Holoalkane dehalogenase engineering

Schanstra, Joost Peter

Summary and Concluding Remarks

Haloalkane dehalogenase from the bacterium *Xanthobacter autotrophicus* GJ10 converts haloalkanes to their corresponding alcohols (Keuning et al., 1985). The dehalogenase is the first enzyme in the degradation route of 1,2-dichloroethane, and is essential for growth of bacteria on this substrate as the sole source of carbon and energy (Janssen et al., 1985; Van den Wijngaard, 1992). The three-dimensional structure and reaction mechanism (Figure 1) of the enzyme are known (see thesis of K. H. G. Verschueren (1993) and thesis of F. Pries (1995) and the publications cited therein). The reaction proceeds via the formation of a covalent alkyl-enzyme which is subsequently hydrolyzed by activated water. Two tryptophans stabilize the chlorine leaving group and histidine 289 acts as a general base to activate a water molecule that hydrolyzes the alkyl-enzyme intermediate (Figure 1). The work described in this thesis is aimed at expanding our knowledge about the kinetics of the dehalogenase reaction and the structure-activity relationships of the enzyme. This should contribute to the basic knowledge that is necessary to improve the characteristics of the enzyme by protein engineering.

Construction of a Vector for Expression and Site-Directed Mutagenesis in Escherichia coli. A good expression and site-directed mutagenesis system can facilitate protein engineering by making it possible to quickly generate mutants and produce enzyme for subsequent studies. The original clone of haloalkane dehalogenase showed that production of the enzyme in *Escherichia coli* was possible to at least 30% of the soluble cellular protein, indicating that *E. coli* was an excellent host for dehalogenase expression. *Chapter 2* described the construction of an expression and site-directed mutagenesis system for haloalkane dehalogenase in *E. coli*. A construct with the dehalogenase gene (*dhlA*) translationally fused behind a bacteriophage T7 promoter resulted in dehalogenase expression at a level of up to 38% of the soluble cellular protein. This plasmid was further modified to allow Kunkel mutagenesis and single stranded DNA-sequencing. The resulting construct thus allowed construction, plate screening, sequencing and large scale expression without recloning. Up to now, the plasmid (pGELAF+) has been used to produce more than 100

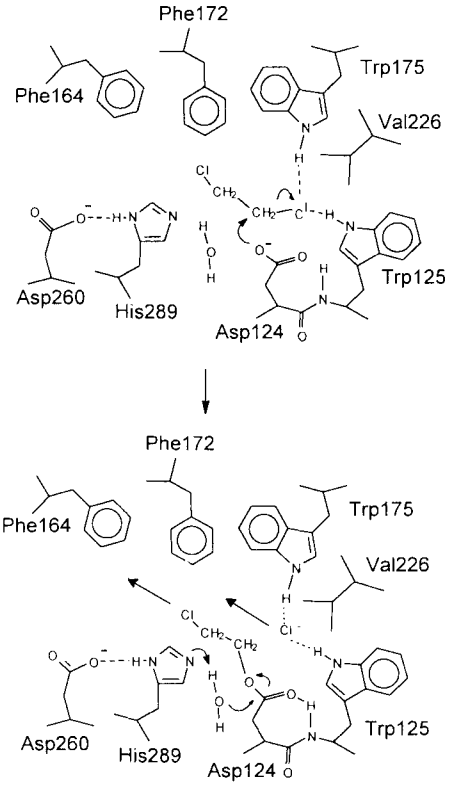


Figure 1: Proposed reaction mechanism of haloalkane dehalogenase.

dehalogenase mutants. Furthermore, the system was also used to construct and express mutants of a bacterial epoxide hydrolase and a fluoroacetic acid dehalogenase (R. Rink, personal communication).

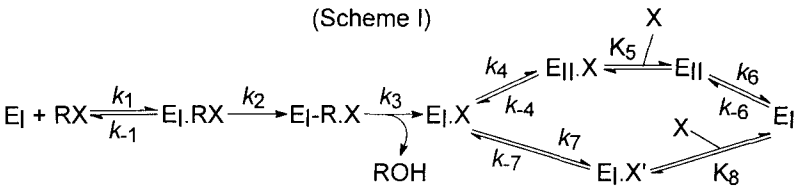
Especially the rapid-quench-flow experiments described in this thesis demanded large quantities of enzyme (50 to 100 mg per rapid-quench curve) and were strongly facilitated by the pGELAF+ expression system. Currently, we can obtain from a 10 liter culture of *E. coli* BL21(DE3)(pGELAF+) in two purification steps about 3 g of purified haloalkane dehalogenase. Expression of haloalkane dehalogenase mutants had to be done at 17°C, however, to prevent the formation of inclusion bodies, which were formed with all mutant proteins at 30°C. Inclusion bodies are protein aggregates often found when heterologous proteins are overexpressed in *E. coli*. The cause of aggregation is unknown, but is clearly related to the high expression level and can often be suppressed by decreasing the temperature or by lowering the concentration of the inducer in inducible systems. All studies described in this thesis were carried out with enzymes produced by the pGELAF+ system.

