Alteration of substrate specificities of thermophilic α/β hydrolases through domain swapping and domain interface optimization

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Protein domain swapping is an efficient way in protein functional evolution in vivo and also has been proved to be an effective strategy to modify the function of the multi-domain proteins in vitro. To explore the potentials of domain swapping for alteration of the enzyme substrate specificities and the structure–function relationship of the homologous proteins, here we constructed two chimeras from a pair of thermophilic members of the α/β hydrolase superfamily by grafting their functional domains to the conserved α/β hydrolase fold domain: a carboxylesterase from Archaeoglobus fulgidus (AFEST) and an acylpeptide hydrolase from Aeropyrum pernix K1 (apAPH) and explored their activities on hydrolyze p-nitrophenyl esters (pNP) with different acyl chain lengths. We took two approaches to reduce the crossover disruptions when creating the chimeras: chose the residue which involved in the least contacts which involved in the least contacts as the splicing site and optimized the newly formed domain interfaces of the chimeras by site-directed mutations. Characterizations of AAM7 and PAR showed that these chimeras inherited the thermophilic property of both parents. In the aspect of substrate specificity, AAM7 and PAR showed highest activity towards short chain length substrate pNPC4 and middle chain length substrate pNPC8, similar to parent AFEST and apAPH, respectively. These results suggested that the substrate-binding domain is the dominant factor on enzyme substrate specificity, and the optimization of the newly formed domain interface is an important guarantee for successful domain swapping of proteins with low-sequence homology.

Keywords domain swapping; thermophilic esterase; α/β hydrolase superfamily

Introduction

Engineering the substrate specificity of critical enzymes is important in many areas, such as metabolic engineering, synthetic biology, and fuel or high-value products industries and so on [1]. Methods used for altering the substrate specificity by mutagenesis are mainly divided into two categories: directed evolution and rational design. The representative techniques of directed evolution are random mutation and DNA shuffling, which have been successfully applied in the functional modification of existing enzymes [2,3]. The directed evolution methods need screening of large libraries of enzyme variants and are time-consuming. Owing to the increasing knowledge about the three-dimensional structures of enzymes, the rational design gradually becomes the main approach to alter specificities of enzymes, such as site-directed mutation and domain recombination [4]. These rational methods play important roles in engineering novel proteins, which have the potential to be used in industry and medical science, and also in the study of structure–function relationships of the proteins [5].

Most proteins have multiple, different functional domains/subdomains, such as substrate-binding domain, catalytic domain, and coenzyme domain. Domain/subdomain recombination is an effective way to change enzyme specificity. Previous works focused on the domain exchange. For example, two phosphofructokinase chimeras were constructed by exchanging the N- and C-terminal fragment of the mammalian M- and C-type isozymes, and the results showed that the N- and C-terminal fragments were responsible for the affinity and allosteric of the enzyme, respectively [6]. In another work, a chimera was constructed by domain swapping of two isoforms of Ferredoxin-NADP+ reductase (FNR), which share 48% sequence identity. This chimera acquired a catalytic
efficiency, as an NADPH-dependent diaphorase, ~2 to 5 folds higher than those of both parents [7].

Interactions on the domain interface are important to maintain the stability of the multi-domain proteins [8]. Recombining domains of proteins with low-sequence identities often results in the destructions of the interactions among residues in domain interface. Therefore, optimization of the newly formed domain interface is necessary for recombination of functional chimeras. Some recent works on multi-domain proteins have shown that the functional change is often achieved through the insertion of protein domains and adjustment of the newly formed interface between them [9]. For example, Huang et al. [10] combined a low-affinity peptide-binding domain and a functionally inert second domain, and optimized the domain interface by directed evolution. These directed evolution processes dramatically enhanced both affinity and specificity for >500 folds and >2000 folds, respectively, which the single domain could not achieve.

