Molecular basis of two novel dehalogenating activities in bacteria

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Chapter 8: Summary
Introduction

It is generally believed that evolution is at the heart of species differentiation of all living organisms, a hypothesis that was first stated by Charles Darwin in his revolutionary work "The Origin of Species". Although this hypothesis was originally based on phenotypic similarities between different species, the current knowledge of the genomes of various species shows that interspecies relationships are even more evident on the genetic level, even when species have very dissimilar phenotypes. Central to the current view of Darwinian evolution and species differentiation are: (i) mutation/recombination of the genetic code and the corresponding phenotypic changes (mutation); (ii) differential survival of altered individuals in the population (selection); and (iii) the propagation of beneficial changes in the population by (a)sexual replication (amplification).

Despite this concise theoretical concept, the mechanisms that gave rise to the current species diversity are poorly understood. This is partly due to the tremendously large timescale of evolutionary changes becoming apparent in higher organisms, making it impossible to get a good idea of the intermediates along the pathway. Often, fossils are the only proof of the past existence of such intermediates ('missing links') that could support the hypothesized evolutionary path between different species, like, for example, the evolution of birds from reptiles and the evolution of Homo sapiens. It is therefore not surprising that the theory of evolution has long been in the realm of theoretical biology, suffering from the inaccessibility to empirical experiments to support hypotheses.

Nowadays this situation is drastically changing. The elucidation of the complete genome sequences of different organisms and the development of modern molecular biology techniques have cleared the road to experimental evolution. Many examples of evolutionary adaptation are being studied in model organisms in the laboratory, like, for example, the adaptation of pathogenic bacteria to antibiotics, or the adaptation of the HIV virus to antiviral drugs. Such studies have made clear that microbes, which divide approximately every 10 to 30 minutes, can rapidly adapt themselves to external factors by mutation and recombination of their gene repertoire. Structure determination techniques like X-ray crystallography and nuclear magnetic resonance (NMR) have revealed the molecular basis of such evolutionary adaptations, in which mutations in a gene can cause changes in the gene-encoded product. Subsequent selection and proliferation of mutated genes thus may result in changes in activity and function of proteins inside the cell, thus providing the basis of evolution at the molecular level.
Dehalogenation of anthropogenic halogenated hydrocarbons by bacteria

From an evolutionary point of view, enzymes that facilitate the growth of certain bacteria on anthropogenic halogenated compounds are especially intriguing, as some of them may have only recently evolved by adaptation of related enzymes in response to elevated concentrations of these compounds in the environment. Although many halogenated compounds occur naturally, compounds like 1,2-dibromoethane and 1,3-dichloro-2-propanol are presumed to be xenobiotic, meaning that they were absent in the environment before their industrial production. The (geno)toxic properties of some man-made halogenated compounds may have put a large evolutionary pressure on bacteria to evolve enzymes that facilitate cleavage of the carbon-halogen bond, and release the halogen as a less toxic halide ion. Various catabolic pathways that depend on such dehalogenases have been identified in bacteria isolated from contaminated soil or water samples, in which they convert specific halogenated compounds into alternative growth substrates for the bacterium.

Different ways to cleave a carbon-halogen bond

Biochemical characterization of several dehalogenases from bacteria revealed a wide variety of different chemical strategies that are used in nature to facilitate cleavage of carbon-halogen bonds. Several anaerobic halorespiring bacteria contain reductive dehalogenases that enable them to use tetrachloroethene, rather than oxygen, as the final electron acceptor in the respiratory chain. Aerobic bacteria, instead, may contain mono- or dioxygenases to oxidatively dehalogenate small haloalkenes, haloalkanes and some aromatic compounds. Most of the structurally characterized dehalogenases, however, utilize cofactor-independent $S_{N}2$-type substitution mechanisms that proceed via a covalently bound intermediate with a nucleophilic aspartate, which is subsequently hydrolyzed by a water molecule. Haloalkane dehalogenase, haloacid dehalogenase and 4-chlorobenzoyl-coenzyme A dehalogenase all make use of such a mechanism, but have different amino acid sequences and different three-dimensional structures. They are evolutionary unrelated to large superfamilies of enzymes with fundamentally different activities. Subtle changes in the active site of the dehalogenases make the dehalogenation activity possible, but the lack of amino acid sequence identity with other
members of the enzyme superfamily make it impossible to identify the likely precursor enzyme.

