University of Groningen

Engineering proteins for environmental applications

Janssen, Dick B.; Schanstra, Joost P.

Published in:
Current Opinion in Biotechnology

DOI:
[10.1016/0958-1669\(94\)90026-4](https://doi.org/10.1016/0958-1669(94)90026-4)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1994

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Janssen, D. B., & Schanstra, J. P. (1994). Engineering proteins for environmental applications. *Current Opinion in Biotechnology*, 5(3). [https://doi.org/10.1016/0958-1669\(94\)90026-4](https://doi.org/10.1016/0958-1669(94)90026-4)

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Engineering proteins for environmental applications

Dick B Janssen and Joost P Schanstra

University of Groningen, Groningen, The Netherlands

Recently, significant new insight has been obtained into the structure and catalytic mechanism of enzymes that convert environmental pollutants. Recent advances in protein engineering make it possible to use this information for improving the catalytic performance of such enzymes to achieve increased stability and expanded substrate range.

Current Opinion in Biotechnology 1994, 5:253–259

Introduction

Biotechnological treatment of waste materials and bioremediation of polluted environments is based on the capacity of microorganisms to transform pollutants to non-toxic products. These microbial conversions require the expression of enzymes that have sufficient catalytic activity towards the compounds of interest. Many synthetic compounds are resistant to biodegradation, however, and persist as pollutants in the environment. The inability of microorganisms to degrade these compounds can be regarded as a problem of enzyme specificity and activity. If microorganisms lack a complete set of catabolic enzymes for mineralization of a particular synthetic compound via a pathway that allows generation of energy for biosynthesis, then that compound will remain recalcitrant in the environment. Interest in the enzymes catalyzing conversion of synthetic compounds has burgeoned with the realisation that several xenobiotics are ubiquitously present in the environment due to their slow rates of biodegradation. The recalcitrance to biodegradation of many chlorinated hydrocarbons and other xenobiotics has also limited the applicability of biotechnological treatment methods.

This review summarizes recently obtained information concerning the structure–function relationships of enzymes that transform xenobiotic compounds. It also discusses the prospects for improving enzyme characteristics, with a view to obtaining more effective detoxification catalysts.

Engineering proteins

With the advance of protein engineering, it has become possible to construct specific mutants of an enzyme on the basis of a prediction of the modifications needed to modify some specific aspect of enzymatic performance [1•,2•]. For proteins that play a role in the detoxification of xenobiotic compounds, characteristics that one would like to modify are catalytic performance (e.g. k_{cat} values, K_m values, and sensitivity to substrate or

product inhibition), resistance to reactive compounds and products, enzyme stability, and substrate range.

Protein engineering requires an understanding of the effect of modifications of primary sequence on the relevant biochemical characteristics of an enzyme [1•]. On this basis, the effect of mutations can be predicted and the mutants can be constructed by *in vitro* mutagenesis. The activity–sequence relationship is best obtained from a three-dimensional structure as determined by X-ray crystallography. Even so, only a few structures are currently available for enzymes catalyzing critical steps in xenobiotic compound degradation pathways [3••,4•,5–7,8••]. In many cases, mechanistic information about enzymes that degrade xenobiotics can also be derived from sequence similarities to enzymes of known structure or mechanism [3••,9–12]. Even without any insight into the structure, mutants may be constructed by careful comparison of activities and sequences between different enzymes [13,14••]. The increasing number of sequences of catabolic genes that are becoming available will extend the possibilities of this approach.

Considerable progress has been made in improving enzyme stability at high temperatures and extreme conditions [2•,15]. Several methods can be used. First, it is possible to construct specific mutants on the basis of a three-dimensional structure and established stabilizing principles, such as α -helix capping [16] and filling internal cavities, although the effect of the latter may differ from one enzyme to another [17,18]. Second, thermostable mutants can be obtained after random mutagenesis by screening methods based on increased enzyme stability, as has recently been accomplished with α -amylase [19]. Third, random mutagenesis may be followed by selection of stabilized enzymes in a thermophilic host organism using conditions under which increased thermostability confers a selectable growth advantage to the host organism. This method has recently been used to obtain a thermostable variant of chloramphenicol acetyltransferase [20].

Modification of the substrate specificity of degradative enzymes by protein engineering has also been achieved in a number of cases [21•,22,23]. Usually, this

is based on the transfer of substrate-binding determinants from one enzyme to another. By changing the binding pockets for peptide side chains, the substrate range of trypsin was converted to that of chymotrypsin, which cleaves carboxy-terminal of large hydrophobic residues instead of basic residues [21•]. In the same way, the specificity of yeast alcohol dehydrogenase was made more similar to that of mammalian alcohol dehydrogenase, which accepts substrates of longer chain length, by constructing a larger substrate binding site [22].

