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Computer modelling of microbial hydrolytic dehalogenation

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Abstract: The biodegradation of organic compounds by microorganisms is an intrinsically complex process. A large number of sub-processes, like penetration of the compounds to the cells, biochemical catalysis and the release of products are taking place during the biodegradation. The separation of these sub-processes from each other and their detailed study is needed for a better understanding of the basic mechanisms of microbial degradation and for the improvement of bioremediation technologies by means of the construction of a new efficient biocatalyst. This contribution attempts to view the biodegradation reaction - hydrolytic dehalogenation - at the level of catalysing biomolecules. Computer modelling is used to extend the knowledge obtained from X-ray analysis, kinetic measurements and site-directed mutagenesis experiments. Among the issues discussed in this article are the determination of the rate-limiting step of the biodegradation reaction, the identification of the active-site amino acids involved in the reaction mechanism, the prediction of proteins' modifications leading to higher activity and the molecular mechanisms of the adaptation of bacteria for the degradation of xenobiotic compounds.

INTRODUCTION

Biodegradation is the conversion of chemical substances by the action of living (micro)organisms. The study of the microbial degradation of organic compounds has been the subject of active research for nearly half of the century (ref. 1), during which biodegradation has been studied by microbiologists, biochemists, geneticists or molecular biologists at the level of cells, enzymes or nucleic acids. More recently, it has become possible to view and study biodegradation processes as the biochemical reactions taking place in the active-sites of proteins, as a result of the technical development of X-ray diffraction and spectroscopic techniques. The static structural information on proteins obtained from X-ray ctrystallography and NMR-spectroscopy can be extended further by the information derived from computer modelling, which provides the basic platform for visualization of proteins in 3D-space (ref. 2), the calculation of reactions in the enzyme active sites (ref. 3) or the simulation of the dynamical behaviour of proteins (ref. 4).

In a recent article (ref. 5), we attempted to summarize computer modelling techniques which are potentially applicable for the study of different sub-processes involved in the microbial degradation. In this contribution, I will demonstrate that computer modelling and bioinformatics can provide useful answers to important questions regarding the mechanisms of biodegradation reactions and the evolution of degradative capabilities in bacteria. The potential use of computer modelling for the design of degradative enzymes with the desired catalytic properties will also be demonstrated. An attempt will be made to answer several important questions: What is the rate-limiting step of particular biodegradation reaction? Which amino acids in the enzyme are involved in the reaction mechanism? How to modify the enzyme in order to achieve a desired activity? How do bacteria adapt to degrade a new compound? The results from the computational analysis of the hydrolytic dehalogenation of halogenated aliphatic compounds by haloalkane dehalogenase, will be used throughout this contribution.

ENZYMATIC DEHALOGENATION BY THE HALOALKANE DEHALOGENASES

Hydrolytic dehalogenation is one the most important mechanisms used by microorganisms to initiatiate the degradation of halogenated aliphatic compounds. The hydrolytic dehalogenases catalyse a nucleophilic displacement reaction with water as the sole co-substrate (ref. 6). Two groups of hydrolytic dehalogenases can be distinguished based on the natural substrate: haloacid dehalogenases and haloalkane dehalogenases. The later group consists of several substrate specificity classes (ref. 7,8,9). The primary structures of three different haloalkane dehalogenases have been published; the enzyme of *Xanthobacter autotrophicus* GJ10, DhlA, (ref. 10), *Sphingomonas paucimobilis* UT26, LinB, (ref. 11) and *Rhodococcus rhodochrous* NCIMB 13064, DhaA (ref. 12). The tertiary structure for a single haloalkane dehalogenase is known, that of the enzyme of *X. autotrophicus* (ref. 13). Crystallographic analysis of this haloalkane dehalogenase under varied pH and temperature led to the following proposal of its reaction mechanism (Fig.1).

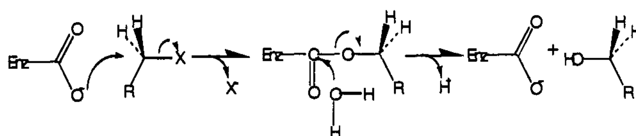

Fig. 1: Reaction mechanism of the haloalkane dehalogenase

STUDY OF THE RATE-LIMITING STEP OF THE DEHALOGENATION REACTION

A number of different processes can potentially be rate-limiting in the biodegradation of synthetic compounds, like: (i) bioavailability, (ii) the penetration or uptake of the compounds through the cell envelope, (iii) the biochemical reaction and (iv) the release of products. Identification of the rate-limiting step of a particular degradation can be important with regards to improving the efficiency of biodegradation for bioremedation purposes (ref. 14), and also for the development of models relating the structure of chemicals to their degradability (ref. 15). Based on kinetic measurements, it has been suggested that the catalytic properties of the first enzyme (haloalkane dehalogenase) in the DCE degradation pathway

