Directed Evolution

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Iterative Saturation Mutagenesis on the Basis of B Factors as a Strategy for Increasing Protein Thermostability**

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Thermostability of proteins is a crucial issue when they are applied as catalysts in organic chemistry, polymer technology, and pollution cleanup, as well as components in detergents, as diagnostic tools (sensors), as bionanotechnological devices, and sometimes even as therapeutic drugs.[1] For this reason, considerable effort has been invested in the quest to enhance thermostability by such techniques as protein engineering,[1] posttranslational enzymatic or chemical modification,[1c,2] use of additives,[1c,3] and/or immobilization.[1c,4] Methods based on protein engineering include site-specific mutagenesis,[1a,c,d,5] which is often guided by the structural differences between mesophilic and thermophilic enzymes,[6] or by computational

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design.[7] Nevertheless, the difficulty in predicting the appropriate sites and the optimal amino acid substitution remains a challenge. Directed evolution,[8] a process comprising random gene mutagenesis, expression, and screening/selection is also appealing.[1b,c,e,9] Accordingly, the gene is targeted by random mutagenesis methods such as repeating cycles of error-prone PCR (epPCR) with formation of relatively large libraries of enzyme variants that have to be screened or selected for the desired but unfortunately rare variants.

We used a new approach to select the sites for amino acid exchanges coupled with an efficient evolutionary strategy, thereby eliminating the need to screen large libraries yet achieving pronounced increases in thermostability. Systematic structural studies regarding mesophilic and thermophilic enzymes have shown that the latter are characterized by higher degrees of rigidity resulting from the accumulation of a variety of different effects, many being rather subtle.[6] These include salt bridges, hydrogen bonds, and π–π interactions at crucial positions in the protein in addition to hydrophobic interactions and possible disulfide bridges, all of which help to prevent unfolding at various stages of denaturation.[1,5–7,9] Enhancing the thermostability of mesophilic enzymes should therefore be possible by increasing the rigidity at appropriate sites. The challenge is to achieve significant degrees of thermostabilization in an efficient manner without losing enzyme activity at room temperature. We anticipated that this goal can be reached in a practical and straightforward process. Firstly, the choice of the appropriate sites at which rigidity is to be increased is made on the basis of atomic displacement parameters obtained from X-ray data, namely the B factors (or B values).[10] They reflect smearing of atomic electron densities with respect to their equilibrium positions as a result of thermal motion and positional disorder.[10] Therefore, we target solely those amino acids in a protein that display the highest B factors, corresponding to the most pronounced degrees of thermal motion and thus flexibility. These sites, comprising one or more amino acids, are then chosen for saturation mutagenesis[8] with the formation of small focused libraries. The gene of the best hit is subsequently used as a template for a second round of saturation mutagenesis at one of the other selected sites and the process is continued by focusing on the remaining sites iteratively (one site may be considered more than once). Such a convergent process maximizes the possibility of additive and/or cooperative effects by combining sensitive amino acid sites (not specific mutations) in an iterative manner. This concept of "iterative saturation mutagenesis" is crucial for success. It is a novel evolutionary strategy that also forms the basis of iterative CASTing (CAST = combinatorial active-site saturation test) as a means to increase the enantioselectivity of enzymes.[11] Iterative saturation mutagenesis as was applied in this case provides variants that are statistically not readily accessible by the traditional random-mutagenesis methods that were used previously to evolve thermostability.[1b,c,e,9]

In a model study, the lipase from *Bacillus subtilis* (Lip A)[12] was chosen as the enzyme for enhancing thermostability. It is a mesophilic lipase composed of 181 amino acids, and it has been characterized by X-ray crystallography several times.[13] We chose the crystal structure from the most recent study, which contains a single independent molecule in the asymmetric unit.[13e,14] Only the resolved amino acids were considered, which means that in the particular case at hand, the nonresolved C and N termini were not addressed. The 10 amino acids that show the highest average B values[15] were then chosen as sites for randomization by using standard saturation mutagenesis[8] (Arg 33: average B factor = 50.9; Lys 69: 44.1; Gln 164: 40.7; Asp 34: 39.9; Lys 112: 39.6; Lys 35: 38.9; Met 134: 38.5; Tyr 139: 37.9; Ile 157: 37.4; Gly 13: 37.0). Eight libraries were then constructed, namely at sites A (Gly 13), B (Arg 33, Asp 34, Lys 35), C (Lys 69), D (Lys 112), E (Met 134), F (Tyr 139), G (Ile 157), and H (Gln 164) (see Figure 1).

