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Review

Evolution of fluorinated enzymes: An emerging trend for biocatalyst stabilization

Nature uses remarkably limited sets of chemistries in its repertoire, especially when compared to synthetic organic chemistry. This limits both the chemical and structural diversity that can ultimately be achieved with biocatalysis, unless the powers of chemical synthesis are merged with biological systems by integrating nonnatural synthetic chemistries into the protoplasma of living cells. Of particular interest, here is the fluorous effect that has recently established the potential to generate enzymes with an increased resistance toward both high temperature and organic solvents. For these reasons, we are witnessing a rapid development of efficient methodologies for the incorporation of fluorinated amino acids in protein synthesis, using both in vivo and in vitro strategies. In this review, we highlight relevant and trendsetting results in the design and engineering of stable fluorinated proteins and peptides along with whole-cell biocatalysis as an economically attractive and convenient application with exclusive focus on industrial biocatalysis. Finally, we envision new strategies to improve current achievements and enable the field to progress far beyond the current state-of-the-art.

Keywords: Biocatalysis / Enzyme stabilization / Fluorinated amino acids / Fluorination / Fluorous effect

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1 Introduction

Enzymes have been used for centuries in technological processes, such as the production of alcohol by fermentation, or enzymatic degradation of milk, but their relevance as biocatalysts in industrial processes has increased dramatically in recent decades [1]. Enzymes, whether in their isolated form or as part of a whole cell fermentation process, have proven to be a sustainable alternative to traditional chemical catalysis for the effective conversion of natural accessible starting materials to high value products, such as biofuels, pharmaceuticals, agrochemicals, etc. Several factors, such as unrivaled chemo-, enantio- and regioselectivity, ability to function under mild conditions, biodegradability, and recyclability are among the main reasons that have prompted the use of enzymes in industrial biocatalysis [2, 3].

All these particular properties displayed by enzymes are a consequence of their complex 3-D structure. In the native state, the polypeptide chain adopts such a conformation that the hydrophobic residues locate themselves in the protein core, minimizing contact with the polar solvent, while hydrophilic residues are located on the surface. Several other interactions between amino acid residues, such as hydrogen bonds, van der Waals's forces, salt bridges, etc., contribute to stabilize the final folded state.

These interactions, which are ultimately responsible for the high enzymatic reactivity and specificity, are however quite sensitive to environmental conditions. Any change in the physical parameters of the aqueous media in which the enzyme has been synthesized and folded can alter its native state and consequently, enzymatic activity can be lost or seriously damaged. This constitutes a clear disadvantage for many biotechnological applications, where organic solvents or high temperature are usually required for the solubilization of highly hydrophobic substrates [4].

Nevertheless, exhaustive research in different fields of enzyme technology has already been conducted in an attempt to overcome these present limitations. Several strategies include

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Abbreviations: AARS, aminoacyl-tRNA synthetases; **APS**, automated peptide synthesis; **CalB**, lipase B from *Candida Antarctica*; **FPhe**, fluorophenylalanine; **FPro**, fluoroproline; **hFLeu**, hexafluoroleucine; **hFVal**, hexafluorovaline; **SPI**, selective pressure incorporation; **tFIle**, trifluoroisoleucine; **tFLeu**, trifluoroleucine; **tFVal**, trifluorovaline

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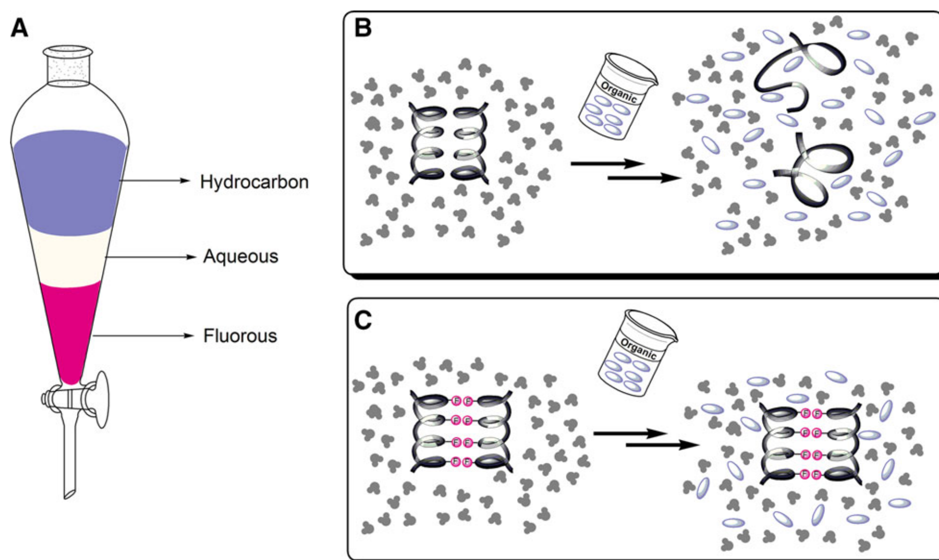

Figure 1. (A) Typical phase separation behavior for highly fluorinated compounds. (B) Effect of the organic solvent addition (blue) to a folded protein. Hydrophobic interactions with the organic solvent induce immediate disruption of the protein native state. (C) Substitution by a fluorous core (pink) prevents denaturation caused by organic solvent or high temperature.

immobilization to solid supports, chemical modification, crystallization, aggregation, protein engineering techniques, and site-directed mutagenesis, which have been widely reviewed [5, 6].

Recent attempts show that enzymatic stabilization could be obtained by introducing fluorinated analogs of hydrophobic amino acids into protein structures in order to imprint these biological biopolymers with some of the properties that characterize fluorinated compounds, such as high temperature resistance, inertness, and extreme hydrophobicity [7–9].

In this review, we aim to present the basis behind this prospect and some of the advances made so far in the development of fluorinated enzymes and peptides that are relevant for industrial biocatalysis. In addition, possible new strategies to improve existing results will also be discussed. For an exhaustive summary of all other possible applications of fluorinated peptides and proteins beyond enzymatic stabilization, the reader is kindly invited to inspect other reviews published elsewhere [10–12].

2 The fluorous effect

Despite the scarcity of naturally occurring organofluorine compounds, chemists have been interested in their synthesis for a long time, mostly due to their unusual physicochemical properties [13]. Extensively fluorinated molecules exhibit resistance to high temperature and an unusual phase separation behavior: they are simultaneously hydrophobic and lipophobic, and they preferentially interact with fluorocarbon compounds. This phenomenon is usually referred to as the *fluorous effect* [7–10].

