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Engineering Lipases with an Expanded Genetic Code

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1.1

Introduction

Lipases (EC 3.1.1.3) are a class of ubiquitous enzymes that catalyze both the hydrolysis and synthesis of acylglycerols with long acyl chains (carbon atoms >10) [1]. Currently, they constitute one of the most important groups of biocatalysts applied in many fields, including foods, detergents, flavors, fine chemicals, cosmetics, biodiesel, and pharmaceuticals owing to their high specificity, regioselectivity, and enantioselectivity [2]. Lipases can be found in a broad range of organisms, including plants and animals, however, it is chiefly the microbial lipases that find immense application. This is because of their high yields and ease of genetic manipulation, as well as wide substrate specificity [3].

Although lipases' properties (molecular weight, pH and temperature optima, stability, substrate specificity) are source dependent, they all share a common structure consisting of a compact minimal α/β hydrolase fold. The hydrolase fold is composed of a central β -sheet consisting of up to eight different β strands connected by up to six α helices [1]. The active site of the α/β hydrolase fold enzymes contains a nucleophilic residue (serine), a catalytic acid residue (aspartate/glutamate), and a histidine residue, always in this order in the amino acid sequence. These residues act cooperatively in the catalytic mechanism of ester hydrolysis [4]. The lipolytic reaction takes place at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Lipases are activated by the presence of this emulsion interface, a phenomenon called *interfacial activation*, which differentiates them from similar esterases. Upon contact with the hydrophobic interface, a lid domain that covers the active site moves away, turning the *closed* form of the enzyme into an *open* form, and leaving the active site available for substrate binding and processing.

1.2

Enzyme Activity of Lipases from Different Sources and Engineering Approaches

So far, several microbial lipases have been purified, characterized, and evaluated for their potentials in biotechnological applications. Bacterial lipases generally can act in a wide range of pH and have temperature optima in the range of 30–65 °C but are mostly unstable in the harsh reaction environments of industrial applications, such as high temperature, organic solvents, strong alkalinity, or acidity [5]. Therefore, more and more attention is being paid to isolating and characterizing novel lipases with high stability from cultivated or uncultivated microbes, and even modifying the lipase structures to improve their catalytic properties through protein engineering strategies [6]. Protein engineers focus not only on increasing the enzyme stability but, given the high versatility of lipases, also on the improvement of promiscuous catalytic activities toward nonnatural substrates, for example, usage of the other enantiomer of a racemic mixture as substrate [2]. Among commonly used strategies, rational protein design and directed evolution are increasingly applied in a synergistic manner. Directed evolution has been used as a preferred method to alter the properties of the enzymes such as thermal stability, enzyme activity, substrate specificity, and enantioselectivity [7, 8]. Bioinformatics tools turn to be helpful in rational protein design. The group of Bornscheuer developed an α/β hydrolase fold 3DM database, which processes genomic and metagenomic information and provides the researcher with structural–functional relationships to carefully choose target residues for mutagenesis as well as the respective amino acid to employ for randomization [9]. Furthermore, comparison of lipases from extremophiles and mesophiles can also play an important role in the prediction of stabilizing mutation [10, 11].

Indeed, extremophiles, microorganisms living at extreme conditions of temperature, pH, and osmosis, are a promising source of robust biocatalysts. Lipases from extremophiles can naturally show considerable resistance to proteases, detergents, chaotropic agents, organic solvents, and to thermal denaturation. Therefore, they are superior to traditional biocatalysts and have become objects of special interest for structural investigations and also for industrial applications [12–15]. Recently, thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* (TTL) and *Caldanaerobacter subterraneus* subsp. *tengcongensis* have been characterized [16]. These enzymes, despite having a very low sequence identity to already known lipases, contain the conserved catalytic triad Ser-Asp-His and share a similar 3D structure with lipases from psychrophiles, mesophiles, and thermophiles (Figure 1.1). In particular, the lipase from *T. thermohydrosulfuricus* shows an excellent combination of high stability with high selectivity; it catalyzes with high enantioselectivity the kinetic resolution of a broad set of industrially relevant substrates and is still active at elevated temperatures and high pH (90 °C, pH 11). In addition, its resistance against organic solvents (up to 99%) makes this enzyme very attractive for biotransformation processes in water-free media.