Methods for improving catalytic rates are still being developed. Some successes have been achieved [24–26], but a general strategy is not yet available. In principle, the modifications needed to accelerate the reaction rate of an enzyme can be derived from an examination of the kinetic mechanism, with identification of the individual kinetic steps that determine the velocity (k_{cat}) and affinity (K_{m}) of an enzyme for its substrate [27]. Calculation of the energy profile along the reaction pathway for a certain step might indicate residues, the modification of which may reduce the transition state energy of this step. Molecular dynamics simulations are essential for the identification of the relevant interactions between atoms of the enzyme and the substrate. These methods require detailed insight into the catalytic mechanism [28,29], which is, however, often not available for environmentally important enzymes. When structural information is lacking, but sequences are available, it may be possible to attribute high catalytic activity for a certain substrate to the presence of specific amino acids by comparison of sequences and activities of related proteins. This has been achieved with a nylon-oligomer hydrolase of a *Flavobacterium* sp. [13] and biphenyl dioxygenase [14••].

The substrate range and catalytic performance of catabolic enzymes can also be modified by *in vivo* selection of mutants, if necessary after performing random or semi-random mutagenesis. An enzyme with a novel substrate range may be selected by growing a microorganism on the substrate of interest, either in chemostat culture or in batch culture. This method was first used for obtaining *Pseudomonas* aliphatic amidase mutants that convert substrates with a longer alkyl chain length [30]. The structure of this enzyme is not known. Continuous culture selection has led to mutants of a *Methylophilus* amidase with improved kinetic constants toward acrylamide [31]. The activity of β -lactamase was modified in a similar way [32].

Environmentally important enzymes

As stated above, the recalcitrance of a xenobiotic compound is usually a result of its failure to be converted at one or more steps in a potentially useful catabolic pathway. Such steps may be initial attack on molecules that have structures that are very different from natural compounds, conversion of chemically reactive intermediates (e.g. as epoxides and aldehydes), and

dehalogenation reactions of inert chlorinated hydrocarbons. In various cases, recalcitrance can indeed be attributed to a specific enzyme in a pathway that lacks the required activity.

Recently, a number of new structures of enzymes involved in the metabolism of xenobiotic compounds have been solved. These include methane monooxygenase, haloalkane dehalogenase and cytochrome P450.

Haloalkane dehalogenase

Haloalkanes are widely used as solvents, degreasing agents, intermediates in chemical synthesis, and pesticides. The cleavage of the carbon–halogen bonds in these compounds is a critical step in their biodegradation. Cleavage can be mediated by enzymes, such as haloalkane dehalogenase or glutathione transferase [33,34•], that catalyze direct nucleophilic displacement.

The best studied dehalogenating enzyme is the haloalkane dehalogenase of *Xanthobacter autotrophicus*. It is a soluble enzyme, 310 amino acids in length, that is produced by several facultatively methylotrophic bacteria that degrade 1,2-dichloroethane [35]. The dehalogenase catalyzes the nucleophilic displacement of halogens from alkylhalides. Haloethers, haloalcohols and halonitriles are also substrates, but the affinity of the enzyme for these compounds is low.

The structure and the mechanism of catalysis have recently been investigated by X-ray crystallography [7,36••]. Catalysis proceeds by nucleophilic attack of a carboxylate oxygen of Asp124 (three-letter amino acid code) on the substrate, yielding a covalent alkyl–enzyme intermediate, in which the nucleophilic aspartate is esterified to a chloroethyl group [36••,37•]. For this step, the presence of two tryptophan groups is essential since they form the halide binding site [38]. The intermediate is subsequently hydrolyzed. A water molecule, activated by His289, attacks the carbonyl carbon of the ester, releasing alcohol. The catalytic residues are arranged in a catalytic triad, with Asp260 providing the carboxylate that activates His289 as a base catalyst. Site-directed mutagenesis studies and oxygen isotope incorporation experiments confirm the proposed mechanism [37•].

The *X. autotrophicus* strain that degrades 1,2-dichloroethane can be used for the treatment of contaminated groundwater and waste gases. The substrate range of the organism is limited, however, by the range of compounds recognized by the dehalogenase. Several chlorinated compounds with a structure similar to 1,2-dichloroethane cannot be degraded because no useful dehalogenase for the initial hydrolytic step has been discovered as yet. This includes compounds such as 1,1-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,2-dichloropropane, and trichloropropanes. Furthermore, the kinetics of the dehalogenase towards 1,2-dichloroethane are not very favourable, with a k_{cat} of 6 s^{-1} and a K_{m} of 0.68 mM . This directly influences the Monod constant of 1,2-dichloroethane degradation, and thus the effi-

ciency of substrate removal in continuously operated reactors [39]. These properties set a number of interesting targets for modifying the dehalogenases towards improved activity with specific compounds.