Members of the α/β hydrolase superfamily catalyze diverse processes as proteolysis, ester hydrolysis, and epoxide hydrolysis [11]. They usually contain multiple domains or subdomains, and the active site usually locates at the interface of domains. The interactions between domains affect the catalytic functions by regulating the substrate binding and accessibility of catalytic residues. In this work, we exchanged the domains of two members of α/β hydrolase superfamily: a carboxylesterase from Archaeoglobus fulgidus (AFEST) and an acylpeptide hydrolase from Aeropyrum pernix K1 (apAPH). AFEST hydrolyzes p-nitrophenyl esters (pNP) with short acyl chain lengths and shows the highest activity toward pNPC4 (para-nitrophenyl-butyrurate) [12]; apAPH is a typical member of the prolyl oligopeptidase family and shows a promiscuous esterase activity with a preference for middle chain length substrates, whose favorable ester substrate is pNPC8 (para-nitrophenyl-caprylate) [13]. The chimera composed of propeller domain of apAPH and catalytic domain of AFEST with an R526 insertion was named PAR, and the opposite one with seven sites mutagenesis for refinement of the newly formed domain interface was named as AAM7. Characterization of the chimeras showed that both of them inherited the high thermophilic property of the parents, but their substrate specificities were similar to the parent which provided the substrate-binding domain.

Materials and Methods

Homology modeling and estimation of the chimeric models

The coordinates of three-dimensional structures of apAPH (PDB ID: 1VE6) and AFEST (PDB ID: 1JJI) were obtained from the protein data bank. Multiple sequence alignment (MSA) of the templates and target chimeric sequences were produced by ClustalW [14], and manually refined, taking into account the structural superimposition of the templates. Structures were superimposed using PyMOL software [15], and used as templates to construct predictive models of chimeras. The chimeric structure models were constructed using the homologous modeling software program Modeller9v7 [16], and scored by PROCHECK program [17]. The models with the lowest energy were selected.

Construction, overexpression and purification of chimeras in Escherichia coli

The chimeras PAR and AAM7 were constructed by overlap extension polymerase chain reaction (PCR) technique [18]. The genes ape1547 and afest were used as the templates. The propeller domain (pp) of ape1547 was amplified using primers up (5′-CAGCAGCATATGGTGCCATTATAAT GC-3′) and down (5′-CGTCTGTCTCCGCCGGCTA TGGAGCC-3′). The catalytic domain (cd1) from ape1547 was amplified using primers up (5′-GGTTGAAAGAG TTAGGCTGTGTTTGGGT -3′) and down (5′-CGAGC C CATTCTCTCTCCTCTCCTC-3′); the cap domain from afest was amplified by primers up (5′-ATGTAAACATATG ATGTTGATATGCA-3′) and down (5′-ACCACACAC CGCCTAACACTTTCAACCAACC-3′); the catalytic domain of afest (cd2) was amplified by primers up (5′-TCCATAGCGGGAGGACAGGAGGATTAAG-3′) and down (5′-CGAGCCGATCCTCTGCAACCAAG AAG -3′), (BamHI and NdeI site are underlined). Then the cd1 and cap were used as templates to amplify full gene of AAM7 and the cd2 and pp were used as templates to amplify the full gene of PAR. The seven mutations of AAM7 were introduced using the modified QuickChange® site-directed mutagenesis protocol [19]. The primers are as follows: L133Q/L136Q up (5′-GGAGGAAAGAG ATCC AGGGGC-3′) and down (5′-GTGCCTGTTAGTTTGG CCTCCA -3′); L264Q up (5′-GAGCTGCAGGCGG CGGG-3′) and down (5′-CCGCCCTGCTGCTAAGC-3′); M285T up (5′-ATACCAAGCACCCGGAGGACG-3′) and down (5′-GTC CTCGTTTGTGTTTAT -3′); L293R/F297Q up (5′-GAGAT CTCGGCCGCGCGTTCTTCCA-3′) and down (5′-TA GGAACCTCAAGGGGCGGAGGAGT-3′) R250V up (5′-CCTCAAGACAGACGCGTTACACCGCTGAAACC C-3′) and down (5′-GGTTTTCAGGCTGTAACGGCTG TTCTAAGGGG-3′). The primers for R526 insertion of PAR are: up (5′-GAATACGACACCCGGTCTAGAGAT- 3′) and down (5′-ATCTTCAAGGGCGCTGTTGTTCTGTT C-3′). The PCR fragments of the complete genes were digested with NdeI and BamHI and inserted into the pET-28a vector (Novagen, Madison, USA). After transformed into E. coli XL1-blue, the cloned gene was completely sequenced by Bio-Basic (Shanghai, China). The proteins were expressed in E. coli BL21-CodonPlus
Native polyacrylamide gel electrophoresis and activity staining

