Protein Stability

DOI: 10.1002/anie.201206708

Engineering Enzyme Stability and Resistance to an Organic Cosolvent by Modification of Residues in the Access Tunnel**

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Biocatalysts produced by mesophilic organisms exhibit high selectivity and operability under environmentally friendly conditions.[1] However, their practical applications can be limited by low resistance to the elevated temperatures and to organic cosolvents required for solubilization of hydrophobic substrates.[2] These problems can be circumvented by identifying and isolating stable biocatalysts from extremophilic organisms.[3] Alternatively, an enzyme's stability can be tailored by modifying its structure by using protein engineering.[3c,4] Generally used methods include mutagenesis towards stable counterparts, consensus-based approaches, optimization of flexible regions, rational introduction of rigidifying and cavity-filling mutations, introduction of specific stabilizing interactions and metal-chelating sites.[2b,4,5] Although application of these methods to particular enzymes has resulted in biocatalysts with enhanced stability, no universal engineering strategy for protein stabilization is currently available.[4b,6]

A large number of chemical reactions are catalyzed in the active sites localized on the protein surface, but many enzymes have their active site buried and connected with the surrounding solvent by an access tunnel.[7] The existence of access tunnels has been described in literature for a wide range of enzymes from all six enzyme classes.[7b] The aim of this study was to identify structural features of the enzymes possessing buried active sites governing their stability and resistance to organic cosolvents. The haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064 (EC 3.8.1.5)[8] has been chosen as a model system for this protein engineering study. DhaA is composed of 293 amino acids and is a representative of the large superfamily of α/β-hydrolases.[8b,9] Here, we constructed a systematic set of DhaA variants (Table 1) by random and site-directed mutagenesis, and characterized them structurally, kinetically, and computationally. The acquired complementary data provided detailed insights into the structure–stability relationships of this enzyme and enabled us to construct a biocatalyst with

Table 1: Substitutions in the DhaA variants.

Variant	Position										
	78	80	95	148[a]	171[a]	172[a]	176[a]	227	240	291	292
DhaA	D	F	L	T	G	A	C	N	W	P	A
DhaA57	D	F	***V***	T	G	***V***	C	N	W	P	A
DhaA60	D	F	***V***	T	G	A	C	N	W	P	A
DhaA61	D	F	L	T	G	***V***	C	N	W	P	A
DhaA63[b]	***G***	***S***	L	***L***	***Q***	***V***	***F***	***T***	***Y***	***A***	***G***
DhaA80	D	F	L	***L***	***Q***	***V***	***F***	N	W	P	A
DhaA82	***G***	***S***	L	T	G	A	C	***T***	***Y***	***A***	***G***
DhaA85	D	F	L	***L***	***V***	***V***	***F***	N	W	P	A
DhaA88	D	F	L	***L***	***L***	***V***	***F***	N	W	P	A

[a] Amino acid lining the access tunnel. [b] Equivalent to Dhla8 described by Gray et al.[8c] Substituted residues are highlighted in bold.

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[**] Dr. Jiri Jarkovsky (Institute of Biostatistics and Analysis, Masaryk University, Brno) is gratefully acknowledged for help with statistical data analysis. T.K. acknowledges scholarships from the Federation of European Microbiological Societies and Ernst-Moritz-Arndt-University for research visits to the Department of Biotechnology and Enzyme Catalysis, Greifswald (Germany). This work was supported by the Ministry of Education of the Czech Republic (LC06010 and MSM0021622412), the Grant Agency of the Czech Republic (203/08/0114, P202/10/1435, and P207/12/0775), the Grant Agency of the Czech Academy of Sciences (IAA401630901) and the European Regional Development Fund (CZ.1.05/2.1.00/01.0001). U.T.B. thanks the German Research Foundation (DFG) for financial support (SPP 1170, Bo1864/4-1). MetaCentrum is acknowledged for providing access to computing facilities, supported by the Czech Ministry of Education of the Czech Republic (LM2010005).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201206708.

substantially improved stability and resistance to organic cosolvent.

One of the universal substrates of haloalkane dehalogenases, 1,2-dibromoethane,[8c] was used for initial activity and kinetic measurements to quantify the effects of dimethyl sulfoxide (DMSO) as a cosolvent on activity and stability of DhaA (Table 2, Figure S1 in the Supporting Information).