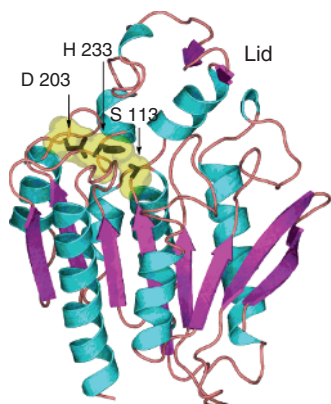


Figure 1.1 3D structure of TTL obtained by homology modeling. The TTL structure contains the α/β hydrolase fold typical of hydrolytic enzymes. The active site with the conserved catalytic triad is shown in yellow. The lid is a mobile loop that modulates the access of the substrate to the active site.

1.3

Noncanonical Amino Acids in Lipase Design and Engineering

Another technique to create biocatalysts that are more robust or exhibit novel properties is by incorporation of noncanonical amino acids (ncAAs), mostly synthetic analogs of the natural amino acids, which greatly expands the set of building blocks of classical protein engineering (Figure 1.2). Using the supplementation-based method (SPI) described in [17], Budisa and coworkers produced three variants of lipase B from *Candida antarctica* (CAL-B) containing different fluorinated amino acids: 5-fluoro-L-tryptophan, *meta*-fluoro-L-tyrosine, and *para*-fluoro-L-phenylalanine. The global fluorination induced moderate

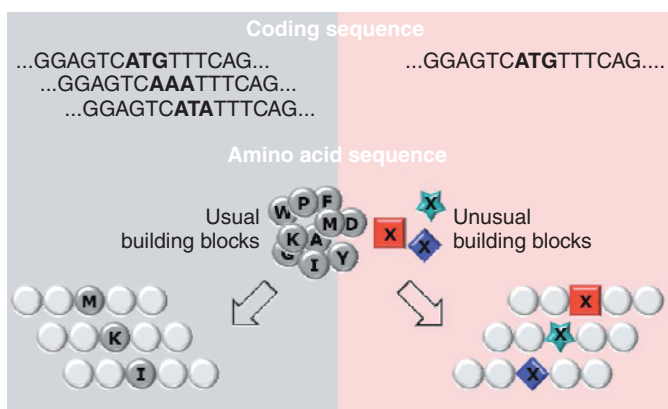


Figure 1.2 Protein engineering by classic mutagenesis versus genetic code engineering. The common site-directed mutagenesis and directed evolution techniques make use of the 20 canonical amino acids or building

blocks. Genetic code engineering, instead, allows the insertion of noncanonical amino acids, which bring new chemical functionalities into the proteins.

changes in the secondary structure of three variants and lowered the catalytic activity. However, the fluorinated variants had a prolonged shelf-life compared with the wild-type protein, which is an important feature for the storage of therapeutic proteins [18]. In another study, the simultaneous incorporation of different ncAAs (six 4(*S*)-fluoroprolines, 16 4-fluorophenylalanines, and two 6-fluorotryptophans) was accomplished in a lipase from TTL. Although this global substitution accounted for about 10% of the total residues, the secondary structure of the modified enzyme was not significantly affected and the enzymatic activity was partially retained [19]. The combination of the method for multiple incorporation of ncAAs with directed evolution strategies might indeed lead to the creation of so-called “teflon proteins” with new interesting properties. Recently, 13 different variants (or congeners) of TTL in which the methionine, proline, phenylalanine, tyrosine, and tryptophan residues