Figure 1. The sites in Lip A that were chosen for saturation mutagenesis. The picture shows a structural model based on the X-ray structure.[13] Library A (dark blue), library B (red), library C (dark green), library D (violet), library E (brown), library F (light blue), library G (light green), library H (yellow). Ser 77 is the catalytically active site.

The generation of the mutant libraries throughout this study was performed by using the QuikChange mutagenesis method from Stratagene.[16] Lip A was expressed in the supernatant of *E. coli* BL21(DE3) cell cultures by using a known expression system. Owing to the difficulties in performing high-throughput assays with triglycerides, which are the natural substrates, *p*-nitrophenyl caprylate was employed. Fast screening for improved thermostability in the initial eight libraries was performed in 96-well microtiter plates by heating the enzyme solutions (diluted supernatants) at 54 °C for 15 min with a PCR thermocycler. The reactions were monitored by a UV/Vis plate reader (405 nm). Under these test conditions, the wild-type (wt) enzyme displays a residual activity of only 9 % in the hydrolysis of *p*-nitrophenyl caprylate. Thermostability was assessed by measuring the residual activity subsequent to the exposure to high temperatures. In the literature, the so-called T_{50} value is often used to quantitatively characterize thermostability.[1,17] It is the temperature required to reduce the initial enzymatic activity by 50 % within a given period of time. T_{50} is near to or at the critical temperature of denaturation. To speed up screening, we applied a 15-min heat treatment, resulting in T_{50}^{15} values.

The hits were then re-examined by applying heat treatment for one hour, resulting in T_{50}^{60} values. Exploratory experiments had shown that Lip A denatures irreversibly at high temperatures, and that $T_{50}^{15} = 50\,^{\circ}C$, whereas T_{50}^{60} was found to be 48 °C. In the case of libraries A, C, D, E, F, G, and H, each originating from a single amino acid site, saturation mutagenesis[8] was applied at the respective single positions and oversampling was performed to ensure > 95 % coverage (about 300 clones).[18] In doing so, we used NNK degeneracy (N: adenine/cytosine/guanine/thymine; K: guanine/thymine) to encode all 20 proteinogenic amino acids. In the case of library B, resulting from cassette mutagenesis that induces simultaneous randomization at positions 33, 34, and 35, a representative sample of only 3000 clones was examined, although about 100 000 clones would be required statistically for 95 % coverage. Variants showing an improved thermostability were grown again, reanalyzed three times, and then sequenced. In the first rounds of mutagenesis, improved variants were identified in libraries A, B, D, E, F, and G but none were found in libraries C and H (Figure 2). The most thermostable variant (IV) resulting from this first evolutionary step originates from library E (Met134Asp) and displays a T_{50}^{15} of 54.3 °C.

To increase thermostability further, two strategies appeared to be logical. One was to produce new variants simply by combining the mutations of the hits from the libraries A–G in various permutational ways. The second strategy, which we chose, involves a stepwise evolutionary approach based on iterative saturation mutagenesis[11] (Figure 2). First, the gene of the best hit, IV, was used as a template for performing saturation mutagenesis (NNK degeneracy) at position 157, the site defining library G in which the second best hit had been found in the first round of mutagenesis.[19] This gives the system the possibility to choose again between the 20 possible amino acids, but this time starting from a different gene. The outcome is mutation Ile 157 Met. Thus, in this case the result is identical to a variant that would have been obtained by combining the mutations of variants IV and VI. Saturation mutagenesis was then continued stepwise following the same criteria by "visiting" the other sites as shown in Figure 2. In the last two steps leading to variants IX, X, and XI, the mutations found were not those induced in the initial mutagenesis rounds. The largest stabilization effect was observed when variant IX, already with a respectable T_{50}^{15} of 62.8 °C, was subjected to cassette mutagenesis (NNK degeneracy) at positions 33, 34, and 35. This resulted in the identification of two hyperthermal variants X and XI (Figure 2) that showed no measurable decrease in activity at room temperature following heat treatment at 100 °C for 15 min. Owing to technical problems with the screening system at temperatures above 100 °C, further optimization by saturation mutagenesis at position 13 or by focusing on the nonresolved C and N termini was not attempted.