The nature of the fluorous effect has remained unclear for several years, but it has been recently explained in terms of the small size and low polarizability of the fluorine atom, which causes fluorinated compounds to exhibit a large apolar surface area, hindering effective packing with other nonfluorinated molecules [14–16]. These characteristics combined with the chemical inertness of fluorinated compounds have been successfully exploited to develop unique materials, such as nonstick coatings (Teflon) [10].

When compared with those interactions operating during protein folding in aqueous solution, the fluorous effect shows noticeable similarities. Both phenomena are driven by the tendency of a certain group of hydrophobic residues to interact with cognates rather than the surrounding polar media. The only intrinsic difference is that the fluorinated molecules are still lipophobic, so the fluorous effect would manifest even in organic solvents and no perturbation of the fluorinated phase is expected.

This led to the idea that, if the hydrocarbon core of an enzyme would be replaced by a fluorinated analog, the resulting fluorinated enzymatic variant should still fold into a structure similar to that of the natural protein and consequently, enzymatic activity should be retained. Interestingly, the fluorous effect also predicts that fluorinated proteins should be resistant to denaturation by addition of standard organic solvents, since the fluorocarbon side chains are also lipophobic [7]. At the same time, a greater resistance to thermal denaturation is expected according to the properties of highly fluorinated compounds (Fig. 1).

This initiative caught the attention of several research groups, due to the fact that the expected properties imprinted by a fluorinated core in enzymes would defeat two of their major drawbacks for biotechnological processes, that is, organic solvent and temperature susceptibilities as well as finding new utilities in various protein-based biotechnologies, such as new biomaterials, biosensors, and protein therapeutics.

3 Bio-incorporation of fluorinated amino acids into proteins and peptides

The substitution of amino acids typically present in the hydrophobic protein core by their fluorinated surrogates was initially conceived, as an approach to preserve biological activity, since those residues are usually not directly involved in the catalytic mechanism but are mostly responsible for maintaining the native structural state [9]. Leucine, isoleucine, valine, glycine and, to less extent, tryptophan, phenylalanine, methionine and

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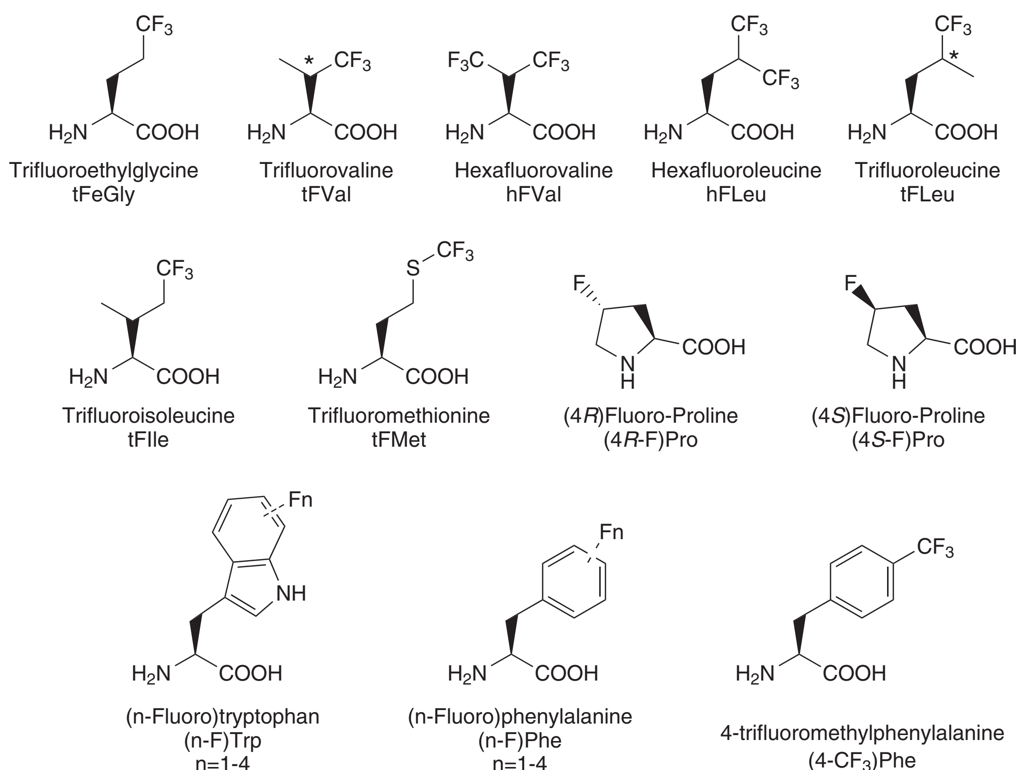

Figure 2. Fluorinated analogs of amino acids that have been incorporated into proteins and peptides. Abbreviations correspond to those used in this review.

proline fulfill this requisite. Various fluorinated analogs of these amino acids, varying the number and position of the fluorine atoms, were incorporated into peptides and proteins (Fig. 2). Some of these are commercially available, while others can be synthesized in a relatively straightforward manner, as reported elsewhere [17, 18].

The incorporation of fluorinated amino acids into proteins was at first a big challenge. The earliest successful reports were accomplished in vivo by employing strains of bacteria that were auxotrophic for the parental amino acid that is intended to be replaced by its fluorinated analog. Under the stress of this metabolic requirement to survive, and in presence of the fluorinated analog, some bacterial strains could incorporate it into their proteins to a certain extent [19, 20]. This method, which is usually called *selective pressure incorporation* (SPI), has been successfully employed to incorporate trifluoromethionine, 4-fluoroproline, trifluoroleucine (tFLeu), and different regioisomers of fluorophenylalanine (FPhe) into proteins expressed mostly in *Escherichia coli*, even though another host microorganisms such as *Saccharomyces cerevisiae* or *Pichia pastoris* have been used [21].

The main disadvantage of this methodology is that fluorination is never 100% effective because of the presence of the natural amino acid derived from cellular metabolism and the requirement for the fluorinated analog to be recognized by the aminoacyl-tRNA synthetases (AARS). These AARS have certain substrate tolerances and some analogs for their natural substrates can be activated and charged, although to a much lesser extent [22, 23]. For this reason, the intracellular concentration of the fluorinated analog must be much higher than that of the natural counterpart, which can be highly detrimental for the host cell growth [24, 25].