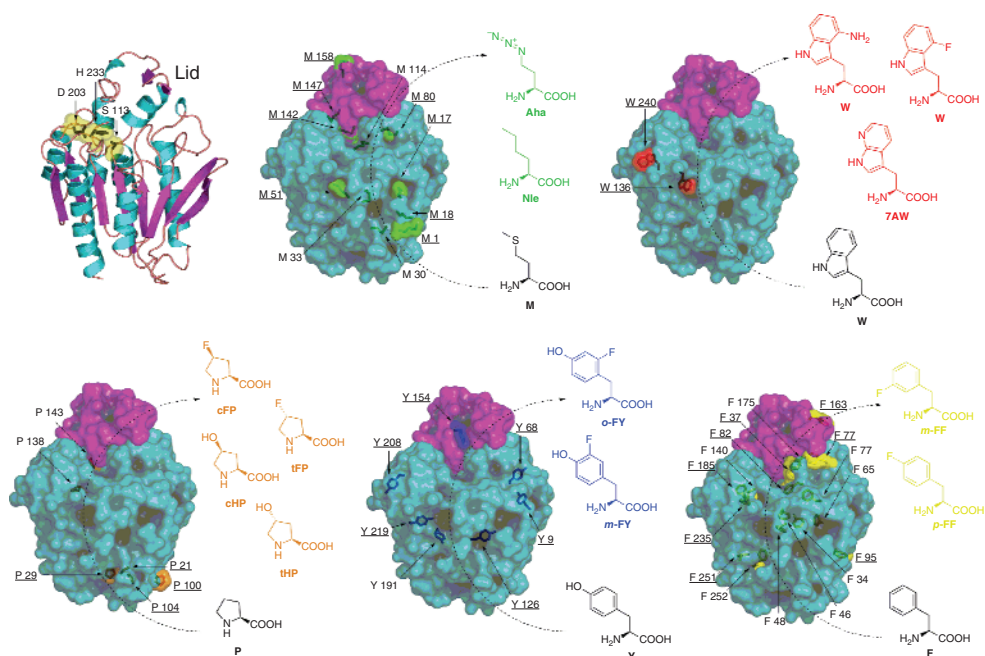


Figure 1.3 Lipase engineering with non-canonical amino acids. Methionine (M), tryptophan (W), proline (P), tyrosine (Y), and phenylalanine (F) side chains were subjected to global replacements with related analogs as follows: Met → Aha (azidohomoalanine) and Nle (norleucine); Trp → 4NW (4-aminotryptophan), 4FW (4-fluorotryptophan) and 7AW (7-azatryptophan); Pro → cFP (*cis*-4-fluoroproline), tFP (*trans*-4-fluoroproline), cHP (*cis*-4-hydroxyproline) and tHP

(*trans*-4-hydroxyproline), Tyr → m-FY (meta-fluorotyrosine) and o-FY (*ortho*-fluorotyrosine), Phe → m-FF (*meta*-fluorophenylalanine) and p-FF (*para*-fluorophenylalanine). Side chains of Met142, Pro143, Met147, Tyr154, Met158, Phe163, and Phe175 belong to the “lid” domain of TTL (in purple) [20]. Note that buried or surface exposed residues are shown un-bold/-underlined or bold/underlined, respectively.

globally substituted with related analogs were generated and characterized (Figure 1.3) [20, 21].

1.4

Case Study: Manipulating Proline, Phenylalanine, and Methionine Residues in Lipase

Proline is an interesting target for modification because it was found that in lipase from *Candida rugosa* its *cis/trans* isomerization plays a role in the lid-closing and lid-opening processes [22]. Replacement of bulky hydrophobic and aromatic residues such as Phe, Tyr, and Trp is also of interest because these residues are lined-up in the substrate-binding cavity and determine the substrate specificity toward different fatty acids. The new variants exhibited the same or lower optimal temperature (until 20 °C less) compared with the wild type and were active at pH 7–9. All the variants, except TTL[tFP], retained quite high resistance to thermal denaturation. The most interesting finding was that the Met → Nle substitution leads to an “always activated” TTL, which does not need thermal activation (Figure 1.4). Thermal activation is a typical feature of thermostable lipases consisting of a gradual increase of activity to reach the maximal level after short incubation at the optimal temperature. A possible explanation for this result is that the Nle hydrophobicity mimics the presence of lipid aggregates and favors the lid opening to expose the active site, resulting in better substrate accessibility in aqueous solutions and no need of thermal activation. Another interesting finding is that the TTL[*m*-FF] congener had 25% more activity and broader substrate