The role of α/β hydrolase fold enzymes in biodegradation

The structure and mechanism of haloalkane dehalogenase is shared with a group of enzymes commonly classified as α/β -fold hydrolases [37]. In these enzymes, the position of the active site residues that form the catalytic triad is conserved along the sequence and follows the order nucleophile–charge relay carboxylate–histidine, each separated by a variable number of other amino acids. Only the histidine is strictly conserved. The nucleophile may be an aspartate, a serine, or a cysteine. An aspartate is used for enzymes that catalyze nucleophilic substitution on a sp^3 hybridized carbon atom. This includes dehalogenases [37], but also epoxide hydrolases [11,37]. The reason that these enzymes have an aspartate as the nucleophile is the requirement for the presence of a carbonyl function in the covalent intermediate [34,37]. In proteolytic enzymes, triacylglyceride lipases, and diene lactone hydrolases [37,40,41], the carbonyl function is provided by the substrate. Several of the other α/β fold hydrolases are also bacterial enzymes involved in degradation of xenobiotic compounds, including aromatics, polychlorinated biphenyls, and haloalkanes [34].

On the basis of considerable sequence similarity to haloalkane dehalogenase, two other hydrolytic dehalogenases are expected to have a similar mechanism, namely the haloacetate dehalogenase (encoded by *debH1*) of *Moraxella* B [10] and the 1,3,4,6-tetrachloro-1,4-cyclohexadiene dehalogenase (encoded by *linB*) of *Pseudomonas paucimobilis* UT26 [9]. Diene lactone hydrolases are responsible for cleavage of the lactones formed after ring cleavage of chlorocatechols by the *ortho*-cleavage route [41]. Hydroxymuconic aldehyde dehydrogenases are involved in the hydrolytic conversion of semialdehydes produced by *meta*-cleavage of substituted catechols during the degradation of toluene, xylenes and polychlorinated biphenyls [42,43].

These α/β -hydrolase fold enzymes share a similar main domain, composed of a large β -sheet surrounded by α -helices. In addition, they have different sequence excursions, often between β -strand 5 and α -helix 3, that form separate domains that influence substrate specificity [37]. As such, the hydrolases are interesting targets for modifying the specificity and activity of catabolic enzymes. Exchange of fragments or modification by site-directed mutagenesis of the cap domain may allow the construction of novel variants with new catalytic activities. We have recently observed that large insertions and deletions in the amino-terminal part of the cap domain can be selected *in vivo* in spontaneous mutants that utilize 1-chlorohexane as a

growth substrate (F Pries, AJ Van den Wijngaard, R Bos, DB Janssen, unpublished data).

Other dehalogenating enzymes

Various other bacterial enzymes involved in dehalogenation reactions are known, and their properties have been reviewed recently [34]. Reaction types include hydration after formation of a coenzyme A activated derivative [44], glutathione substitution [45,46], monooxygenation of aromatic substrates, elimination of HCl direct hydration, and a lyase type reaction of *vic*-haloalcohols [35]. Although considerable biochemical work has been done, protein engineering of these enzymes is still difficult because structural insight is limited. As yet, the X-ray structure of only glutathione transferase has been determined [47,48]. The sequence similarity to the bacterial dehalogenating enzymes, however, is too limited to draw conclusions about the structure of the binding site of the halogenated substrate.

Methane monooxygenase

Several bacterial monooxygenases that are involved in the initial oxidation of hydrocarbons can dehalogenate chlorinated hydrocarbons by converting them to chemically unstable epoxides or *gem*-chloroalcohols. The highest activity and broadest substrate range is shown by the soluble methane monooxygenase of methanotrophic bacteria. This enzyme catalyzes formation of epoxides from halogenated ethylenes, and dehalogenation of short chain halogenated alkanes is also observed. This makes methanotrophs promising biocatalysts for the removal of chlorinated pollutants from groundwater and air. Limitations for their application are the toxicity of degradation products, the kinetics of substrate conversion (especially the relatively high K_s value), the substrate range, and the fact that the enzyme is only expressed under copper limitation. However, mutants with constitutive expression of the enzyme have been isolated [49]. Similar mutants have been isolated from *Pseudomonas* G4, another promising candidate for trichloroethylene removal [50].

The biochemical properties and gene sequences of the enzymes from *Methylosinus trichosporium* and *Methylococcus capsulatus* are very similar. They are hexameric proteins ($\alpha_2\beta_2\gamma_2$) with subunits of 60.6 kDa (α), 45.0 kDa (β), and 19.8 kDa (γ). The X-ray structure of the *M. capsulatus* enzyme has recently been solved [8]. The catalytic sites are composed of a dinuclear iron centre and are located in the α subunits. Each iron has a histidine, two glutamates, and a water molecule as ligands. In addition, two non-protein ligands are in the crystal structure, presumably a hydroxide and an acetate ion. The two iron atoms and the coordinating ligands line a buried internal cavity that is surrounded by hydrophobic residues. Since the cavity has no direct opening to the surface of the enzyme, dynamic motions must be required for entrance of substrates. Some insight into the catalytic mechanism also

comes from the sequence similarity to ribonucleotide reductase [12]. The position of the catalytic tyrosine in this enzyme, which is transformed to a tyrosyl radical during catalysis, is occupied by a cysteine in methane monooxygenase [8**].