**Molecular basis of two novel dehalogenating activities in bacteria**

This thesis describes the X-ray crystallographic elucidation of the molecular structures of two novel, unrelated bacterial dehalogenases, the haloalcohol dehalogenase HheC from *Agrobacterium radiobacter* AD1, and a trans-3-chloroacrylic acid dehalogenase (CaaD) from *Pseudomonas pavonaceae* 170. Structures of the native enzymes, and of complexes of the enzymes with products, substrate analogues, or inhibitors provide conclusive insights into the mechanistic details of two novel dehalogenation strategies, which fundamentally differ from the mechanisms of previously characterized haloalkane-, haloacid-, and 4-chlorobenzoyl-Coenzyme A dehalogenases. Chapter 2 gives a detailed overview of the structures and mechanisms of the different bacterial dehalogenases, emphasizing the differences and similarities with evolutionary related enzyme families, and on the possible evolutionary origins of the dehalogenases.

In chapter 3, 4 and 5, the crystallization, the structure elucidation and the catalytic mechanism of haloalcohol dehalogenase HheC are described. HheC catalyzes the dehalogenation of vicinal haloalcohols like 2,3-dichloro-1-propanol via an one-step intramolecular substitution mechanism, yielding the corresponding epoxide and HCl. The results show that HheC is structurally and mechanistically related to a widespread family of NAD(P)H-dependent redox enzymes, the short-chain dehydrogenases/reductases (SDR family). X-ray structures of HheC with bound products and a substrate analogue demonstrate that catalysis depends on a conserved Ser-Tyr-Arg catalytic triad. The SDR NAD(P)H-binding site has changed, however, into a halide-binding site that supports the cofactor-independent dehalogenation reaction. Structure-based sequence alignments further evidence that the six haloalcohol dehalogenases that are currently known have evolved from two different NAD-binding SDR enzymes, rather than from NAD(P)-binding enzymes.

In chapter 6, I present X-ray structures of the trans-3-chloroacrylic acid dehalogenase CaaD from *P. pavonaceae* 170, both of the native enzyme and of a form that is covalently inactivated by a mechanism-based inhibitor. The results provide conclusive evidence that CaaD cleaves an sp²-hybridized carbon-halogen bond via a Michael addition of water to trans-3-chloroacrylate, a degradation product of the soil
fumigant 1,3-dichloropropene. Subsequent decomposition of the product, an unstable halohydrin, results in the release of malonate semialdehyde and HCl. CaaD is related to the well-studied *Pseudomonas* enzyme 4-oxalocrotonate tautomerase (4-OT). Whereas this tautomerase uses an N-terminal catalytic proline (Pro1) as a base to transfer protons in tautomerization reactions, the Pro1 in the dehalogenase is acidic, stabilized in a hydrophilic active site. Together with a buried glutamate base, the acidic proline facilitates a hydratase activity, rather than a tautomerase activity.

**On the origin of dehalogenating activities in bacteria**

Although the evolutionary origins of most dehalogenases remain unclear, the structural information on haloalcohol dehalogenase HheC and trans-3-chloroacrylic acid dehalogenase CaaD offers insights into their likely evolutionary origins. In contrast with the haloalkane dehalogenases and haloacid dehalogenases, only a few amino acid sequences of haloalcohol dehalogenases and 3-chloroacrylic acid dehalogenases are known. Their rare occurrence in soil bacteria, and their absence in other organisms, suggests that they may have evolved from related enzymes with different activities in response to elevated concentrations of their halogenated substrates in the environment. Catalytic promiscuity of a precursor enzyme may have played an important role in their emergence in bacteria. An SDR precursor of the haloalcohol dehalogenases may fortuitously have bound a haloalcohol substrate, possibly with the halogen atom positioned in the NADH cofactor-binding site. The catalytic tyrosine may have been able to deprotonate the haloalcohol hydroxyl group, thereby catalysing the intramolecular substitution reaction. Such a rudimentary dehalogenase activity may subsequently have been optimized by evolutionary selection. A similar scenario may apply to the emergence of 3-chloroacrylic acid dehalogenase activity in bacteria. In contrast to the SDR enzymes, a few tautomerases have been experimentally shown to possess a rudimentary dehalogenase activity. It will be a scientific challenge to verify such hypotheses on the evolutionary origin of dehalogenases using directed evolution.