From this point on, we focused our attention on variants X and XI. Following purification of both variants, the respective residual activity curves, the T_{50}^{60} values (60-min heating time), the kinetic constants, as well as the substrate and selectivity profiles were determined. Figure 3 shows the residual activity curves of the wt and of variants X and XI, demonstrating the enormous differences in thermostability. The half-lives ($t_{1/2}$) at 55 °C were measured to be 905 and 980 min, respectively, whereas $t_{1/2}$ of the wt is < 2 min. The enhanced thermostability is also revealed by the T_{50}^{60} values of variants X and XI, which are 89 and 93 °C, respectively, compared with 48 °C for the wt. This means that ΔT_{50}^{60} values of 41 and 45 °C, respectively, have been achieved. We are not aware of other cases of such dramatic increases in thermostability.[20]

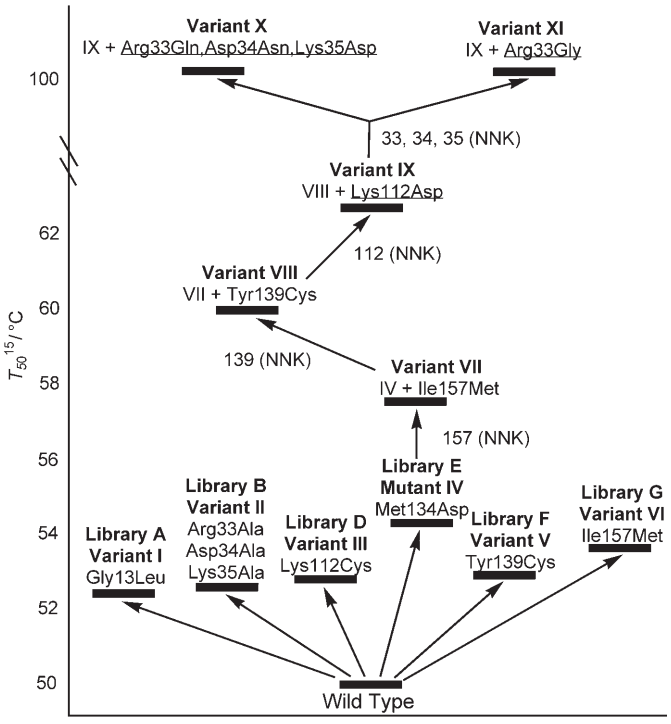

Figure 2. Thermostability diagram. Enzyme variants were characterized after preliminary screening by measuring T_{50}^{15}. The underlined amino acid exchanges indicate new mutational changes in the respective mutagenesis experiment; those that are not underlined appeared earlier in the evolutionary tree.

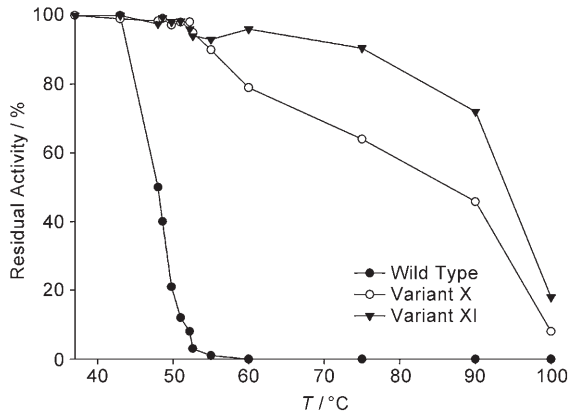

Figure 3. Thermostability of the purified wt and mutants X and XI of Lip A as displayed by the residual-activity curves. Activity in the hydrolysis of *p*-nitrophenyl caprylate was measured after the enzyme solutions were treated at various temperatures (37–100 °C) for 1 h.

