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Rational design of a more stable penicillin G acylase against organic cosolvent

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Abstract

We have used a simple and efficient approach by combining the known functional and structural properties of penicillin G acylase (PGA) from *E. coli*, and tried to mutate PGA of *Bacillus megaterium* with the goal of increasing the stability of the enzyme in organic solvents or at acidic pH. The PGA mutants Kβ427A, Kβ430A and Kβ427A/Kβ430A obtained have higher stability in DMF than the wild-type PGA.

Keywords: Penicillin G acylase; Protein stability; Protein engineering

1. Introduction

Since the end of the 1960s, the majority of *N*-deacylations of penicillin and cephalosporin G for industrial production of 6-APA and 7-ADCA has been carried out by a group of penicillin G acylases (PGAs, EC 3.5.1.11) to accelerate the commercialization of semi-synthetic β-lactam antibiotics. In the last 20 years, more efforts were made to employ the same enzymes in the synthesis of new cephalosporins based on the catalysis of the reverse reaction, i.e. the acylation of the β-lactam nucleus, such as 7-ADCA and 7-ACA with the appropriate D-amino acid analogs [1]. PGA can be used in the synthesis of β-lactam antibiotics following the kinetic-controlled synthesis. However, following this strategy, stable enzymes are needed to withstand the unfavorable reaction conditions (i.e. acidic pH in favor of the synthetic process, cosolvents to influence the p*K* of the carboxyl group) [2].

Protein engineering has been used in many ways to improve the properties of enzymes. Natural enzymes are likely to be suboptimal in biocatalytic applications because of their pH optima and pH-dependent instabilities. The isoelectric point (IP) of a protein is related to surface charge [3] and it has been observed that proteins tend to be most stable near the IP [4]. We have thought that lowering the IP of the PGA through altering its surface charge by site-directed mutagenesis may obtain stable variants of PGA in acidic pH. Meanwhile, by replacing the charged residue by less polar amino acid, the hydrophobicity

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of the protein surface is enhanced, and the mutant protein may be more stable in organic solvent [5].

Therefore, we have searched and replaced the non-conserved basic residues on the protein molecular surface with neutral residues to improve the stability of PGA.

2. Methods and reagents

2.1. Software

PMODELING was for homology-based protein structure modeling [6]. MSI Insight II version 95 and RASWIN 2.7.0.1 was for structure analysis. Hbplus was for hydrogen bonding calculation [7].

2.2. Strains and plasmids

PGA expression plasmid pEES102 was constructed as described previously [8].

2.3. Site-directed mutagenesis

The following six primers, corresponding to mutations Kβ427A, Kβ430A and Kβ427A/Kβ430A were used for site-directed mutagenesis by overlap extension PCR [9]:

1. 5′-GAAAAAGGGTTGGCAGCAGAAAAATG-3′;
2. 3′-CTTTTTCCCAACCGTCGTCTTTTTAC-5′;
3. 5′-TGAAAGCAGAAGCATGGCGTATGCCT-3′;
4. 3′-ACTTTCGTCTTCGTACCGCATACGGA-5;
5. 5′-GAAAAAGGGTTGGCTGCAGAAGCATGG-3′;
6. 3′-CTTTTTCCCAACCGACGTCTTCGTACC-5′;

The upstream primer is 5′-ATCATCGCTATGGGGCTTCA-3′ and the downstream primer is 3′-ATACTCATTCATCTTTCAGCTGTAAA-5′.

PCR fragments containing mutations were cloned into pEES102 by *Sca*I and *Sal*I to replace the wild-type fragment and were confirmed by DNA sequencing. PGA expression, purification and activity assay were described previously [8]. Purified enzyme was used as sample for the study of enzyme stability.

2.4. Reagents

All restriction enzymes, Taq polymerase and T4 ligase were from GIBCO BRL. Dimethylformamide (DMF) was AR grade.

3. Results and discussion

Structure-based protein rational design is demonstrated as a powerful tool to improve the biological functions of proteins [10–12]. However, the three dimensional (3D) structure of *Bacillus megaterium* PGA (BPGA) is still unknown. Fortunately, there exists a homologous protein of BPGA, *E. coli* PGA (EPGA), with known structure (protein data bank, PDB [13] code 1AI4). Their sequence similarity is 33%, within the normal application scope of protein comparative modeling. [14] The structure of BPGA was thus modeled based on the single template EPGA with the in house software package PMODELING of protein comparative modeling [6]. The modeled structure (Fig. 1) is evaluated by ASSESSOR, a protein structure evaluation program in PMODELING, and the result compatibility score is 262.4, highly exceeding the minimum acceptable score 79.3. Meanwhile, the modeled structure also had acceptable stereochemistry parameters. All these showed that the protein structure model has acceptable quality of protein comparative modeling.

