X-ray structural studies on two dehalogenating enzymes from Xanthobacter autotrophicus GJ10
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Summary and outlook

Many of the environmental pollutants known to date are man-made halogenated hydrocarbons. These compounds accumulate in the biosphere because the stability of the carbon–halogen bond hampers degradation. The most widely produced organohalogen is 1,2-dichloroethane (DCE), an intermediate in the production of vinylchloride and polyvinylchloride (PVC). It is considered one of the major environmental pollutants. Biotechnological DCE removal has been applied since it was found that the microorganism Xanthobacter autotrophicus GJ10 is capable of utilizing 1,2-dichloroethane (Cl-CH₂-CH₂-Cl) as its sole source of carbon and energy. To convert DCE to glycolate, the bacterium employs an four-step enzymatic degradation route, which contains two dehalogenases, haloalkane dehalogenase (DhIA) and L-2-haloacid dehalogenase (DhIB). These enzymes hydrolytically cleave one carbon–halogen bond of the substrate.

Haloalkane dehalogenase is a globular, 35 kDa enzyme with an α/β-hydrolase fold and a catalytic triad of Asp124-His289-Asp260. It catalyses the conversion of 1,2-dichloroethane to 2-chloroethanol and chloride in a two-step mechanism: the S_n2 nucleophilic displacement of the chlorine atom by Asp124 of the enzyme yielding an enzyme-ester intermediate, followed by hydrolysis of the ester by a water molecule which is activated by His289. During the reaction the halide ion is bound between Trp125 and Trp175 and kinetic experiments have shown that halide release is the rate-limiting step in the dehalogenation reaction.

L-2-Haloacid dehalogenase catalyses the fourth step in DCE breakdown, the conversion of chloroacetate (Cl-CH₂-COOH) to glycolate and chloride. DhIB stereospecifically acts on the L-stereomer of chiral 2-haloalkanoate compounds to produce the corresponding D-2-hydroxyalkanoate. The enzyme (molecular mass 2 × 27.5 kDa) is dimeric and several homologues are found in Pseudomonas species.

This thesis describes the crystallization, structure determination and elucidation of the reaction mechanism of L-2-haloacid dehalogenase from Xanthobacter autotrophicus as well as the relevance of the structure for other members of the haloacid dehalogenase (HAD) superfamily. A second part of the thesis is devoted to ligand-binding studies and mutant structures of haloalkane dehalogenase from the same organism, which contributed to an attempt to improve the catalytic properties of this enzyme.
Chapter 1 provides a general introduction to the world of enzymatic dehalogenation. The relevance of organohalogen compounds is described as well as their potential threat to the environment. Biotic detoxification techniques rely on the availability of microorganisms with an appropriate enzyme to catalyse cleavage of the carbon–halogen bond. Several possible dehalogenation mechanisms are discussed and all dehalogenases of which the three-dimensional structure is known are reviewed in detail. Haloalkane dehalogenase, L-2-haloacid dehalogenase and 4-chlorobenzoyl-CoA dehalogenase have a completely different fold, yet their hydrolytic dehalogenation mechanisms are amazingly alike. All three involve a covalent enzyme-ester intermediate formed after nucleophilic attack of an aspartate residue, which is hydrolysed in the next step of the reaction.

**L-2-Haloacid dehalogenase**

The crystallization of L-2-haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 is described in Chapter 2. Crystals could be grown from hanging drops, applying PEG and sodium formate as precipitant. The use of macroseeding techniques improved the growth rate and quality of the crystals considerably. The three-dimensional structure of DhlB has been determined through multiple isomorphous replacement with incorporation of anomalous scattering information, as presented in Chapter 3. Diffraction data from two mercury, one osmate and one iridium derivatized protein crystal provided sufficient phase information to produce an interpretable electron-density map. The structure was refined at a resolution of 1.95 Å. The enzyme is a homodimer, and each monomer consists of a mixed α/β main domain with a Rossmann-folding motif and two helical subdomains which form the dimerization interface. The structure is homologous to that of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL. In DhlB, the catalytically important residues surround the active-site cavity, which is completely shielded from the solvent. Inside this cavity, a formate ion is bound and a substrate-binding model is proposed on the basis of the position of this analogue.

In Chapter 4, the structure of the enzyme refined against new data at a resolution of 1.52 Å is described as well as the structures of the complexes of DhlB with L-2-monochloropropionate (L-MCPA) and with monochloroacetate. The two complex structures provided crystallographic evidence of the enzymatic mechanism. By lowering the pH of the experiment, the enzyme was inactivated and both complexes clearly show that a covalent bond has formed between Asp8 Oδ1 and the C2 atom of the dechlorinated substrates. The chloride ion is bound in a halide-stabilising cradle formed by Arg39, Asn115, and Phe175, and the carboxylate moiety of the substrate is held in position by Ser114, Lys147, and several main-chain amide groups. These structures demonstrate that the reaction proceeds through a two-step mechanism: first, a nucleophilic attack of the methyl group which is stereospecific, forming a covalent bond with the substrate, and secondly, the methyl group is released in a hydrolytic reaction. Furthermore, the three-dimensional structure of CheY, allowing the identification of the methyl group, shows that the side chain in the P-site is present as the methyl group and not as a phospho-ester.

**Haloalkane dehalogenase**

Crystals of L-2-haloacid dehalogenase were grown at 1.15 Å resolution with a solvent content of 65%, 10.5% with anomalous scattering information, and a crystal to data collection ratio of 1.15 Å resolution with a solvent content of 65%, 10.5% with anomalous scattering information. The structure was refined at a resolution of 1.95 Å. The enzyme is a homodimer, and each monomer consists of a mixed α/β main domain with a Rossmann-folding motif and two helical subdomains which form the dimerization interface. The structure is homologous to that of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL. In DhlB, the catalytically important residues surround the active-site cavity, which is completely shielded from the solvent. Inside this cavity, a formate ion is bound and a substrate-binding model is proposed on the basis of the position of this analogue.

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Enzymatic dehalogenation. The potential threat to the viability of microorganisms with an ether bond. Several possible mechanisms, of which the three-dimensional structure of L-2-haloacid dehalogenase suggests, are presented. A covalent enzyme-ester intermediate, which is hydrolysed in the next step, results in a covalent enzyme-ester intermediate, which is subsequently hydrolysed by a water molecule. The limited substrate specificity and stereospecificity of the enzyme is explained by the size and location of a small pocket in which the methyl group of L-MCPA is bound.

L-2-Haloacid dehalogenase is a member of the HAD superfamily of hydrolases, which furthermore comprises P-type ATPases, phosphatases and epoxide hydrolase. In Chapter 5, the three-dimensional structure of DhlB is compared with that of the response regulator protein CheY, allowing the assignment of a conserved pair of aspartate residues as the Mg²⁺-binding site in the P-type ATPase and phosphatase members of the superfamily. A model of their active site is presented and an ATPase/phosphatase catalytic mechanism is proposed comprising a phospho-aspartate intermediate, analogous to the dehalogenase mechanism.

**Haloalkane dehalogenase**

Crystals of haloalkane dehalogenase from Xanthobacter autotrophicus GJ10 diffract to atomic resolution when synchrotron radiation is used. The refinement and analysis of the structure at 1.15 Å resolution is the subject of Chapter 6. The structure has been refined to an R-factor of 10.5% with an estimated coordinate error of 0.038 Å for all atoms in the model. It shows all characteristics of an atomic resolution structure, including individual anisotropic displacement parameters, alternative conformations for side-chain and main-chain atoms, hydrogen atoms (at calculated positions), alternative solvent structure, and rigorous error estimates of individual parameters. The structure has been compared to previously reported DhlA structures. The analysis of the protein’s geometry and stereochemistry has revealed that several bond lengths and angles deviate significantly from Engh & Huber values, that the main-chain co torsion angle has a wide spread around its ideal trans value, and that C–H...O interactions assist in satisfying the hydrogen-bond acceptor capacity of main-chain carbonyl O atoms in the central ß-sheet.

Several mutants of haloalkane dehalogenase have been characterized for their kinetics and three-dimensional structure. Together with the ligand-binding studies on DhlA, they are presented in Chapters 7, 8, and 9. In Chapter 7, the structure of Phe172Trp dehalogenase is discussed in correlation with the higher catalytic efficiency of the mutant towards longer-chained substrates. It shows a local conformational change in the helix-loop-helix region that covers the active site. This change allows larger substrates to bind more easily in the active site cavity. Furthermore, kinetic experiments have demonstrated that the enzyme isomerization step associated with halide release has an increased rate in the mutant. This suggests that the isomerization might be a conformational change which takes place in this part of the cap domain.

To explore the rate-limiting step of halide release in the dehalogenation reaction, the structure of the Trp175Tyr mutant has been investigated in Chapter 8. The overall structure has remained...
intact and the tyrosine side chain is in a similar orientation as the tryptophan in the wild-type enzyme. Owing to its smaller size however, the volume of the active site cavity has increased and the halide-stabilising aromatic rings are further apart. The resulting weaker interactions with the halogen atom / halide ion explain the decreased rates of substrate binding and carbon-halogen bond cleavage as well as the faster halide release. This is further evidenced by DhlA complex structures with acetic acid, which binds with one of its oxygen atoms to both Trp125 and Trp175 in wild-type and only to Trp125 in the mutant where the tyrosine ring has rotated away.

A second possible route of halide release has been studied in Chapter 9 in which the structure of wild-type haloalkane dehalogenase at high bromide concentration is presented. Kinetics of halide import demonstrated the formation of an initial 'collision complex' of enzyme and halide before actual import. The X-ray structure revealed one bromide ion firmly bound in the active site and two more bromide ions weakly bound on the enzyme surface. The ion close to Thr197 and Phe294 most likely indicates the position where the collision complex is formed, as it is close to the earlier proposed tunnel for substrate import and since DhlA mutants of the two residues showed different halide-binding kinetics.

Outlook

The work described in this thesis has resolved many questions about the structure and mechanism of L-2-haloacid dehalogenase, it has shed light on the rate-limiting step of DCE conversion by haloalkane dehalogenase, halide release, and it has shown what we can learn about protein structure in general from atomic-resolution data. Yet, other questions remain to be answered and new questions have come up.

The atomic-resolution structure of DhlA has shown that protein geometry and stereochemistry is different from that of small molecules. These differences may be small, yet they are significant. Therefore, it is of general chemical interest to analyse all atomic-resolution protein structures for the exact nature of these differences in order to find an explanation. Certainly, geometry- and stereochemistry-library development will benefit from such crystal-structure statistics, and thereby all techniques that rely on these libraries, for instance refinement of X-ray and NMR structures, molecular dynamics, protein structure prediction and molecular modelling.

The challenge in haloalkane dehalogenase research still is to find a way to speed up the crucial step of the mechanism without interfering with the other steps. More three-dimensional structures of DhlA mutants and different ligands bound to DhlA are an essential contribution that can be made by crystallographic techniques. Unfortunately, these structures cannot be obtained routinely. Molecular modelling might circumvent these problems, but first it should be validated with the help of the mechanisms and calculation of the electronic nature of the active site.

Furthermore, it would be valuable to understand the role of dimers and larger structures. Only for a detailed understanding of the mechanism would we be able to exploit the different pathways of other types of DhlA. More studies of other types of DhlA are necessary to find out which mechanisms are optimal for biocatalysis. This is also important for understanding other enzymes and finding new applications.
Summary and outlook

Validation of whether this approach would yield a sufficient level of structural detail. The structures in this thesis could constitute a starting point for such a validation.

The mechanism of L-2-haloacid dehalogenase is known in detail for the first step. However, in the second step, hydrolysis of the enzyme-ester intermediate, the origin of the water molecule or hydroxyl ion is still uncertain. An enzyme rearrangement is probably required, and to access the nature of this rearrangement, a structure of DhlB at its optimum pH would be helpful. Furthermore, not much is known yet about substrate import and product export and the possible role of dimer formation in these processes, which was suggested on the basis of preliminary calculations of the electrostatic potential of the enzyme.

Only for a few enzymes which catalyse hydrolytic dehalogenation, structure-based knowledge of the mechanism is available. Although the fold of the enzymes is very different, their mechanisms are remarkably alike. Does this hold for all hydrolytic dehalogenases? Moreover, it would be very interesting to obtain structures and a detailed comprehension of the mechanism of other types of dehalogenases, not in the least because many of the organohalogen pollutants are not degraded by hydrolytic dehalogenases. A more complete understanding of enzymatic carbon–halogen cleavage would be a major step forward in choosing and/or (re-)designing the optimal way of bioremediation of individual organohalogen compounds. The new structural insights in DtrlA and DhlB presented in this thesis are an important contribution to this understanding.