SUMMARY AND CONCLUDING REMARKS

Summary
Halogenated carbon compounds represent a major class of recalcitrant environmental pollutants. Even for many of these often xenobiotic chemicals, however, the highly dynamic and adaptive microbial world has managed to develop suitable degradation pathways. A particular critical step in the degradation pathway of organohalogens appears to be the cleavage of carbon-halogen bonds. Several distinct mechanisms have been identified for this reaction (Fetzner, 1998) and they may serve as a base for the development of new degradation pathways (Bosma *et al.*, 2002). Haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* (Janssen *et al.*, 1985) appears to be an attractive candidate for the biodegradation of halogenated aliphatic compounds, since in addition to its preferred substrate 1,2-dichloroethane (DCE), it is capable of converting a broad range of brominated and chlorinated alkanes *via* a simple hydrolytic mechanism (Keuning *et al.*, 1985, Schanstra *et al.*, 1996b). The structure and mechanism of DhlA have been characterized extensively in the past (Franken *et al.*, 1991, Ridder *et al.*, 1999, Verschueren, 1993). The enzyme consists of an alpha/beta-hydrolase main domain (Ollis *et al.*, 1992), with an additional alpha-helical cap domain covering the hydrophobic active site. The catalytic triad located in this cavity is composed of Asp124-His289-Asp260 and its action is supported by stabilizing interactions of hydrogens bound to the indole nitrogens of Trp125 and Trp175. The exact positioning of the substrate in a low-dielectric environment and the stabilization of the leaving anion in the S_N2 displacement reaction are critical factors for substrate conversion (Devi-Kesavan & Gao, 2003, Hur *et al.*, 2003, Lau *et al.*, 2000, Lightstone *et al.*, 1997, Lightstone *et al.*, 1998). The reaction proceeds via the formation of a covalent alkyl-enzyme intermediate, which is hydrolyzed by an activated water molecule (Verschueren *et al.*, 1993b) (Fig. 1). The main rate-limiting step in the conversion of DCE, however, is halide release (Schanstra *et al.*, 1996b). Halide transport occurs *via* two distinct routes, which operate in parallel and have been subjected to detailed kinetic and thermodynamic characterization (Fig. 2) (Krooshof *et al.*, 1999, Schanstra & Janssen, 1996). When halide binding is measured, at low concentrations the 'upper' route is prevailing. This route involves a slow enzyme isomerization followed by a rapid binding step. At increasing concentrations, halide import shifts to the 'lower' route, which proceeds via a fast bimolecular binding

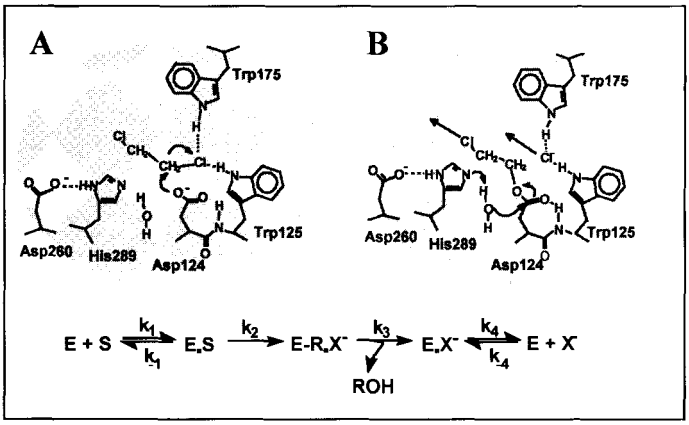

Fig. 4. Catalytic mechanism of haloalkane dehalogenase.
A. Cleavage of the carbon-halogen bond. **B.** Hydrolysis of the covalent intermediate.