The Kinetics of Substrate Conversion by Wild Type Haloalkane Dehalogenase. Wild type haloalkane dehalogenase was subjected to a thorough kinetic analysis using steady-state and pre-steady-state kinetic techniques (*Chapter 3 and 4*). This resulted in the reaction mechanism shown in Scheme I.

The first goal was to identify the steps determining the low steady-state k_{cat} of the enzyme for both 1,2-dichloroethane and 1,2-dibromoethane. X-ray crystallographic studies in the group of Prof. BW Dijkstra (University of Groningen) on haloalkane dehalogenase crystals soaked in 1,2-dichloroethane showed that the chloride ion remained bound in the active site of the dehalogenase after completion of the chemical part of the reaction (Verschueren et al., 1993b). This indicated that halide release, under the conditions applied, could be rate-limiting. However, if halide release were rate-limiting, it is puzzling that the steady-state k_{cat} values for 1,2-dichloroethane and 1,2-dibromoethane are similar, although the dissociation constants for the corresponding halides are very different (Verschueren et al., 1993c; Kennes et al., 1995). Since the intrinsic protein fluorescence of the dehalogenase is quenched by binding of halides between the active site tryptophans (Figure 1, Verschueren et al., 1993c, Kennes et al., 1995), the kinetics of halide binding and release under pseudo first-order conditions could be studied using stopped-flow fluorescence. Analysis of the pre-steady-state data was done using numerical simulation since the concentration dependency was found to be unusually complex (Johnson, 1986; Mendes, 1993).

These studies revealed that there are two parallel routes for halide release in the wild type enzyme (Scheme I, from $E_I.X$ to E_I). Halide release was found to follow mainly the upper route in Scheme I, in which a slow enzyme isomerization that could limit the k_{cat} precedes actual halide dissociation, which is very fast. Furthermore, halide binding and release in ^2H_2O was slower than in ^1H_2O,

(Scheme I)



showing that the solvent kinetic isotope effect found on the steady-state k_{cat} for 1,2-dichloroethane and 1,2-dibromoethane conversion could be caused by a lower rate of the enzyme isomerization preceding halide release rather than by slowing down a chemical step. Halide binding via the lower route in Scheme I (from $E_I.X$ to E_I) was found to occur only at high halide concentrations and is a more common ligand binding mechanism involving the formation of an initial collision complex (Johnson, 1986). Only a small amount of halide will exit via this route.

Further kinetic analysis of substrate conversion by rapid-quench-flow and stopped-flow fluorescence showed that both for 1,2-dichloroethane and 1,2-dibromoethane conversion, hydrolysis of the alkyl-enzyme intermediate (k_3, Scheme I) is only about two-fold faster than the rate of halide release. The rate of cleavage of the carbon-halogen bond (k_2, Scheme I) was not found to be rate-limiting for these two substrates in the wild type enzyme. In rapid-quench-flow experiments, the rate of hydrolysis of the alkyl-enzyme intermediate was affected by the use of ^2H_2O as the solvent. Thus, besides the effect of ^2H_2O found on the rate of halide release (via the enzyme isomerization), ^2H_2O also contributes to the lower steady-state k_{cat} in ^2H_2O by lowering the rate of dealkylation of the covalent intermediate.

The main difference in the kinetics of conversion between 1,2-dibromoethane and 1,2-dichloroethane was located at the beginning of the reaction sequence (steps 1 and 2, Scheme I). Both substrate binding and carbon-halogen bond cleavage were found to be slower with 1,2-dichloroethane than with 1,2-dibromoethane. This was in agreement with the observation that the enzyme has a higher affinity for bromide than for chloride and that bromine is a better leaving group.

Steady-state kinetic analysis of the dehalogenation of a number of other substrates showed that the rate-limiting step for most brominated compounds is at the end of the reaction sequence (i.e. hydrolysis of the alkyl-enzyme intermediate and/or halide release). Rate-limiting steps for chlorinated compounds at saturating substrate concentration switched to carbon-halogen bond cleavage upon increasing alkyl-chain length and introducing more polar R-groups.