Native polyacrylamide gel electrophoresis (PAGE) was performed at basic pH (separating gel, pH 8.8) with 8% polyacrylamide slab gels. Nondenaturing gels were stained with coomassie brilliant blue. For activity staining, gels were incubated in a solution (100 ml) of 100 mM Tris–HCl, pH 7.5, containing 5 mg of β-naphtylacetate (predissolved in 0.5 ml of methanol) and 25 mg of Fast Blue RR at room temperature. Reactions were stopped after 15–30 min by rinsing with tap water and placing gels in 7.5% (v/v) acetic acid.

Measurement of steady-state kinetics

Esterase activities were determined using pNP with different chain length as substrates, respectively. The amount of p-nitroaniline released from p-nitrophenyl was monitored at 405 nm using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of esterase activity was defined as the amount of protein that released 1 μmol p-nitrophenol per minute. The standard assay was performed in 20 mM wide range buffer (containing 20 mM acetic acid, 20 mM MES, 20 mM HEPES, 20 mM TAPS and 20 mM CAPS, and the pH was adjusted to the optimum pH at corresponding temperature with 1 M NaOH) at 80°C. The assay for AFEST was performed in 40 mM Na2HPO4/NaH2PO4 buffer pH 8.0 containing 1% (v/v) Tween-20. The kinetic parameters Km and kcat of the parents and the chimera AAM7 were determined using pNP-substrates. The initial steady-state velocities of substrate hydrolysis were monitored at 9–12 different substrate concentrations. All measurements were made in duplicate or triplicate. The kinetic parameters were determined from the rates of hydrolysis by fitting the initial velocities to the Michaelis–Menten equation \[
\frac{v_0}{[S]_0} = \frac{k_{cat}[E]_0[S]_0}{(S)_0 + K_m}\.
\]

Far-UV circular dichroism spectra of enzymes

Measurements were carried out using a Jasco J-815 circular dichroism (CD) spectropolarimeter and 5 mM wide range buffer at the pH optimum for each enzyme, with a protein concentration of 0.1 mg/ml, at room temperature. The path length was 0.1 cm; scan range of wavelength was from 260 nm to 190 nm. The analysis of the CD spectra was performed using the CDPro software package [20].

Results

Design of the chimeras AA and PA

The sequence identity of AFEST and apAPH is only 15%, but the catalytic domains of them are very similar, with rmsd 2.6Å. The substrate-binding domains of AFEST and apAPH are very different, a 50 residues cap domain constituted of three α helices of AFEST and a seven blades β propeller domain of apAPH, respectively. We set to construct chimeric enzymes by exchanging the propeller domain of apAPH with the cap domain of AFEST.

We analyzed the number of contacts the residues in domain boundaries of the two enzymes involved in by using the LPC/CSU server (http://ligin.weizmann.ac.il/lpccsu/) [21]. The results showed that the residues involved in the minimum contacts are the Gly 332 of apAPH and Val 56 of AFEST (Supplementary Fig. S1). To minimize disruption of the domain interface interactions, we chose these two residues as splicing sites to construct the chimeras. We named the chimera constructed of cap domain of AFEST and catalytic domain of apAPH as AA, and the opposite one as PA. The model structures of the chimeras were constructed using the program Modeller9v7, joining the cap domain of AFEST (amino acids 1–56) with the catalytic domain of apAPH (amino acids 333–581) and the propeller domain of apAPH (amino acids 1–332) with the catalytic domain of AFEST (amino acids 57–311). After evaluating the quality of the models by Procheck and profile_3D program, we chose the best conformation for subsequent experiments (Fig. 1).