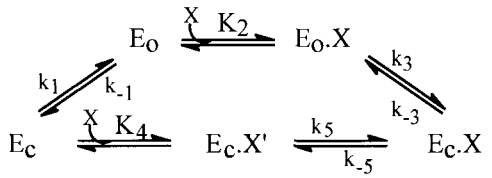

Fig. 2. Halide binding and release occurs via two parallel routes. The "upper route" involves a conformational change ($E_c \rightarrow E_o \rightarrow E_c$) and is the main route at low halide concentrations, at higher concentrations transport via the "lower route" becomes more important. This route involves the formation of a surface located collision complex.

step, followed by a slow isomerization. Under normal reaction conditions the export of halide ions will mainly occur via the 'upper' route. Chapter 2 of this thesis focuses on the nature of the initial enzyme-substrate complex in the 'lower' route. Analysis of the X-ray structure in the presence of high bromide concentrations revealed two additional weakly bound ions at the surface of the enzyme. One of them is located close to the proposed substrate entrance tunnel, at the interface of the cap and main domain (Verschueren *et al.*, 1993a). Two of the residues involved in this surface-located binding site, Phe294 and Thr197, were mutated to alanine. Stopped flow fluorescence binding studies of these mutants established the elimination of the binding site, simplifying the lower route into a simple bimolecular association exhibiting a linear concentration dependence. The combination of X-ray crystallography and kinetic analysis thus revealed that the initial enzyme-substrate complex in the 'lower' route comprised a weak transient collision complex and eluded its exact location.

Chapter 3 and 4 describe a molecular dynamics simulation (MD) of DhlA and the characterization of a disulfide bond mutant that was constructed based on these MD observations. The MD simulation in chapter 3 showed high mobility in a particular helix-loop-helix region in the cap domain, involving residues 184-211. In order to stabilize this region, it was cross-linked to the main domain by the introduction of two cysteines that can form a disulfide bond between of the cap domain and the main domain. The positions of cysteine residue 201 and 16 were determined using the computer program SSBOND (Hazes & Dijkstra, 1988). The constructed double mutant contained a very stable disulfide bond and the oxidized form showed a significantly enhanced stability towards thermal and urea denaturation. Introduction of the disulfide bond also enhanced the kinetic stability of DhlA, indicating that the flexible region identified in the MD simulations, is probably involved early in the unfolding process. In general, the results show that MD simulations are capable of identifying targets for stability enhancement.

Apart from an enhanced stability, the Asp16Cys/Ala201Cys mutant also was the first mutant that showed an improved catalytic efficiency towards DCE. So far the only mutants that showed increased activity toward DCE were Val226Ala (Schanstra *et al.*, 1997) and Pro168Ser (Krooshof, 2000), but in these cases the specificity constant k_{cat}/K_m turned out to have decreased because of a major increase in K_m. The specificity constant for DBE for these particular mutants on the other hand did improve. We could not find an obvious structural explanation for the improved catalytic function of the disulfide bond mutant, but in chapter 4 the kinetic basis is elucidated. Stopped-flow fluorescence halide binding measurements revealed an increased rate of halide export. Additionally, the increase of k_{cat} toward other small chlorinated substrates, for which halide export is not rate limiting, indicated higher rate of cleavage of carbon-chlorine bonds. These combined effects resulted in a unique mutant with improved catalytic efficiency towards DCE, but a virtually unaffected efficiency towards the brominated equivalent.

For rational design aimed at improving enzyme characteristics, extensive knowledge on structure-activity relationships is required. In many cases such detailed information is not available and even when it is, accurate prediction of the precise effects of a mutation often proves

to be impossible. For these reasons *in vitro* directed evolution coupled to phenotypic screening methods presents an attractive alternative to rational design. This approach has turned into a key technology since convenient shuffling methods have become available that enable the *in vitro* recombination of genes. Chapter 5 describes the development of a mutagenesis method that provides an effective tool for further expansion of the explorable sequence space.

The presence of a direct and indirect repeat in the cap domain of DhlA suggests that the adaptation to DCE has occurred by the generation of segmental mutations in the cap domain. Former *in vivo* experiments also support that this type of mutation played an important role in the adaptation of DhlA to other substrates (Pries *et al.*, 1994). Chapter 5 presents a method that enables the *in vitro* generation of this type of mutations. Deletions and repeats arise from the pairwise fusion of two gene fragments that are truncated by exonuclease BAL-31 at either their 3' or the 5' side. The library was generated from a constructed hypothetical ancestor protein, lacking the wild-type repeats and no longer capable of converting DCE. Screening revealed ten individual mutants with increased activity towards 1,2-dibromoethane (DBE), six of them were also capable of hydrolyzing DCE. This new *in vitro* mutagenesis method complements the directed evolution toolbox and might release DhlA from its presumed evolutionary local optimum.

