Engineering the thermostability of a TIM-barrel enzyme by rational family shuffling

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**A B S T R A C T**

A possible approach to generate enzymes with an engineered temperature optimum is to create chimeras of homologous enzymes with different temperature optima. We tested this approach using two family-10 xylanases from *Thermotoga maritima*: the thermophilic xylanase A catalytic domain (TmxAcat, $T_{opt} = 68\,^\circ C$), and the hyperthermophilic xylanase B (TmxB, $T_{opt} = 102\,^\circ C$). Twenty-one different chimeric constructs were created by mimicking family shuffling in a rational manner. The measured temperature optima of the 16 enzymatically active chimeras do not monotonically increase with the percentage of residues coming from TmxB. Only four chimeras had a higher temperature optimum than TmxAcat, the most stable variant ($T_{opt} = 80\,^\circ C$) being the one in which both terminal segments came from TmxB. Further analysis suggests that the interaction between the N- and C-terminal segments has a disproportionately high contribution to the overall thermostability. The results may be generalizable to other enzymes where the N- and C-termini are in contact.

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Microorganisms occur in almost all environments on Earth, including high-temperature environments such as hot springs. In most cases, proteins from (hyper)thermophilic organisms have been found to be structurally similar to their mesophilic counterparts, except for minor differences [1–3]. Recent studies have shown that compactness, contact order, and the density of salt bridges may be the primary factors that result in enhanced thermal stability of proteins [4–6].

The (β/β)8-barrel fold, which was found in triose-phosphate isomerase, and is therefore also known as the TIM-barrel fold, is the most common enzyme fold [7]. (β/β)8-barrel enzymes cover five of the six enzyme classes defined by the Enzyme Commission, acting as oxidoreductases, transferases, lyases, hydrolases and isomerases [7]. Overall, hydrolases (especially glycosidases) are the dominating class, comprising about half of the known (β/β)8-barrels [7]. Structure-thermostability relationships and the engineering of glycoside hydrolases have attracted considerable current interest [8–10]. Family-10 xylanases perform glycosidic-bond hydrolysis with net retention of anomeric configuration via the double displacement mechanism involving two glutamate residues [11–13]. Hydrolysis of the β-1,4-glycosidic bonds of xylan, the major constituent of hemicellulose in the plant cell wall, is important in the paper, food and animal feed industries. When xylanases are used in biotechnological processes operating at elevated temperatures, a high thermal stability of the enzyme is a desirable property because it reduces costs by extending the life of the biocatalyst. Ideally, the optimum working temperature of the enzyme should be the temperature where the biotechnological process operates, also considering the thermal stabilities of the other components.

In the present study, we used two family-10 xylanases from the hyperthermophilic eubacterium *Thermotoga maritima* MSB8 with widely different temperature optima as starting points for rational family shuffling [14] to construct chimeric enzymes with various optimum working temperatures in the thermophilic temperature range, in order to test this approach and to find the key stabilizing regions of the protein. Xylanase B (TmxB) is a hyperthermophilic enzyme with an optimum working temperature $T_{opt} = 102\,^\circ C$, while the catalytic domain of xylanase A (TmxAcat) is thermophilic with $T_{opt} = 68\,^\circ C$. TmxB is a well characterized enzyme with a known three-dimensional structure [5,15,16]. TmxAcat, the middle domain in a five-domain chain, has been cloned with various N- and C-terminal boundaries by Winterhalter et al. [17]. Twenty-one chimeras were constructed and several of them were found to have a higher thermal stability than TmxAcat, and it was found that the two terminal segments have a disproportionately large contribution to thermal stability.

**Materials and methods**

*Construction of chimeric enzymes.* Genes *xynAcat* (GenBank Accession No. Z46264, basepairs 1340–2323) and *xynB* (GenBank Accession No. AAD35164) encoding TmxB and TmxAcat were PCR
amplified from the genomic DNA of T. maritima MS88, which was provided by Robert Huber (Universität Regensburg, Germany).

Chimeric enzymes were constructed by shuffling segments of the nucleotide sequences of the parental genes using a self-priming PCR reaction. Five highly similar regions suitable as shuffling sites were identified in a nucleotide sequence alignment prepared by the SIM algorithm (http://expasy.ch/tools/sim.html) [18] (see Fig. S1 in the Supplementary Material). To select suitable sites, the similarity of the two aligned sequences was checked in a 19-basepair window sliding along the alignment. The criterion for selection was that the number of differing nucleotides within the window must be less than three, and the differing nucleotides must not be in the first or the last three positions within the window. Sites meeting these criteria can be used as self-priming sites at the annealing temperature of the first PCR step.