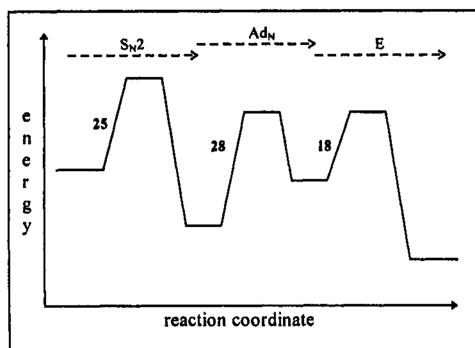

Fig. 2: Energy profile for the three-steps dehalogenation of 1,2-dichloroethane by haloalkane dehalogenase. The numbers quantify activation energy barriers (in kcal mol^{-1}) derived from AM1 semi-empirical quantum-mechanic calculation

influence the overall degradation rate for short-chain halogenated substrates (ref. 16,17). The dehalogenation reaction of the haloalkane dehalogenase involves three consecutive reaction steps with different reaction mechanisms: nucleophilic substitution (S_N2), nucleophilic addition (Ad_N) and elimination (E). Semi-empirical quantum-mechanic (QM) calculations of all three reactions were performed with the microscopic model of the enzyme active site, composed of 13 amino acid residues, and 1,2-dichloroethane as a substrate (ref. 18,19). The derived energy pathway shown in figure 2 was used to approximate the kinetic rates of a particular reaction, and led us to propose the Ad_N reaction to be the slowest out of three examined.

This conclusion is in agreement with the results from the transient kinetic measurements of Schanstra and co-workers (ref. 20), who postulated Ad_N to be the slowest reaction. Additional stopped-flow kinetic experiments indicated that the product (halide) release from the enzyme active site can limit the overall catalytic performance of the enzyme. Involvement of the slow enzyme isomerization in the halide release was proposed by Schanstra and Janssen (ref. 21). This isomerization is a slow conformational change and should take place in the so-called cap domain of haloalkane dehalogenase. Our working hypothesis on the possible mechanism of the conformation change in the haloalkane dehalogenase considers the involvement of *cis->trans* isomerization of the Gln167-Pro168 peptide bond. *Cis-trans* isomerization is an intrinsically slow process occurring in a millisecond range (ref. 22,23), thereby fitting the time scale deduced for the conformational change of DhlA (6 s^{-1}) from the kinetic experiments of Schanstra and Janssen. We have recently applied the conformational analysis CICADA (ref. 24) to study *cis-trans* isomerization in the haloalkane dehalogenase (Garcic, L., Damborský, J., Koca, J.,

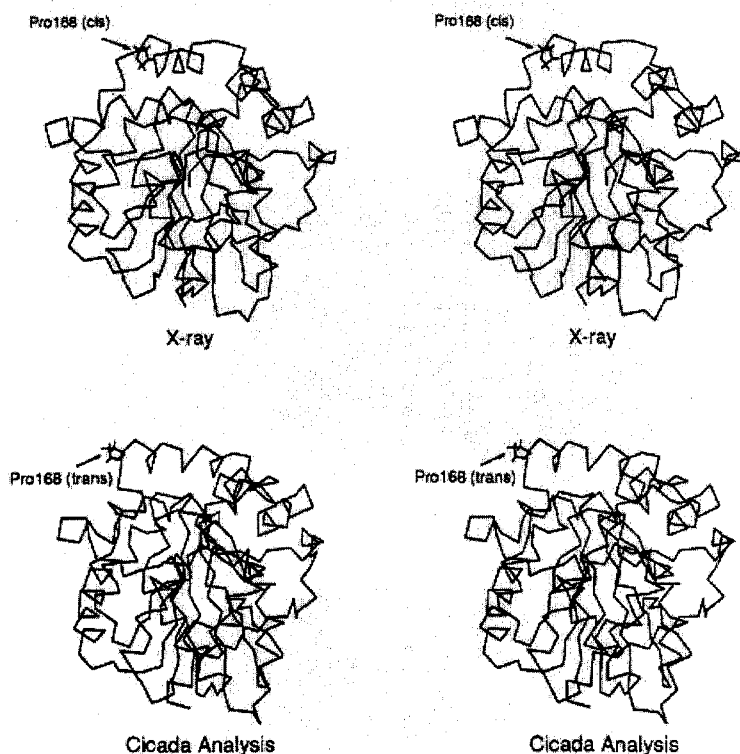

Fig. 3: Crystal structure of haloalkane dehalogenase and its hypothetical 'open' conformation obtained from the CICADA conformational analysis. Only Cα atoms are shown for clarity (stereo view). The position of the Pro168 is indicated by the arrow. Note that the secondary structural elements are fully preserved in the calculated structure