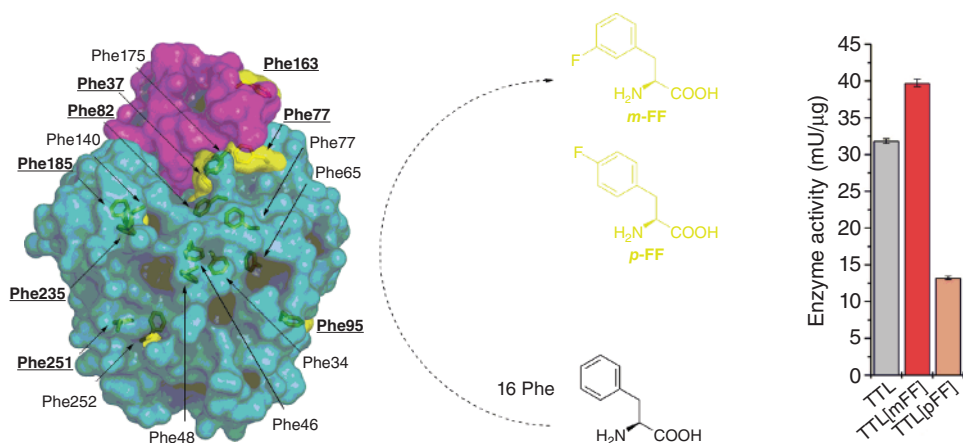


Figure 1.4 Lipase variants generated by the substitution of 16 Phe residues with *p*-FF and *m*-FF. The TTL[*m*-FF] congener showed a 25% increase in activity whereas TTL[*p*-FF] displayed only 40% of the enzymatic activity

when compared with the parent TTL. Lipase activity was determined by measuring the hydrolysis of *p*-nitrophenyl palmitate (pNPP) according to Winkler and Stuckmann [23].

specificity toward triglycerides with longer as well as shorter acyl chains when compared with the parent lipase (Figure 1.4).

1.5

“Unnatural” Lipases Are Able to Catalyze Reactions under Different Hostile Environments

In a following work [20], the residual activity of both parent TTL and of the 13 congeners was measured after pretreatment with several organic solvents, metal ions, surfactants, inhibitors, and protein reducing, alkylating, and denaturing agents. Noteworthy is the finding that, contrary to the wild type, the Trp congeners TTL[4NW], TTL[4FW], and TTL[7AW], as well as TTL[Aha] remain active after treatment with 90% pyridine, an organic solvent used for the synthesis of biodegradable polymers. Another interesting finding is that among the Pro congeners, the fluorinated ones, TTL[cFP] and TTL[tFP], were generally more active than the hydroxylated ones upon treatment with several organic solvents commonly used in industries (*tert*-butanol, ethanol, isopropanol, acetone, DME, acetonitrile, *n*-hexane, hexadecane, isooctane, and *n*-decylalcohol). In particular, TTL[cFP] was the most active congener exhibiting 3.2-fold higher activity in acetonitrile, 1.6-fold higher in *n*-hexane, and 4.4-fold higher in *tert*-butanol, a solvent used in the biodiesel production. The other fluorinated congener, TTL[tFP], also showed higher activity in several organic solvents, as a 2.6-fold increase in acetone and a 1.9-fold increase in DMF, two industrial solvents used in the production of synthetic fibers and plastics (Figure 1.5). TTL[4NW] as well as TTL[mFF] showed an unprecedented resistance against the denaturant guanidinium chloride (0.5 M) and TTL[7azaW] retained twofold more activity than the WT upon 2 M urea treatment. Finally, TTL[cFP] and TTL[mFF] were not inactivated by the protein inhibitor Pefabloc in contrast to TTL[tFP] and TTL[oFF] bearing the isomeric analogs. These results clearly demonstrate that ncAAs can confer advantages to lipase-catalyzing reactions in the hostile industrial environments.

Global replacement methods are a valuable tool for discovering new features of lipases and other biocatalysts, especially due to the synergistic effect of the substitutions. However, the drawbacks are that the output is mostly unpredictable and, when a high number of residues are involved, their substitution is difficult and sometimes can lead to a misfolded or inactive enzyme. A more selective and controlled modulation of the properties of a biocatalysts is achieved by site-specific modifications. The conjugation of synthetic polymers to proteins by covalent attachment has significantly improved different properties such as stability, activity, biocompatibility, and so on [24a,b]. For example, the activity of a modified CAL-B bearing at the surface a low-molecular-weight dextran improved more than twofold in the hydrolysis of *p*-nitrophenylbutyrate (*p*-NPB) and (±)-methyl mandelate [25].