The availability of an X-ray structure for methane monooxygenase makes it, in principle, feasible to construct specific mutants of the enzyme that may have an altered kinetic performance. It is likely, however, that further kinetic and mechanistic insight is needed before protein engineering towards specific properties is feasible. Useful goals would be to expand the substrate range to compounds that are hardly hydrolyzed by the present enzyme and to improve the kinetics of conversion of environmentally important substrates, such as trichloroethylene and 1,1,1-trichloroethane.

Cytochrome P450

Cytochrome P450s form a broad group of monooxygenases that catalyze oxidation of a wide range of substrates. They are produced by some bacteria, yeasts, and by higher organisms, where they play a role in detoxification of xenobiotics, bioactivation reactions, and metabolism of various endogenous compounds. Much work has been done to unravel the catalytic mechanism of these heme-containing enzymes [6]. Three-dimensional structures have so far been obtained only for two bacterial cytochrome P450s [6,51*]. In higher organisms, the enzymes are membrane bound, which makes their purification and crystallization difficult.

The environmental relevance of cytochrome P450 is related both to the wide range of reactions that it can perform and to its broad substrate range. This makes cytochrome P450 an interesting protein to engineer when constructing organisms that degrade chlorinated hydrocarbons. The enzyme from higher organisms can catalyze oxygen incorporation in various haloalkanes and haloalkenes, but this activity is low in the bacterial enzymes. Under anaerobic conditions, the *Pseudomonas putida* enzyme involved in camphor oxidation rapidly catalyzes dehalogenation by two-electron reduction and elimination [52].

The X-ray crystal structures of the camphor-oxidizing *P. putida* P450 [6] and the *Bacillus megaterium* P450 that oxidizes fatty acids are now known [51*]. The *Pseudomonas* enzyme requires an FAD-containing reductase and an iron-sulphur redox protein for activity. The *Bacillus* enzyme is more similar to the eukaryotic microsomal cytochrome P450. It combines the hydroxylase and reductase functions in a single polypeptide chain that can be cleaved into a hydroxylase fragment of 55 kDa and an FAD and FMN containing hydroxylase domain.

Recently, Paulsen *et al.* [53**] have described a promising combination of molecular dynamics and site-directed mutagenesis for modification of the activity of *P. putida* cytochrome P450. The hydroxylation of camphor is coupled to the reduction of NADH, which

is only 12% efficient in the case of norcamphor. Uncoupling results from the movement of norcamphor, which is much smaller than camphor, in the active site of the enzyme [54]. Molecular dynamics predicted an increase in the coupling in a mutant carrying a tyrosine 185 to phenylalanine substitution. Construction of this mutant showed, indeed, that the coupling for norcamphor was doubled [53**]. Another study combined the knowledge gathered from molecular dynamics and static modelling of the unusual substrate ethylbenzene in the active site of P450 [55]. On this basis, mutants were constructed that had increased coupling and stereospecificities [56*]. The above studies show the strength of molecular dynamics and modelling combined with site-directed mutagenesis, and should be equally applicable for substrates of cytochrome P450 that are of more environmental importance.

Ligninase

The white rot fungus *Phanerochaete chrysosporium* is capable of lignin degradation by extracellular lignin peroxidases and manganese peroxidases. The lignolytic enzymes also convert several chlorinated polycyclic aromatic hydrocarbons [57,58]. Ligninases catalyze lignin breakdown by the formation of cation radicals that mediate chain radical reactions leading to cleavage of the lignin backbone. Hydrogen peroxide and a veratryl alcohol are used as the substrates [59,60]. During the reaction, a cation radical is generated that diffuses out of the active site and can react with various aromatic compounds. This mechanism explains the broad substrate range of the enzyme and its potential in the degradation of complex organic molecules [61].

The X-ray structure of lignin peroxidase of *P. chrysosporium* has been recently determined and refined [4*,5,62]. The structure is similar to that of cytochrome *c* peroxidase, including the active-site regions. The active site contains both a histidine residue that accepts a proton from the peroxide and an arginine that facilitates O—O bond cleavage. A site where veratryl alcohol can bind has also been identified [4*]. Veratryl alcohol is converted by the enzyme to an aryl cation radical and gains access to active site by a small opening from the active site to the solvent.

As ligninase is an extracellular enzyme, it is particularly sensitive to environmental conditions in a waste treatment or bioremediation process. The availability of the X-ray structures will facilitate the development of enzymes that are more stable under various process conditions.

