Intramembrane proteases regulate a growing number of biological processes emanating from biological membranes. The structures of bacterial homologues of all four catalytic types of these integral membrane proteins have been solved, but their mechanism is incompletely understood, mainly because of the difficulty of gaining structural information about how they bind substrates. To gain insight into substrate binding to rhomboid proteases, we have synthesized a series of covalently binding peptide chloromethylketones and analysed their interactions with E.coli rhomboid GlpG enzymologically and structurally. We show that tetrapeptidyl chloromethylketones derived from a natural rhomboid substrate bind GlpG covalently in a mechanism-based and sequence-dependent, substrate-like manner. We have solved the crystal structures of several tetrapeptidyl chloromethylketones, which reveal, for the first time, the molecular interactions of a substrate peptide with an intramembrane protease. The overall binding mode differs from previous speculative models. The structures explain the observed amino acid preferences in substrates and show the S1 to S4 subsites of rhomboid active site. The S1 site is well formed and extends into a water filled cavity (“water retention site”) previously proposed to be important for acyl-enzyme hydrolysis. Unexpectedly, the S4 subsite is formed by residues from the peripheral structure of the L1 loop. Mutational and crystallographic analysis of the S4 subsite shows that it is plastically formed by three residues of the L1 loop. Given that the L1 loop region has diversified and expanded in rhomboid-like proteins including iRhomts, we propose that this element of the rhomboid fold plays a role in substrate or client protein binding in the broader rhomboid-like superfamily. Finally, using molecular dynamics and the published structural and enzymatic data we propose a model of the P4 to P3’ region of the substrate that is in contact with the active site of GlpG. The mode of binding of the rest of the transmembrane domain of the substrate is less clear and we propose two possible scenarios.
were solved. Structural analyses, site-directed mutagenesis experiments, and phylogenetic studies allowed us to identify the residues responsible for the observed differences in substrate specificity between the NRH isoforms. The presence of a tyrosine at position 249 (PpNRH1 numbering) conferred high hydrolase activity for purine ribosides, while an aspartate residue in this position conferred high activity for uridine. To analyze the physiological role of the PpNRHs, single knockout mutants were generated. NRH deficiency caused delayed bud formation and under conditions of nitrogen shortage, PpNRH1-deficient plants cannot salvage adenosine-bound nitrogen. NRH deficiency was accompanied by significant changes in the levels of purine, pyrimidine and cytokinin metabolites relative to those seen in the wild-type, illustrating the importance of these enzymes in nucleoside and cytokinin metabolism.

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**ENGINEERING ENZYME ACCESS TUNNELS FOR PROTEIN STABILIZATION**

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Enzymes have a great potential in many areas of biotechnology. Natural enzymes have not evolved for hostile industrial environment such as extreme pH, elevated temperature or presence of co-solvents, thus stabilization of enzymes against unnatural conditions has become an important target for protein engineers. Most of the studies focused on enzyme stabilization focused on active site or its vicinity, on modification of protein surface or highly flexible protein regions [1-6]. Haloalkane dehalogenases are predominantly microbial enzymes with great potential in biotechnology and biocatalysis. However, these practical applications are limited due to low resistance of haloalkane dehalogenases to organic co-solvents and elevated temperature [7]. Recently constructed variant of haloalkane dehalogenase DhaA exhibited 4000-fold improved kinetic stability in 40 % (v/v) DMSO, enhanced thermostability by 16.4 °C, but 100-fold lower catalytic activity with 1,2-dibromoethane in a pure buffer compared to the wild type enzyme. Enzyme stabilization was achieved by introduction of four bulky and mostly hydrophobic residues into the enzyme access tunnel. These residues improved a contact with other residues of the access tunnel, enhanced packing of hydrophobic core and prevented the entry of DMSO into the active-site cavity [8]. Objective of this follow up study was focused on improvement of catalytic activity of highly stable DhaA variant in pure buffer with minimum loss of its stability. Saturation mutagenesis in two of the four tunnel positions resulted in a single point variant, whose catalytic activity was increased 32- and 10-fold in pure buffer and in 40 (v/v) % DMSO, respectively, while thermal stability was lowered just by 4 °C. Structural analysis and molecular dynamics revealed that the newly introduced mutation (F176G) reopened previously closed tunnel in stable DhaA and thus restored enzymatic activity, while remaining tunnel mutations maintained protein stability. Optimization of amino acid residues lining the access tunnels thus represents a generally-applicable strategy for fine-tuning stability-function trade-off of enzymes with buried active sites.

INVESTIGATION OF GATING MECHANISMS IN THE ACCESS TUNNEL OF HALOALKANE DEHALOGENASES

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Molecular gates are structural features present in many diverse biological systems, namely enzymes, ion channels, protein-protein and protein-nucleic acid complexes. In enzymes, gates may control the ligands’ entry to and egress from buried active sites, regulate the access of solvent molecules, or even synchronize molecular events taking place in different parts of the protein. However, the mechanism of gating is not well understood in spite of their broad occurrence [1], and their rational engineering is challenging [2].

The haloalkane dehalogenases (HLDs) are bacterial enzymes that catalyze the hydrolysis of a wide variety of halogenated organic compounds into the corresponding alcohols. This property makes them very interesting for a number of biotechnological applications, such as bioremediation, biocatalysis, and biosensing. Redesign of dehalogenase tunnels has been accomplished in previous works and has proven successful to increase enzyme activity, enantioselectivity and stability. DhaA31 mutant is an example in which the narrowing of the access tunnels increased $k_{cat}$ by 32-fold towards an anthropogenic pollutant, 1,2,3-trichloropropane (TCP) [3].

In the present work we investigated eventual gating processes present in the access tunnels of the DhaA31 mutant [4]. We have performed four 1 μs molecular dynamics simulations using AMBER 12 [5] and analyzed tunnel dynamics by CAVER 3.0 [6]. From these calculations, a gating process could be identified on the main tunnel of DhaA31. This gating is ruled by an alpha helix movement, associated with the movement of the side chains of key residues Phe149, Phe168, Ala172, and Tyr176. The observed gate may regulate not only the exchange of the ligands, but also the number of solvent molecules accessing the active site during the catalytic cycle. Periodic desolvation may explain the observed increase in the catalytic activity for DhaA31 with respect to the wild type. Furthermore, these studies point out the direction for additional improvements in the next round of protein engineering by a rational re-design of this gate.

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