unpublished results). In this study the Gln167-Pro168 dihedral angle was continuously moved from the *cis* to *trans* conformation while relaxing the rest of the protein by simulated annealing. The hypothetical structure of the haloalkane dehalogenase in its 'open' configuration has been obtained (Fig. 3) with calculated barrier of the conversion 25 kcal mol^{-1}. These theoretical results however still need experimental validation.

IDENTIFICATION OF THE FUNCTIONALLY IMPORTANT AMINO ACIDS OF THE HALOALKANE DEHALOGENASE

Verschueren and co-workers proposed several active site amino acids as being essential for the catalytic mechanism of haloalkane dehalogenase based on the crystallographic analysis (ref. 13,25). The catalytic triad of the protein consists of Asp124, Asp260 and His289, while the oxy-anion pocket is composed of Glu56 and Trp125. It has been suggested that two tryptophans, Trp125 and Trp175, stabilize the halide ion formed during the dehalogenation reaction and this proposal was subsequently confirmed by fluorescence studies (ref. 26) and site-directed mutagenesis experiments (ref. 27). Quantum-mechanical calculations at the semi-empirical level of theory were able to quantitatively describe this stabilizing effect of the tryptophan residues (ref. 18). An additional active site residue, Phe172, has been identified as being an important contributor to the halide ion stabilization (Fig. 4).

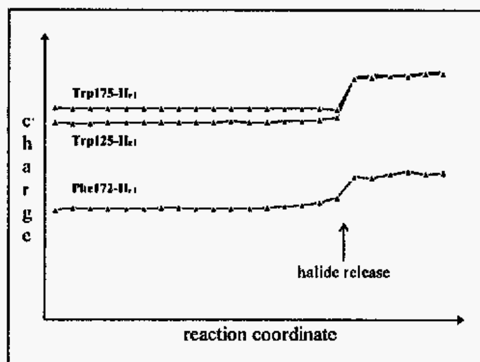

Fig. 4: Changes in atomic charges during the first step (S_N2) of the dehalogenation reaction. The development of the positive charge on selected hydrogen atoms of the residues Trp125, Phe172 and Trp175 clearly supports their role in the electrostatic stabilization of the halide ion released during the reaction

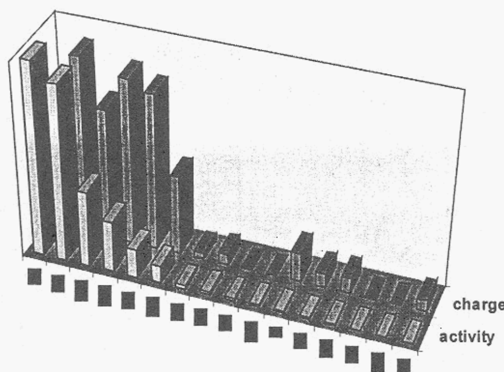

Fig. 5: Comparison of experimentally observed activities of 16 variants of haloalkane dehalogenase with the calculated charge difference of educt and product on atom Phe172-H_{e1} and its equivalents. Without exception, all mutants with significant activity in the experiment (Phe172Tyr, Phe172Trp, wild type, Phe172Met, Phe172His and Phe172Cys) have the charge difference one order of magnitude higher than mutants with low activity

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LinB   - - - - - - - - - - - M S L G A K - P F G E K K F I E I K - - - - - G R R M A Y I D E G - - T G D P I L F Q - H G N P T 40
Mtcy1  - - - - - - - M T A F G V E P Y G Q P K Y L - E I A - - - - - - - - - G K R M A Y I D E G K - - G D A I V F Q - H G N P T 41
DhaA   - - - - - - - - - - - M S E I G T G F P F D P - H Y V E V L - - - - - G E R M H Y V D V G P R D G T P V L F L - H G N P T 43
Mtcy3  - - - - - - - - - - - M S I D F T P D P Q L Y - P F E S R W F D S S R G - R I H Y V D E G T - - G P P I L L C - H G N P T 45
Mtcy2  M D - V L R T P D S R F E H L V G Y P F A P - H Y V D V T A G D T Q P L R M H Y V D E G P G D G P P I V L L - H G E P T 57
DhlA   M I N A I R T P D Q R F S N L D Q Y P F S P - N Y L D D L P G Y P G L - R A H Y L D E G N S D A E D V F L C L H G E P T 58
       β1 β2 β3

