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RARE-PAL/Rational redesign of phenylalanine ammonia-lyases for reversing their natural selectivity

Abstract: according to the proposed plan, during the first financial period *Activity 1.1*, the rational design-driven mutagenesis, has been implemented, while *Activity 1.2*, the Ala-scan of *Pc*PAL active site residues, has been started.

Objective/Activity 1. *Rational design driven site-directed mutagenesis for identification of D-selectivity modulating residues*

Subactivity 1.1. Site-directed mutagenesis driven by rational design concepts (months 1-3)

Based on the proposed rational design approach for the selectivity reversal of PALs the following the following envisaged mutations were performed: H396K, H396Y, N384A (involved in the H-bonding network involving R354, responsible for the fixation of the substrate`s carboxylic group), combined or not with mutations of hydrophobic residues L206, L134, L256 to bulky phenylalanine (F), restricting the substrate accomodation in the conformation leading to the L-amino acid, combined or not with the mutations of residues F137, I460, K456 to smaller residues (alanine or valine), providing more space for the substrate accomodation in conformation providing the D-enantiomer (for active site residues see Figure 1, while the obtained/envisaged mutant list is presented in Table 1.)

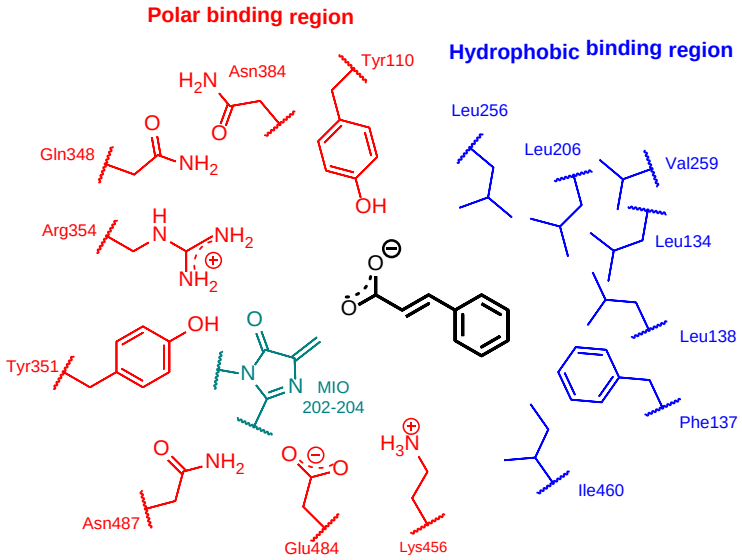


Figure 1. Active site model of *Pc*PAL with (*E*)-cinnamic acid as ligand, showing the hydrophobic binding pocket (blue), the polar substrate binding region (red) and the catalytically essential MIO-group

The desired mutations were introduced using the site-directed mutagenesis protocol developed by Naismith et al.[1] Therefore designed primers which contain non-overlapping sequence and primers which contain overlapping sequence (primer-primer complementary) with a $T_{m\ no}$ 5 to 10 ^{o}C higher than the $T_{m\ pp}$ so that the non-overlapping sequences can bridge the nick and bind to the newly synthesized DNA efficiently.

Generally, the PCR amplification using these primers showed high efficiency (Figure from Table 1), only in some cases low amplification eficiency could be observed. In these cases optimization the of the PCR procedures (addition of DMSO, modification of template and primer concentration) lead to succesfull mutation.

The PCR reactions with a total volume of 50 µl consisted of 2 ng of DNA template (plasmids containing the gene of the wild-type enzyme, readily available through the host research group), 1 µM primer pair, 200 µM dNTPs and 3 units of Phu Hot-Start DNA polymerase. The PCR cycles were initiated at 95 ^{0}C for 5 minutes to denature the template DNA, followed bz 20 amplification cycles. Each amplification cycle consisted of 95 ^{0}C for 1 minutes, T $T_{m\ no}$ - 5 ^{0}C for 1 minute and 72 ^{0}C for 10 minutes (500 bp/min). The PCR cycles were finished with a final annealing step at $T_{m\ pp}$- 5 ^{0}C for 1 minute and 72 ^{0}C for 30 minutes.

The PCR products were further treated with 5 units of DpnI at 37 ^{0}C for 2 hours and then 10 µl of each PCR reaction was analyzed by agarose gel electrophoresis. An aliquout of 3 µl of the above PCR products was transformed through heat-shock into *E.coli* XL-1 Blue competent cells. The transformed cells were spread on Luria-Bertani (LB) plates containing antibiotics and incubated at 37 ^{0}C overnight. Two colonies from each plate were grown and the plasmid DNA was isolated. To verify the mutation 300 ng of plasmid DNA was mixed with 50 pmol T7 or home-designed sequencing primers in a volume 12 µl. DNA sequencing was carried out using the sequencing services of Biomi (Gödöllő, Hungary), confirming the desired mutation.

Further, the plasmids with the mutant genes were succesfully transformed into different *E.coli* host cells (*E.coli* Rossetta DE3, pLysS) suitable for protein expression.

Milestones: the collection of multiple *Pc*PAL variants (Table 1) has been obtained and will be used within the D-selectivity screens.

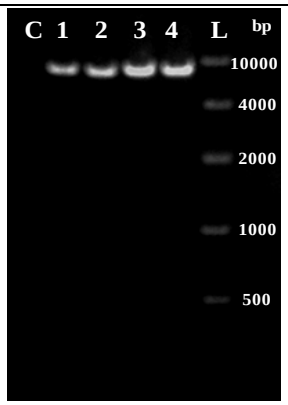
Entry	***Envisaged PcPAL mutants***	***Number of mutations introduced***	***Stage of experiments***	***Representative*** agarose gel electrophoresis of the PCR products
1	H396K	1	Mutations succesfully performed	 **C.** DNA control before PCR, **1.** *Pc*PAL L134FV mutant **2.** *Pc*PAL H396K mutant. **3**. *Pc*PAL N384A mutant **4**. *Pc*PAL Q488N mutant **L.** DNA Ladder
2	H396Y	1		
3	N384A	1		
4	Q488N	1		
5	E484D	1		
6	K457S	1		
7	K457A	1		
8	H396K/F137V	2		
9	H396Y/F137V	2		
10	H396K/F137A	2		
11	H396Y/F137A	2		
12	L134F	1		
13	L206F	1		
14	L256F	1		
15	L134F/F136A	2		
16	E484D/H396K	2		
17	E484D/L134F	2		
18	E484D/L256F	2		
19	E484D/K456S	2		

Table 1. Mutant *Pc*PAL variants obtained by site-directed mutagenesis of active site residues selected through rational design.

Subactivity 1.2. Ala-scan of active site residues combined with monodental substrate fixation

As second approach using variants H396K, H396Y, N384A as template, Ala-scan of all residues from both the hydrophobic substrate-binding pocket as well as the polar binding pocket of *Pc*PAL will be performed. At the current stage of the project the primer design has been performed and will be followed by the mutagenesis experiments.

References

1. Liu, H., Naismith, J. H.: An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnology*, **2008**, 8:91