Conclusions

Rational enhancement of catalytic activity.

Over the years, enhancement of DhlA catalytic activity for its natural substrate DCE by protein engineering has proven to be a very difficult task. This is mainly caused by the so-called cavity dilemma (Krooshof, 2000). The conversion of small hydrophobic substrates requires a very precise positioning of the substrate and strong stabilization of the leaving group in the transition state. DhlA appears to have adapted to DCE by evolving an active site that is located in a small internal cavity, forming a tight hydrophobic reaction chamber, shielded from the solvent (Verschueren *et al.*, 1993a). This enables the nucleophile and the electrophile to approach in a low-dielectric environment in an orientation that allows the reaction to occur without much structural reorganization (Lightstone *et al.*, 1997, Lightstone *et al.*, 1998). Stabilization of the leaving group is provided by hydrogen bond interactions with the indole nitrogens of Trp125 and Trp175, and to a lesser extend with Phe172 (Damborsky *et al.*, 1997, Lau *et al.*, 2000). They bind the halogen moiety of the substrate and also form a binding site for the released halide ion. Compared to DhlA, the closely related dehalogenases DhaA and LinB exhibit a much larger and more open active site with a less defined halide-binding site (Marek *et al.*, 2000a, Newman *et al.*, 1999, Oakley *et al.*, 2002), which clearly explains their preference for much bulkier substrates (Damborsky & Koca, 1999, Nagata *et al.*, 1997).

Although this cavity concept provides an optimal micro-environment for the conversion of the substrate, it suffers from the disadvantage of complicating the exchange of substrate and product, since the reaction chamber is isolated from the solvent. The small uncharged alkylhalide substrates and their alcohol products are probably able to diffuse more or less freely through the enzyme. For the charged halide ion, however, the cavity acts as a trap, requiring an intricate structural mechanism to enable solvation by water molecules and subsequent export (Krooshof *et al.*, 1999, Schanstra & Janssen, 1996). For the most favorite substrates of DhlA, DCE and DBE, halide release has even become the rate-limiting step (Schanstra *et al.*, 1996b). Ironically, increasing halide transport appears to be very simple, as will be discussed in more detail in the next paragraph, it simply requires a destabilization (or increased flexibility) of the cap domain, the 'lid' that covers the active site cavity. However, the enzyme appears to have evolved a delicate balance between carbon-halogen bond cleavage and halide release. Mutations promoting halide

	1,2-dibromoethane			1,2-dichloroethane		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
Wild type	3.0	0.010	3.0×10^5	3.3	0.53	6.2×10^3
Val226Ala	8.2	0.033	2.5×10^5	3.8	1.5	2.5×10^3
Phe172Trp	5.9	0.025	2.4×10^5	2.9	5.13	5.6×10^2
Phe172Tyr	5.3	0.12	4.4×10^4	1.5	4.96	3.0×10^2
Trp175Tyr	5.0	0.06	8.5×10^4	0.33	2.85	1.0×10^2
Pro168Ser	5.8	0.06	9.7×10^4	4.8	2.5	1.9×10^3
Asp16Cys/ Ala201Cys	3.0	0.010	3.0×10^5	4.7	0.5	8.9×10^3

Table 1. Steady-state kinetic parameters of wild type and mutant haloalkane dehalogenases

transport always appear to have a negative effect on the preceding reaction steps (Krooshof *et al.*, 1998, Schanstra *et al.*, 1996a, Schanstra *et al.*, 1997). Several factors are believed to contribute to this effect. The binding energy of the substrate, which is an essential factor contributing to the catalytic power of the enzyme (Lightstone *et al.*, 1997), may be reduced by the increased flexibility, thereby lowering the bimolecular binding rate. The electrostatic contributions of the cap domain residues Trp175 and Phe172 to the stabilization of the transition state probably have diminished,resulting in a less efficient C-X bond cleavage and also hydrolysis of the covalent intermediate often becomes more difficult (Lau *et al.*, 2000, Lightstone *et al.*, 1998).