This also implies that there is no control over which amino acid positions will be substituted and supposedly all intrinsic occurring amino acid locations will be replaced. In practice this does not happen though due to the high toxicity of the fluorinated analog, which prevents its presence at an early stage of the cell culturing process and accordingly, bacterial strains must be first proliferated with the full suite of natural amino acids. When an acceptable cell density is reached (normally close to the stationary phase), the cells are collected and transferred to new media where the natural amino acid has been completely depleted and replaced by the fluorinated analog (Fig. 3). Due to the remaining intracellular concentration of the parental amino acid, substitution efficiencies of 70–90% are typically found.

Alternatively, fluorinated amino acids can be incorporated in vitro by automated peptide synthesis (APS) [26, 27]. This approach makes use of a solid support and appropriately protected derivates of amino acids in order to synthesize a polypeptide chain by successive cycles of activation and deprotection, chemically mediated by suitable activators and deprotecting agents. Despite the fact that precise control over polypeptide sequence and substitution sites can be achieved, this technique is unfortunately limited to polypeptide chains up to approximately 50 amino acids and results too expensive for generating a relatively large amount of biocatalyst.

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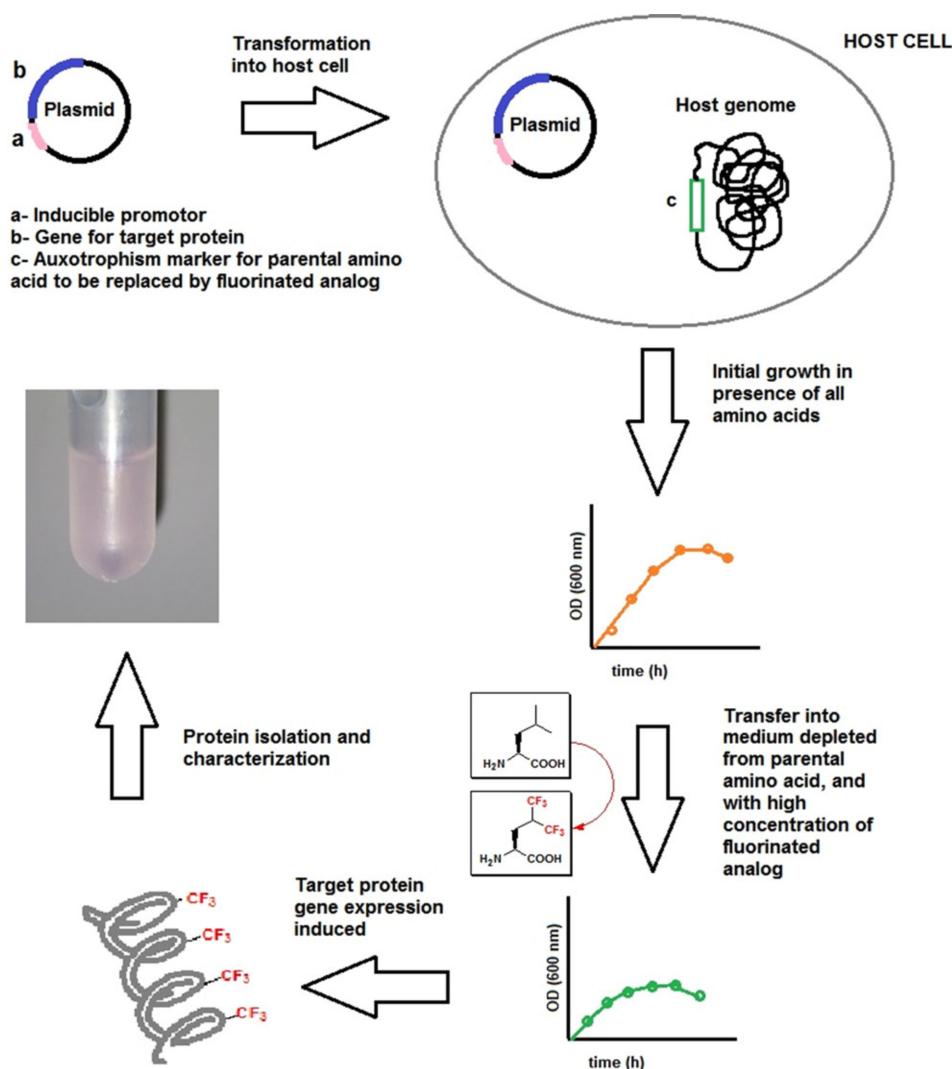

Figure 3. Scheme for the selective pressure incorporation (SPI) method. Strong host auxotrophism and control of fermentation conditions are crucial for effective fluorinated amino acid incorporation.

4 Effect of fluorination on enzymatic stability and reactivity

The first reports of the incorporation and effect on enzymatic activity of fluorinated amino acids into proteins in vivo were made in the late 1950s, employing some bacterial strains that were able to grow in presence of *p*-FPhe and incorporate this amino acid into some of their proteins [20–28]. Boyer was even able to feed rabbits with this fluorinated analog that was then incorporated into proteins of muscle, blood, and liver [29]. Aldolase with a replacement of 25% and glyceraldehyde 3-phosphate dehydrogenase with a replacement of approximately 16% of their Phe residues were crystallized and assayed enzymatic activities were similar to the corresponding wild-type enzymes. No difference in stability was found after heat denaturation of glyceraldehyde 3-phosphate dehydrogenase, as one of the first proofs that protein fluorination can preserve enzymatic activity.

The amino acid tFLeu was also successfully incorporated into *E. coli* proteins by means of the SPI method by Rennert and Anker in 1963 [19]. They initially cultivated the corresponding auxotrophic bacteria in presence of both leucine and the toxic tFLeu. When growing only in presence of the fluorinated amino acid, all leucine residues in proteins were replaced by tFLeu. No abnormal enzymatic activity was detected, but a slower cell growth was observed.

After these first results, several amino acids have been incorporated in vivo with dissimilar efficiencies and other aims than enzymatic stabilization for biocatalysis [10–12, 30]. It was not before the late 1990s that the fluorous effect was envisioned and subsequently explored [7].