Aromatic dioxygenase

Aromatic dioxygenases catalyze the initial steps in the degradation of monocyclic aromatic hydrocarbons and polychlorinated biphenyls. The limited activity of these enzymes to highly chlorinated biphenyls contributes to the environmental recalcitrance of these compounds.

The enzyme from *Pseudomonas* LB400, one of the most efficient biphenyl degraders, is an octameric protein composed of 33.2 kDa subunits [63]. It inserts two oxygen atoms of molecular oxygen into biphenyls to produce 2,3-dihydrodiols. The substrate range of biphenyl dioxygenases influences the range of compounds that can be degraded by a biphenyl-degrading organism [64]. Erickson and Mondello [14**] have recently engineered an enhanced biphenyl dioxygenase. On the basis both of sequence comparison and of differences in activities between the *Pseudomonas* LB400 and the *Pseudomonas pseudoalcaligenes* KF707 enzymes, they replaced four residues of the LB400 dioxygenase subunit with corresponding residues from the KF707 dioxygenase. The resulting engineered enzyme showed the broad substrate range of LB400 dioxygenase combined with the better activity of the KF707 enzyme for double *para*-substituted congeners. The substrate range of the toluene dioxygenase has also been modified by random mutagenesis, followed by selection [65] (see this issue, pp 249–252).

Conclusions

The development of genetic engineering, and the increasing number of known three-dimensional structures, has opened the possibility of engineering enzymes with improved applicability in environmental biotechnology. The number of successes is still very small, but the prospect is certainly there. When detailed structural information is lacking, recombinant proteins can, instead, be constructed on the basis of predictions made by comparison of catalytic activities and sequences of related enzymes. Even without this information, the performance of biocatalysts may be improved by constructing efficient selection systems in which the desired modified enzymes can be screened or isolated on the basis of a selective growth advantage. The simplicity of the last approach will make it the method of choice in many cases where structural information is not yet available.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Fersht A, Winter G: Protein Engineering. *Trends Biochem Sci* 1992, 17:292–294.
A review describing the development and status of protein engineering, including an evaluation of strategies for modifying enzyme specificity.
2. Arnold FH: Engineering Proteins for Nonnatural Environments. *FASEB J* 1993, 7:744–749.
Discusses a variety of strategies to obtain stabilized proteins, including enzymes that are resistant to organic solvents.

3. Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolov F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J *et al.*: The α/β Hydrolase Fold. *Protein Eng* 1992, 5:197–211.
A similar overall structure and catalytic mechanism, which can be identified by sequence alignment, is reported for several enzymes involved in biodegradation of xenobiotics.
4. Poulos TL, Edwards SL, Wariishi H, Gold MH: Crystallographic Refinement of Lignin Peroxidase at 2 Å. *J Biol Chem* 1993, 268: 4429–4440.
Shows that the crystal structure of lignin peroxidase is similar to cytochrome *c* peroxidase. Veratryl alcohol can be bound close to the active-site cavity.
5. Piontek K, Glumoff T, Winterhalter K: Low pH Crystal Structure of Glycosylated Lignin Peroxidase from *Phanerochaete chrysosporium* at 2.5 Å Resolution. *FEBS Lett* 1993, 315:119–124.
6. Poulos TL, Raag R: Cytochrome P450cam: Crystallography, Oxygen Activation and Electron Transfer. *FASEB J* 1992, 6:674–679.
7. Verschuere KHG, Franken SM, Rozeboom HJ, Kalk KH, Dijkstra BW: Refined X-Ray Structures of Haloalkane Dehalogenase at pH 6.2 and pH 8.2 and Implications for the Reaction Mechanism. *J Mol Biol* 1993, 232:856–872.
8. Rosenzweig AC, Frederick CA, Lippard SJ, Nordlund P: Crystal Structure of a Bacterial Non-Haem Iron Hydroxylase that Catalyses the Biological Oxidation of Methane. *Nature* 1993, 366:537–543.
Describes the active site of a bacterial non-haem hydroxylase, which contains two irons bound as ligands to water, a histidine, and glutamate residues. The substrate binding is somewhat similar to that of cytochrome P450.
9. Nagata Y, Nariya T, Ohtomo R, Fukuda M, Yano K, Takagi M: Cloning and Sequencing of a Dehalogenase Gene Encoding an Enzyme with Hydrolase Activity Involved in the Degradation of γ -Hexachlorocyclohexane in *Pseudomonas paucimobilis*. *J Bacteriol* 1993, 175:6403–6410.
10. Kawasaki H, Tsuda K, Matsushita I, Tonomura K: Lack of Homology between Two Haloacetate Dehalogenase Genes Encoded on a Plasmid from *Moraxella* sp. Strain B. *J Gen Microbiol* 1992, 138:1317–1323.
11. Lacourciere GM, Armstrong RN: The Catalytic Mechanism of Microsomal Epoxide Hydrolase Involves an Ester Intermediate. *J Am Chem Soc* 1993, 115:10466–10467.
12. Nordlund P, Dalton H, Eklund H: The Active Site Structure of Methane Monooxygenase is Closely Related to the Binuclear Iron Center of Ribonucleotide Reductase. *FEBS Lett* 1992, 307:257–262.
13. Kato K, Fujiyama K, Hatanaka HS, Priyambada ID, Negoro S, Urabe I, Okada H: Amino Acid Alterations Essential for Increasing the Catalytic Activity of the Nylon-Oligomer-Degradation Enzyme of *Flavobacterium* sp. *Eur J Biochem* 1991, 200:165–169.
14. Erickson BD, Mondello FJ: Enhanced Biodegradation of Polychlorinated Biphenyls after Site-Directed Mutagenesis of a Biphenyl Dioxygenase Gene. *Appl Env Microbiol* 1993, 59:3858–3862.
Mutagenesis of the dioxygenase, based on sequence comparison, yields an enzyme of broad specificity and higher activity against polychlorinated biphenyls that have chlorines at the 4 and 4' positions.
15. Fagain CO, O'Kennedy R: Functionally-Stabilized Proteins — A Review. *Biotechnol Adv* 1991, 9:351–409.
16. Chakrabarty A, Doig AJ, Baldwin RL: Helix Capping Propensities in Peptides Parallel those in Proteins. *Proc Natl Acad Sci USA* 1993, 90:11332–11336.
17. Kimura S, Oda Y, Nakai T, Katayanagi K, Kitakuni E, Nakai C, Nakamura H, Ikehara M, Kanaya S: Effect of Cavity-