In the past, several mutations were found to enhance the specific activity towards the best known substrate, DBE. Val226Ala (Schanstra *et al.*, 1997), Phe172Trp and Phe172Tyr (Schanstra *et al.*, 1996a), Trp175Tyr (Krooshof *et al.*, 1998) and Pro168Ser (Krooshof, 2000) all showed a significant increase in k_{cat} for DBE (Table 1), but at the same time suffered from a negative effect on K_m values, which resulted in a decreased catalytic efficiency k_{cat}/K_m. Only two of these mutants, Val226Ala and Pro168Ser, also showed a higher k_{cat} for DCE. This reflects the fact that chlorine is a poorer leaving group and requires a stronger stabilization for the reaction to proceed, so in general it will be more difficult to enhance the enzyme towards DCE. Although all the mutants mentioned in table 1 showed strongly enhanced halide release rates, overall k_{cat} values only marginally increased, indicating the mutations always appear to slow down other steps in the mechanism. In view of this history of failing attempts to enhance DhlA towards its natural substrate DCE, the improved k_{cat}/K_m of the disulfide mutant Asp16Cys/Ala201Cys (Chapter 4) appeared as a surprise. This mutant manages to enhance k_{cat} without affecting K_m. The mutant showed enhanced halide release rates, but also a higher rate of cleavage of carbon-chlorine bonds, which was indicated by the increase of k_{cat} toward other small chlorinated substrates. It is interesting to note that it would have been very unlikely for this disulfide mutant to have evolved *in vivo*, since the introduction of this disulfide requires two independent mutations that are not beneficial by themselves. Although this mutant proves that enhancement is possible, the result is only small and the chances of designing other mutations that can significantly enhance the activity of this enzyme for DCE are probably very limited.

Halide transport

Chapter 2 of this thesis unveils the nature of the so-called 'lower' route, one of the two parallel routes haloalkane dehalogenase can use for halide transport (Schanstra & Janssen, 1996). The 'upper' route, which prevails at low concentrations, however, still remains enigmatic. From kinetic analysis we know that the actual halide release is preceded by a slow isomerization step, that shows a significant solvent kinetic isotope effect (Schanstra &

Janssen, 1996). Unlike the uncharged substrate and the alcohol product, release of the halide requires solvation of the charged ion prior to transport. In the paper dealing with the kinetics of halide binding of DhlA (Schanstra & Janssen, 1996) several suggestions were done concerning the nature of the isomerization. One of the possibilities was that it could involve a specific export route assisted by charged residues, as is common in transport via membrane ion channels. The most obvious candidate for this would be Lys176, since this is the only positively charged residue that is in the near vicinity. Mutating Lys176 to valine, serine, glutamate and arginine had no significant influence on k_{cat}, but for all mutants the k_{obs} values for bromide binding appeared to be higher compared to the wild type enzyme (Pikkemaat, unpublished results). However, at low concentrations k_{obs} still showed the characteristic initial decrease upon increasing concentrations, showing that the mutations did not eliminate transport via the upper route and establishing that Lys176 is not involved in the 'upper' route.

Another hypothesis concerning the physical route for halide export involved a small tunnel connecting the cavity with the solvent (Verschueren *et al.*, 1993a). The entrance is formed by Lys259, Asp260 and Lys261, but the tunnel seems to be blocked by Leu262. The flexibility of this side chain, however, is believed to allow effective transport through this tunnel. Residues Lys259 and Lys261 were also identified as a potential halide ion binding sites by calculation of molecular interaction fields (GRID) (J. Damborsky, pers. comm.). Removing the positive charge of these residues by mutating Lys259 to leucine and Lys261 to isoleucine had no significant effect on k_{cat}. Similar to the Lys176 mutants these mutations also did not eliminate transport via the 'upper' route (Pikkemaat, unpublished results). The concentration dependence of k_{obs} for Lys259Leu was very similar to wild type, but Lys261Ile showed much higher k_{obs} values. This is probably caused by an increased flexibility of the cap domain, since this mutation causes the disruption of the salt bridge between Lys261 and Asp170, one of the few stabilizing interactions between the main and the cap domain. Although the tunnel does not seem to be involved in halide transport, it would be interesting to find out whether it has a physical function in the catalytic process, for example by mutating the obstructing Leu262 to a smaller residue.