Since then, fluorinated variants of several proteins with different sizes, varying α-helix/β-sheet contents and overall folds were obtained and characterized in order to determinate to what extent this possibility could be regarded as a plausible enzymatic stabilization methodology [9–12]. Annexin V, Barstar, Green Fluorescent Protein, and Mini-IGFBP-5 are some of the early enzymes used as models [31]. These studies clearly showed that in some cases extensive fluorination results in an increased

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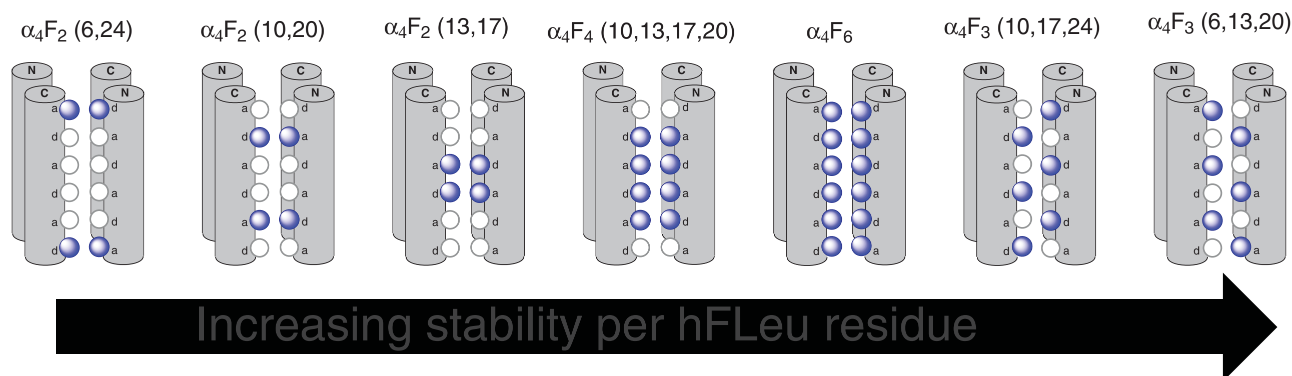

Figure 4. Thermodynamic stability of a series of α_4 proteins with different Leu (white) and hFLeu (blue) motifs, as determinated by GuHCl-induced unfolding. Adapted with permission from [38] with permission. Copyright 2009 American Chemical Society.

stability with retention of biological activity while in other cases a destabilization or misfolding was observed, with subsequent loss of enzymatic activity. It was also shown that incorporation efficiency of fluorinated derivatives was dependent on the fluorine content in the amino acid analog. The higher the content of fluorine, the lower the substitution level in the target protein was accomplished. It is clearly obvious that the bulkiness of the fluorine-containing residues plays an important role during the accommodation of amino acid residues in the folded state and therefore, reactivity [32–34].

This observation inspired many groups to try to comprehend the packing effects caused by fluorination, and to this purpose, peptides and small proteins synthesized by APS are particularly enlightening, since they permit access to homogenous samples with complete control of fluorination sites and amino acid sequence.

The main structural models used to study the packing effects following fluorination were 4-helix bundles. This 3-D arrangement is commonly present in proteins and it has been the topic of extensive de novo design efforts [35, 36]. It has been demonstrated that the hydrophobic core of a 4-helix bundle can be built from contact interactions between hydrophobic residues at the "a" and "d" positions of a helical heptad $(abcdefg)_n$ repeat. Substitution of this hydrophobic core by fluorocarbon interactions should be, in principle, conservative of the 3-D structure.

One of the first reports of protein stabilization through fluorination made on parallel coiled-coil protein domain synthesized by APS was simultaneously conducted by the groups of Kumar and coworkers [8–37]. They used a de novo designed coiled-coil domain from a GCN4-bZip eukaryotic transcriptional activator, a small protein capable of targeting DNA binding, as a simple model to study fluorous effect. After substitution of all leucine and valine residues by tFLeu and trifluorovaline, respectively, an increase in the melting point from 47°C to 61°C was observed. Similarly, substitution of solely leucine residues by tFLeu resulted in a value of 62°C and an increase of approximately 1 kcal/mol for the free energy of unfolding, as suggested by denaturation experiments. Remarkably, the fluorinated GCN4-bZip could retain its capability to bind target DNA as another early indication that protein fluorination is conservative of biological activity.

Other important studies on a de novo designed antiparallel 4-α-helix bundle protein were conducted by Marsh and coworkers [38,39]. The protein model, namely α_4H, contains Leu at the three a and three d positions of the heptad repeat. The authors studied the physicochemical properties of a series of fluorinated analogs for these tetramers by progressively increasing the number of incorporated hFLeu at various positions within the core. All the fluorinated analogs, namely α_4F_n, retained the structure of the native α_4H. They observed that the stability of the α_4F_n proteins increased with the number of hFLeu residues. For example, the protein α_4F_6, in which all the Leu residues are replaced by hFLeu, was 14.8 kcal/mol more stable than α_4H. Interestingly, they observed that the increased stability depends not only on the number of hFLeu, but also on the position and pattern of the substitutions (Fig. 4). The most stabilizing arrangement seems to be an alternating pattern in which hFLeu is incorporated at a positions and Leu at the d positions, or vice versa.

The results obtained with these model peptides clearly demonstrated the usefulness of the fluorous effect in protein stabilization and encouraged several research groups to achieve fluorinated amino acid incorporation into highly relevant enzymes for biocatalysis.

5 Evolved methodologies for generating fluorinated proteins

Due to the limitations of APS to generate highly pure proteins in a cost-effective manner, SPI is still the most promising methodology for manufacturing scalable quantities of fluorinated enzymes or proteins for industrial applications. Different efforts have been made in order to "evolve" in vivo incorporation of fluorinated amino acids trying to overcome the disadvantages exhibited by SPI method, such as low protein yields, heterogeneous samples, and noncontrol of substitution sites [40].

One of the methodologies to improve the substitution of a natural amino acid by a noncanonical surrogate consists of engineering the translational machinery of the host cell. This approach is normally called *residue-specific evolution* since it results in the global substitution of all natural amino acid occurring sites by its analog [41].