To obtain more-detailed information regarding the crucial question of whether or not increased thermostability was evolved at the expense of activity,[1,6,7,9] further kinetic measurements were made by using *p*-nitrophenyl acetate (PNPA) as the substrate at 25 °C in potassium phosphate buffer solution (10 mM; pH 7.0). The values of K_m and k_{cat} of the wt and of the purified variants X and XI were derived from the corresponding Michaelis–Menten plots. The data show that no significant differences in catalytic parameters exist (wt: $K_m = 1.1$ mM; $k_{cat} = 6.5 \times 10^{-2}$ min^{-1}; $k_{cat}/K_m = 5.8 \times 10^{-2}$ mM^{-1} min^{-1}. Variant X: $K_m = 0.62$ mM; $k_{cat} = 5.8 \times 10^{-2}$ min^{-1}; $k_{cat}/K_m = 9.4 \times 10^{-2}$ mM^{-1} min^{-1}. variant XI: $K_m = 0.73$ mM; $k_{cat} = 6.3 \times 10^{-2}$ min^{-1}; $k_{cat}/K_m = 8.6 \times 10^{-2}$ mM^{-1} min^{-1}). Thus, the thermostability of the variants has been increased significantly without influencing enzyme activity. This conclusion was corroborated by measuring the activity and, when relevant, the enantioselectivity of the wt and variants X and XI as catalysts in the hydrolysis of 12 other *p*-nitrophenyl esters, some of which are chiral. Significant differences in the catalytic profile were not observed in any of the cases. For example, in the hydrolytic kinetic resolution of *p*-nitrophenyl-2-[4-(2-methylpropyl)phenyl]propanoate, the selectivity factor (E) in favor of the *R* enantiomer remains constant when going from the wt to the variants (wt: $E = 8.1$; variant X: $E = 8.2$; variant XI: $E = 8.5$).

The X-ray structure of Lip A exhibits a compact minimal α/β hydrolase fold with a six-stranded parallel β sheet flanked by five α helices, two of them on one side of the sheet and three on the other side.[13] The catalytic triad (Ser 77, Asp 133, and His 156), as well as the amino acids forming the oxyanion hole, reside in positions very similar to those of other lipases. A sound discussion regarding the source(s) of enhanced thermostability requires X-ray structural data of the variants coupled with a detailed theoretical analysis, which are not yet available. At this time we note that most of the mutations (positions 13, 33, 34, 35, 112, 134, and 157) lie in surface loops (Figure 4) where flexibility is expected to be highest. The respective amino acid exchanges help in stabilizing these loops and thus the protein through a variety of interactions.[1,6,9e] Solvent exposure of the randomized positions may also play a role. The power of our approach, from a practical point of view, has to do with the fact that these specific factors need not be assessed to obtain improved mutants.

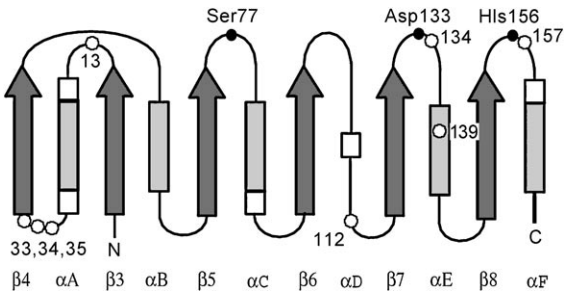

Figure 4. Secondary-structure topology of Lip A modified from the representation that was published earlier.[13] The nomenclature of the strands is taken from the canonical α/β hydrolase fold. All the positions that lead to thermostable variants are represented as white circles, the amino acids of the catalytic triad as black circles, and the termini of the protein as C and N. 3_{10} helical turns are represented as white boxes.