Analyzing and optimization of domain interfaces of chimeras

The genes of chimeras AA and PA were constructed by overlap extension PCR technique using genes of apAPH and AFEST as the templates, and expressed in E. coli (DE3)-RIL, and soluble protein was purified by nickel-chelating chromatography (Qiagen, Valencia, USA).
Unfortunately, we only obtained the soluble chimera PA, whereas the AA was mainly existed in the precipitation components. We compared the model structures of chimeras with the parental structures to find the reason for this phenomenon. We analyzed the interactions formed by the residues on the domain interfaces of parent apAPH, AFEST, and chimeras AA, PA, respectively, by using Ligplot software [22]. Comparison of AA and apAPH showed that on domain interface of apAPH, there are many residues involved in hydrophobic interactions and buried inside the structure [Supplementary Fig. S3(A)]. However, after the construction of chimera AA, some of the buried residues are exposed, such as L133, I136, L264, M285, L293, and F297 (equivalent to L409, I412, L540, M561, L569, and F573 of apAPH) [Fig. 2(A) and Supplementary Fig. S3(B)]. The exposure of these hydrophobic residues might cause the chimera misfolding and inactivation. So we mutated these residues into hydrophilic residues based on the according residues of parent AFEST, such as L133Q, I136Q, L264Q, M285T, L293R, and F297Q, respectively. Besides, in our previous studies, we found that the mutant R526V enhanced esterase activity of apAPH remarkably [23]. In order to obtain the chimera with high esterase activity, we introduced this site-mutagenesis on the chimera, the R250V. We term this seven-site mutant as AAM7. The model structure of AAM7 was constructed in silico.

The results of chimera PA showed that there were a large number of hydrophobic and hydrogen bonding interactions between the propeller domain from apAPH and the catalytic domain from AFEST [Supplementary Fig. S3(C)]. Hydrophobic interaction between the domains is an important factor for the enzyme maintaining the correct conformation and catalytic activity. It is noteworthy that, between residues on catalytic domain and residues E1, F2, I5, L12, I13, V9, E10, K17, there existed hydrophobic interaction and also formed many hydrogen bonds. Previous work in our lab reported the important role of the N-terminus 21 residues helix of apAPH [23]. Residues of this helix formed a number of hydrophobic and polar interactions with the residues on adjacent part of the catalytic domain to stabilize the protein structure. These results suggested that PA maintained the similar domain interface interactions with the parent apAPH, indicating that the PA can be correctly folded, and functionally expression. In our previous work, we have found the critical role of the inter-domain salt bridge formed by Arg526 and Glu88 of apAPH for protein stability [24,25]. Therefore, we introduced the Arg526 into the chimera PA to increase its stability. We superimposed and compared the model structure of PA with the structure of apAPH, and selected the 526 site of PA to insert the

Figure 1 The structures of parents AFEST (red, PDB ID: 1JJI) and apAPH (blue, PDB ID: 1VE6), and the model structures of chimera PA, AA. The crossover points are indicated. The coordinates of parents’ structures were obtained from the protein data bank. The model structures of chimera PA and AA are constructed by homology modeling software Modeller 9v7.

Figure 2 The model structures of the chimeras (A) The mutated residues of AAM7 (left) and PAR (right). The residues mutated are shown as red sticks and the residue numbers are labeled. The blue dash lines shown the polar contacts formed by R526 and E81 of PAR. (B) The superimposition of catalytic sites of AAM7 with apAPH (left) and PAR with AFEST (right). The pNPC4 substrates are shown in magenta; the chimeras AAM7 and PAR are shown in green, the parent apAPH and AFEST are shown in cyan; the catalytic triads and the oxyanion hole Gly are shown in sticks and the residue numbers are indicated.
Arg. After homology modeling, we selected the model structure with minimized energy and named it as PAR [Fig. 2(A)].