The first PCR steps amplified the selected regions between two shuffling sites. Denaturation and annealing steps were performed at 98 °C for 2 min and 57 °C for 30 s, respectively, and primer extension was carried out at 68 °C for 30 s-2 min, repeating for 15-20 cycles. The specific combinations of primers (see Table S1 in the Supplementary Material) were used to construct the individual chimeric genes. Purified PCR products were used as template DNAs for the second overlapping PCR without primers. Finally, combined fragments were amplified in the third PCR using respective forward and reverse primers.

Bacterial strains and plasmids. Escherichia coli cultures were grown in LB medium. When appropriate ampicillin or chloramphenicol were added at a final concentration of 100 mg/l and 30 mg/l, respectively. Parental and amplified chimeric genes were cloned into the Ndel and BamHI sites of the pET21c vector system (Novagen), then transformed into E. coli.

Enzyme production and purification. Plasmids harboring a wild-type or a chimeric gene were used to transform E. coli BL21-CodonPlus(DE3)-RIL cells. LB ampicillin culture was inoculated with a final concentration of 100 mg/l and grown in LB medium. When appropriate ampicillin or chloramphenicol were added at a final concentration of 100 mg/l and 30 mg/l, respectively. Parental and amplified chimeric genes were cloned into the Ndel and BamHI sites of the pET21c vector system (Novagen), then transformed into E. coli.

Assay of xylanase activity and protein determination. Xylanase activity was determined by measuring the increase in reducing groups during the enzymatic hydrolysis of xylan by the dinitrosalicylic acid method of Bernfeld [19]. Standard assay mixtures (500 μl) contained 1% oat spelt xylan in buffer A, plus appropriately diluted enzyme. Incubation conditions were 10 min at appropriate temperature. The kinetic parameters kcat and km were determined at the optimum working temperatures of the enzymes by the Eadie-Hofstee plot from three independent experiments, and at five substrate concentrations.

Protein concentration was determined by the bicinchoninic acid (BCA) method using BSA as standard as well as by UV absorbance at 280 nm. Respective theoretical molar extinction coefficient calculated by ProtParam [20] was used.

CD spectropolarimetry and DSC. Thermal unfolding curves and the apparent melting temperatures were determined with a differential scanning calorimeter (Microcal VP-DSC) and with a CD spectropolarimeter (Jasco J720). The heating rate was set to 2 and 1 °C/min, respectively. Priory to the measurement protein samples were dialyzed overnight in 45 mM potassium-phosphate, pH 6.0 and diluted to 0.1–0.4 mg/ml.

Homology modeling. Homology models were built for TmxAcAt and the chimeras BA2B, BA2A, and AB2A using the X-ray structure of TmxB (PDB entry 1vbu) as a template. Sequence alignments were prepared with the T-Coffee program [21]. The models were built by version 9.3 of the Modeller program [22]. The automodel protocol was used with very thorough variable target function optimization followed by thorough molecular dynamics optimization. Twenty-five models were generated for each target, and the one having the lowest value of the objective function was selected as the final model.

Estimation of effective contact energies. Effective side chain contact energies were calculated using the Miyazawa-Jernigan contact energy matrix [23]. Contacts were defined as residue pairs with side chain centers of mass closer than 6.5 Å to each other. Effective interaction energies between two protein regions were calculated by summing up the side chain contact energies.