Table 2: Steady-state kinetic parameters of DhaA variants with 1,2-dibromoethane in 40% DMSO.[10]

Variant	K_m or $K_{0.5}$[a] [mM]	K_{si} [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
DhaA	14.17 ± 1.58	ND[d]	2.25 ± 0.13	0.16 ± 0.02
DhaA57	4.09 ± 0.29[b]	ND[d]	0.50 ± 0.02	0.12 ± 0.01
DhaA61	5.47 ± 0.34[c]	ND[d]	1.01 ± 0.04	0.18 ± 0.01
DhaA63	1.08 ± 0.26	41.44 ± 14.14	0.72 ± 0.06	0.67 ± 0.16
DhaA80	0.88 ± 0.16	13.14 ± 2.73	0.25 ± 0.02	0.29 ± 0.05
DhaA85	1.40 ± 0.30	6.69 ± 1.61	0.36 ± 0.04	0.26 ± 0.06
DhaA88	5.10 ± 3.29	2.34 ± 1.54	0.86 ± 0.44	0.17 ± 0.07

[a] K_m for all variants except DhaA57 and DhaA61; $K_{0.5}$ for DhaA57 and DhaA61, where cooperativity was observed. [b] Cooperativity with Hill coefficient $n_H = 2.43 \pm 0.28$. [c] Cooperativity with Hill coefficient $n_H = 1.93 \pm 0.13$. [d] ND, not detected.

DMSO was chosen for this purpose, since it caused the lowest perturbation of DhaA structure in a preliminary screen of fourteen water-miscible organic cosolvents. Addition of 40% (v/v) DMSO led to an order of magnitude drop in the initial catalytic rate of DhaA and dramatic reduction of its stability at 37°C (Figure 1, Table S1 in the Supporting Information), quantified by the half-life of the enzyme.[2b] The structural changes of DhaA induced by the organic cosolvent were revealed by fluorescence spectra, which were acquired in the presence of DMSO at various concentrations, providing the half-concentration.[2b] Although the melting temperature[2b] of DhaA in buffer is 50°C, presence of 40% (v/v) DMSO caused early protein denaturation at 37°C.

Unless the molecular basis of enzyme resistance to organic cosolvent is known, random mutagenesis, followed by screening or selection, is an appropriate method for generating desired variants.[2a] To improve the stability of DhaA, the recombinant gene *dhaA* (Table S2 in the Supporting Information) was randomly modified by error-prone PCR, according to the protocol by Schmidt et al.[11] at the average mutation rate of 1.5 nucleotides per sequence. Colonies of the error-prone PCR library were screened by using a pH-indicator-based colorimetric assay,[12] optimized for the presence of the organic cosolvent DMSO. Altogether, we examined the activity of 5362 colonies towards 1,2-dibromoethane in 42% (v/v) DMSO and identified four positive hits (Figure S2 in the Supporting Information).

One of these hits, the double mutant DhaA57, exhibited significantly improved stability compared to the wild-type enzyme, while keeping a similar level of catalytic efficiency (Figure 1, Table 2). Mapping of these substitutions on the structure of wild-type DhaA[8b] revealed that one of them (Leu95Val) is located on the protein surface, whereas the other (Ala172Val) lines the access tunnel. The single-point mutants DhaA60 and DhaA61, constructed by site-directed mutagenesis, showed that entire stabilization of DhaA57 is due to the mutation located in the access tunnel (Figure 1). Circular dichroism spectroscopy measurements revealed that this mutation also improved stability of the protein structure at elevated temperatures. Thermostable enzymes with improved resistance to other denaturing factors have been reported.[3c,13] The observed correlation between the resistance to DMSO and to elevated temperatures prompted us to reconstruct the thermostable ten-fold mutant of DhaA described by Gray et al.,[8c] here named DhaA63 (Table 1). Characterization of DhaA63 revealed a substantially higher melting temperature (by 18°C) and also exceptional resistance to the organic cosolvent, with the half-life in 40% (v/v) DMSO extended to more than three months (Figure 1). The catalytic efficiency of DhaA63 in the aqueous buffer was 6-fold lower, but in 40% DMSO it showed 4-fold higher catalytic efficiency than the wild-type enzyme (Table 2). To dissect the contributions of mutations to the stability of DhaA63, we constructed DhaA80, carrying the four substitutions in the access tunnel (Thr148Leu, Gly171Gln, Ala172Val, and Cys176Phe), and DhaA82 with six mutations on the protein surface (Asp78Gly, Phe80Ser, Asn227Thr, Trp240Tyr, Pro291Ala, and Ala292Gly). Essentially all structural stabilization came from the four mutations in the access tunnel and their effects were additive (Table S3 in the Supporting Information). Tunnel and surface mutations jointly contributed to the enzyme's kinetic stability and catalytic efficiency towards 1,2-dibromoethane (Table 2, Figures S1 and S3 in the Supporting Information). Subsequent saturation mutagenesis of DhaA80 focused on a variable tunnel residue 171, which showed three different stabilizing substitutions in the study of Gray et al.[8c] This mutagenesis resulted in two variants, DhaA85 and DhaA88, with further improved stability (Figure 1). Altogether, our results demonstrate that as few as four point mutations in the tunnel residues can improve enzyme's melting temperature in aqueous buffer by 19°C, double its resistance to DMSO, and prolong the half-life in the presence of organic cosolvent from minutes to weeks.