The Procheck analysis of the energy minimized model of PAR and AAM7 showed that almost all the residues were found in allowed regions of the Ramachandran plot. Moreover, among the total amino acids, over 92% of residues were positioned in the most favorable regions of the Ramachandran plot (Supplementary Fig. S4). The pNPC4 substrate was docked into the binding site of PAR and AAM7 by using the CDOCK module of Accelrys Discovery Studio software. Figure 2(B) shows the catalytic triads residues (Ser169, His280, and Asp248 in AAM7; Ser429, His555, and Asp524 in PAR) of the chimeras that are consistent with the parents (Ser445, His556, and Asp524 in apAPH; Ser160, His285, and Asp255 in AFEST). As the inter-domain interactions significantly contribute to the protein structure stability, we also calculated the domain interaction energy of the chimeras to examine the domain interface stabilities of the chimeras (Supplementary Table S1). The results calculated by the Accelrys Discovery Studio software showed that the recombinant domains of the chimeras could form strong van der waal’s and electrostatic interactions as parents.

Construction, expression, and purification of the chimeras PAR and AAM7 in vitro
The mutant PAR and AAM7 were constructed by Quick-change PCR and expressed in E. coli as soluble enzyme (Supplementary Fig. S2). The native PAGE electrophoretic characterization of the chimeras showed that the apparent molecular masses of PAR and AAM7 in nondenaturing conditions are about 180,000 and 70,000 daltons (Fig. 3). This result suggested that they were in the active trimer and dimer form, respectively. A comparison of the far-UV CD spectra of chimeras and their parent enzymes indicated that PAR and AAM7 retained the parental folds (Fig. 4).

Effect of temperature on the activities of the parent and chimeras
Activities for the parents AFEST, apAPH and chimeras PAR, AAM7 were determined with the substrate pNPC8 over a wide range temperature (40–90°C). Similar to apAPH, the optimal temperature of PAR was over 90°C, and its activity was undetectable under 50°C. The optimal temperature for the enzymatic activity of the AAM7 was 80°C [Fig. 5(A)], which is similar to AFEST. The chimera maintained about 90% of the maximal activity even at 90°C. At lower temperature, AAM7 still showed activity, exhibiting about 20% of the maximal activity at 50°C. Below 60°C, the activity of AAM7 increased quickly with temperature increasing, whereas above 60°C, the activity increased slower. This trend is similar to the parent AFEST, which has an inflection point at 70°C. This result implies that the rigidity of the whole molecule reduced due to the substitution of the propeller domain with the cap domain, and benefited the catalysis at low temperatures.

pH dependence of the activities of the parent and chimeras
In order to investigate the influence of domain recombination on the active-site microenvironment of the chimeras, we compared the pH dependence of the hydrolytic activities of the parents and chimeras. PAR and AAM7 displayed high activity at alkaline pH, and their activities were undetectable at pH below 7.0. The optimal pH of PAR was 9.0, similar to parent apAPH, while the optimal pH of AAM7 was ~9.5, shifted to the alkaline region relative to both the parents [Fig. 5(B)]. The pKₐ values of catalytic...
Histidines of AFEST, apAPH, PAR, and AAM7 calculated by PROPKA web server [26] were 6.19, 6.25, 6.35, and 6.88, respectively. We speculate that the electrostatic environment around catalytic histidines changed after domain recombination caused by subtle changes in active site and affected their deprotonation.

Thermostability of the chimeras

We measured the thermal inactivation of the chimeras at 70°C (Fig. 6). The results showed that the half-lives of PAR and AAM7 are about 11 and 13 h, respectively (Table 1), which demonstrates that these chimeras maintain the thermostability of the parents. To examine the effect of insertion of R526 on the structure stability, we also measured the thermostability of PA at 70°C and the results are shown in Table 1. As expected, marked increasing of stability was observed in PAR mutant compared with PA, as the half-life of PA at 70°C was only 6.8 h. These results revealed that the R526 insertion did make an important contribution to the thermostability of the chimera.