LinB   S S Y L W R N I M P H C A G L G - R L I A C D L I G M G D S D K L D P S - G P E R Y A Y A E H R D Y L D A L W E A L D L 98
Mtcy1  S S Y L W R N I M P H L E G L G - R L V A C D L I G M G A S D K L S P S - G P D R Y S Y G E Q R D F L F A L W D A L D L 99
DhaA   S S Y L W R N I I P H V A P S H - R C I A P D L I G M G K S D K - - P D - L - D - Y F F D D H V R Y L D A F I E A L G L 97
Mtcy3  W S F L Y R D I I V A L R D R F - R C V A P D Y L G F G L S E R - - P S - G F G - Y Q I D E H A R V I G E F V D H L G L 100
Mtcy2  W S Y L Y R T M I P P L S A A G H R V L A P D L I G F G R S D K - - P T R I E D - Y T Y L R H V E W V T S W F E N L D L 114
DhlA   W S Y L Y R K M I P V F A E S G A R V I A P D F F G F G K S D K - - P V D E E D - Y T F E F H R N F L L A L I E R L D L 115
       α1 Δ β4 α2

LinB   G D R V V L V V H D W G S A L G F D W A R R H R E R V Q G I A Y M E A I A M - - P I E W A D F P E Q D - - - R D L F Q A 153
Mtcy1  G D H V V L V L H D W G S A L G F D W A N Q H R D R V Q G I A F M E A I V T - - P M T W A D W P P A V - - - R G V F Q G 154
DhaA   E E - V V L V I H D W G S A L G F H W A K R N P E R V K G I A C M E F I R P - I P - T W D E W P E F A - - - R E T F Q A 151
Mtcy3  - D R Y L S M G Q D W G G P I S M A V A V E R A D R V R G V V L G N - - - - - - - - - T W - F W P A D T L A M K - A F S R 149
Mtcy2  H D - V T L F V Q D W G S L I G L R I A A E H G D R I A R L V V A N G - - - F L P A A Q G R T P L P F Y V W R - A F A R 169
DhlA   R N - I T L V V Q D W G G F L G L T L P M A D P S R F K R L I I M N A C L M T D P V T Q P A F S A F V T Q P A D G F T A 174
       β5 α3 β6 α4

LinB   F R S Q A G E E L V L Q D - N V F V E Q V L P G L I L R P L S E A E M A A Y R E P F L A A G E A R R P T L S W P R Q I P 212
Mtcy1  F R S P Q G E P M A L - E H N I F V E R V L P G A I L R Q L S D E E M N H Y R R P F V N G G E D R R P T L S W P R N L P 213
DhaA   F R T A D V G R E L I I D Q N A F I E G A L P K C V V R P L T E V E M D H Y R E P F L K P V D - R E P L W R F P N E L P 210
Mtcy3  V M S S P P V Q Y A I L R R N F F V E R L I P A G T E H R P S S A V M A H Y R A V Q P N A A A - R R G V A E M P K Q I - 207
Mtcy2  Y S P V L P A G R L V - - - N F G T V H R V P A G V - - R A G - - - - - - Y D A P F P D K T Y - Q A G A R A F P R L V P 217
DhlA   W K Y D L V T P S D L R L D Q F M K R - - - - - W A P T - L T E A E A S A Y A A P F P D T S Y - Q A G V R K F P K M V A 227
       α5 α6 Δ α7 α8

LinB   I A G T P A D V V A I A R D Y A G W L S E S P I P K L F I N A E P G A L T T - G R M R D F C R T W P N Q - - - - T E I T 267
Mtcy1  I D G E P A E V V A L V N E Y R S W L E E T D M P K L F I N A E P G A I I T - G R I R D Y V R S W P N Q - - - - T E I T 268
DhaA   I A G E P A N I V A L V E A Y M N W L H Q S P V P K L L F W G T P G V L I P P A E A A R L A E S L P N C K - - T V D I G 268
Mtcy3  L A A R P L L A R L A R E V P A T L G T - - - K P T L L I W G M K D V A F R - P K T I I P R L S A T F - - P D H V L V E 261
Mtcy2  T S P D D P A V P A N R A A W E A L G R W - D K P F L A I F G Y R D P I L G Q A D G P L I K H I P G A A G Q P H A R I K 276
DhlA   - Q R D Q A C I D I S T E A I S F W Q N D W N G Q T F M A I G M K D K L L G - P D V M Y P M K A L I N G C P E P L E I A 285
       Δ α9 β7 α10 β8