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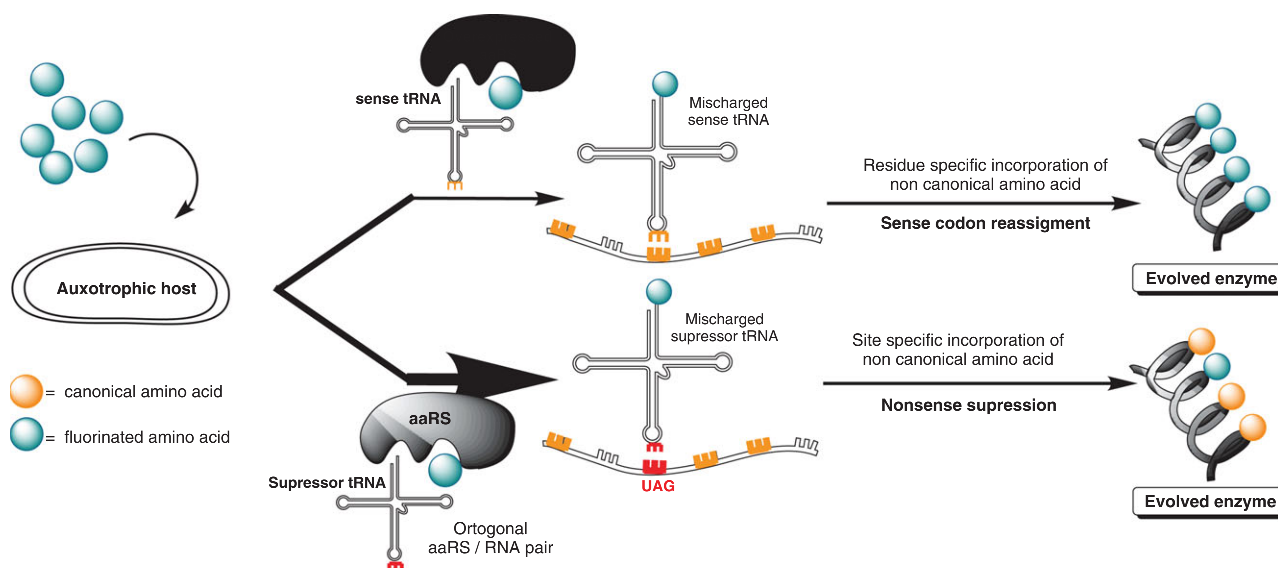

Figure 5. Scheme for the sense codon reassignment and *nonsense* suppression strategies for evolution of fluorinated proteins. It should be noted that orthogonal pair does not necessary need auxotrophy of the host cell.

The first results in this direction came from the laboratory of Tirrell and coworkers [42]. Based on the previous observation that tFLeu could be incorporated in vivo by *E. coli* without any requisite to evolve the host cell but in the case of the highly fluorinated hFLeu, normal activation levels by wild-type leucyl-tRNA synthetase were not high enough to achieve protein synthesis [32], they engineered extra copies of the *E. coli leuS* endogenous gene under a constitutive promoter to overexpress the tRNA synthetase and achieve effective incorporation. By this mechanism, enzymatic activity of the corresponding Leucyl-tRNA synthetase was increased by eight-fold compared to the wild-type strain. This evolved *E. coli* mutant was able to incorporate in vivo hFLeu and the incorporation efficiency was tested on a coil–coil dimer, reaching a 74% replacement of all Leu residues. This substitution induced protein structure stabilization, and the increment on the melting temperature was even higher than that of the analog that incorporates tFLeu (76°C and 67°C, respectively). Both were more stable than the wild-type protein (54°C melting point). The same stability results were obtained during denaturation studies with urea. Incorporation of 5,5,5-trifluoroisoleucine and trifluorovaline into a DNA-binding bzip model protein and murine dihydrofolate reductase was also achieved in evolved bacterial hosts with high incorporation efficiency (85–92%) by overexpressing an amino acyl tRNA synthetase. In both cases, fluorine incorporation resulted in good protein yields, increased stability against temperature denaturation and unchanged biological activity [43–45].

Although this methodology allows a higher level of incorporation of the fluorinated amino acid into the tRNA and subsequently improved yields for the fluorous protein, all existing codons for the parental amino acid could be potentially recognized by this mischarged tRNA, resulting in a general fluorination of the cellular proteome. This is mainly avoided by overexpressing the gene for the target protein whose transduction then makes use of nearly all fluorinated analog, preserving cellular viability. Still no control on substation sites can be accomplished following this methodology.

As discussed above, studies with peptide models obtained by APS led to the conclusion that a compromise should be achieved linking fluorine content and enzyme stability and activity, in view of the fact that for many folding motifs excessive fluorination results in a destabilization of the tertiary structure. For this reason, a desirable goal would be to achieve site-substitution by in vivo procedures in order to be able to biosynthesize larger amounts of fluorinated enzymes for biocatalysis with control on substitution sites and fluorine content.

This possibility has been developed by recent advances created by synthetic biologists to reprogram the genetic code in order to expand the number of amino acids that can be genetically encoded beyond those 21 considered canonical or standard amino acids. This approach, known as *nonsense suppression* (Fig. 5), consists in inserting a noncanonical amino acid during protein synthesis in response to one of the stop codons by chemically charging the corresponding suppressor tRNA [46].

For example, *E. coli* amber stop codon (UAG) can be introduced at a desired location on a gene that codifies for a protein of interest. If we expressed this gene into a cell-free expression system in presence of the corresponding amber suppressor tRNA charged with the desired noncanonical amino acid, the resulting protein will incorporate this amino acid at the specific sites of the polypeptide chains where the amber codon have been introduced [47]. Generally, the expression of the new protein could also be accomplished in vivo without dramatically affecting the expression of other proteins, due to the scarcity of the amber codon in the *E. coli* genome.

Nonsense suppression evolution effectively expanded the flexibility for incorporation of fluorinated amino acids, but the method has restrictions for big scale applications. Suppressor aminoacyl-tRNA is a stoichiometrically consumed reagent and its synthesis is troublesome and expensive.

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Further research to overcome this limitation was conducted; efforts were focused on engineering an AARS/suppressor tRNA pair by directed mutagenesis in order to make it capable of charging the suppressor tRNA with the noncanonical amino acid in vivo, eliminating the need to synthesize this component. At the same time, this engineered pair must be completely noncross reactive and orthogonal to the endogenous AARS/tRNA pairs within the host cell. Furter et al. used an engineered tRNA/AARS pair, originally responsible for charging phenylalanine in *S. cerevisiae,* for site-specific incorporation of *p*-fluorophenylalanine in *E. coli* [48]. Similarly, a tRNA/tyrosyl-tRNA synthetase pair from *Methanococcus jannaschii*, established through several rounds of negative and positive screening from a mutant library by the group of Schultz, was successfully employed for the incorporation of *p*-trifluoromethylphenylalanine in *E. coli* [49, 50].

So far this technique is limited to one of the *nonsense* codons, though efforts are under way to use sense codons ("codon reassignment"), and five base codon–anticodon pairs in order to synthesize proteins incorporating multiple noncanonical amino acids in vivo in one single experiment [51, 52].

Other components of the protein synthesis machinery could be also evolved to achieve efficient enzymatic fluorination. Mutation on the ribosome is an alternative currently under early investigation for improving noncanonical amino acid incorporation [53].