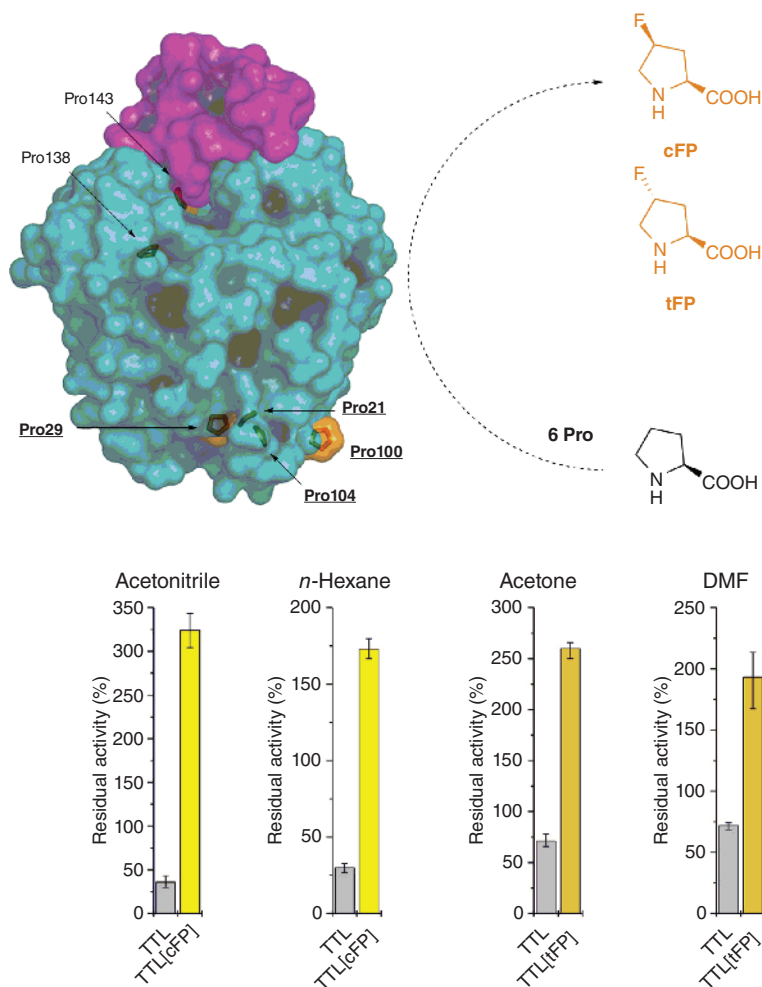


Figure 1.5 Improved lipase congeners generated by the substitution of Pro residues with cFP and tFP. The global replacement of six Pro residues in TTL with the fluorinated counterparts cFP and tFP conferred activity enhancement upon treatment with

several organic solvents used in industries. Residual activity was measured using the *p*-nitrophenyl palmitate assay after 1 h incubation at 25 °C with 90% of organic solvent. (Data taken from [20]).

1.6

Lipase Engineering via Bioorthogonal Chemistries: Activity and Immobilization

Especially the recent development of a wide range of novel site-directed bioorthogonal transformation techniques is permitting the fine control of the installation and manipulation of various chemical groups on the protein surface [26]. Site-specific modifications are increasingly benefiting from the development

of orthogonal aminoacyl-tRNA synthetase/tRNA pairs, which install ncAAs at amber stop codon inserted into the protein sequence, a methodology called stop codon suppression (SCS) [27, 28]. The global replacement and site-specific technologies, of course, can also be combined. Hoels and Budisa [29] showed that TTL[Nle] congener generated by SPI can be further site-specifically modified by the insertion of the unnatural photoreactive amino acid *p*-benzoyl-phenylalanine (Bpa). The congener TTL(D221Bpa)[Nle] showed the significantly enhanced enzyme performance delivered by the incorporation of Nle as described in [21] and additionally the possibility for photocrosslinking given by Bpa.