The crystal structures of DhaA57 and DhaA80 were determined to 1.2 Å and 1.5 Å resolution,[14] respectively, to understand the molecular basis of the observed stabilization. The visual inspection of the structures revealed that the bulkier and mostly hydrophobic side chains of introduced substitutions display improved contact with the other residues of the access tunnel (Figure 2). Stabilization at elevated temperatures is most probably due to enhanced packing of the hydrophobic core caused by the residues lining the access tunnel (Leu148, Asn171, Val172, and Phe176). Molecular dynamics simulations of DhaA, DhaA57, and DhaA80 in 40% (v/v) DMSO were carried out to explore the molecular basis of the resistance to organic cosolvent. Comparison of the trajectories revealed that mutations in the tunnel influence the access of cosolvent molecules to the active-site cavity (Figure 2). The introduced substitutions sealed the tunnel and possibly prevented destabilization of the protein structure by DMSO molecules entering the active site. This observation is consistent with reports describing the importance of shielding

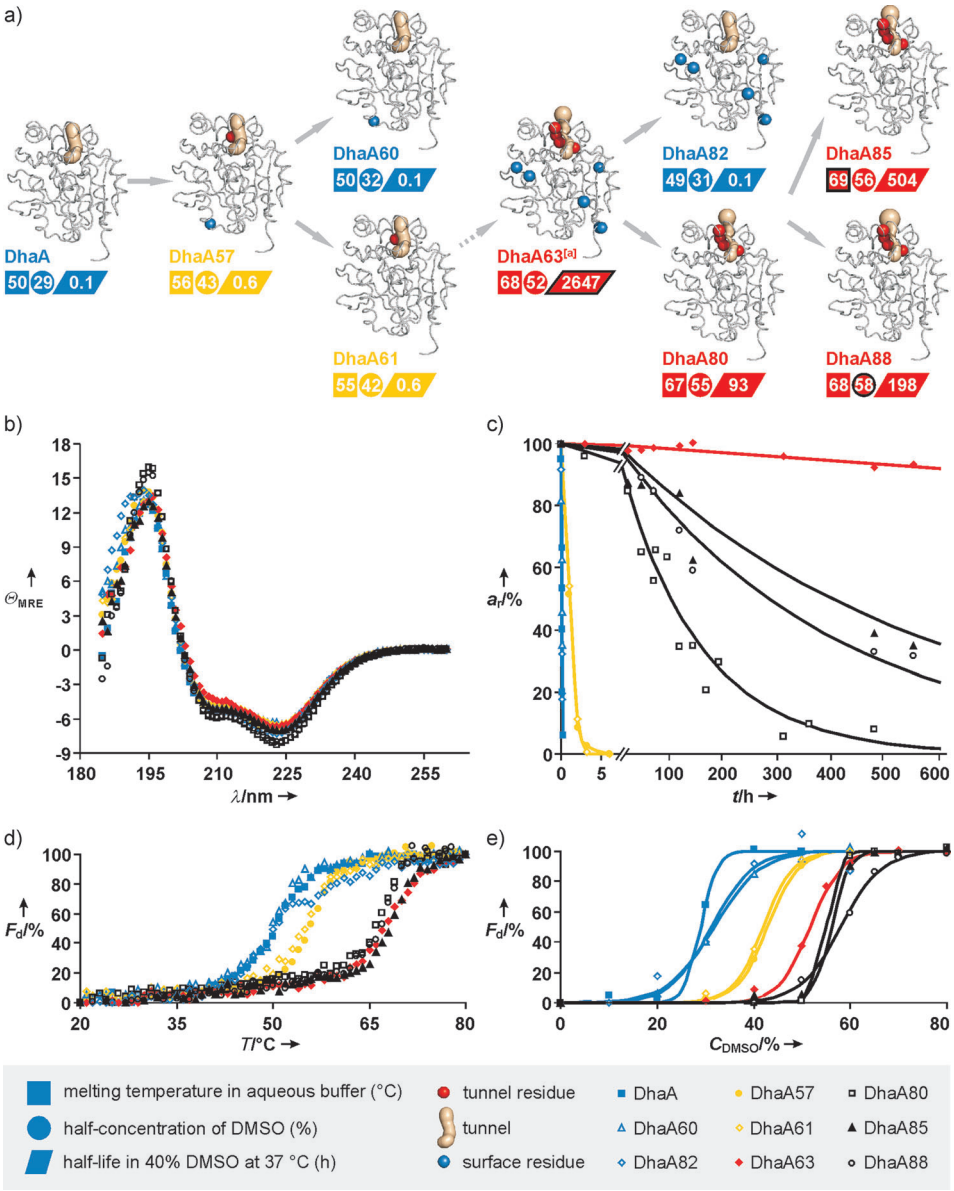

Figure 1. Stabilization of DhaA by engineering of the access tunnel. a) Mutations located in the access tunnel were essential for improving the structural and kinetic stability of DhaA variants, while solely surface mutations did not contribute to protein stabilization. Thermostability, structural resistance to DMSO, and kinetic stability in 40% (v/v) DMSO were quantified by the melting temperature (T_m), half-concentration ($C_{1/2}$), and the half-life ($\tau_{1/2}$), respectively.[2b] The color of shapes indicates relative stability compared to the wild-type enzyme; blue: no improvement, yellow: improvement, red: substantial improvement, and black frame: the greatest improvement. The scheme was created using PyMOL 1.2.8 (DeLano Scientific, San Francisco, CA, USA). [a] Mutant equivalent to Dhla8 from gene site saturation mutagenesis.