Alternatively it was suggested that the kinetically observed isomerization might involve a conformational change of helices in the cap domain (Schanstra & Janssen, 1996), similar to the conformational changes that occur during the interfacial activation of several lipases, another representative group within the α/β-hydrolase fold family (Jaeger *et al.*, 1994). Displacement of one or more helices could expose the buried active site to the solvent and allow water to solvate the halide ion. Thermodynamic analysis of halide binding confirmed that the isomerization is associated with large movements (Krooshof *et al.*, 1999). The possibility that the flexible area identified by the MD simulations (helices 6 and 7, res.185-211, chapter 3 of this thesis) would be involved was ruled out, since stabilizing the loop by the introduction of a disulfide bond did not significantly affect halide binding (chapter 4 of this thesis).

Several lines of evidence suggest that the conformational change might involve helices 4 and 5 (res. 159-181). This was first proposed by Schanstra *et al.* based on the structure and kinetic properties of the Phe172Trp mutant (Schanstra *et al.*, 1996a). This mutant shows strongly enhanced halide release rates, which were attributed to an increased flexibility in this particular helix-loop-helix region. Supporting evidence was provided by another mutagenesis study, that demonstrated the involvement of Trp175 in the upper route (Krooshof *et al.*, 1998), furthermore a *cis-trans* isomerization of Pro168 is presumed to be involved or might even trigger the conformational change in helices 4 and 5 (Krooshof, 2000). Although the aforementioned papers seem to provide a strong

indication that helices 4 and 5 are somehow responsible for the isomerization in the 'upper' route, the evidence is far from conclusive. Other (non-published) results of mutagenesis studies indicate that virtually every mutation in the cap domain causes a small or large accelerating effect on the 'upper' route. In view of the cavity dilemma, this observation is well explainable. Since the cap domain exhibits a carefully balanced structural stability, it is easily disturbed by mutations, thereby spontaneously facilitating halide release. Thus, any suggested evidence concerning mutations in the cap domain has to be treated with great caution. More conclusive evidence can only be obtained via the opposite approach: establishing a mutation that abolishes the 'upper' route. An attempt to achieve this by introducing a disulfide bond connecting helices 4 and 5 to the main domain failed. Substitution of the wild type salt-bridge between Asp170 and Lys261 by a disulfide bond as well as the closest match provided by the SSBOND program (Hazes & Dijkstra, 1988), Gly171Cys/Lys261Cys, both revealed mutant enzymes that were very unstable and suffered from disulfide bond mismatching (H. Wijma, unpublished results).

A covalent link would obviously represent the most straightforward approach, but maybe more subtle methods like introducing saltbridges or hydrogen bonds between the two domains, or removing unfavorable interactions in the mainly hydrophobic contact area, can also succeed in overruling the destabilizing effect of a cap domain mutation and diminish or even completely block the upper route. For this approach to be successful, more sophisticated modeling programs, preferably the availability of an SSBOND-like program for introduction of other types of interactions, are needed.

Directed evolution

As already mentioned in the introduction, directed evolution is rapidly evolving as a powerful alternative for rational design (Ness *et al.*, 2000). Screening large libraries of randomly mutagenized genes and combining the beneficial mutations, or direct shuffling of two or more closely related genes, allows a rapid accumulation of desired mutations without the requirement of structural information. Enzymatic properties such as substrate specificity, enantioselectivity, thermostability, solvent or protease resistance and protein solubility can be altered in a predefined direction, making the engineered enzymes more suitable for industrial or pharmaceutical applications.