It is also important to mention that even though fluorination without rational design can result in a partial loss of biological activity, the artificial fluorinated enzymes can still be submitted to directed evolution in order to recover full activity or even enhance it [54, 55]. This approach was used by Tirrell to recover biological activity of chloramphenicol acyltransferase (CAT) after incorporation of tFLeu, which resulted in enhanced thermal stability but partial activity loss. After screening a library of error-prone PCR *E. coli* mutants auxotrophic for Leu, a fully active fluorinated chloramphenicol acyltransferase with enhanced thermal stability was identified [56].

6 Fluorination effect in stability and activity of proteins and peptides with relevance to biocatalysis

The industrial application of biotechnological routes for the generation of highly valuable chemicals has spread worldwide and the discovery of novel biosystems capable of innovative chemical transformation is a long-established research field under permanent expansion. At the same time, finding new methodologies for stabilizing these biocatalysts in order to permit their application under nonnative reaction conditions represents a scientific area with equivalent growth.

As discussed previously, introduction of fluorinated hydrophobic amino acids together with rational design constitutes a new promising alternative for enzymatic stabilization against the effect of organic solvents and high temperature, but this possibility still needs to be fully explored and developed.

Despite our limited knowledge of how to achieve effective stability by fluorination with minimum compromise on biological activity, some successful examples have been reported of stabilization of enzymes with strong relevance for industrial biocatalysis.

One of the first studies involving high-value biocatalysts was conducted by Wiltschi on the residue specific fluorination of aromatic residues for lipase B from *Candida Antarctica* (CalB) [57] This highly relevant industrial lipase has multiple applications, including polymerizations, ring opening reactions of β-lactams, resolutions of chiral alcohols and amines, modifications of sugars and related compounds, etc. [58, 59]. Wild-type CalB contains 319 amino acids, from which five are Trp residues, nine are Tyr, and 11 correspond to Phe. A CalB expression vector inducible by methanol and transformed into an auxotrophic *P. pastoris* yeast as host cell was used for large-scale protein expression. The introduction in parallel of the fluorinated amino acids 5-fluorotryptophan, 3-fluorotyrosine, and 4-FPhe by SPI method generated the corresponding fluorinated proteins with yields between 32 and 64% of the parental nonfluorinated lipase.

The MS analysis of the cell lysate after protein expression in presence of the fluorinated amino acid shows the successful incorporation of all three analogs at multiple sites (Fig. 6A). The incorporation was stochastic: a mixture of differentially fluorinated proteins was obtained and, even though complete substitution was observed in all three cases, the dominant species in the mass spectra corresponded to those carrying three to four replacements (Fig. 6B). Similar results were reported for the incorporation of trifluoromethionine in *P. pastoris* and *S. cerevisae.* As previously described, this seems to be a consequence of the intracellular availability of the canonical amino acid during fluorination in response to metabolic turnover.

Physicochemical characterization of the fluorinated proteins by circular dichroism, far UV light absorption, and fluorescence showed some alterations of the secondary structure for the fluorinated variants (Fig. 6C), but their behavior against proteolysis by protein quinase K remained intact. Lipase activity assays showed retention of the enzymatic properties with a similar profile of activity versus temperature but with a lower efficiency (Fig. 6D). Interestingly, the fluorinated variants retained their lipase activity even after several months under storage at 4°C, against a substantial detriment observed for the wild-type protein.

Further studies were conducted by Merkel et al. on another highly relevant industrial lipase from *Thermoanaerobacter thermohydrosulfuricus* [60]. This enzyme consists of 267 amino acids, including six proline, 16 phenylalanine, and two tryptophan residues. In an attempt to study how global fluorination affects structural integrity and enzymatic activity, simultaneous substitution of all mentioned residues by their fluorinated analogs 4-fluoroproline, 4-FPhe, and 6-fluorotryptophan was attempted in a single experiment. This results in 10% fluorination of the primary sequence. After expression protein in an *E. coli* strain and SPI incorporation of the fluorinated analogs, the fully fluorinated variant was obtained as the main species on the MS profile, but protein production was achieved with a low yield (11.5 mg/L culture compared to 47 mg/L for the nonfluorinated protein). This lower production yield could be attributed to the formation of inclusion bodies that lowers expression levels for the fluorinated soluble variant.

CD spectroscopy showed no significant alteration of the secondary structure as well as biological studies, which

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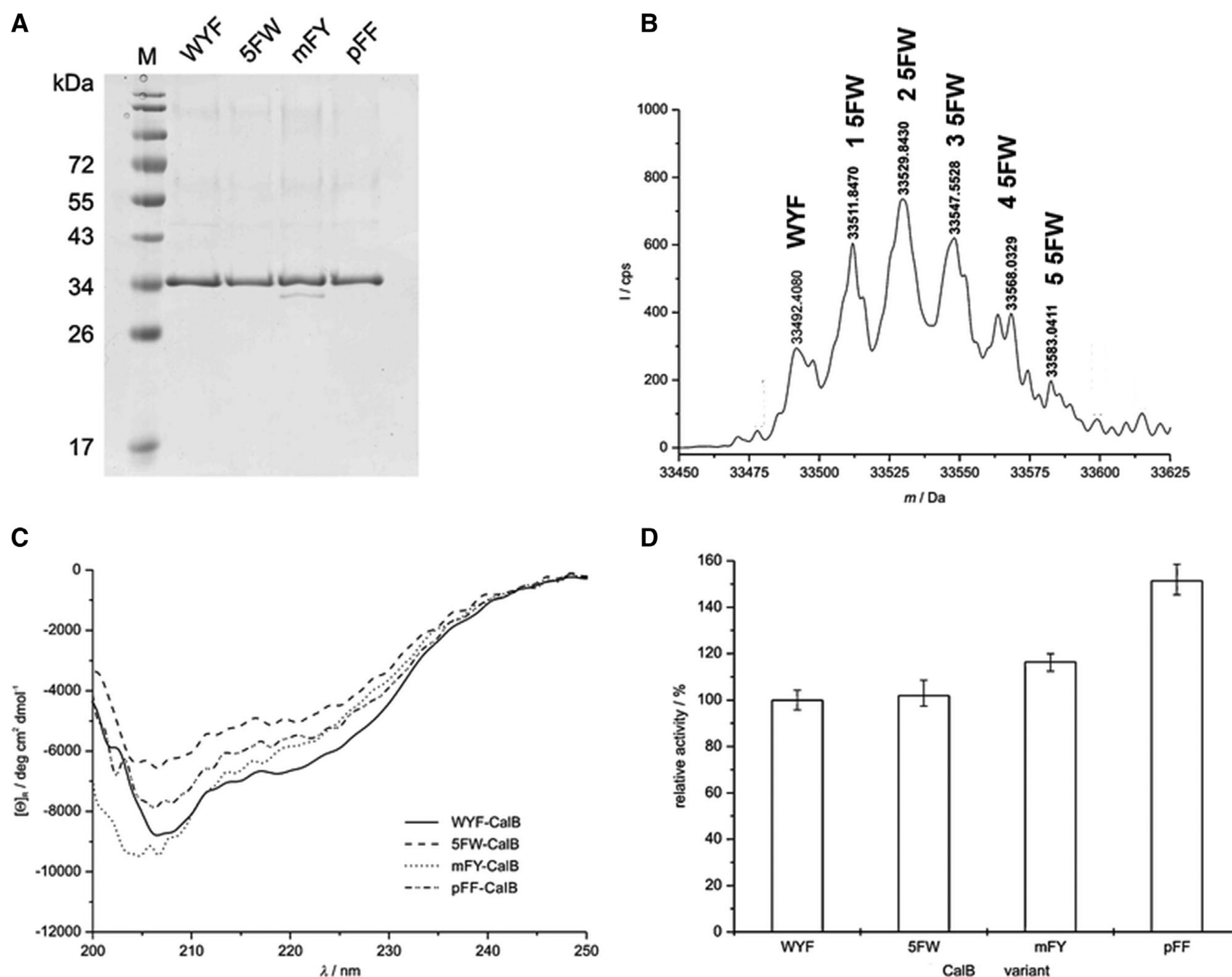