Another approach, which has been extensively used for the improvement of lipase activity, has been the application of immobilization techniques. Different strategies considering the nature of the support (e.g., polysaccharides, epoxy-acrylic resins, silica, and glass slides), the pore size of the support or the binding methodology (ionic exchange, hydrophobic adsorption, entrapment, crosslinking, etc.) have been critical for a particular set of enzyme properties. Recently, a growing interest toward the use of nanomaterials (especially nanoparticles and nanofibers) as carriers for enzyme immobilization has been observed [30]. Although immobilization of lipases may produce some improvements in enzyme features, such as stability, activity, selectivity, or specificity [5], this process has a cost that in certain cases may cause some difficulties in the implementation of the processes. To overcome this drawback, some groups focused on a whole-cell biocatalyst, which enables the direct use of lipase-producing microorganisms. For example, *Aspergillus oryzae* and *Pichia pastoris* strains, which have been genetically engineered to express several microbial lipases, have been developed for use as whole-cell biocatalysts in biodiesel production and enantioselective transesterification [31, 32].

1.7

Conclusions and Perspectives

Although recently developed metagenomic strategies are expanding our knowledge about biocatalysts, helping us to isolate and identify new enzymes with novel biocatalytic activities from the uncultivable microbial communities living in various terrestrial environmental niches [33], there is no way to bypass enzyme engineering in the near and distant future. Enzyme engineering has been mainly applied so far to improve lipases catalytic properties, mostly based on classical protein engineering or more advanced directed evolution. These approaches, however, are limited to the 20 natural building blocks. New frontiers in the engineering of lipases and other biocatalysts can be envisioned by the usage of an expanded genetic code, which, in theory, can provide an unlimited set of synthetic ncAAs with new chemical functionalities.

The synthetic enzyme examples (i.e., lipases designed with ncAAs) discussed earlier clearly demonstrate that ncAAs have potential advantages in biocatalysis. Still, synthetic biology remains a field of many undisclosed opportunities. Incorporating different ncAAs with various physicochemical properties can

provide not only insights into the protein activity and stability but also lead to the increased resistance to denaturation induced by organic solvent, enhanced heat stability, prolonged half-life, and so on. In general, the use of ncAAs will lead to new findings that might have a practical return, as they will enable us to sharpen the tools for the design of more active, specific, and stable enzymes. We anticipate that these efforts will dramatically increase the future applications in the field of natural and synthetic biocatalysis.

References

1. Jaeger, K.E., Dijkstra, B.W., and Reetz, M.T. (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu. Rev. Microbiol.*, **53**, 315–351.
2. Widersten, M. (2014) Protein engineering for development of new hydrolytic biocatalysts. *Curr. Opin. Chem. Biol.*, **21C**, 42–47.
3. Rogalska, E., Nury, S., Douchet, I., and Verger, R. (1997) Microbial lipases: structures, function and industrial applications. *Biochem. Soc. Trans.*, **25**, 161–164.
4. Ollis, D.L., Goldman, A. *et al.* (1992) The α/β hydrolase fold. *Protein Eng.*, **5**, 197–211.
5. Gupta, R., Gupta, N., and Rath, P. (2004) Bacterial lipases: an overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.*, **64**, 763–781.
6. Glogauer, A., Krieger, N. *et al.* (2011) Identification and characterization of a new true lipase isolated through metagenomic approach. *Microb. Cell Fact.*, **10**, 54. doi: 10.1186/1475-2859-10-54
7. Reetz, M.T. (2004) Controlling the enantioselectivity of enzymes by directed evolution: practical and theoretical ramifications. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 5716–5722.
8. May, O., Nguyen, P.T., and Arnold, F.H. (2000) Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. *Nat. Biotechnol.*, **18**, 317–320.
9. Kourist, R., Bornscheuer, U.T. *et al.* (2010) The alpha/beta-hydrolase fold 3DM database (ABHDB) as a tool for protein engineering. *ChemBioChem*, **11**, 1635–1643.
10. Cambillau, C. and Claverie, J.M. (2000) Structural and genomic correlates of hyperthermostability. *J. Biol. Chem.*, **275**, 32383–32386.
11. Vogt, G., Woell, S., and Argos, P. (1997) Protein thermal stability, hydrogen bonds, and ion pairs. *J. Mol. Biol.*, **269**, 631–643.
12. Elleuche, S., Schroder, C., Sahm, K., and Antranikian, G. (2014) Extremozymes-biocatalysts with unique properties from extremophilic microorganisms. *Curr. Opin. Biotechnol.*, **29**, 116–123.
13. Vieille, C. and Zeikus, G. (2001) Hyperthermophilic enzymes: source, uses, and molecular mechanism for thermostability. *Microbiol. Mol. Biol. Rev.*, **65**, 1–43.
14. Antranikian, G. (2008) in *Thermophiles: Biology and Technology at High Temperatures* (eds F. Robb *et al.*), CRC Press, Boca Raton, FL, pp. 113–160.
15. Haki, G.D. and Rakshit, S.K. (2003) Developments in industrially important thermostable enzymes: a review. *Bioresour. Technol.*, **89**, 17–34.
16. Royter, M., Antranikian, G. *et al.* (2009) Thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* SOL1 and *Caldanaerobacter subterraneus* subsp. *tengcongensis*. *Extremophiles*, **13**, 769–783.
17. Budisa, N. (2004) Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire. *Angew. Chem. Int. Ed.*, **43**, 6426–6463.