The widely used method to predict the stability of protein variants based on structure is free energy perturbation (FEP) [15]. However, we had a number of reasons not using this strict method in this example. First, in this case, we were interested in the stability of enzyme in the acidic and organic cosolvent, which is still remaining a difficult task for FEP method. Second, we had only the modeled protein structure in hand, which was supposed to be less accurate than the crystal structure. This might be an essential flaw if we want to use it for free energy calculation. To avoid the “common error”, stated by van Gunsteren and Mark [16], “to attempt to improve a basically crude model by performing one aspect of the calculation in a rigorous manner.”, we finally adopted the empirical method to select the adequate protein mutants. Refer to the results of Rio and Rodriguez [17] and Rio et al. [18], we

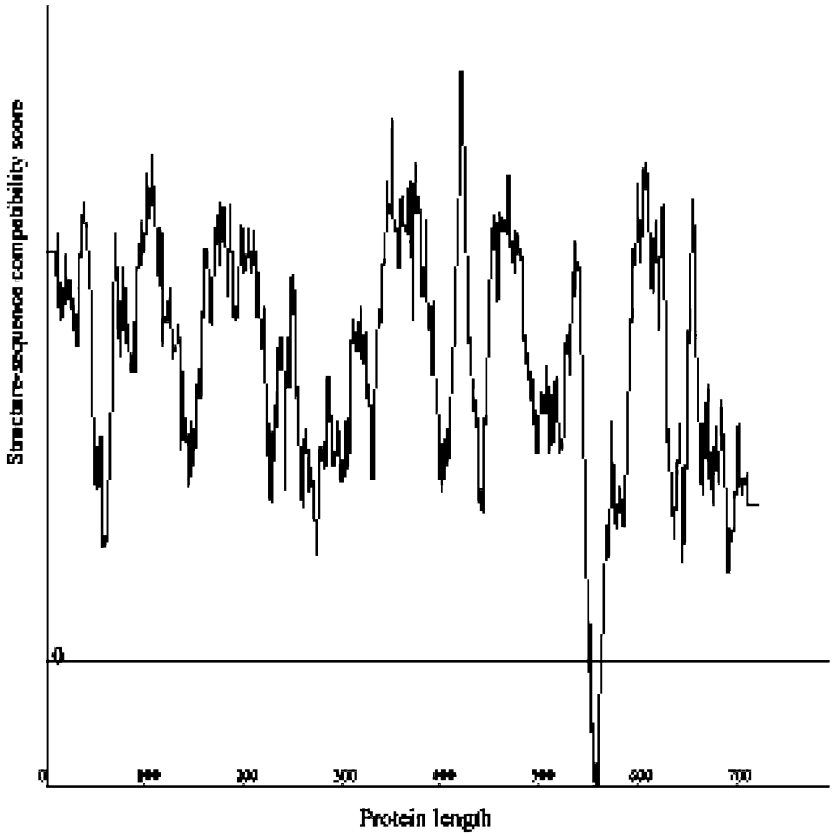

Fig. 1. The profile plot of modeled structure evaluated by ASSESSOR. The plot was calculated using a sliding window size of 21 to average the comparability scores of individual amino acids.

Table 1
Selection of residues to be replaced by alanine according to the rules to engineer the protein

Candidate site	Number of salt bridges	Number of hydrogen bonds	Distance to active center (Å)	Results
B314Lys	0	4	44.3	Too many hydrogen bonds
B427Lys	0	1	40.9	✓
B115Lys	0	0	38.4	Close to active site
B116Lys	0	0	36.5	Close to active site
B430Lys	0	1	35.8	✓
A021Lys	0	2	33.4	✓
B492Lys	0	1	32.4	✓
A128Lys	0	1	31.9	✓
B277Arg	1	3	27.6	Salt bridge exist
B334Lys	1	2	21.8	Salt bridge exist
B450His	0	2	21.4	Not on the surface
B436Lys	1	2	20.6	Salt bridge exist
B512Lys	0	0	20.5	✓
A149His	0	1	19.6	Close to active site
B199Arg	0	1	19.6	Not on the surface
B247Lys	0	3	13.6	Too many hydrogen bonds

determined the following thumb rules to engineer the protein:

1. mutate the basic amino acids on surface, which are not in the fatal structure elements (i.e. α-helix, β-sheet and β-turn);
2. mutate non-conservative amino acids in the multiple alignment of PGA sequences from others source [19];
3. mutate the amino acids involving in few interactions, i.e. no salt bridge and only few side chain hydrogen bonds (<3) to the other amino acids;
4. mutate the amino acids far away from the active site of the enzyme, to minimize the effect of the mutagenesis on the activity;
5. mutate the basic amino acids to the neutral amino acids, to enhance the stability of enzyme in non-aqueous medium;
6. mutate the amino acids to smaller ones (i.e. alanine), to minimize conformation.

There are 31 basic amino acids in the surface loops, in which only 16 are not conservative (see Table 1). These amino acids were further selected by evaluating their salt bridges, side chain hydrogen bond and distance to active center. Finally, six amino acids at positions β427K, β430K, α021K, β492K, α128K and β512K were considered to be most suitable for mutagenesis. However, only two amino acids distant from active center were mutagenesised at first cycle of protein engineering, i.e. Kβ427A, Kβ430A and Kβ427A/Kβ430A.

Table 2
Yield and the half-lives of wild-type and mutant PGAs in 40% DMF and at 55 °C

PGA	Yield (U/ml)	Stability ($t_{1/2}$) (min)	
		40% DMF[a]	55 °C[b]
Wild type	30	31	8.6
Kβ427A	28	32	7.4
Kβ430A	28	54	15.0
Kβ427A/Kβ430A	25	80	16.6

[a] Samples were incubated in 50 mM sodium phosphate buffer (pH 7.5) with 40% DMF at 25 °C.
[b] Samples were incubated in 50 mM sodium phosphate buffer (pH 7.5) at 55 °C.

As shown in Table 2, the half-lives of Kβ430A and Kβ427A/Kβ430A are longer in 40% DMF at 25 °C. However, although the thermostability of Kβ430A and Kβ427A/Kβ430A are also increased, the half-life of Kβ427A dropped a little in comparison with the wild-type enzyme. In addition, we found that all these mutants still kept the same expression levels of the wild-type PGAs.

To investigate the effect of DMF on different PGAs, the samples were incubated in DMF of different concentrations at 0 °C (Fig. 2). As shown

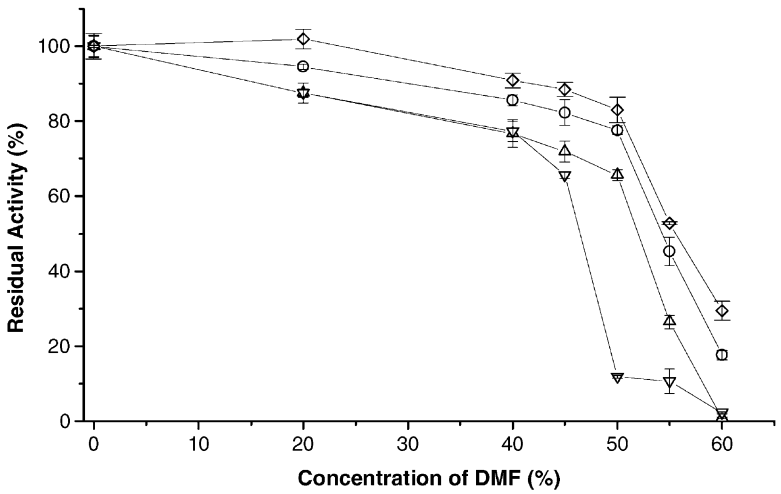

Fig. 2. Stabilities of wild-type and mutant PGAs in DMF of different concentrations. Samples with the same initial enzyme activity of 5 U/ml were incubated in ice bath in DMF of different concentrations. The residual activities were assayed after 3 h. The enzyme activity in 0% DMF was described as 100%. Symbol: (◇) Kβ427A/Kβ430A; (○) Kβ427A; (△) Kβ430A; (▽) wild type.

in Fig. 2, the residual activity of Kβ427/Kβ430A in 60% DMF is 29.4%, and Kβ427A with 17.6%, while the wild-type enzyme only has 2.2% residual activity.

4. Conclusion

β-Lactamic antibiotic industry is undergoing a huge reformation by enzymatic synthesis. Two different strategies, kinetic-controlled synthesis and thermodynamic-controlled synthesis, were brought forward. Thermodynamic-controlled synthesis is contrastively direct way toward synthetic reaction. But highly stable enzymes are necessary in this strategy, especially in cosolvent condition, which increase the difficulty to create. Two mutative PGAs reported in this article showed higher stability than the wild-type enzyme in organic solvent. They make it possible to control the synthesis by thermodynamic strategy. They also showed as successful examples for rational design.

Using comparative modeling system based on the conservativeness of the 3D structure of protein and the stereo-conformation of PGA from *E. coli*, a rational design is useful to find the mutagenic site(s) for enhancing an enzyme's stability. Our design approach is rather simple to obtain the mutants with expected properties. There is clearly considerable potential for engineering enzymes to function in unusual environmental conditions. Combination of single mutagenesis would further increase the stability of enzyme.

The results of site-directed mutagenesis of PGA reported have strongly recommended that enzymes can be rationally engineered to improve their stabilities in organic solvents. Small alterations, even single amino acid substitution can enhance the stability of the enzyme in high concentrations of DMF. Mutation of non-conserved surface residues appears to be a generally applicable strategy for enhancing enzyme stability in organic solvents. Furthermore, single mutations can be combined to get the double mutant (Kβ427A/Kβ430A) with higher stability. The thermostabilities of Kβ430A and Kβ427A/Kβ430A were significantly higher than those of the wild type and Kβ427A. The half-lives of the double mutant increased about 100% to 16.6 min at 55 °C, which is comparable to the most thermostable PGA ever reported (almost 15 min at 55 °C) [19]. Kβ427A seemed to only contribute to the stability in DMF but not thermostability.

In short, we have obtained three mutants with the same hydrolytic activities as those of the parent PGA. Among these, two mutants have enhanced stability in DMF or at 55 °C. Another mutant only has improved stability in DMF.

No obvious stability enhancement of PGA in acidic pH was observed; perhaps the lowering of IP was not enough. We hope that multiple mutations of amino acids may bring about certain change.

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References

[1] A. Bruggink, E.C. Roos, E.D. Vroom, Org. Process Res. Dev. 2 (1998) 128–133.
[2] R. Fernandez-Lafuente, C.M. Rosell, Enzyme Microbiol. Technol. 19 (1996) 9–14.
[3] A.J. Russell, A.R. Fersht, Nature 328 (6) (1987) 496–500.
[4] K.A. Dill, Biochemistry 29 (31) (1990) 7133–7155.
[5] F.H. Arnold, Trends Biotechnol. 8 (1990) 244–249.
[6] D.F. Ding, H.X. Tang, Acta Biophys. Sinica 11 (1995) 416–428.
[7] I.K. McDonald, J.M. Thornton, J. Mol. Biol. 238 (1994) 777–793.
[8] S. Yang, H. Huang, R. Zhang, X. Huang, S. Li, Z. Yuan, Protein Expr. Purif. 21 (1) (2001) 60–64.
[9] S.N. Ho, H.D. Hunt, Gene 77 (1989) 51–59.
[10] F. van de Velde, F. van Rantwijk, R.A. Sheldon, Trends Biotechnol. 19 (2) (2001) 73–80.
[11] F. Cedrone, A. Menez, E. Quemeneur, Curr. Opin. Struct. Biol. 10 (4) (2000) 405–410.
[12] M. Lehmann, M. Wyss, Curr. Opin. Biotechnol. 12 (4) (2001) 371–375.
[13] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, Nucleic Acids Res. 28 (2000) 235–242.

[14] R. Sanchez, U. Pieper, F. Melo, N. Eswar, M.A. Marti-Renom, M.S. Madhusudhan, N. Mirkovic, A. Sali, Nat. Struct. Biol. 7 (2000) 986–990.
[15] W.F. Van Gunsteren, F.J. Luque, Annu. Rev. Biophys. Biomol. Struct. 23 (1994) 847–863.
[16] W.F. van Gunsteren, A.E. Mark, J. Mol. Biol. 227 (2) (1992) 389–395.
[17] G.D. Rio, M.E. Rodriguez, Biotechnol. Bioeng. 48 (1995) 141–148.
[18] G.D. Rio, A. Lopez-Munguia, X. Soberon, Ann. N. Y. Acad. Sci. 799 (1996) 61–64.
[19] R.M.D. Verhaert, A.M. Riemens, Appl. Environ. Microbiol. 63 (9) (1997) 3412–3418.