Results and discussion

Characterization of parental and chimeric enzymes

In this work, we have generated twenty-one out of the possible 62 (2^4+64, minus the two parental enzymes) chimeric xylanases by combining segments from a thermophilic and a hyperthermophilic xylanase variants, thereby obtaining a range of active enzymes with various temperature optima ranging from 61 to 80 °C (Fig. 1). After experimental characterization of these chimeras, we noticed the likely importance of the interaction between the N- and C-termini. Based on the activity screening measurements, the six chimeric enzymes in which the N- or C-terminal segments or both had been replaced were characterized in detail by circular dichroism (CD) and differential scanning calorimetry (DSC). These chimeric enzymes were compared with TmxAcAt and TmxB with respect to their optimum working temperatures and melting temperatures (Table 1). BA2B was the most stable chimera, with a temperature optimum 12 °C higher than that of TmxAcAt. The optimum temperatures of the other five chimeras were around or even less than that of TmxAcAt (Fig. 2 and Fig. 3). Those chimeras (BA2A and B2A) of which the thermophilic N-terminus was replaced by the respective hyperthermophilic segment(s) were inactive regardless of the length of the replaced fragment. The calorimetric curves of chimeric enzymes and thermophilic TmxAcAt displayed two melting temperatures. The first melting temperature (Tm, in Table 1), which is usually near the optimum working temperature, corresponds to a reversible change in tertiary structure (CD and DSC data not shown) that results in a rapid inactivation of the enzyme. The second peak corresponds to the irreversible unfolding of the overall structure. The DSC scans showed that the thermal unfolding of each enzyme is irreversible, and there was evident precipitation of the proteins. The results show that replacing only one of the two terminal segments of TmxAcAt by the corresponding segment from TmxB (i.e. chimeras BA2B/AB2B and AB2B/AB2B) results in destabilization, but when both terminal segments are replaced (chimera BA2B and AB2A), a significant stabilization can be observed (see data in Table 1).

In a previous study [24], Numata et al. constructed chimeric isopropylmalate dehydrogenases by combining fragments from a thermophilic and a mesophilic variant. They found that the thermal stability of the chimeric enzymes was nearly proportional to the fraction of the sequence coming from the thermophilic variant, suggesting that the residues having a stabilizing effect distribute nearly evenly along the sequence. In contrast to these results, the stability of our chimeric xylanases is not proportional to the content of the
amino acid sequence from the hyperthermophilic TmxB, and the relationship is not even monotonically increasing (see Fig. 1). As Fig. 1 shows, most chimeras (12 out of 16) have a lower temperature optimum than even the thermophilic TmxA cat enzyme. Our findings suggest that in family-10 xylanases, stabilizing residues are not evenly distributed along the sequence; rather, the terminal regions, and especially the interactions between the two termini, contribute more to the overall stability of the enzyme than the rest of the protein.

Kinetic parameters

Out of the six chimeras selected for detailed characterization, four had an enzymatic activity. The enzyme kinetic parameters of these constructs were determined at their optimum working temperatures. Kinetic constants for the parental and chimeric enzymes are shown in Table 2. The $K_m$ values obtained for the chimeric enzymes are comparable to those of the parental enzymes, with a variation below 30%. A larger variation was observed in $k_{cat}$ values. The $k_{cat}$ values for chimeric xylanases with terminal segments from the same parental enzyme (AB4A and BA4B) are comparable to those of TmxA cat and TmxB, but for the two constructs with terminal segments from different parental enzymes (A5B and AB5), $k_{cat}$ values are about half of those of the parental enzymes. A similar statement can be made with regard to the $k_{cat}/K_m$ values.

Interaction energies between the N- and C-termini

Using the available three-dimensional structure of TmxB and homology models of TmxA cat as well as the chimeras BA4B and BA5, we calculated effective interaction energies between the N-terminal 50 residues and the C-terminal segment after residue 250, roughly corresponding to segments 1 and 6 in each structure. Fig. 4 shows the negatives of the calculated energies (i.e. a higher value means stronger interaction) for each structure, and it also shows the contributions of various interaction types to the total interaction energies. For TmxB, the interaction energy between the termini is much more favorable than in TmxA cat ($\Delta G^0 = 189$ vs. $\Delta G^0 = 126$ U). It appears that this interaction is far from optimal in TmxA cat, and can be made stronger by introducing new aromatic-aromatic and aromatic-hydrophobic contacts. As expected, the total interaction energy and its composition are nearly the same in the BA5 chimera as in TmxB. In the BA5 chimera, the total interaction energy is less favorable ($-120$ U) than even in TmxA cat, and the relative contributions of various interaction types are similar to those of TmxA cat, with few hydrophobic-hydrophobic and hydrophobic-aromatic contacts. This indicates that the N-terminal hyperthermophilic segment and the C-terminal thermophilic segment do not match each other very well. Replacing both termini, however, results in the N-to-C interface being transferred along with the two segments, and results in a net gain in thermal stability. For the TmxA cat and TmxB molecules as well as the BA4B and BA5 chimeras, the calculated interaction energies correlate well with the temperatures belonging to the first peaks observed in

Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$T_{opt}$ (°C)</th>
<th>$T_{m1}$ (°C)</th>
<th>$T_{m2}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TmxA cat</td>
<td>68</td>
<td>70.4</td>
<td>91.1</td>
</tr>
<tr>
<td>BA5</td>
<td>Inactive</td>
<td>58.7</td>
<td>69.8</td>
</tr>
<tr>
<td>A5B</td>
<td>61</td>
<td>63.2</td>
<td>78.3</td>
</tr>
<tr>
<td>BA4B</td>
<td>80</td>
<td>84.1</td>
<td>91.0</td>
</tr>
<tr>
<td>TmxB</td>
<td>102</td>
<td>102.5</td>
<td>—</td>
</tr>
<tr>
<td>B5A</td>
<td>Inactive</td>
<td>65.3</td>
<td>80.8</td>
</tr>
<tr>
<td>AB5</td>
<td>71</td>
<td>72.3</td>
<td>85.5</td>
</tr>
<tr>
<td>AB4A</td>
<td>73</td>
<td>75.5</td>
<td>79.6</td>
</tr>
</tbody>
</table>

Optimum working temperatures and melting temperatures were determined by xylanase activity assay and differential scanning calorimetry (DSC), respectively. Calorimetric measurements resulted in two-peak curves except for TmxB. The first melting temperature ($T_{m1}$) corresponds to a reversible change in tertiary structure that inactivates the enzyme rapidly, whereas the second peak ($T_{m2}$) corresponds to the irreversible unfolding of the overall structure.
the heat capacity curves. For the A5B chimera, our calculations yield a more favorable interaction energy than expected on the basis of the measured melting temperature; however, they are in accord with the finding that A5B is more stable than BA5.

Conclusions

The structural determinants of the stability of (β/α)8-barrel proteins have been studied extensively [25]. An analysis of ~70 structures found that most stabilizing residues contribute to the eight-stranded β-sheet [26]. A study using combinatorial mutagenesis [27] and a computational study [28] showed that the loops at the bottom of the barrel (the αβ-loops) are more important for
stability than those at the top side (βα-loops), suggesting a “division of labor” between the two faces of the protein. The ring-like architecture of the (βα)8-barrel entails that the two terminal (βα) segments are in contact, forming similar interactions as neighboring (βα) units. A few studies showed that these regions may also be important for stability. In indoleglycerol phosphate synthase from \textit{T. maritima}, the stabilizing role of a salt bridge fixing the N-terminus to the core, and that of another salt bridge serving neighboring (βα) units. A few studies showed that these regions may also be important for stability. In indoleglycerol phosphate synthase from \textit{T. maritima}, the stabilizing role of a salt bridge fixing the N-terminus to the core, and that of another salt bridge serving as a clamp between helices α4 and α8 was demonstrated [29].

Investigations of detailed unfolding pathways of (βα)8-barrel proteins suggested various unfolding mechanisms including the 6 + 2 and the 3 + 3 + 2 mechanisms, which both involve the disruption of the last two strands and three helices early during unfolding [30–32]. By strengthening the interactions between the terminal regions, the activation energy of this initial step can be increased, resulting in an enhanced resistance against global unfolding.

Our results suggest that combining and mixing segments from a thermophilic and a hyperthermophilic (βα)8-barrel enzyme can be an effective and simple way to generate enzymes with an engineered optimum working temperature and structural stability. However, not all chimeric constructs had the expected higher thermal stability, which teaches us the lesson that the network of interactions between the replaced segments and their environment should be examined when selecting segments for replacement; even the introduction of a segment with a high intrinsic stability may lead to overall destabilization when its interactions with its surroundings are unfavorable. We have successfully shifted the optimum working temperature of a thermophilic xylanase into the hyperthermophilic range. The analysis of the results obtained with selected chimeric enzymes showed that the interactions between the two terminal regions have a major contribution to thermostability. The results may be generalizable to other enzymes where the N- and C-termini are in contact.

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\section*{Appendix A. Supplementary data}


\section*{References}

catalytic core of the family F xylanase from *Pseudomonas fluorescens* and identification of the xylopentaose-binding sites, Structure 2 (1994) 1107–1116.