Haloalkane Dehalogenase Mutants. The properties of both the Val226Ala (*Chapter 5*) and the Phe172Trp (*Chapter 6*) dehalogenase confirmed that an enzyme isomerization preceding halide release is the main rate-limiting step for 1,2-dibromoethane conversion in the wild type enzyme. In both mutants, the rate of bromide release increased substantially, resulting in a two to three-fold higher steady-state k_{cat} for 1,2-dibromoethane. Since halide release had become much faster, hydrolysis of the alkyl-enzyme intermediate was now rate-limiting for 1,2-dibromoethane conversion in these mutant enzymes. An increase in k_{cat} was not found for 1,2-dichloroethane conversion although the rate of chloride release increased. This was caused by a significantly lower rate of alkylation (k_2), which became rate-limiting for the Phe172Trp enzyme, resulting in a steady-state k_{cat} for 1,2-dichloroethane in this mutant that was even lower than with the wild type enzyme. The rate of substrate binding was also lower in the mutant enzymes than in the wild type dehalogenase.

Phenylalanine 172 and valine 226 make up part of the hydrophobic wall lining the active site cavity of the dehalogenase. We have proposed in *Chapter 5* that the characteristics of the halide release kinetics found for the Val226Ala enzyme are mainly caused by repositioning of Phe172 by the Val226Ala mutation since in the wild type enzyme these two residues are in close contact. This is in agreement with the observation that the Val226Ala enzyme and the Phe172Trp enzyme show similar properties with respect to the

kinetics of halide release. The X-ray structure of the Phe172Trp dehalogenase indicates that the helix (helix 4, 159-161)-loop-helix (helix 5, 171-181) structure which is part of the cap domain and covers the active site of the dehalogenase, could be more flexible in this mutant than in the wild type enzyme. This would result in an improved accommodation of large substrates in the hydrophobic active site cavity, but less efficient binding of small hydrophobic substrates. The stabilization of the transition state during carbon-halogen bond cleavage could also be weakened by increased flexibility, e.g. by disturbing a specific interaction between the Cl_β of 1,2-dichloroethane and position 172 that is seen in the alkyl-enzyme of wild type enzyme.

An interesting feature of the Phe172Trp enzyme is the 10-fold higher k_{cat}/K_m for 1-chlorohexane compared to the wild type enzyme. Both the k_{cat} and K_m were improved. The wild type dehalogenase has a very low activity for 1-chlorohexane and the absence of a solvent kinetic isotope effect indicated that for this substrate the rate-limiting step is before hydrolysis of the alkyl-enzyme intermediate (*Chapter 4*), most likely cleavage of the carbon-halogen bond. How 1-chlorohexane is bound is unknown, but that a more flexible active site cavity as suggested by the Phe172Trp X-ray structure increases the binding energy of the substrate in this mutant is likely. Furthermore, bound substrate may be in a more reactive position, increasing the rate of formation of the covalent intermediate. A more detailed kinetic analysis of 1-chlorohexane is difficult to perform due to the low solubility of the compound in water.

The Phe172Trp enzyme and spontaneous cap domain mutations in haloalkane dehalogenase that were selected *in vivo* on 1-chlorohexane in our laboratory (Pries et al., 1994b) have in common that they have an increase in activity and affinity for 1-chlorohexane. No X-ray structures have been obtained so far for these cap domain mutants. The diverse nature of the cap domain mutations and their similar effect on 1-chlorohexane conversion indicate a rather global effect, such as an increase in flexibility of a part of the cap domain and/or increase in active site cavity size (Pries et al., 1994b, thesis K. H. G. Verschueren, chapter 7). The X-ray structure of the *in vitro* constructed Phe172Trp dehalogenase may serve as a model for the *in vivo* obtained cap domain mutants in a sense that in both cases global effects, i.e. the loss of stabilizing interactions in the helix-loop-helix region between residues 159-181 explain the increased flexibility.