- Modulating Mutations on the Stability of *Escherichia coli* Ribonuclease HI. *Eur J Biochem* 1992, 206:337–343.
18. Eijssink VGH, Dijkstra BW, Vriend G, Van der Zee JR, Veltman OR, Van der Vinne B, Van den Burg B, Kempe S, Venema G: The Effect of Cavity-Filling Mutations on the Thermostability of *Bacillus stearothermophilus* Neutral Protease. *Protein Eng* 1992, 5:421–426.
 19. Joyet P, Declerck N, Gaillardin C: Hyperthermostable Variant of a Highly Thermostable Alpha-Amylase. *Biotechnology* 1992, 10:1579–1583.
 20. Turner SL, Ford GC, Mountain A, Moir A: Selection of A Thermostable Variant of Chloramphenicol Acetyltransferase (Cat-86). *Protein Eng* 1992, 5:535–541.
 21. Hedstrom L, Szilagyi L, Rutter WJ: Converting Trypsin to Chymotrypsin: The Role of Surface Loops. *Science* 1992, 255:1249–1253.
- The surface loops and amino acids forming the S1-binding site of trypsin are modified. This results in an enzyme with altered specificity that displays increased binding of peptides with large hydrophobic residues.
22. Green DW, Sun H-W, Plapp BV: Inversion of the Substrate Specificity of Yeast Alcohol Dehydrogenase. *J Biol Chem* 1993, 268:7792–7798.
 23. Rheinhecker M, Baker G, Eder J, Fersht AR: Engineering a Novel Specificity in Subtilisin BPN'. *Biochemistry* 1993, 32:1199–1203.
 24. Chaidaroglou A, Kantowitz ER: Alteration of Aspartate 101 in the Active Site of *Escherichia coli* Alkaline Phosphate Enhances the Catalytic Activity. *Protein Eng* 1989, 3:127–132.
 25. Grunert H-P, Landt O, Zirpel-Giesebrecht M, Backmann J, Heinemann U, Saenger W, Hahn U: Trp59 to Tyr Substitution Enhances the Catalytic Activity of RNase T1 and of the Tyr to Trp Variants in Positions 24, 42 and 45. *Protein Eng* 1993, 6:739–744.
 26. Getzoff ED, Cabelli DE, Fisher CL, Parge HE, Viezzoli MS, Banci L, Hallewell RA: Faster Superoxide Dismutase Mutants Designed by Enhancing Electrostatic Guidance. *Nature* 1992, 358:347–351.
 27. Benner SA: Enzyme Kinetics and Molecular Evolution. *Chem Rev* 1989, 89:789–806.
 28. Mulholland AJ, Grant GH, Richards WG: Computer Modelling of Enzyme Catalyzed Reaction Mechanisms. *Protein Eng* 1993, 6:133–147.
 29. Warshel A: Computer Simulation of Enzymatic Reactions. *Curr Opin Struct Biol* 1992, 2:230–236.
 30. Clarke PH: The Evolution of Degradative Pathways. In *Microbial Degradation of Organic Compounds*, Edited by Gibson DT. New York: Marcel Dekker Inc; 1985:11–27.
 31. Silman NJ, Carver M, Jones CW: Directed Evolution of Amidase in *Methylobacillus methylotrophus*: Purification and Properties of Amidases from Wild-Type and Mutant Strains. *J Gen Microbiol* 1991, 137:169–178.
 32. Oliphant AR, Struhl K: An Efficient Method for Generating Proteins with Altered Enzymatic Properties: Application to β -Lactamase. *Proc Natl Acad Sci USA* 1989, 86:9094–9098.
 33. Leisinger T, Bader R: Microbial Dehalogenation of Synthetic Organohalogen Compounds: Hydrolytic Dehalogenases. *Chimia* 1993, 47:116–121.
 34. Janssen DB, Pries F, Van der Ploeg JR: Genetics and Biochemistry of Dehalogenating Enzymes. *Annu Rev Microbiol* 1994, in press.
- An overview of the diversity of enzymes that cleave carbon-halogen bonds.
35. Van den Wijngaard AJ, Van der Kamp KWHJ, Van der Ploeg JR, Pries F, Kazemier B, Janssen DB: Degradation of 1,2-Dichloroethane by *Ancyllobacter aquaticus* and Other Facultative Methyiotrophs. *Appl Env Microbiol* 1992, 58:976–983.