LinB   V A - G A H F I Q E D S P D E I G A A I A A F V R R L R P A
Mtcy1  V P - G V H F V Q E D S P E E I G A A I A Q F V R R L R S A A G V
DhaA   - P - G L H Y L Q E D N P D L I G S E I A R W L P A L
Mtcy3  L P N A K H F I Q E D A P D R I A A A I I E R F G
Mtcy2  - - - A S H F I Q E D S G T E L A E R M L S W Q Q A T
DhlA   D - - A G H F V Q E - F G E Q V A R E A L K H F A E T E
       α11
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Fig. 6: Sequence alignment of haloalkane dehalogenase of Sphingomonas paucimobilis UT26 (LinB), Rhodococcus rhodochrous NCIMB 13064 (DhaA), Xanthobacter autotrophicus GJ10 (DhlA) and putative dehalogenases of Mycobacterium tuberculosis H37Rv (Mtcy1, Mtcy2, Mtcy3). The initial alignment was prepared using an automatic multiple alignment algorithm with consecutive manual re-alignment using the information from secondary-structure predictions and the homology models built from this alignment. Conserved regions are in grey. The residues forming the active site of DhlA are framed. Crystallographically determined secondary structure elements of DhlA are indicated by the lines below the sequence. Direct repeats (VTQPA) in the sequence of DhlA are indicated by the arrows. Catalytic triad is marked by triangles above the sequences

The stabilizing effect of the residue at position 172 was further probed by the QM calculations performed with the exhaustive set of single-point mutants of the haloalkane dehalogenase bearing the substitution at the position 172 (ref. 29). The calculated changes in charge on the stabilizing hydrogen atoms were compared with relative activities derived experimentally (ref. 28) for the same set of mutants. These results clearly indicate that only mutants with good stabilization achieved good activity in the experiment (Fig. 5). A similar computational procedure could be used for the combinatorial screening of a large number of mutants for their potential activity *prior* to their construction and experimental testing in the laboratory.