The Route of Halide Release. In the dehalogenase mutants studied in this thesis, the rate of halide release increased significantly due to an elevated rate of the enzyme isomerization preceding actual release ($E_I.X \rightarrow E_{II}.X$, Scheme I). We have hypothesized for the wild type enzyme that this isomerization is a step necessary to allow water to enter the buried active site cavity and solvate the charged halide ion. A possible route could involve a tunnel extending from the active site towards the solvent, as suggested by X-ray crystallography (Verschueren et al., 1993a). This tunnel was also proposed to be used for substrate binding, which we found to be extremely fast, however. Probably, release of the polar alcohol product out of the hydrophobic active site cavity is also fast since the alcohol was never observed in the X-ray structure and alcohols bind poorly to the enzyme. The proposed tunnel seems to be blocked by Leu262, and if substrate binding and product release occur *via* this route Leu262 has to move away (Verschueren et al., 1993a). However, the kinetics of substrate binding and halide release are fundamentally different. Halide release is much slower, although a halide ion is smaller than the corresponding dihaloethane. Since halide ions cannot easily diffuse through a hydrophobic protein environment and since the process requires a

slow enzyme isomerization it is likely that a more extensive motion is involved.

A possible route for halide release could be formed by a conformational change in a part of the cap domain that shields the active site from the solvent. This may expose part of the active site, allowing water to enter and solvate the halide ion. The effect of the mutations Val226Ala and Phe172Trp was that the rate of the enzyme isomerization increased drastically and suggested that the conformational change could be located in the helix-loop-helix structure covering the active site. The possible increase in flexibility of this helix-loop-helix structure of the Phe172Trp enzyme supports the idea that motions required for halide release take place there. The effects of the mutations and solvent isotope effects (*Chapter 3*) also support a larger conformational change than the movement of a single residue in the interior of the enzyme such as Leu262. Large conformational changes that accompany substrate binding have also been observed in lipases (Tilbeurgh et al., 1993) and aldose reductase (Rondeau et al., 1992; Grimshaw et al., 1995). However, in these cases the enzyme switches from an open to a closed conformation, which is different from rapid transient events that occur in haloalkane dehalogenase.

The loss of the only hydrogen bond between helix 4 and 5 ($Thr173O_{\gamma 1}$-Val165O) in the Phe172Trp enzyme indicates that the route for halide release could involve movement of these two helices. Furthermore, the X-ray structure of the Phe172Trp enzyme showed that the two tryptophan residues between which the halogen-moiety of the substrate and the halide is bound were at nearly identical positions in mutant and wild type enzyme, indicating that altered positions of these tryptophans were not responsible for the large difference in rate of halide release. Clearly, more information is necessary to understand the nature of the proposed conformational change.

Connections Between Haloalkane Dehalogenase Reaction Steps. The increase in the rate of halide release in the mutant dehalogenases was always accompanied by a decrease in the rate of substrate binding and the rate of cleavage of the carbon-halogen bond. The rate of hydrolysis of the alkyl-enzyme intermediate, however, was hardly affected. This 'uncoupling' between the rates of alkylation and dealkylation in haloalkane dehalogenase is allowed since these two processes are not the reverse of each other, unlike acylation and deacylation in the serine proteases (Stryer, 1988) and in α/β-hydrolase fold enzymes that have a Ser as the nucleophile (Ollis et al., 1992). Alkylation occurs by displacement of the halogen on the C_α of the substrate with a carboxylate oxygen of Asp124, while dealkylation occurs by attack of activated water on the C_γ of alkylated Asp124 (Verschueren et al., 1993b; Pries et al, 1994a, 1995b). Furthermore, dealkylation is largely determined by the main domain of the enzyme. Activation of a water molecule by the Asp260-His289 couple and stabilization of the tetrahedral intermediate by the oxyanion hole are all functions provided mainly by main domain residues located at the hydrophilic bottom of the active site (Verschueren et al., 1993b) which are not strongly influenced by mutations that affect halide release.

The observed decrease in the rate of substrate binding and carbon-halogen bond cleavage and the increase in the rate of halide release in the mutants suggests that these steps are linked in the dehalogenase reaction. The proposed involvement of the helix-loop-helix structure that covers the active site cavity of the enzyme in these processes can explain this observed coupling. An increase in the flexibility of this region would facilitate the conformational change required for halide release, but destabilize bound substrate and the transition state during carbon-halogen bond cleavage, resulting in a net increase in both k_{cat} and K_m for 1,2-dibromoethane conversion. A

rigid hydrophobic active site cavity would stabilize smaller hydrophobic substrates, but also the halide product. Especially for 1,2-dichloroethane the rigidity of the active site of the dehalogenase seems to be important for substrate binding and carbon-halogen bond cleavage. The kinetic mechanism of the wild type enzyme seems to be optimized towards 1,2-dichloroethane conversion in the sense that there is an optimal balance between the rates of steps that cannot be changed independently. This makes elevating the k_{cat} of the dehalogenase towards a compound like 1,2-dichloroethane a difficult and complex task. We found that a significant increase in k_{cat} could still be accomplished for the substrates 1,2-dibromoethane and 1-bromo-2-chloro-ethane, both 'nonnatural' substrates in which the balance between C-X cleavage and X-release is not optimal.

Prospects for Engineering. The studies described in this thesis showed that simple point mutations can result in an increase in the catalytic rate of haloalkane dehalogenase for specific substrates such as 1,2-dibromoethane, and an increase in the affinity and catalytic rate for 1-chlorohexane. However, an increase in the k_{cat} for shorter halogenated alkanes was accompanied by an increase in K_m, for reasons discussed above.

But what is the rate constant of haloalkane dehalogenase that should be improved in order to make the enzyme a more efficient biocatalyst for the degradation of halogenated compounds? The concentration of 1,2-dichloroethane at spillage sites can range from 20 to 150 μM and has to reduced to environmentally acceptable values of below 0.1 μM (Stucki and Thüer, 1995). At these low 1,2-dichloroethane concentrations, the conversion rate is depending on the first-order rate constant k_{cat}/K_m, which is for a four-step reaction mechanism (Scheme II in *Chapter 4*) equal to $k_1k_2/(k_{-1}+k_2)$. Engineering of the dehalogenase towards more efficient conversion of low concentrations 1,2-dichloroethane should thus not focus on t slow step under saturating 1,2-dichloroetha concentrations, i.e. halide release, but on ti rate of substrate binding and cleavage of ti carbon-halogen bond.

Substrates poorly converted by the wi type enzyme such as 1,2-dichloropropane ar 1-chlorohexane have their rate-limiting step(before hydrolysis of the alkyl-enzym intermediate (*Chapter 4*). Longer substrate such as 1-chlorohexane do not fit in the activ site of the wild type dehalogenase, but a goo substrate such as 1-bromohexane indicates tha even the wild type enzyme possesse considerable flexibility to accommodate suc larger substrate molecules. Improve degradation of the longer substrat 1-chlorohexane was accomplished b increasing the flexibility of the cap domai even more (*Chapter 6*; Pries et al., 1994b). A more precise definition of the interactions tha determine the flexibility of the cap and thei effect on binding of larger substrates could hel fine tuning the dehalogenase towards thes longer substrates.

In contrast, for shorter halogenate compounds such as 1,2-dichloropropane an dichloromethane it is likely that the flexibilit of the hydrophobic substrate binding site in th wild type enzyme is too large for stron binding of these substrates and for lowering th energy of the transition state during carbon chlorine bond cleavage. Sterically, thes substrates do fit in the active site, as shown b the high activity with the brominated analog 1,2-dibromopropane and dibromomethane Decreasing the flexibility of the helix-loop helix structure that forms part of th hydrophobic substrate binding site migh improve the affinity for such shorte chlorinated substrates. This could b accomplished by introducing additiona interactions between the main and the ca domain of the enzyme. For example, th surface located salt bridge (Lys261N_ζ

Asp170$O_{\delta 2}$) which is located in the loop of the helix-loop-helix structure may be modified to a disulfide bridge by introduction of Cys at these positions. This might reduce the flexibility of the substrate binding site. However, it may also impair substrate binding as Asp170 and Lys261 are located at the entrance of the proposed tunnel (Verschueren et al., 1993a). Additional interactions between helix 4 and 5 might also provide more stability. Besides stabilization of the helix-loop-helix structure, introduction of additional hydrophobic residues in the active site cavity could also reduce the mobility for dichloromethane. Such an approach has been successful for pentachloroethane conversion by P450cam. A molecular dynamics simulation showed that a P450 mutant with a more bulky hydrophobic residue reduced the mobility of pentachloroethane in the active site cavity and created more 'active conformations' close to the heme (Manchester and Ornstein, 1995a). This mutant had a three-fold higher conversion rate for pentachloroethane than the wild type enzyme.

It is clear that additional knowledge about the dehalogenase reaction is necessary. What is the nature of binding of other substrates than 1,2-dichloroethane? How do substrates enter? What does the proposed conformational change look like and how is it related to the substrate binding site? How are the transition states during the reaction stabilized? These questions need to be answered before starting real design of the dehalogenase, but the work in this thesis has identified new directions to pursue for improving the dehalogenase activities for specific substrates.