 36. Verschueren KHG, Seljée F, Rozeboom HJ, Kalk KH, Dijkstra BW: Crystallographic Analysis of the Catalytic Mechanism of Haloalkane Dehalogenase. *Nature* 1993, 363:693–698.
- Different steps in the conversion of 1,2-dichloroethane by haloalkane dehydrogenase are visualized by X-ray crystallography of intermediates.
37. Pries F, Kingma J, Pentenga M, Van Pouderoyen G, Jeronimus-Stratingh CM, Bruins AP, Janssen DB: Site-Directed Mutagenesis and Oxygen Isotope Incorporation Studies of the Nucleophilic Aspartate of Haloalkane Dehalogenase. *Biochemistry* 1994, 33:1242–1247.
- Presents biochemical evidence for the catalytic mechanism of haloalkane dehalogenase.
38. Verschueren KHG, Kingma J, Rozeboom HJ, Kalk KH, Janssen DB, Dijkstra BW: Crystallographic and Fluorescence Studies of the Interaction of Haloalkane Dehalogenase with Halide Ions. Studies with Halide Compounds Reveal a Halide Binding Site in the Active Site. *Biochemistry* 1993, 32:9031–9037.
 39. Van den Wijngaard AJ, Wind RD, Janssen DB: Kinetics of Bacterial Growth on Chlorinated Aliphatic Compounds. *Appl Environ Microbiol* 1993, 59:2041–2048.
 40. Derewenda ZS, Sharpe AM: News from the Interface: The Molecular Structure of Triacylglyceride Lipases. *Trends Biochem Sci* 1993, 18:20–25.
 41. Chaeh E, Ashley GW, Ollis D: Catalysis by Diene Lactone Hydrolase: A Variation on the Protease Mechanism. *Proteins* 1993, 16:64–78.
 42. Menn F-M, Zylstra GJ, Gibson DT: Location and Sequence of the *todF* Gene Encoding 2-Hydroxy-6-oxohepta-2,4-dienoate Hydrolase in *Pseudomonas putida* F1. *Gene* 1991, 104:91–94.
 43. Nordlund I, Shingler V: Nucleotide Sequence of the *Meta*-cleavage Pathway Enzymes 2-Hydroxymuconic Semi-Aldehyde Dehydrogenase and 2-Hydroxymuconic Semialdehyde Hydrolase from *Pseudomonas* CF600. *Biochim Biophys Acta* 1990, 1049:227–230.
 44. Liang P-H, Yang G, Dunaway-Mariano D: Specificity of 3-Chlorobenzoyl Coenzyme A Dehalogenase Catalyzed Dehalogenation of Halogenated Aromatics. *Biochemistry* 1993, 32:12245–12250.
 45. Orser CS, Dutton J, Lange C, Jablonski P, Xun L, Hargis M: Characterization of a *Flavobacterium* Glutathione Transferase Gene Involved in Reductive Dechlorination. *J Bacteriol* 1993, 175:2640–2644.
 46. Xun L, Topp E, Orser CS: Purification and Characterization of a Tetrachloro-p-Hydroquinone Reductive Dehalogenase from a *Flavobacterium* sp. *J Bacteriol* 1992, 174:8003–8007.
 47. Ji X, Zhang P, Armstrong RN, Galliland GL: The Three-Dimensional Structure of a Glutathione-S-Transferase from the Mu Gene Class. Structural Analysis of the Binary Complex of Isoenzyme 3-3 and Glutathione at 2.2-Å Resolution. *Biochemistry* 1992, 31:10169–10184.
 48. Reinemer P, Dirr HW, Ladensten R, Huber R, Lo Bello M, Frederici G, Parker MW: Three-Dimensional Structure of Class π Glutathione S-Transferase from Human Placenta in Complex with S-Hexylglutathione at 2.8 Å Resolution. *J Mol Biol* 1992, 227:214–226.
 49. Phelps PA, Agarwal SK, Speitel GE Jr, Georgiou G: *Methylosinus trichosporium* OB3b Mutants Having Constitutive Expression of Soluble Methane Monooxygenase in the Presence of High Levels of Copper. *Appl Env Microbiol* 1992, 58:3701–3708.