18. Budisa, N., Wenger, W., and Wiltshi, B. (2010) Residue-specific global fluorination of *Candida antarctica* lipase B in *Pichia pastoris*. *Mol. Biosyst.*, **6**, 1630–1639.
19. Merkel, L., Schauer, M., Antranikian, G., and Budisa, N. (2010) Parallel incorporation of different fluorinated amino acids: on the way to 'teflon' proteins. *ChemBioChem*, **11**, 1505–1507.
20. Acevedo-Rocha, C.G., Antranikian, G., Budisa, N. *et al.* (2013) Non-canonical amino acids as a useful synthetic biological tool for lipase-catalysed reactions in hostile environments. *Catal. Sci. Technol.*, **3**, 1198–1201.
21. Hoesl, M.G., Budisa, N., Antranikian, G. *et al.* (2011) Lipase congeners designed by genetic code engineering. *Chem-CatChem*, **3**, 213–221.
22. Andreotti, A.H. (2003) Native state proline isomerization: an intrinsic molecular switch. *Biochemistry*, **42**, 9515–9524.
23. Winkler, U.K. and Stuckmann, M. (1979) Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.*, **138**, 663–670.
24. (a) Kochendoerfer, G.G. (2005) Site-specific polymer modification of therapeutic proteins. *Curr. Opin. Chem. Biol.*, **9**, 555–560; (b) Heredia, K.L. and Maynard, H.D. (2007) Synthesis of protein-polymer conjugates. *Org. Biomol. Chem.*, **5**, 45–53.
25. Siddiqui, K.S. and Cavicchioli, R. (2005) Improved thermal stability and activity in the cold-adapted lipase B from *Candida antarctica* following chemical modification with oxidized polysaccharides. *Extremophiles*, **9**, 471–476.
26. Sletten, E.M. and Bertozzi, C.R. (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem. Int. Ed.*, **48**, 6974–6998.
27. Wang, L. and Schultz, P.G. (2004) Expanding the genetic code. *Angew. Chem. Int. Ed.*, **44**, 34–66.
28. Hoesl, M.G. and Budisa, N. (2012) Recent advances in genetic code engineering in *Escherichia coli*. *Curr. Opin. Biotechnol.*, **23**, 751–757.
29. Hoesl, M.G. and Budisa, N. (2011) Expanding and engineering the genetic code in a single expression experiment. *ChemBioChem*, **12**, 552–555.
30. Marciello, M., Filice, M., and Palomo, J.M. (2012) Different strategies to enhance the activity of lipase catalysts. *Catal. Sci. Technol.*, **2**, 1531–1543.
31. Adachi, D., Kondo, A. *et al.* (2011) Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. *Bioresour. Technol.*, **102**, 6723–6729.
32. Jin, Z., Han, S.Y., Zhang, L., Zheng, S.P., Wang, Y., and Lin, Y. (2013) Combined utilization of lipase-displaying *Pichia pastoris* whole-cell biocatalysts to improve biodiesel production in co-solvent media. *Bioresour. Technol.*, **130**, 102–109.
33. López-López, O., Cerdán, M.E., and González-Siso, M.I. (2014) New extremophilic lipases and esterases from metagenomics. *Curr. Protein Pept. Sci.*, **15**, 445–455.