UNDERSTANDING OF THE MOLECULAR MECHANISMS OF ADAPTATION FOR DEGRADATION OF XENOBIOTIC SUBSTRATES

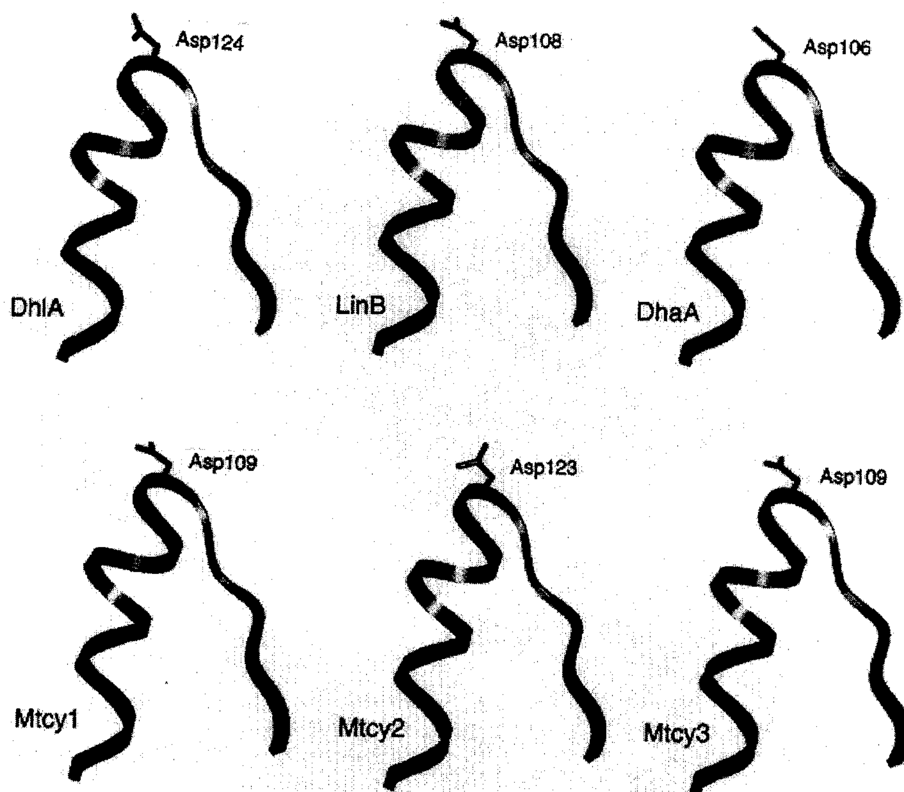

Fig. 7: Cα-ribbon representation of the nucleophile elbows of DhlA (X-ray), LinB, DhaA, Mtcy1, Mtcy2 and Mtcy3 (homology models). The nucleophile elbow is a sharp γ-like turn bearing the nucleophilic residue (Asp) which initiates the reaction. The sharp nature of this turn results in Asp having unfavourable (φ,ψ)–angles and forces the side-chain of this amino acid to point into the active site cavity. The nucleophile elbow is a characteristic for α/β hydrolase fold enzymes (ref. 32)

The adaptation of microorganisms to xenobiotic substrates represents a good model system for the study of natural evolution. Such research is attractive both from the scientific and the practical point of view. It is generally accepted that a missing catalyst could be one of the reasons for the recalcitrance of the environmental pollutants. Comparisons of the sequences and tertiary structures of haloalkane dehalogenases originating from different strains were used to obtain information on mechanisms of adaptation at a molecular level. As described

above, primary sequences are known for three haloalkane dehalogenases (DhlA, LinB and DhaA) and a search of the genomic databases identified an additional three protein sequences (assigned Mtcy1, Mtcy2, Mtcy3) showing a large similarity to haloalkane dehalogenases. All three Mtcy sequences originate from a single organism *Mycobacterium tuberculosis* H37Rv (ref. 30) and it can be expected, that these proteins also have potential for dehalogenation. The alignment of the sequences for all six proteins is shown in figure 6. Three-dimensional models of LinB (ref. 31), DhaA, Mtcy1, Mtcy2 and Mtcy3 were constructed by homology modelling using the crystal structure of DhlA as a template. The same reaction mechanism and membership of the α/β hydrolases family can be deduced from the presence of the nucleophile elbow in all six studied proteins (Fig. 7).

DhlA is the only enzyme which can catalyse the dehalogenation of 1,2-dichloroethane, but on the other hand it can not attack longer-chain substrates (>C6). LinB and DhaA dehalogenases prefer larger substrates, but are inactive with DCE. What are the structural reasons for the differences in substrate specificity of these enzymes? Efficient catalysis of the dehalogenation of DCE requires the formation of the tight Michaelis-Menten complex, stabilization of the transition states and also stabilization of the halide ion formed as a reaction product. To fulfil these requirements, the reaction catalysed by DhlA dehalogenase is situated in a small, highly hydrophobic cavity, which is completely shielded by protein from the solvent. The above discussed residues Trp125, Phe172 and Trp175 to a large extent ensure good stabilization of the transition states and the products *via* electrostatic interactions. A much larger and less hydrophobic active site cavity, with less efficient stabilization is expected for LinB and DhaA proteins. The size of the active sites of haloalkane dehalogenases is mainly modified by the indels in the N-terminal part of the cap domain (the region around the helix 4 and the helix 5 in Fig. 6). This was demonstrated in elegant adaptation experiment by Pries and co-workers (ref. 33) and corresponds well to large gaps in the loops of the discussed region of LinB, DhlA proteins seen in the alignment (Fig. 6). Comparison of the homology models with the X-ray structure of DhlA further suggest, that loop insertions in the later protein (residues 166-168) closed the entrance to the active site thus creating a small buried cavity suitable for DCE dehalogenation. Direct repeats noted by Pries and co-workers in the DhlA sequence are situated in the same region. According to this proposal, the substrates enter the DhlA protein from the other side then in LinB, DhaA and Mtcy1. The different orientation of the substrate molecule bound to the enzyme active site with regards to the nucleophile could consequently result in migration of the catalytic acid within these proteins. In DhlA, Mtcy2 and Mtcy3, the second catalytic acid (Asp) is positioned after the strand 7, while in LinB, Mtcy1 and DhaA the analoguous catalytic acid (Glu) is positioned after the strand 6 (Fig. 6). Such repositioning of the second catalytic acid was experimentally probed by Krooshof and co-workers using site-directed mutagenesis and molecular mechanics calculations (ref. 34).

SPECULATION ON THE FURTHER EVOLUTION OF THE DHLA HALOALKANE DEHALOGENASE

Can we say also something about possibilities for the further natural evolution of the haloalkane dehalogenase of *X. autotrophicus* GJ10? Its evolutionary youth has been implicated from its catalytic imperfection (the enzyme converts only 6 substrate molecules per second) and simple catalytic cycle without use of any co-factors or metal ions (ref. 6). The industrial production of its natural substrate, 1,2-dichloroethane, started only 75 years ago giving insufficient time for optimization of its structure and reaction mechanism. The enzyme is presently trapped in the local evolutionary minimum with simultaneously optimized rates for the carbon-halogen bond cleavage and the halide export. Fine tuning of the dehalogenase structure, e.g., by point mutations, might not lead to further improvement of the enzyme and a more fundamental change could be necessary for the protein to escape from this local minimum. A large number of *in vitro* point mutants of haloalkane dehalogenase have already been prepared in the laboratory of Professor D.B. Janssen (University of Groningen), some of

them with either faster C-X bond cleavage or faster halide export (ref. 21,28,34,35), but none of them with improved overall kinetic performance for DCE. Modification of the reaction mechanism could lead to the simultaneous improvement of both C-X bond cleavage and the halide export. Using QM calculations, we have demonstrated (ref. 29) that coupling of the S_N2 dehalogenation with hydrogenation of the halide ion can result in faster reaction kinetics of the dehalogenation reaction and the formation of the HCl (Fig. 8). Fast export of electroneutral HCl from the enzyme active-site would be expected. Evolutionary event leading to the development of such an enzyme would involve incorporation of the hydrogen-donating co-factor to the molecule of haloalkane dehalogenase.

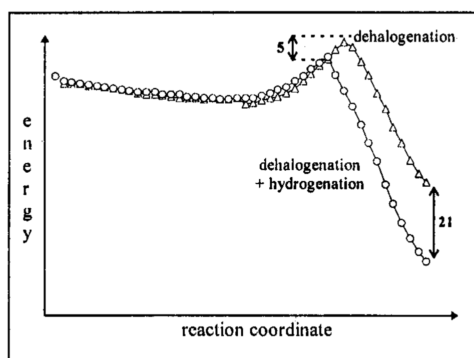

Fig. 8: Comparison of the energy profiles calculated for the S_N2 dehalogenation and the S_N2 dehalogenation coupled with hydrogenation of the halide ion. Doubly protonated histidine served as a hydrogen donor. The numbers quantify the differences between the activation energy barriers and between the energy of products (in kcal mol^{-1}) derived from AM1 semi-empirical quantum-mechanic calculation. The dehalogenation coupled with hydrogenation is kinetically favourable and results in the formation of the electroneutral HCl

GENERAL CONCLUSIONS

The application of computer modelling and bioinformatics to the study of the molecular mechanisms of biodegradation reactions has been illustrated in this contribution. Theoretical calculations can guide experiments or help with their interpretation. Computer modelling provides the tools enabling us to study problems which are experimentally outside the range of present technical possibilities, like localization of the transition states of chemical reactions, study of dynamic behaviour of biomolecules or structural analysis of the proteins which can not be crystallized and are too large or unstable for NMR measurements. The haloalkane dehalogenase is just one enzyme involved in biodegradation reactions on which computational study is being undertaken. Other calculations were conducted with cytochrome P450cam by the group of Rick L. Ornstein (Pacific Northwest Laboratory). Several other biodegradative proteins with known tertiary structures, like protocatechuate 3,4-dioxygenase, methane monooxygenase, dihydroxybiphenyl dioxygenase, L-2-haloacid dehalogenase, 4-chlorobenzoyl-CoA dehalogenase, represent suitable targets for future theoretical studies which will broaden our understanding of degradation reactions at a molecular level.

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REFERENCES

1. M. Alexander. *Biodegradation and Bioremediation*, p. 308. Academic Press, San Diego (1994).
2. J.P. Tollenaere, P.A.J. Janssen. *Med. Res. Rev.* 8, 1-25 (1988).
3. A.J. Mulholland,G.H. Grant, W.G. Richards. *Prot. Engng.* 6, 133-147 (1993).
4. M. Karplus, G.A. Petsko. *Nature* 347, 631-639 (1990).
5. J. Damborsky, M. Kuty, M. Nemec, J. Koca. In *QSAR in Environmental Sciences - VII* (F. Chen, G. Schuurmann, eds.), SETAC Press, Pensacola (1997).
6. D.B. Janssen, F. Pries, J.R. Van der Ploeg. *Annu. Rev. Microbiol.* 48, 163-191 (1994).
7. J.H. Slater, A.T. Bull, D.J. Hardman. *Biodegradation* 6, 181-189 (1995).
8. J. Damborsky, M.G. Nyandoroh, M. Nemec, I. Holoubek, A.T. Bull, D.J. Hardman. *Biotech. Appl. Biochem.* 26, 19-25 (1997).
9. Y. Nagata, K. Miyauchi, J. Damborsky, K. Manova, A. Ansorgova, M. Takagi. *Appl. Environ. Microbiol.* 63, 3707-3710 (1997).
10. D.B. Janssen, F. Pries, J. Ploeg, B. Kazemier, P. Terpstra, B. Witholt. *J. Bacteriol.* 171, 6791-6799 (1989).
11. Y. Nagata, T. Nariya, R. Ohtomo, M. Fukuda, K. Yano, M. Takagi. *J. Bacteriol.* 175, 6403-6410 (1993).
12. A.N. Kulakova, M.J. Larkin, L.A. Kulakov. *Microbiology* 143, 109-115 (1997).
13. K.H.G. Verschueren, S.M. Franken, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra. *J. Mol. Biol.* 232, 856-872 (1993).
14. I. Singleton. *J. Chem. Tech. Biotechnol.* 59, 9-23 (1994).
15. W.J.G.M. Peijnenburg, J. Damborsky (eds.) *Biodegradability Prediction*, p. 143. Kluwer Academic Publishers, Dordrecht (1996).
16. A.J. Van den Wijngaard, R.D. Wind, D.B. Janssen. *Appl. Environ. Microbiol.* 59, 2041-2048 (1993).
17. J. Damborsky, K. Manova, M. Kuty. In *Biodegradability Prediction* (W.J.G.M. Peijnenburg, J. Damborsky, eds.), pp. 75-92. Kluwer Academic Publishers, Dordrecht (1996).
18. J. Damborsky, M. Kuty, M. Nemec, J. Koca. *J. Chem. Inf. Comp. Sci.* 37, 562-568 (1997).
19. M. Kuty, J. Damborsky, M. Prokop, J. Koca. *J. Chem. Inf. Comp. Sci. in revision.*
20. J.P. Schanstra, J. Kingma, D.B. Janssen. *J. Biol. Chem.* 271, 14747-14753 (1996).
21. J.P. Schanstra, D.B. Janssen. *Biochemistry* 35, 5624-5632 (1996).
22. P.A. Evans, C.M. Dobson, R.A. Kautz, G. Hatfull, R.O. Fox. *Nature* 329, 266-268 (1987).
23. W.J. Chazin, J. Kordel, T. Drakenberg, E. Thulin, P. Brodin, T. Grundstrom, S. Forsen. *Proc. Natl. Acad. Sci. USA* 86, 2195-2198 (1989).
24. J. Koca. *J. Mol. Struct. (Theochem)* 308, 13-24 (1994).
25. K.H.G. Verschueren, F. Seljee, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra. *Nature* 363, 693-698 (1993).
26. K.H.G. Verschueren, J. Kingma, H.J. Rozeboom, K.H. Kalk, D.B. Janssen, B.W. Dijkstra. *Biochemistry* 32, 9031-9037 (1993).
27. C. Kennes, F. Pries, G. . Krooshof, E. Bokma, J. Kingma, D.B. Janssen. *Eur. J. Biochem.* 228, 403-407 (1995).
28. J.P. Schanstra, I.S. Ridder, G.J. Heimeriks, R. Rink, G.J. Poelarends, K.H. Kalk, B.W. Dijkstra, D.B. Janssen. *Biochemistry* 35, 13186-13195 (1996).
29. J. Damborsky, M. Bohac, M. Prokop, M. Kuty, J. Koca. *submitted.*
30. W.J. Philipp, S. Poulet, K. Eiglmeier, L. Pascopella, V. Balasubramanian, B. Heym, S. Bergh, B.R. Bloom, W.R. Jacobs, S.T. Cole. *Proc. Natl. Acad. Sci. USA* 93, 3132-3137 (1996).
31. J. Damborsky, A.T. Bull, D.J. Hardman. *Biologia* 50, 523-528 (1995).
32. D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verschueren, A. Goldman. *Prot. Engng.* 5, 197-211 (1992).
33. F. Pries, A.J. VandenWijngaard, R. Bos, M. Pentenga, D.B. Janssen. *J. Biol. Chem.* 269, 17490-17494 (1994).
34. G.H. Krooshof, E.M. Kwant, J. Damborsky, J. Koca, D. B. Janssen. *Biochemistry* 36, 9571-9580 (1997).
35. J.P. Schanstra, A. Ridder, J. Kingma, D.B. Janssen. *Prot. Engng.* 10, 53-61 (1997).