50. Shields MA, Reagin MJ: Selection of a *Pseudomonas* Strain Constitutive for the Degradation of Trichloroethylene. *Appl Env Microbiol* 1992, 58:3977–3983.
 51. Ravichandran KG, Boddupalli SS, Hasemann CA, Peterson JA, Deisenhofer J: Crystal Structure of Hemoprotein Domain of P450BM-3, a Prototype for Microsomal P450's. *Science* 1993, 261:731–736.
- Describes the crystal structure of the haemoprotein domain of P450 BM-3, which hydroxylates fatty acids. This is the second cytochrome P450 for which the structure has been determined. A hydrophobic channel that leads to the substrate-binding site is identified.
52. Li S, Wackett LP: Reductive Dehalogenation by Cytochrome P450_{CAM}: Substrate Binding and Catalysis. *Biochemistry* 1993, 32:9355–9361.
 53. Paulsen MD, Filipovic D, Sligar SG, Ornstein RL: Controlling the Regiospecificity and Coupling of Cytochrome P450_{CAM}: T185F Mutant Increases Coupling and Abolishes 3-Hydroxynorcamphor Product. *Protein Sci* 1993, 2:357–365.
- A tyrosine 185 to phenylalanine substitution in cytochrome P450_{CAM} resulted in a doubling of coupling for norcamphor and an abolishment of 3-hydroxynorcamphor product. Demonstrates the application of molecular dynamics for modifying enzyme selectivity.
54. Bass MB, Paulsen MD, Ornstein RL: Substrate Mobility in a Deeply Buried Active Site: Analysis of Norcamphor Bound to Cytochrome P450_{CAM} as Determined by a 201-psec Molecular Dynamics Simulation. *Proteins* 1992, 13:26–37.
 55. Filipovic D, Paulsen MD, Loida PJ, Sligar SG, Ornstein RL: Ethylbenzene Hydroxylation by Cytochrome P450_{CAM}. *Biochem Biophys Res Comm* 1992, 189:488–495.
 56. Loida PJ, Sligar SG: Engineering Cytochrome P450_{CAM} to Increase the Stereospecificity and Coupling of Aliphatic Hydroxylation. *Protein Eng* 1993, 6:207–212.
- A combination of molecular dynamics, static modelling and site-directed mutagenesis is used to engineer an enzyme with increased stereospecificities and coupling.
57. Field JA, De Jong E, Feijoo-Costa G, De Bont JAM: Screening for Lignolytic Fungi Applicable to Biodegradation of Xenobiotics. *Trends Biotechnol* 1993, 11:44–49.
 58. Hammel KE: Organopollutant Degradation by Lignolytic Fungi. *Enzyme Microb Technol* 1989, 11:776–777.
 59. Harvey PJ, Floris R, Lundell T, Palmer JM, Schoemaker HE, Wever R: Catalytic Mechanisms and Regulation of Lignin Peroxidase. *Biochem Soc Trans* 1992, 20:345–349.
 60. Higuchi T: Biodegradation Mechanism of Lignin by White-Rot Basidiomycetes. *J Biotechnol* 1993, 30:1–8.
 61. Reddy CA: An Overview of the Recent Advances on the Physiology and Molecular Biology of Lignin Peroxidases of *Phanerochaete chrysosporium*. *J Biotechnol* 1993, 30:91–107.
 62. Edwards SL, Raag R, Wariishi H, Gold MH, Poulos TL: Crystal Structure of Lignin Peroxidase. *Proc Natl Acad Sci USA* 1993, 90:750–754.
 63. Eltis LD, Hofmann B, Hecht H-J, Lünsdorf H, Timmis KN: Purification and Crystallization of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase. *J Biol Chem* 1993, 268:2727–2732.
 64. Yates JR, Mondello FJ: Sequence Similarities in the Genes Encoding Polychlorinated Biphenyl Degradation by *Pseudomonas* Strain LB400 and *Alcaligenes eutrophus* H850. *J Bacteriol* 1989, 171:1733–1735.
 65. Ramos JL, Wasserfallen A, Rose K, Timmis KN: Redesigning Metabolic Routes: Manipulation of TOL Plasmid Pathway for Catabolism of Alkylbenzoates. *Science* 1987, 235:593–596.

DB Janssen and JP Schanstra, Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands.