Enzyme Catalysis

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Systematic Optimization of Interface Interactions Increases the Thermostability of a Multimeric Enzyme**

*Andreas Bosshart, Sven Panke, and Matthias Bechtold**

With their exceptional selectivity, biocatalysts play an increasing role in diverse chemical fields including food processing, production of fine, specialty, and bulk chemicals, and fuel production.[1] A frequently remaining fundamental limitation is operational stability,[2] in particular at higher reaction temperatures, which are often preferred because of an increase in reaction rate or reactant solubility, a decrease in medium viscosity or risk of microbial contamination, or a more favorable position of the reaction equilibrium.[3] However, the structural integrity of an enzyme, especially of mesophilic origin, is often impaired under these high-temperature operational regimes. Inactivation typically starts with a loss of integrity of the quaternary (for multimeric enzymes) or tertiary structure (for monomeric enzymes) and is followed by an irreversible denaturation step.[4] The situation can be improved by immobilization,[5] additives,[6] or enzyme engineering,[7] preferably employing semi-rational approaches to prevent the often tedious development of high-throughput assay formats. Such approaches include increasing the similarity to a consensus amino acid sequence derived from sequence alignments[8] or are based on available crystal structures, which can direct the variation of presumably very flexible amino acids identified by their high atomic displacement parameters (B-factors).[7a] In the case of multimeric enzymes, the placement of intersubunit ionic interactions or disulfide bridges was shown to prevent subunit dissociation and increase biocatalyst stability.[9] However, successful placement of such strong links to prevent multimer dissociation[10] as well as proper disulfide formation in model expression hosts such as *E. coli* is nontrivial.[11] Consequently, generic strategies to systematically improve the thermostabilty of multimeric enzymes are still lacking.

We reasoned that a systematic variation of the non-conserved residues of a protein–protein interface should rapidly reveal those positions in the amino acid sequence of an enzyme, the mutation of which can contribute to strengthening the inter-subunit interface and thus counter disintegration. To test this hypothesis, we selected the homodimeric D-tagatose 3-epimerase of *P. cichorii* (PcDTE).[12] PcDTE catalyzes the reversible C3-epimerization of all four ketohexose epimer pairs[13] and is hence of strategic importance for preparative production of rare hexoses, which on industrial levels takes place at elevated temperatures to reduce viscosity and microbial contamination. However, wild-type (wt) PcDTE degrades rapidly at elevated temperatures. To exclude that disintegration of the tertiary structure of one of the monomers was actually the rate-determining step in thermal inactivation, we varied the 10 amino acids with the highest B-factors extracted from the analysis of 3 PcDTE crystal structures (PDB ID 2QUN, 2QUM, 2QUL) as described by Reetz et al.[14] Of these, only substitution of K122 (K122V) showed a slight impact on the thermostability (conversion of D-fructose to D-psicose, $T_{50}^{20} = 67.2\,^{\circ}C$ ($+1.2\,^{\circ}C$ compared to wt), with T_{50}^{20} being the temperature at which 50% activity is lost after 20 minutes of incubation (see the Supporting Information for details). Interestingly, K122 is part of the dimer interface, whereas six of the other residues are not, suggesting that disintegration of the quaternary structure is the more likely rate-limiting step in thermal inactivation.

As a first attempt towards stabilization of the dimeric interface of PcDTE, we introduced intersubunit disulfide bonds at positions F157 or position W160 and W262. These sites were selected by the program "MODIP"[15] as the only potentially suitable locations at the interface, albeit already with a low quality ranking. Correspondingly, the subsequent mutagenesis and expression in *E. coli* did not lead to enzyme variants with increased stability (see Figure S1 in the Supporting Information).

Instead, when we explored structure-guided systematic strengthening of interactions between the two subunits throughout the entire dimer interface, variants with improved thermostability could be readily isolated. For this, we conducted first a thorough analysis of one PcDTE crystal structure (PDB ID 2QUN) using the software PDBePISA[16] to identify the residues involved in interface formation. As a homodimer, PcDTE has a virtually symmetric interaction pattern, suggesting a maximum of 44 sites for engineering (Figure S3 and Table S3). Three of the 44 residues make only negligible contributions to the buried surface area of the interface and were discarded. Ten highly conserved residues in the interface (Figure 1), likely to be crucial for function or structural integrity and thus unlikely to yield mutants with improved stability, were identified from a sequence alignment with 28 other DTE sequences (listed in the Uniprot database as DTEs, Figure S2) and discarded as well.

Each of the 31 remaining sites was randomized in a first round of variation separately by site-saturation mutagenesis with NNK degeneracy primers allowing sampling of all

[*] A. Bosshart, Prof. Dr. S. Panke, Dr. M. Bechtold
Bioprocess Laboratory, Department of Biosystems Science and Engineering, ETH Zurich
Mattenstrasse 26, 4058 Basel (Switzerland)
E-mail: matthias.bechtold@bsse.ethz.ch
Homepage: http://www.bsse.ethz.ch/bpl

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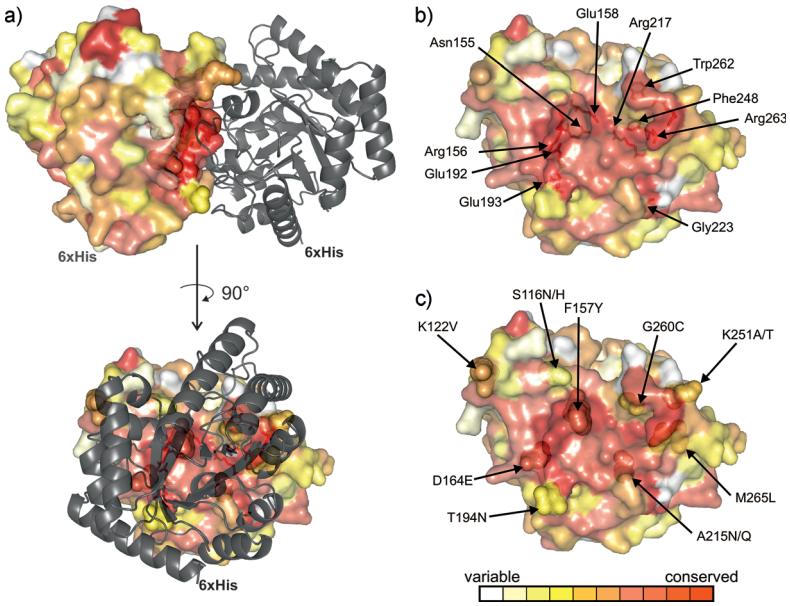

Figure 1. Localization of strictly conserved amino acid residues and those affording more thermostable PcDTE variants. a) PcDTE dimer with chain A in surface representation and chain B (dark gray) shown in cartoon representation, the C-terminal 6xHis-tag is marked. b) Chains A/B with all 10 strictly conserved interface residues shown as sticks. c) Chains A/B with the nine interface sites that afforded an improved mutant during the initial stability screening, highlighted as spheres. The coloring of each residue corresponds to its degree of conservation in 10% increments.

possible amino acids with a reduced set of codons (see Supporting Information for details). Nine of the 31 libraries produced at least one improved variant (i.e. affording at least 120% conversion after heat treatment when compared to the heat-treated wt), the best mutants being S116N, K122V, F157Y, D164E, T194N, A215N, K251A, G260C and M265L (Figure 1c and Figure 2a).

The T_{50}^{20} for each of these variants varied from 67°C (D164E, wt + 1.4°C) up to 78.1°C (F157Y, wt + 12.5°C). The sites for the nine stabilizing mutations were located mostly around the border of the interface except for mutation F157Y which is positioned right in the center of the dimeric interface and produced the biggest increase in thermostability. Computational modeling suggested that the new Y157 residue can form a hydrogen bond with the strictly conserved residue N155 of the other subunit, thus contributing to a stabilized dimer interface (Figure 3).

Another interchain hydrogen bond is probably established between the side-chain nitrogen atom of the new N194 and the carbonyl oxygen atom of E222. Mutation S116N might allow the formation of intrachain hydrogen bonds with the carbonyl oxygen atom of residue F157 and similarly, mutation A215Q is expected to lead to a hydrogen-bond network with E215 and R224. The stabilizing effect of the other mutations was more difficult to rationalize: we speculate that mutations K122V and K251T reduce the flexibility of the side chains and mutation G260C remedies packing defects within the protein-dimer interface.[17]

To accumulate beneficial mutations and also capture nonadditive effects,[18] we proceeded by iterative site-directed mutagenesis (ISM),[14] which goes through iterative cycles of saturation mutagenesis, targeting one previously identified beneficial site after another and using the best variant of each cycle as template for the next round. ISM was started on the most improved mutant, F157Y (re-named to Var1, Figure 2), as template. G260, having resulted in the second best improvement in the single site investigation, was randomized and screened as described above except that the heat-treatment was performed at 80°C for 20 minutes. The resulting Var2 had the same mutation as in the initial screening (G260C) and a T_{50}^{20} of 80.0°C. Next, position A215 was randomized in Var2 but yielded no improved

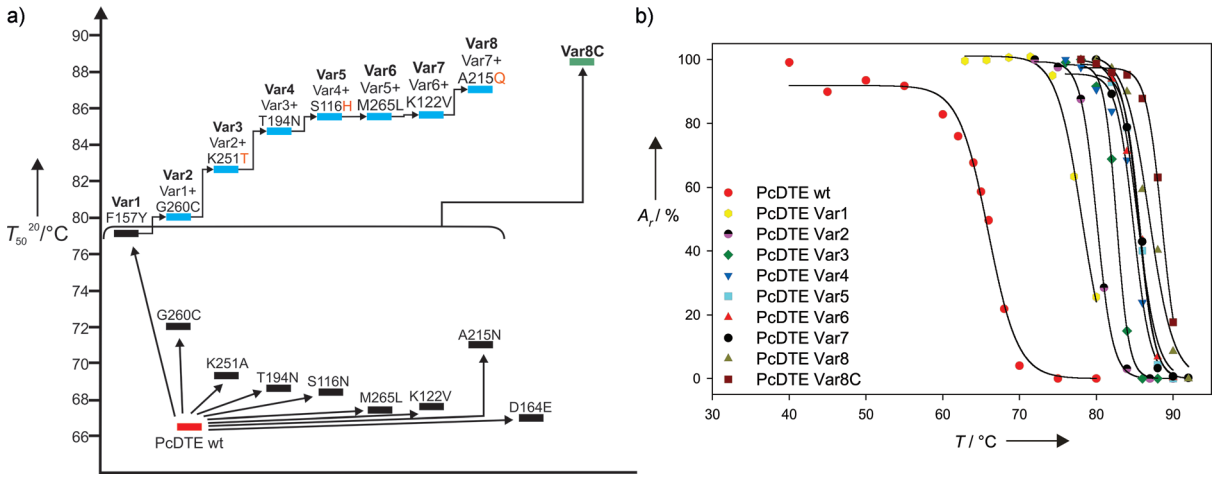

Figure 2. a) Thermostability, expressed as T_{50}^{20} value, of all variants involved in this study: PcDTE wild-type (red bar), hits obtained in the first variation round (black bars), variants 2–8 obtained by ISM (blue bars), and variant 8C obtained by combination of the 8 mutations from the first round (green bar). Mutation D164E was excluded in combinations as no improved variant could be identified during ISM. b) Residual activity (A_r) curves of PcDTE wt, variants 1 to 8, and variant 8C, fitted to a second-order sigmoidal function.

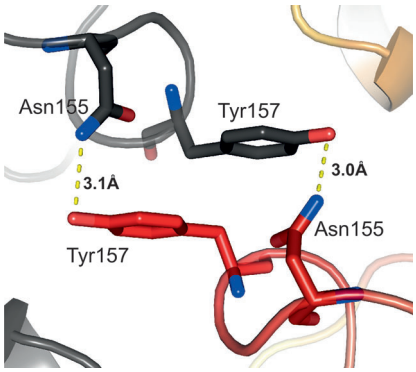

Figure 3. PyMol model of the best single improvement in PcDTE thermostability (F157Y), based on PDB 2QUN. Chain A is colored in red, chain B is colored in dark grey. The hydroxyl group of Y157 of chain A enables hydrogen bond formation to the highly conserved residue N155.

variant, even though randomizing this position had resulted in a significantly more stable variant in the initial screening. Therefore, the probing of this site was re-visited at the last stage of this iterative procedure. Randomization of Var2 at position 251 yielded K251T in Var3 and a T_{50}^{20} of 82.6 °C. In the next iteration stages positions 194 (Var4, T194N, T_{50}^{20} = 84.7 °C) and 116 (Var5, S116H, T_{50}^{20} = 85.5 °C) were addressed with increasing selection pressure in form of increased temperature during the heat-treatment step (Figure 2). Randomization of site 265 in Var5 resulted in Var6 (M265L) that showed no significant increase in T_{50}^{20} but a considerable increase in enzyme activity of heat-treated lysate. Randomization of position 122 of Var6 lead to K122V (Var7, T_{50}^{20} = 85.6 °C). Randomizing D164 of Var7 did not lead to further improvement. Finally, site 215, previously postponed, was again randomized in Var7 and led to a more stable Var8 (A215Q T_{50}^{20} = 87 °C), representing a very respectable increase of T_{50}^{20} of 21.4 °C over wt.

Var8 contained only three mutations that had not already been found in the first round of variation (K251T, S116H, A215Q, Figure 2a). This high conservation of mutations prompted us to change also the amino acids in these three sites to those found in the first round, leading to Var8C (as Var8 but with K251A, S116N, and A215N). Surprisingly, this variant exhibited an even higher T_{50}^{20} (88.5 °C vs. 87 °C), albeit at a lower specific activity.

To demonstrate that the achieved thermal stabilization actually translates into superior performance in a process-like setting, we calculated total turnover numbers (TTN) of wt protein, Var8, and Var8C at different temperatures (40–70 °C). TTN equals $k_{cat,obs}/k_{inact,obs}$ if deactivation is controlled by unfolding at elevated temperatures (i.e. unfolding is a first order process). Under that condition TTN is defined as the product of catalytic proficiency, expressed as the apparent catalytic constant $k_{cat,obs}$ (from Michaelis–Menten plots), and operational stability, expressed as the inverse of the apparent first-order deactivation constant $k_{inact,obs}$ (obtained from the enzyme half-life time $t_{1/2}$ in an isothermal continuous enzyme membrane reactor, EMR).[4a] As expected the wt PcDTE half-life time decreased rapidly with increasing temperature

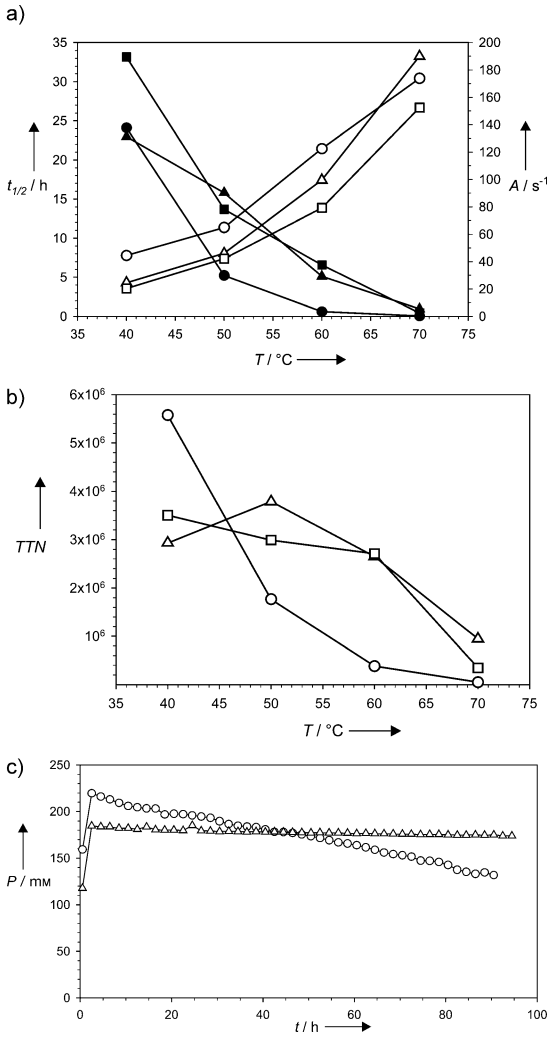

Figure 4. Enzyme kinetic and stability characteristics of PcDTE variants at different temperatures. a) Half-life time ($t_{1/2}$) (full symbols) and catalytic activity (A, $k_{cat,obs}$; empty symbols) of PcDTE wt (circle), Var8 (triangle), and Var8C (square) and b) total turnover number TTN for PcDTE wt (circle), Var8 (triangle), and Var8C (square) are plotted against temperature. c) Long-term stability experiment under D-psicose (P) production conditions (EMR at 50 °C, 1 M D-fructose feed) for PcDTE wt (circle) and PcDTE Var8 (triangle). The same initial amount of enzyme was applied in both runs (0.18 mg mL^{-1}).

while half-life times for Var8 and Var8C remained above 5 h even at 60° (Figure 4A), resulting in a TTN for Var8 that is between more than 2 (at 50 °C) and 18 times (70 °C) higher than the TTNs for wt PcDTE (Figure 4b). The performance improvement was confirmed by the nearly constant conversion during a four day EMR operation for PcDTE Var8 (50 °C, 1 M D-fructose feed, 180 μg mL^{-1} enzyme) compared to a 40 % decrease observed for the wt (Figure 4c). Interestingly, both enzymes exhibited considerably longer half-life times (wt: 24-fold; Var8: 80-fold, Table S5) under production than under the initial analytical conditions (substrate and enzyme concentrations approx. 20-fold lower). We attribute this to the stabilizing effect of higher enzyme and/or higher substrate concentrations in the reactor.

Next, substrate specificity and selectivity of Var8 remained comparable to wt (Table S6).

We conclude that a straightforward semi-rational surface engineering strategy in multimeric enzymes, based only on a crystal structure and a limited number of homologous sequences, can lead to drastically improved thermostability with a limited screening effort (< 4000 clones were screened in total for the interface engineering). The improved thermostability translated directly into superior operational stability under production-like conditions. In fact, variants Var8 and Var8C obtained here have acquired characteristics typically associated with enzymes from a thermophilic organism, that is, high thermostability and high activity at elevated temperatures. We argue that the approach adopted here is a potentially generic method for increasing the stability of multimeric biocatalysts.

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