[8c] b) Far-UV circular dichroism spectra of folded enzymes used for analyses measured in phosphate buffer (50 mM, pH 7.5). The mean residual ellipticity (θ_{MRE}) is given in $10^3 \times \text{deg cm}^2\,\text{dmol}^{-1}$. c) Kinetic stability was measured as changes with time of residual activity (a_r) towards 1,2-dibromoethane in the presence of 40% (v/v) DMSO at 37°C. d) Structural resistance to elevated temperatures was determined as thermal denaturation in phosphate buffer (50 mM, pH 7.5). Only every tenth point is shown. F_d refers to the unfolded fraction. e) Structural resistance to DMSO was tested in mixtures of phosphate buffer (50 mM, pH 7.5) and DMSO after incubation for 30 minutes at 37°C. F_d refers to the unfolded fraction.

the interior of proteins from organic solvents.[15] To further explore this phenomenon, we tested the stability of wild-type DhaA and four variants with cumulatively introduced substitutions to the access tunnel in the presence of three cosolvents of a different size: acetonitrile < DMSO < *tert*-butyl alcohol. The differential scanning calorimetry experiments revealed that the resistance against individual solvents does not reflect their molecular size and at the same time significantly correlates with the thermal stability of the variants (Table S4 and Figure S4 in the Supporting Information). The same molecular basis most probably determines stability at higher temperatures and in the presence of organic cosolvents.

To validate general applicability of the concept of tunnel engineering, the stability effects of all possible single point mutations in 26 different proteins from all six enzyme classes were analyzed (Table S5 and Figure S5 in the Supporting Information). The effect of 227924 mutations, out of which 43681 were localized in the access tunnels, was evaluated computationally using FoldX.[16] At least one stabilizing mutation ($\Delta\Delta G \leq -1$ kcal mol^{-1}) was identified for 29.2% (95% confidence interval, 27.3–31.1%) of the tunnel residues, compared to 21.3% (20.5–22.1%) of the residues located in other protein regions (Figure 3, Table S6 in the Supporting Information). The difference between the tunnel and other protein regions becomes even more profound if one considers highly stabilizing mutations ($\Delta\Delta G \leq -2$ kcal mol^{-1}): the highly stabilizing mutation was found for 9.2% (8.0–10.4%) of the tunnel residues, compared to only 4.7% (4.3–5.2%) of the residues in other protein regions. This implies that saturation mutagenesis targeting the tunnel residues has a two times better chance to produce protein variants with significantly improved stability than mutagenesis targeting other protein regions.