Figure 6. (A) Protein preparations of CalB expressed in the presence of the amino acids 5-fluorotryptophan (5FW), 3-fluorotyrosine (mFY), and 4-FPhe (pFF). Wild-type CalB (MW = 33 kDa) is indicated as WFY. Equal amounts of protein were loaded onto each lane. (B) ESI-MS spectrum for the variant containing 5-fluorotryptophan (5FW). The peaks correspond to the congeners containing 1–5 5FW residues. (C) Far-UV CD spectra for the fluorinated CalB variants. (C) Lipase activity of the variants (determinated by monitoring *p*-nitrophenylpalmitate hydrolysis at 410 nm) after several months storage at 4°C compared to the wild type (WYF, set as 100%). Adapted from [57] with permission.

demonstrated the presence of lipase activity, but with a maximum that was only about 60% of the parental lipase. Interestingly, the fluorinated variant showed an optimal temperature value of approximately 60°C, 10°C lower than the wild-type protein. This constitutes a very desirable feature for the industrial design of so-called "cold-wash" lipases.

A more systematic study on the same lipase was presented by Hoesl et al. [61]. They incorporated fluorinated derivatives of Phe, Pro, and Tyr at multiple positions and characterized their structure, stability, and activity. The results indicated that for the fluorinated Pro and Phe analogs, the protein yield was higher than for the corresponding wild-type protein. However, none of the congeners showed higher temperature resistance compared to the parent lipase, even though the variant containing 3-FPhe displayed a broader substrate tolerance than the wild type.

Other relevant enzymes for industrial biocatalysis are DNA polymerases. These enzymes catalyze the synthesis of DNA copies from a DNA template. Thermostable DNA polymerases have been applied for the detection and amplification of DNA sequences of interest with the aim of identifying microorganism contamination in food or pharmaceuticals, virus detection, medical diagnosis, gene synthesis for molecular biology applications, DNA sequencing, etc. [62–65]. All these applications rely on the fidelity and stability of DNA polymerases.

The group of Marx and coworker globally replaced 32 proline residues on a 540 amino acid residue KlenTaq DNA polymerase by (4*R*)-fluoroproline ((4*R*)-FPro) following the SPI method on a *E. coli* auxotrophic for Proline [66]. According to the previous example, in this case the yield of fluorinated protein was lower compared to the wild type (0.2–0.5 vs. 8 mg/L). After MS-ESI analysis, they found that about 92% of all Pro residues were replaced by (4*R*)-FPro. Interestingly, all the efforts for incorporating (4*S*)-fluoroproline failed. It has been shown previously that

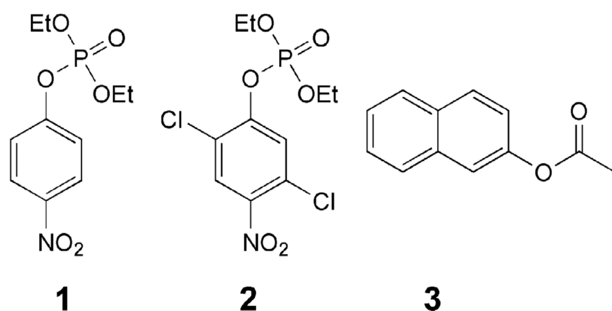

Figure 7. Chemical structures for assayed substrates for fluorinated S5 phosphotriesterase: paraoxon (1), chlorpyrifos (2), and 2-naphthylacetate (3). Adapted with permission from [74]. Copyright 2011 Wiley-VCH.

introduction of a fluorine atom in position 4 of Pro can strongly affect the *endo*/*exo* conformation equilibrium of the proline ring and this explains that usually only one of the stereoisomers of 4FPro can be tolerated as substitute for proline in certain protein structures [67,68]. As a consequence of fluorination, a small decrease in thermal stability was detected after incubation for several periods at 95°C. More importantly, no loss in fidelity, activity, or sensitivity could be detected after PCR experiments to amplify DNA fragments, compared to the parental wild-type enzyme.

Organophosphate esters are highly toxic compounds, which became widely dispersed on the Earth's surface due to the indiscriminate misuse of agricultural insecticides and during development of chemical warfare agents. If ingested or adsorbed, they cause respiratory failures and even death to humans and other vertebrates [69]. Several approaches have been applied to decontaminate the soil, such as incineration, acid or base hydrolysis, chemical oxidation, etc., but most of these methodologies are expensive or environmentally hazardous [70,71]. Stable phosphoesterases constitute a promising biocatalytic alternative, since they catalyze the hydrolysis of organophosphates esters into harmless products. These enzymes can also find application in the production of biofuels and the hydrolysis of other interesting organic esters [72,73].

