \text{ \AA}^2$, whereas for the side-chain atoms it is, as expected, somewhat higher, $15.8 \text{ \AA}^2$. The mean B -value for all solvent molecules is $35.1 \text{ \AA}^2$. As can be noticed from this Figure, the secondary structural elements other than turns, have the lowest isotropic temperature factors, compared to the rest of the protein.

There is a good correlation between the mobility of the atoms in the dehalogenase structure and the solvent accessibility of the residues. Solvent-exposed residues have a higher degree of mobility than buried residues, especially for the side-chains. This feature can also be seen by comparing the B -factors of the β -sheet and the helices of the first domain (Table 6). The eight-stranded β -sheet

Table 6

B-factors and accessible surface area of the secondary structure elements in the dehalogenase model at pH 6.2

α -Helix	β -Strand	B -factors ($\text{\AA}^2$)			Accessible surface area for all atoms ($\text{\AA}^2$)	Mean accessible area per residue ($\text{\AA}^2$)
		All atoms	Main-chain	Side-chain		
H ₁	β_a	20.62 (21)†	18.41 (12)†	23.57 (9)†	181	60.33 (3)‡
	β_1	14.21 (28)	14.22 (12)	14.19 (16)	124	41.33 (6)
	β_2	8.16 (52)	7.83 (24)	8.44 (28)	112	18.67 (6)
	β_3	12.16 (48)	10.57 (24)	13.75 (24)	46	7.67 (6)
		11.14 (119)	11.14 (56)	11.15 (63)	409	29.21 (14)
	β_4	7.39 (46)	7.76 (24)	6.99 (22)	44	7.33 (6)
H ₂	β_b	9.50 (23)	9.79 (12)	9.19 (11)	38	12.67 (3)
	β_5	14.27 (136)	12.73 (60)	15.48 (76)	519	34.60 (15)
H ₃		9.44 (37)	8.85 (20)	10.13 (17)	2	0.40 (5)
		9.53 (88)	9.63 (48)	9.41 (40)	97	8.08 (12)
	β_6	12.54 (63)	10.57 (28)	14.12 (35)	78	11.14 (7)
H ₄		10.59 (59)	10.45 (32)	10.75 (27)	333	41.63 (8)
H ₅		10.10 (92)	8.58 (44)	11.49 (48)	438	39.82 (11)
H ₆		10.27 (78)	8.20 (32)	11.70 (46)	471	58.88 (8)
H ₇		10.18 (61)	7.91 (36)	13.44 (25)	304	33.78 (9)
H ₈		10.57 (83)	8.66 (44)	12.73 (39)	216	19.64 (11)
H ₉		15.14 (135)	13.46 (68)	16.84 (67)	840	49.41 (17)
	β_7	13.91 (52)	11.56 (28)	16.65 (24)	56	8.00 (7)
H ₁₀		13.33 (83)	11.43 (44)	15.47 (39)	469	42.64 (11)
	β_8	16.55 (25)	14.06 (12)	18.85 (13)	142	47.33 (3)
H ₁₁		23.85 (147)	21.34 (72)	26.26 (75)	847	47.06 (18)
Overall B -factor for β -sheet:					Mean accessible surface per residue for β -sheets	
All β -strands		11.83 (395)†	10.72 (196)†	12.92 (199)†	All β -strands	16.80 (49)‡
β_1 - β_8		11.46 (351)	10.25 (172)	12.62 (179)	β_1 - β_8	14.05 (43)
β_a - β_b		14.81 (44)	14.10 (24)	15.62 (20)	β_a - β_b	36.50 (6)
Overall B -factor for α -helices					Mean accessible surface per residue for helices	
All helices		13.52 (1081)	12.02 (536)	14.99 (545)	All helices	36.89 (134)
Domain I (H ₁ -H ₃ /H ₉ -H ₁₁)		15.20 (708)	13.81 (348)	16.54 (360)	Domain I	36.56 (876)
Domain II (H ₄ /H ₈)		10.33 (373)	8.73 (188)	11.96 (185)	Domain II	37.04 (47)

† Number of atoms.

‡ Number of residues.

(average B -factor, $11.5 \text{ \AA}^2$) forms the central, hydrophobic core of domain I, whereas the helices are located around this sheet with some of them consequently more exposed to solvent (mean B -factor of these helices, $15.3 \text{ \AA}^2$). This correlation between mobility and solvent accessibility is, however, not seen for the helix residues of the cap domain. The average isotropic temperature factor of the helices of domain II is similar to that of the main β -sheet in domain I (mean B -value, $10.0 \text{ \AA}^2$), although the solvent accessibility of the residues in the cap domain is almost twice as high. This could either indicate that domain II has a more rigid structure or that the accessible surface area of the cap domain in the crystal is reduced due to crystal contacts. Indeed, the solvent accessibility of the helix residues in domain II drops from $37 \text{ \AA}^2$ to $21 \text{ \AA}^2$ per amino acid residue, if one considers the packing of the dehalogenase molecules in the crystal lattice, indicating that this structural arrangement of the molecules in the lattice can have quite an influence on the rigidity of the cap domain.

(vi) Solvent structure

In the pH 6.2 haloalkane dehalogenase model 258 solvent molecules have been included. They were all assigned as water molecules and have B -factors below $70 \text{ \AA}^2$. Since 110 water molecules have a temperature factor below $30 \text{ \AA}^2$, they are considered to be an integral part of the protein (see e.g. Blevins & Tulinsky, 1985; Karplus & Schulz, 1987). Among them, 18 are internal water molecules with zero solvent accessibility (average temperature factor is $11.6 \text{ \AA}^2$). Of the remaining 240 solvent molecules, 195 belong to the first hydration shell, directly hydrogen-bonded to the protein surface (average B -factor, $34.9 \text{ \AA}^2$); 45 molecules are located in the second hydration shell (distance to the nearest protein atom $> 3.50 \text{ \AA}$) and are fixed by solvent-solvent hydrogen bonds (see e.g. Blake *et al.*, 1983). Their average temperature factor is $47.6 \text{ \AA}^2$.

The 205 water molecules in the pH 8.2 structure were assigned independently from the pH 6.2 model. The 18 internal water molecules are identical and the first hydration shell contains 163 water

molecules, of which 141 are identical to the solvent molecules of the first hydration shell in the pH 6.2 dehalogenase model.

(vii) Crystal packing

Figure 13 gives an illustration of the packing of the dehalogenase molecules in the crystal lattice. There are only a few contacts between neighboring units in the crystal. They are listed in Table 7. Except for four contacts, the interactions are always between the cap domain residues of one molecule and domain I amino acid residues of a crystallographically related molecule. The importance of the cap domain for the formation of crystals is also shown by crystallization experiments of mutants with mutations located in the cap domain region that affect the crystal contacts. Attempts to crystallize such mutant enzymes, under a variety of conditions, have not been successful.

(c) The internal active site cavity

The two domains of haloalkane dehalogenase contribute to the formation of an internal cavity of approximately $37 \text{ \AA}^3$ in size. This cavity is predominantly surrounded by hydrophobic residues. These residues are Glu56 (only C^β and C^γ of the side-chain, the carboxyl oxygen atoms point into the interior of the protein), Trp125, Phe128, Leu262 and Leu263 of domain I. The cap domain provides mostly aromatic rings: Phe164, Phe172, Trp175, Phe222, Pro223 and Val226. The only charged residues in the cavity are the active-site residues Asp124 and His289. The former residue is pointing into the cavity. At pH 6.2, its side-chain is stabilized by hydrogen bonds of the $O^{\delta 2}$ atom with the backbone amide groups of Glu56 ($2.93 \text{ \AA}$) and Trp125 ($2.78 \text{ \AA}$), respectively, and by a salt-bridge between the $O^{\delta 1}$ atom and the $N^{\epsilon 2}$ atom of His289 ($2.71 \text{ \AA}$). A small tunnel, of which Lys259, Asp260 and Lys261 constitute the entrance, connects the cavity with the solvent outside. The side-chain of Leu262 seems to block this entrance, but this leucine side-chain must be flexible, since even in the crystal structure, a heavy-atom derivative such as

Figure 13. Stereo view of a unit cell to show the packing of the dehalogenase molecules in the crystal. The 4 molecules are displayed as C^α tracing.

Table 7
Inter-molecular contacts in the dehalogenase crystal (< 3.5 Å)

Symmetry transformation	Residue 1	Residue 2	Closest distance (Å)		Domains
			pH 6.2	pH 8.2	
$-x, -y, z$	Pro279 C ^{β}	Gln10 N ^{ϵ^2}	—	3.5	I-I
	Glu280 N	Gln10 O ^{ϵ^1}	2.9	3.0	I-I
	Glu308 O	Arg11 N ^{η^1}	3.4	3.5	I-I
	N	C ^{β}	—	2.9	I-I
	O ^{ϵ^1}	Pro211 C ^{β}	3.3	3.5	I-II
$-x+1/2, y+1/2, -z$	Gln167 N ^{ϵ^2}	Asn14 C ^{β}	2.8	—	II-I
		O ^{δ^1}	—	3.0	II-I
	Ala169 N	Ser13 O	2.8	2.8	II-I
	Ser183 O ^{γ}	Glu72 O ^{ϵ^1}	3.1	2.7	II-I
	Arg186 N ^{η^2}	Asn43 N ^{δ^2}	—	3.0	II-I
	Gln189 N ^{ϵ^2}	Asn43 O ^{δ^1}	2.8	2.7	II-I
	O ^{ϵ^1}	Ser44 N	3.1	3.0	II-I
	Arg193 N ^{η^2}	Asp40 O	2.7	2.8	II-I
		Glu41 O ^{ϵ^2}	3.3	3.2	II-I
	N ^{η^1}	O ^{ϵ^2}	3.3	3.2	II-I
$x+1/2, -y+1/2, -z$	Val156 O	Gln245 N ^{ϵ^2}	3.2	3.1	II-I
	Gln245 N ^{ϵ^2}	Val156 O	3.2	3.1	I-II
	Asp266 C ^{β}	Tyr269 O ^{δ^1}	3.2	—	I-I
	Tyr269 C ^{δ^1}	Asp266 C ^{β}	3.2	—	I-I

Au(CN)₂⁻ could easily be soaked into this cavity (Franken *et al.*, 1991). Two solvent molecules occupy the space of this cavity in the native structure. One water molecule is stabilized between the ring nitrogen atoms of the two tryptophan residues (Trp125 and Trp175). The other is hydrogen-bonded to the O ^{δ^1} atom of Asp124. This can be seen in Figures 4(b) and 6(b). Another two water molecules are located at the opposite side of the Asp124 side-chain. They fill up a space of approximately 14 Å³. The refinement of the 1.9 Å structures at pH 6.2 as well as at pH 8.2 clearly revealed that this space is occupied by two water molecules, instead of one as was previously seen in the 2.4 Å pH 6.2 structure (Franken *et al.*, 1991). These water molecules make hydrogen bonds with the backbone nitrogen atom of Gly55 and the side-chain atoms of Gln123 and Thr58 for the one; and with the N ^{ϵ^2} atom of His289 (3.02 Å at pH 6.2; 2.78 Å at pH 8.2) for the other. This latter water molecule most likely acts as the hydrolytic water molecule in the reaction mechanism.

(d) *Comparison of the pH 6.2 and the pH 8.2 structures and the reaction mechanism*

The structure of haloalkane dehalogenase has been refined with data from crystals at two different pH values: pH 6.2, the pH of the crystal growth; and pH 8.2, the pH of optimum activity. Comparison of the dehalogenase structures at the two different pH values revealed almost no differences. The r.m.s. deviation between the two refined models is 0.12 Å for the 1240 main-chain atoms, whereas it is 0.28 Å for all 2479 atoms. Apart from some flexible side-chains on the surface of the protein, the two structures superimpose very well,

except for a small, but significant, movement of the Asp124 side-chain. At pH 6.2, His289 is hydrogen-bonded to the O ^{δ^1} atom of Asp124 (N ^{ϵ^2} His289-O ^{δ^1} Asp124, 2.71 Å, Table 4), although the bond angle (N-H...O, 94°) indicates that this hydrogen bond is not very strong. At pH 8.2 the distance between the side-chains of the histidine and the aspartate residues becomes significantly larger (N ^{ϵ^2} His289-O ^{δ^1} Asp124, 3.30 Å). From this, one can conclude that the N ^{ϵ^2} atom of His289 is mostly deprotonated at pH 8.2, which results in a loss of the salt-bridge with the Asp124 side-chain. The only hydrogen bond the O ^{δ^1} atom of Asp124 then still makes, is that to a water molecule. This observation is in agreement with the reaction mechanism as postulated by Franken *et al.* (1991).

Asp124 of the catalytic triad was proposed to act as the nucleophile. It stands proud of the rest of the active-site surface in order to have easy access on one side by the substrate and on the other side by a hydrolytic water molecule. This is achieved by situating the residue at the extremely sharp nucleophile elbow, present in all members of the α/β hydrolase fold family (Ollis *et al.*, 1992). The substrate enters the cavity and the two not very tightly bound solvent molecules originally filling up the space must be displaced upon substrate binding. This means that at pH 8.2, after the substrate has bound in the active-site cavity, the negative charge of the internal Asp124 has presumably no interaction with His289 or the water molecule. The N-terminal helix dipole (Hol *et al.*, 1978) of helix H₃ could stabilize the negatively charged side-chain of Asp124. Its O ^{δ^1} atom is very close to the axis of this helix. The effect of the helix macrodipole could be increased by the non-polar nature of the active-site cavity. The O ^{δ^1} atom of Asp124 can then perform a

nucleophilic attack on the carbon atom of the substrate, which, from docking experiments, would be 3.1 Å away (Franken *et al.*, 1991). The resulting negatively charged five-coordinated carbon atom in the transition state and the released halide ion could be stabilized by the helix dipole as well as by the ring nitrogen atoms of two Trp residues (Trp125 and Trp175). These tryptophan residues point with their side-chains into the internal cavity, and preliminary soaking experiments with halides clearly indicated that these residues are important for binding chloride and iodide groups. A covalent intermediate ester is formed that is subsequently cleaved by a water molecule (Wat402 in Fig. 4(b)). This hydrolytic water molecule, which was not yet visible in the electron density of the refined pH 6.2 model at 2.4 Å (Franken *et al.*, 1991), is in the refined 1.9 Å pH 8.2 structure 2.78 Å away from the N^{ε2} atom of His289, and in the plane of the ring, so it can donate its proton to the ring nitrogen atom of the histidine. Subsequently, the hydroxyl group can perform a nucleophilic attack on the Asp124 C^γ atom of the intermediate, which is 3.11 Å away, releasing the alcohol product and restoring the Asp124 side-chain. The higher pH can subsequently most likely promote the movement of the proton on the N^{ε2} atom of the histidine towards the chloride product, which finally can leave the cavity. The site of the hydrolytic water is then most probably replenished by an incoming water molecule.

4. Conclusions

The refinement of haloalkane dehalogenase at two different pH values, the original pH of the crystallization (pH 6.2) and the pH of optimum activity for the enzyme (pH 8.2), revealed a small, but significant movement in the position of the catalytic triad residues, which is in agreement with the proposed reaction mechanism (Franken *et al.*, 1991). At pH 8.2, the active-site His289 is mostly neutral, which results in a loss of the salt-bridge between the histidine residue and the nucleophilic Asp124, originally present in the pH 6.2 structure. This leaves the negatively charged aspartate side-chain ready to perform a nucleophilic attack on the carbon atom of the substrate, situated in the internal active-site cavity. An ester bond between the Asp124 side-chain and the substrate is formed. This ester was proposed to be hydrolyzed by a hydrolytic water molecule attacking the C^γ atom of Asp124 (Franken *et al.*, 1991). The collection of a high resolution data set has now revealed the proper position of the putative hydrolytic water molecule. It is located in a perfect position to perform the nucleophilic attack on the ester. Its nucleophilicity is enhanced by the presence of His289 nearby, which can easily pick up a proton from the water molecule.

Further detailed investigation of the two refined models showed a number of unusual structural features, of which one could be related to the existence of a bend in the central β -sheet. A novel helix-

turn-strand motif, the $\alpha\beta_4$ turn, has been observed between helix H₃ and β -strand 5, the strand that forms the hinge of this bend in the main β -sheet. It is a turn of four residues, composed of two inner amino acid residues with (ϕ , ψ) angles in the β -region of the Ramachandran plot, flanked by two residues with left-handed α -helical main-chain torsion angles. Two hydrogen bonds between these outer residues with residues in the β -sheet can help stabilize this bend in the sheet. Another remarkable observation, rarely seen in other refined globular protein structures, is the occurrence of a proline residue other than in the first turn in four out of 11 helices. Two other α -helices have adopted a 3_{10} configuration in the central part of their α -helical hydrogen-bonding pattern.

These present refined models may serve as a solid framework for further investigations to obtain more insight into the structural and biochemical properties of this haloalkane dehalogenase, in view of protein engineering experiments geared towards an environmental "restorer" with an improved catalytic efficiency and altered substrate specificity.

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