Clearly, a hyperthermophilic enzyme has been created, yet its catalytic profile is one of a mesophile. In some previous studies, enhanced thermostability also did not reduce activity, although the reported increases in thermostability were much smaller than in the present study.[1,9] Natural hyperthermophiles evolved not just to withstand extreme temperatures, but also to display optimal reaction rates under such conditions.[6] Excessively high activity at high temperatures would adversely effect the balance of metabolic transformations, which could be detrimental to the organism. This suggests that natural hyperthermophilic enzymes contain built-in structural features that slow down reaction rates, perhaps mainly in the interior of their globular structures. These are the regions that remain unaffected in our approach.[21] To engineer proteins for wide industrial applications, we suggest that after the improvement of thermostability by the present approach or through another method,[1,9] a second-step enhancement of substrate scope and/or enantioselectivity can be addressed (if needed) by applying such techniques as epPCR,[8] DNA shuffling,[8] or CASTing.[11,22] Indeed, this goes hand in hand with the recent conclusion that enhanced thermostability promotes the ease of evolvability.[23]

The strategy we have described constitutes a combination of rational design and directed evolution. In the present model study, less than 8000 clones were screened, yet it resulted in an impressive enhancement of thermostability without compromising the activity or enantioselectivity. The efficiency of the method is derived from the rapid build up of evolutionary pressure exerted by the iterative cycles of saturation mutagenesis at properly chosen sites in the enzyme. In contrast, repeating cycles of epPCR, previously used most often in the enhancement of thermostability,[1,9] would not be expected to result in the hyperthermophilic variants X and XI (from the present study) owing to statistical reasons. Our approach uses a physical parameter directly related to the known correlation between flexibility and stability. Nevertheless, it is currently not clear how general the method is because only one example has been reported so far.

Application of the strategy requires the availability of the X-ray structure of the wt. However, protocols for computing B factors[24] have emerged that can, in principle, be used in those cases in which crystallographic data is not available. Moreover, B factors correlate with the NMR relaxation data.[25] Finally, based on the same principle described herein, the targeted reduction in thermostability, which is desired for special applications,[26] should be possible by following a similar strategy, e.g., by focusing on sites with low B factors in nonregular structural units.

Experimental Section

Enzyme expression and screening system: Saturation mutagenesis libraries were generated by using the QuikChange mutagenesis strategy from (Stratagene, USA) by using NNK degenerate primers.[16]

Culture conditions: *Bacillus subtilis* lipase A was expressed in the supernatant of *E. coli* BL21 (DE3; Novagen, Madison, USA) cell cultures by using the expression system based on the pET22 vector previously described.[13b] Supernatant expression avoids the use of whole cells, which lead to background reactions. The colonies were picked with a colony picker QPIX (Genetix, Boston, MA), and precultures were grown in deep-well plates by using 800 μL per well of lactose-free 505 media[11] (formula per liter: 20 mL of 50 × 505 solution (250 g L^{-1} glycerol, 25 g L^{-1} glucose, and distilled water), 50 mL of 20 × NPS solution (66 g L^{-1} $(NH_4)_2SO_4$, 136 g L^{-1} KH_2PO_4, and 142 g Na_2HPO_4), 2 mL of 1 M $MgSO_4$, and filled to a volume of 1 L with ZY media (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract)). This preculture was used to inoculate, using a manual plate replicator, an expression culture with 900 μL per well of 5052 media[27] containing lactose as inducer (formula per liter: 20 mL of 50 × 5052 solution (100 g L^{-1} α-lactose, 250 g L^{-1} glycerol, 25 g L^{-1} glucose, and distilled water), 50 mL of 20 × NPS solution, 2 mL of 1 M MgSO4, and filled to a volume 1 L with ZY media).[27] In both media carbenicillin was used as antibiotic (100 μg mL^{-1}). The cultures were grown at 37 °C and 800 rpm for 24 h. The cultures were then centrifuged at 4000 rpm for 20 min.

Gene sequencing: Mutant genes were sequenced by using the standard T7-3T7 primer by Medigenomix (Martinsried, Germany).