We conclude that saturation mutagenesis of the residues lining the access tunnels is a widely applicable strategy for

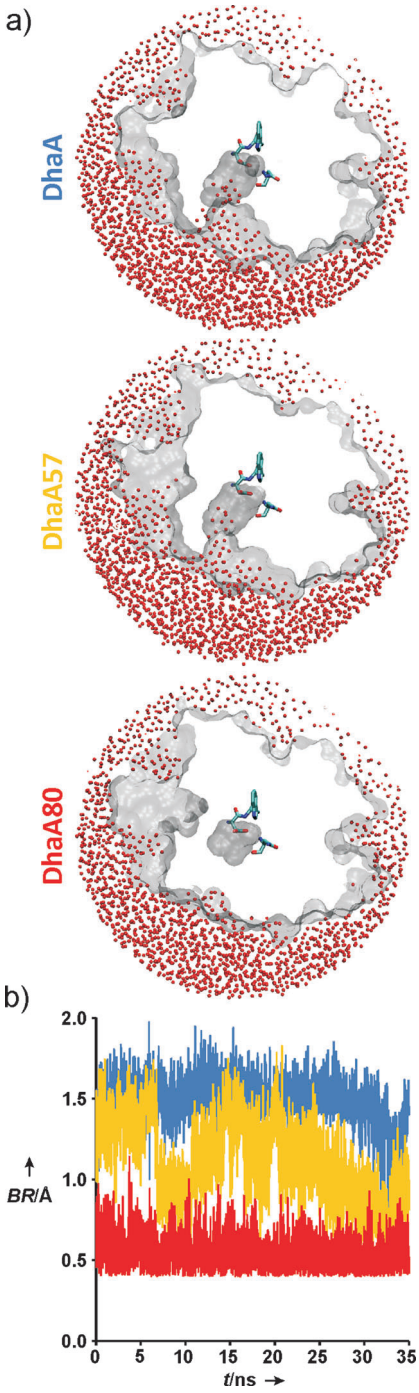

Figure 2. Structural analysis and molecular dynamics simulation of DhaA and its variants. a) The penetration of DMSO molecules into the active sites. The surfaces of the crystal structures of DhaA (PDB ID 1CQW),[8b] DhaA57 (PDB ID 4F5Z; this study), and DhaA80 (PDB ID 4F60; this study) are shown in gray, the nucleophile and halide-stabilizing residues as green sticks, and the cumulative positions of DMSO molecules obtained from MD simulations as red dots. b) Time evolution of the tunnel bottleneck radius (*BR*) in DhaA (blue), DhaA57 (yellow), and DhaA80 (red) during 35 ns MD simulations. The average bottleneck radii decreased from (1.5 ± 0.1) Å in DhaA to (1.1 ± 0.3) Å in DhaA57 and (0.5 ± 0.1) Å in DhaA80.

engineering resistance to organic cosolvents and elevated temperatures in the proteins with buried active sites. This

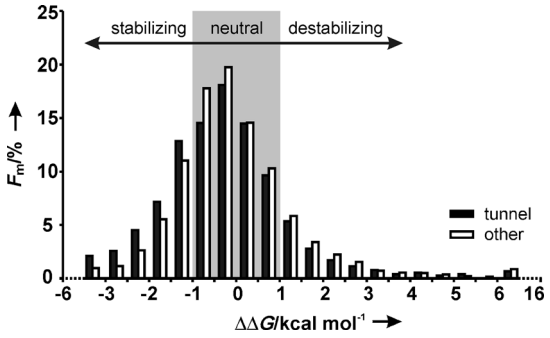

Figure 3. Evaluation of the stability effects of mutations located in the access tunnels and in other protein regions. For each protein from the test set, the $\Delta\Delta G$ values of all possible single point mutations were calculated by FoldX.[16] The mutation with the lowest $\Delta\Delta G$ was selected for each amino acid position. The selected mutations were divided into 20 bins according to their $\Delta\Delta G$ values. F_m refers to mutation fraction.

strategy consists of: 1) computer-assisted identification of the tunnel residues,[17] 2) construction of small focused libraries by variation of the amino acid composition at these sites,[5a,18] and 3) biochemical screening of the libraries for mutants showing high activity and improved stability. Computationally identifiable tunnel residues represent good targets for focused evolution. Substitutions in these natural hotspots do not disrupt the active-site architecture and provide high yields of functional variants.[8d] Fine-tuning of the access tunnels leads to the enzymes with enhanced thermal stability, but also the ability to selectively discriminate between substrate, product, and solvent molecules, and thus greater resistance to organic cosolvents.

Received: August 19, 2012
Revised: December 5, 2012
Published online: January 9, 2013

Keywords: directed evolution · enzyme catalysis · enzymes · protein engineering · protein stability

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