Chapter 5 of this thesis presents a strategy for the random introduction of repeats and deletions aimed at introducing DCE hydrolyzing capacity in a primitive form of DhlA. One of the key factors in the success of this directed evolution experiment, was the switch to another expression system. The original expression vector, pGELAF(+), designed by Schanstra et al., is a pET-3d derived vector in which the gene is under control of a T7 promoter (Schanstra *et al.*, 1993). The expression in *E. coli* BL21(DE3) is induced by IPTG. A major drawback of this vector-host combination is the instability of the system. Because of the leakiness of the promoter, even uninduced cell cultures produce significant levels of protein. Due to some unknown recombination event, the cells manage to disrupt the expression while retaining the ampicillin resistance. In case of high-yield production purposes the problem can easily be circumvented by using freshly transformed cells and start off with a sufficiently high cell density, but for directed evolution experiments it appeared impossible to obtain reproducible results with this particular expression system. Another problem, which is probably caused by the high expression levels that can be reached, is the formation of inclusion bodies. Screening for colonies with an apparently higher activity could easily result in selecting mutants with improved solubility, which can also be considered an interesting aim, rather than enhanced catalytic activity. Formation of inclusion bodies could partially be prevented by lowering the growth temperature after induction, but it would be much more convenient to employ a system with more controllable expression levels. For the directed evolution experiments we therefore decided to switch to a more reliable and stable system, the pBAD expression system

(Invitrogen). This system is composed of a pUC derived vector and allows expression under control of the *araBAD* promoter, which regulates the arabinose metabolic pathway in *E. coli*. In combination with *E. coli* TOP10, a strain capable of transporting arabinose, but not metabolizing it, the system provides a tightly regulated, dose-dependent expression.

The original solid state assay for detecting haloalkane dehalogenase activity was based on the use of broomthymolblue as a pH indicator (Schanstra *et al.*, 1993). Acidification caused by the dehalogenation reaction will turn the blue colonies into yellow and they are surrounded by halos. A major complication of such a pH-based detection system is that it suffers from diffusion and subsequent risks of signal overlap between neighboring colonies. In this respect, the solid state assay based on methylene blue and eosin B (chapter 5), forms an attractive alternative, since indicator action depends on mobilization of the dyes at low pH and their movement into the acid-producing colonies (Loos, 1975). In the current experimental set up mutants with a DCE conversion rate about ten times lower than that of the wild type could not be identified, so for many potential applications involving worse substrates, the sensitivity of the system will have to be improved. In chapter 5 the mutant libraries were screened by simple visual inspection of the color developing in the colonies. Digital and kinetic imaging would probably provide a much more accurate detection method, likely to enhance resolution significantly. Combined with a new microcolony method also a large capacity increase will be achievable (Delagrave *et al.*, 2001).

One could also consider alternative screening methods. The most effective types of assays are probably growth-based selection systems, in which the host is only able to grow in the presence of the desired reaction product or the enzymatic activity that is under selection. However, at this stage one is stuck to *E. coli* as a host organism for screening large libraries. The problem with a growth-based assay for dehalogenase screening in *E. coli* is, that this bacterium is not capable of using the hydrolysis product as carbon and energy source, unless an additional pathway for further conversion into components supporting central metabolic pathways is also introduced. This could, for example, involve an alcohol dehydrogenase → aldehyde dehydrogenase → haloacid dehalogenase, analogous to the system the original *Xanthobacter* uses to degrade DCE (Janssen *et al.*, 1985). However, this approach not only requires a lot of additional engineering, various factors can play a role in the success of such an engineered system: the balance between the expression levels of the different enzymes in the pathway, unforeseen toxicity of intermediates, substrate spectra of the separate enzymes etc.

A potential alternative is to use a host that is already capable of growth on the product of the desired conversion. Theoretically this approach is much more attractive, but due to a much lower transformation efficiency, the applicability of organisms other than *E. coli* is limited to small libraries. For haloalkane dehalogenase such a growth based selection system is available. In the past several *Pseudomonas* strains have been isolated, which are capable of growing on a variety of haloalcohols (Strotmann *et al.*, 1990, Stucki & Leisinger, 1983). Introducing a library of DhlA mutants in a suitable strain, i.e. a strain that can use the alcohol product of the substrate of interest as a carbon and energy source, would allow screening for DhlA variants that can use new substrates. This growth-based selection principle was already successfully employed *in vivo* for adaptation of wild type DhlA to chlorohexane and yielded several mutants with enhanced activity towards chlorohexane (Pries *et al.*, 1994). A similar attempt to adapt the putative ancestor DhlA (chapter 5), which is incapable of hydrolyzing DCE, *in vivo* to this substrate, however, was not successful (Pikkemaat, unpublished results). As already mentioned in chapter 5, the first step in the evolutionary process from the 'primitive' form to wild type DhlA, the introduction of the initial repeat,