Substrate specificity of chimeras

We investigated the significance of substrate-binding domains on specificity of ester substrate chain length. The kinetic parameters for parent apAPH, AFEST, and the chimeras PAR and AAM7 were initially assayed using pNP esters with acyl chains of various lengths at 80°C, and the results are shown in Table 2. Both the $K_m$ and $k_{cat}$ values for PAR decreased with increasing acyl chain length of the pNP esters. The great decrease of $K_m$ and slight decrease of $k_{cat}$ gave the chimeras a preference for middle chain esters, with the best substrate as pNPC8, consistently with apAPH. The activity of AAM7 on the long chain substrates is very low and the kinetic parameters for pNP-C12, C14 and C16 are undetectable. The chimera AAM7 had a clear preference for substrates with a short acyl chain, with pNPC4 being the best, similar to the parent AFEST. These results indicated that the cap and propeller domains are important for the substrate chain length specificities of AFEST and apAPH.

Discussion

Esterases have been widely used in industry to catalyze the stereospecific hydrolysis, transesterification, and conversion
Table 2 Kinetic parameters for hydrolysis of several pNP substrates by the parent enzymes and chimeras

<table>
<thead>
<tr>
<th>pNP-substrates</th>
<th>apAPH</th>
<th>PAR</th>
<th>AAM7</th>
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<tbody>
<tr>
<td></td>
<td>$k_{cat}$/K_m</td>
<td>$k_{cat}$/K_m</td>
<td>$k_{cat}$/K_m</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
<td>$K_m$ (µM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
</tr>
<tr>
<td>pNP-2C</td>
<td>4.5 ± 0.2</td>
<td>178 ± 1.1</td>
<td>25.5 ± 3</td>
</tr>
<tr>
<td>pNP-4C</td>
<td>5.9 ± 0.1</td>
<td>115 ± 6.1</td>
<td>51.7 ± 3</td>
</tr>
<tr>
<td>pNP-8C</td>
<td>7.5 ± 0.2</td>
<td>107 ± 6.1</td>
<td>70.2 ± 3</td>
</tr>
<tr>
<td>pNP-12C</td>
<td>22 ± 0.1</td>
<td>0.2 ± 1</td>
<td>0.3 ± 1</td>
</tr>
<tr>
<td>pNP-16C</td>
<td>1.3 ± 0.2</td>
<td>0.2 ± 1</td>
<td>0.3 ± 1</td>
</tr>
<tr>
<td>pNP-18C</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 1</td>
<td>0.3 ± 1</td>
</tr>
<tr>
<td>pNP-C4</td>
<td>0.05 ± 0.01</td>
<td>0.9 ± 0.1</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>pNP-C8</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
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The trend of the $K_m$ values of PAR for the short-chain substrates (C2, C4) are improved about 5 folds, while for the longer chain substrates (C8–C16), the $K_m$ values are reduced by 2–40 times. The trend of $K_m$ values of PAR is similar to apAPH. These results suggest that the propeller domain is more suitable for the combination of long-chain substrates, while the cap domain prefer short-chain substrates. Consistent with this result, after substituted the propeller domain with the cap domain, the $K_m$ values of AAM7 reduced 2–25 times for short-chain substrates (C2, C4) and improved 5–18 folds for longer-chain substrates (C8, C10), relative to parent apAPH. Also the trend of the $k_{cat}$ values of PAR and AAM7 is similar to apAPH and AFEST, respectively. These results indicate that the substrate-binding domains are the dominant factor for their substrate specificities. Besides, we calculated the
substrate-binding pockets of AFEST and PAR using CASTp web server (http://sts.bioengr.uic.edu/castp) [29]. The results showed that the substrate-binding pocket became larger when the cap domain of AFEST substituted by propeller domain of apAPH (Fig. 7). We speculate that the larger pocket in the hybrid domain interface of the chimera PAR is suitable for the catalysis of larger substrates, and prefer the middle-chain substrate.

In summary, we constructed two active chimeric esterases through domain swapping. The characterization of chimeras indicated that the substrate-binding domains are important to the substrate specificities of α/β hydrolase family members. Our results also show that domain swapping combined with domain interface optimization is an effective way to recombine parent proteins with low-sequence identities.

Supplementary Data
Supplementary data are available at ABBS online.

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