Baker et al. reported the improved refoldability of a phosphotriesterase after residue-specific substitution of Phe by *p*-FPhe [74]. This is a globular dimeric protein where dimerization is controlled by particular contact interactions between seven Phe residues. Introduction of fluorinated Phe should enhance the interaction force dimer formation (due to the increased apolar surface area) and hence, stability. After 4FPhe supplementation to an auxotrophic *E. coli* strain and protein isolation and purification, 89.4% incorporation efficiency into the phosphotriesterase was achieved, as determined by MALDI-TOF MS. Circular dichroism studies revealed that introduction of 4FPhe improved refoldability after heating at 70°C. While the wild-type phosphotriesterase exhibited a complete loss in structure, the fluorinated variant still retained around 30% of the native state. Determination of melting temperature for both proteins showed a slightly improved stability, with melting temperature values about 1.3–2.5°C higher in favor of the fluorinated variant.

The activity of the phosphotriesterases was tested against two organophosphates and one ester as model substrates (Fig. 7). Outstandingly, fluorination led to 5.8-fold higher turnover for two of these substrates and a residual 40% activity after heating at 55°C was detected for the fluorinated phosphotriesterase, while the wild-type variant retained only 13% of the activity after analogous treatment (Table 1).

These selected examples clearly demonstrate that introduction of fluorinated amino acids constitute an alternative approach to traditional protein engineering for achieving stable biocatalysts for industrial applications. Despite these being preliminary results and none of these fluorinated biocatalysts have been applied so far beyond laboratory experiments, we trust that further investigations will indubitably lead to future technological applications, at least in some cases.

7 Concluding remarks

The impact of biocatalysis has been remarkably amplified during the last few decades, driven by a global necessity for transforming our current production systems into more sustainable methodologies, but also as a result of the attractive advantages that biosystems have to offer to the manufacture of fine chemicals. The considerable advances made in this field by molecular biologist have remained so far limited to the possibilities offered by the 20 natural amino acids.

Nowadays, the expansion of the pool of amino acids for ribosomally mediated protein synthesis, in combination with the latest genomic and proteomic methods, has made it possible to incorporate new properties into proteins and biomaterials, which will radically transform our traditional view of biocatalysis.

Table 1. Kinetic parameters for wild-type S5 phosphotriesterase (PTE) and its fluorinated variant (pFF-PTE).

		Substrate 1	Substrate 2	Substrate 3
PTE	K_M [$\times 10^{-4}$M]	0.0031 ± 0.0007	2.6 ± 0.6	1.5 ± 0.4
	k_{cat} [s^{-1}]	4.3 ± 0.1	8.3 ± 0.6	3.3 ± 0.8
	k_{cat}/K_M [$\times 10^4$ $M^{-1}s^{-1}$]	1400 ± 300	3.3 ± 0.8	2.3 ± 0.2
pFF-PTE	K_M [$\times 10^{-4}$M]	0.0075 ± 0.0012	8.7 ± 2.6	1.9 ± 0.5
	k_{cat} [s^{-1}]	29.7 ± 0.8	7.8 ± 0.6	20.2 ± 0.4
	k_{cat}/K_M [$\times 10^4$ $M^{-1}s^{-1}$]	400 ± 800	0.9 ± 0.2	10.8 ± 0.7

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In this sense, the discovery of the fluorous effect has been an inspiration for uninterrupted efforts trying to design efficient incorporation methodologies for fluorinated amino acids and to understand how fluorination affects structure and reactivity.

Encouraged by our partial success hitherto, the initial results in the stabilization of highly relevant biocatalysts for industrial applications stimulate even more the interest of the scientific community to improve these outcomes and it is not inconceivable to anticipate that we will be eventually able to generate complete functional highly stable enzymes and even cells with a high fluorine content in their proteomes.

But, as we approach this point, what will be the next direction on our way to fluorinated biocatalysts? From our discussion here, it results obvious to envisage that the limiting steps for future industrial application of fluorinated enzymes will be the ability to produce fluorinated amino acids on a large-scale and at reasonable price. So far, all these fluorinated analogs have to be synthesized chemically and supplemented into the culture medium during cell host growth. Would it be possible to evolve the bacterial metabolism in order to biosynthesize the fluorinated amino acid in vivo?

The group of Schultz has been able to evolve an *E. coli* strain that is capable of biosynthesizing *p*-aminophenylalanine and incorporate this noncanonical amino acid into proteins in response to amber stop codons [75]. It is clear that metabolic engineering is the most advantageous approach to generate noncanonical amino acids of interest directly in the protein synthesizing cell.

However, it should be kept in mind that metabolic engineering of fluorinated amino acids is, by far, a much larger challenge due to the intrinsic toxicity of the fluorinated metabolites and the lack of a biocatalyst capable of fluorine activation. The recent isolation of a fluorination enzyme from the soil bacterium *Streptomyces cattleya* constitutes one exception [76,77]. The fluorinase catalyzes the conversion of (S)-adenosyl-L-methionine and inorganic fluoride ion to 5′-fluoro-5-deoxyadenosine, which is afterwards metabolically transformed into fluoroacetate and 4-fluorothreonine. This is an exceptional natural mechanism revealing the inimitable potential of enzymes as catalysts since chemical activation of fluoride anion is particularly difficult.

Some of these fluorinated metabolites have been subsequently biotransformed into other highly valuable pharmacological compounds, such as salinosporamide [78] and fluorinated polyketides, employing polyketide synthase and fluoroacetate [79]. Chemo-enzymatic methoxy to fluorine substitution has been accomplished as well [80].

Although there is still a long way to go until we could biosynthesize the desired fluorinated amino acid while simultaneously incorporating it into the target enzyme in vivo, this topic constitutes a very promising research field that may have a deep future impact on biotechnological processes and protein-based technologies.

We believe the evolution of fluorinated proteins could indeed transform our perception of industrial biocatalysis and several studies are currently conducted in our group in order to generate highly stable fluorinated variants of valuable industrial enzymes.

Practical application

Protein evolution in order to develop stable enzymes for industrial applications constitutes a field in continuous expansion. Previously, ribosome-mediated protein synthesis was restricted to a repertoire of 20 canonical amino acids. Recently, the emerging field of synthetic biology has the potential to enrich protein biosynthesis with synthetic amino acids that carry new functionalities not developed during the evolution of the living world.

Introduction of fluorinated amino acids into the hydrophobic core of enzymes imprints these biocatalysts with some outstanding properties, such as resistance to denaturation caused by organic solvents or high temperatures as a consequence of the so-called “fluorous effect.” These chemical properties represent very valuable features for industrial applications of enzymes.

In this review, we present some advances in our understanding of the fluorous effect and we discuss some literature examples showing promising practical relevance. We anticipate that further research on this topic will allow us to design a novel generation of uniquely stable biocatalysts for industrial and therapeutic use.

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