probably yields a mutant with only a very low activity towards DCE, which may be too low to support growth of the *Pseudomonas* host. Apart from the apparently inadequate sensitivity, the use of this growth-based selection method for screening large libraries is also hampered by the transformation procedure, since it requires a laborious and inefficient triparental mating step. When these problems could be overcome, however, the method would represent a very attractive alternative. Since there are many proteins of industrial or pharmaceutical interest that cannot be functionally expressed in *E. coli*, it is to be expected that in the near future also more efficient transformation procedures for hosts other than *E. coli* will be developed.

The main conclusion concerning the catalytic enhancement of DhlA for DCE, is that it will be very hard, if not impossible, to improve the enzyme by rational design. The most obvious alternative would be a directed evolution approach. Random mutagenesis and subsequent screening of huge numbers of transformants, however, did also not yield significantly improved mutants for 1,2-dichloroethane. Screening 50,000 colonies of a library generated using an *E. coli* mutator strain yielded only a mutant with a lower pH optimum (Chang *et al.*, 1999). Analysis of over 100,000 colonies of an error-prone PCR library prepared in our own lab, also failed to identify mutants with enhanced catalytic properties toward DCE (Pikkemaat, unpublished results). These results are very remarkable. Considering the recent appearance of DCE in the biosphere and the relatively poor catalytic performance of the enzyme, one would expect the enzyme to be standing only at the beginning of its evolutionary track. In chapter 5 of this thesis, it is therefore suggested that DhlA might be trapped on a local evolutionary peak, which means that every single amino acid substitution will result in a less efficient protein. Although conclusive evidence for such a statement would require saturation mutagenesis on every amino acid residue in the wild type protein, the available random mutagenesis results ((Chang *et al.*, 1999) and Pikkemaat, unpublished) support the assumption that the DCE converting performances of DhlA cannot be enhanced by a simple point-mutation.
An intriguing strategy to release DhlA from this evolutionary pitfall could be to go back in its evolutionary history. There are strong indications that the adaptation of DhlA to DCE involved the introduction of a repeat in the cap domain (chapter 5). Since the putative ancestor enzyme will lack this repeat, it is not able to convert DCE (figure 3). Using a directed evolution approach, several randomly created segmental mutants capable of hydrolyzing DCE were isolated, which all contained repeats in the same region of the cap domain. The length of the repeats ranged from 5 amino acids to as many as 21 and the exact location was also variable. It is difficult to speculate on the structural effects of the insertions since there are no 3D-structures available, but it is remarkable that all the repeats are located in front of Trp175 (wild type numbering). In the wild type enzyme, Trp175 is an important residue in the stabilization of the transition state. In the structures of the dehalogenases DhaA and LinB, which are not able to dechlorinate DCE, the stabilizing function of Trp175 seems to be fulfilled by phenylalanine residues (Marek *et al.*, 2000b, Newman *et al.*, 1999). Also in DhlA substitution of Trp175 does not always abolish dehalogenating activity, but it is affecting the activity towards DCE stronger than towards DBE (Kennes *et al.*, 1995, Krooshof *et al.*, 1998). Despite its inability to hydrolyze DCE, the putative ancestor enzyme is still catalytically active with DBE (app. 55% of wild type), so it is unclear whether Trp175 is still functional. Although only crystal structures of the segmental mutants can provide conclusive evidence, the inserted repeats might serve to push this tryptophan residue downstream and allow it to adopt a proper orientation to assist catalysis.

With the introduction of the particular repeat present in the wild type enzyme, DhlA seems to have run into a dead-end track, blocking the way for further improvement. It might therefore be worthwhile to go one step back in evolution, by

starting with one or more of the new sequential mutants isolated in chapter 5. Subjecting these mutants to additional rounds of random mutagenesis and shuffling, might liberate DhlA from its evolutionary trap and lead to a much more efficient catalyst. The putative ancestor of DhlA might also provide a useful scaffold for the adaptation towards other halogenated substrates, offering the possibility to develop custom-made enzymes for the detoxification of many xenobiotic compounds.

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