Screening: The screening for thermostability was performed by using the supernatants in 96-well microtiter plates. *p*-Nitrophenyl caprylate was selected as the substrate for the preliminary screening. Stock solutions of the substrate (50 mM substrate in acetonitrile) were stored at −20 °C. The supernatants were diluted in double-distilled water in a 1:3 ratio (supernatant/water) to adjust the lipase activity, and 30 μL of this solution was tested against a solution of *p*-nitrophenyl caprylate in K_2HPO_4 (50 mM), Triton X-100 (0.1 %); pH 8 buffer solution (100 μL of 2.5 mM). The hydrolytic reaction was followed at 405 nm over 3 min by using a Spectramax Plus^{384} from Molecular Devices (Sunnyvale, USA). No significant background hydrolysis was observed when the supernatant was used under these working conditions.

Thermostability was assessed based on the residual activity subsequent to the exposure to high temperatures. 100 μL of diluted supernatants from the same deep-well plate was transferred to 200-μL PCR tube 96-well plates that were then heated for 15 min at a defined temperature; after cooling to 4 °C for 5 min (stop the heat shock), the samples were kept at room temperature for 15 min. A volume of 30 μL was transferred from each well to a microtiter plate, and the hydrolytic reaction **1**→**2** was repeated to compare the activity before and after the heat shock.

Exploratory experiments have shown that the lipase A from *Bacillus subtilis* denatures at high temperatures. The wt Lip A shows a T_{50}^{15} of 50 °C and a T_{50}^{60} of 48 °C.

According to these characteristics, a preliminary screening was developed to identify thermally stable variants from the designed libraries: heating the enzyme solutions at 54 °C for 15 min by using a PCR thermocycler. According to this test, the wt enzyme has an average residual activity of ≈10 % (average of 96 tests) in the hydrolysis of the *p*-nitrophenyl caprylate. Mutants showing more than 20 % retained activity (established as threshold for screening purposes) were considered as hits of the screening, and their thermostability was subsequently reproduced. The T_{50}^{15} was determined by measuring the activity after heating at different temperatures for 15 min. For this purpose, a gradient PCR thermocycler was used. The heat-shock temperature was then increased stepwise, according to the T_{50}^{15} of the variant selected as the template.

The kinetic constants of the purified mutants were calculated by using PNPA as the substrate. Initial rates of hydrolysis of PNPA at various concentrations were determined at 25 °C in potassium phosphate buffer (10 mM; pH 7.0). The values of K_m and k_{cat} of the purified variants were derived from the corresponding Michaelis–Menten plots.

The $t_{1/2}$ values of the mutants at 55 °C were determined by taking samples at different times and their hydrolytic activities then evaluated. The percentage of retained activity of each sample was then plotted against time. The half-life was determined from this plot as the time at which the activity is reduced to 50 % of the initial activity.

Enzyme purification: The enzyme variants were purified from the culture supernatant as previously reported[12a] and by means of a double ultrafiltration process by using Amicon Ultra Centrifugal filters of 10 NMWL (NMWL = nominal molecular weight limit) and 50 NMWL cut offs (Millipore, USA). The purity of the 19.4-kDa enzyme was confirmed by SDS-PAGE electrophoresis (10 % acrylamide).

Reaction conditions: To expression culture supernatant (100 μL), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer solution (400 μL, 100 mM; pH 7.5) containing substrate (20 μL, 10 mg mL^{-1}) in acetonitrile were added. The reaction mixture was shaken at 800 rpm for 4 h at 30 °C. The solution was extracted with dichloromethane (500 μL). A second extraction step was performed by adding dichloromethane (500 μL) and HCl (20 μL, 10 %) to optimize acid extraction. The extracted organic phase was evaporated and redissolved in dichloromethane (100 μL) and analyzed by GC.

GC analysis: The chiral analyses of the kinetic resolution reactions of *p*-nitrophenyl-2-[4-(2-methylpropyl)phenyl]propanoate were performed by using a DiMePeBETA-Ivadex-1 chiral column (25 m, 0.25 mm, 0.15 μm) from IVA Analysentechnik, (Meerbusch, Germany). Conditions: Carrier (N_2) 1.4 mL min^{-1}. Temperature profile: 140 °C, 30 min, 10 °C min^{-1}, 200 °C for 90 min. Absolute configuration was determined by comparison with commercial enantiopure products.

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