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Enhancing Protein Stability with Genetically Encoded Noncanonical Amino Acids

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Abstract

The ability to add noncanonical amino acids to the genetic code may allow one to evolve proteins with new or enhanced properties using a larger set of building blocks. To this end, we have been able to select mutant proteins with enhanced thermal properties from a library of *E. coli* homoserine *o*-succinyltransferase (*metA*) mutants containing randomly incorporated noncanonical amino acids. Here, we show that substitution of Phe 21 with *p*-benzoylphenylalanine (pBzF), increases the melting temperature of *E. coli metA* by 21°C. This dramatic increase in thermal stability, arising from a single mutation, likely results from a covalent adduct between Cys 90 and the keto group of pBzF that stabilizes the dimeric form of the enzyme. These experiments show that an expanded genetic code can provide unique solutions to the evolution of proteins with enhanced properties.

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All known organisms use the same twenty canonical amino acids with the only known exceptions being selenocysteine and pyrrolysine, which are found in a small number of proteins. This fact raises the question as to whether twenty is the optimal number, or whether additional amino acids might provide an evolutionary advantage to an organism. We and others have begun to explore this notion by randomly incorporating noncanonical amino acids (ncAAs) into proteins and carrying out screens or selections to identify proteins with

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ASSOCIATED CONTENT

Supporting Information

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Supplementary figures (S1-S6), gels and mass spectrometry (S7), strains and plasmids used (Table S1), experimental methods, supplementary references

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enhanced properties. To this end, cyclic peptides, antibodies, enzymes and phage have been identified in which a noncanonical amino acid provides improved affinity, catalytic activity, or fitness^{1–6}. We now ask whether ncAAs can enhance the physical properties of proteins – specifically, their thermal stability.

Protein stability generally depends on a large number of van der Waals, hydrogen bonding, and electrostatic interactions and a limited number of disulfide bonds. With the exception of the latter, these interactions are relatively weak in energy, but collectively can lead to a significant favorable free energy of folding. As a result, efforts to increase the thermal stability of proteins often require the additive effects of multiple mutations^{7–8}. Due to their covalent nature, engineered cysteine crosslinks have been extensively explored as a means of increasing thermal stability, although they are subject to stringent distance and conformational constraints which limit their application^{9–11}. With the introduction of additional functional groups in the side chains of ncAAs that can form new noncovalent or covalent interactions not accessible to the common 20 amino acids, we asked whether additional energetically stabilizing interactions can be realized^{12–15}. Here we show that introduction of a single benzophenone containing ncAA into the *Escherichia coli* (*E. coli*) protein homoserine o-succinyltransferase (*metA*) leads to a 21°C increase in thermal stability.

Previously, it has been shown that the growth of *E. coli* is greatly reduced at temperatures of 44°C and above in minimal medium, and that the addition of methionine to the media is able to partially rescue growth^{16–17}. This effect is largely attributed to the thermal instability of a single enzyme, homoserine o-succinyltransferase, an essential enzyme in methionine biosynthesis which begins to unfold and aggregate above 40°C¹⁸. Mutations that moderately increase *metA* stability and reduce aggregation have been found to confer enhanced growth rates at 44°C^{19–20}. This property of *metA* provides a rational basis for selection, as *metA* mutants containing a noncanonical amino acid which increases the enzyme's thermal stability should have a significant growth advantage.

To test this notion, a comprehensive amber nonsense TAG scanning library (containing 261 mutants out of 308 possible positions) was generated in which almost every position in *metA* not directly adjacent to the active site was mutated, as determined using a homology model of *E. coli metA* (generated based on the previously solved crystal structure of *metA* in *B. cereus*)²¹. The resulting library was then transformed into *E. coli* strain JW3973–1 (*metA*) along with a plasmid bearing a polyspecific amber nonsense suppressor tRNA/ aminoacyl-tRNA synthetase (aaRS) pair that is orthogonal to the endogenous *E. coli* tRNA/ aaRS pairs (i.e., does not cross react with host tRNA/aaRS pairs) and is able to selectively and efficiently incorporate a variety of ncAAs, including p-acetylphenylalanine, p-fluoroacetylphenylalanine, p-azidophenylalanine, o-methyltyrosine, p-iodophenylalanine, p-bromophenylalanine, o-t-butyl tyrosine, o-allyl tyrosine, p-biphenylalanine, and p-acrylamido-phenylalanine. Three additional tRNA/synthetase pairs that specifically incorporate p-vinylsulfonamido-phenylalanine, p-boronophenylalanine, and p-benzoylphenylalanine, were also co-transformed separately. Transformants were grown at 44°C in liquid minimal media containing each of the ncAAs for 20–40 hours. We expected that *E. coli* containing *metA* mutants which confer increased thermal stability to outgrow other bacteria. Cultures were

subsequently plated and colonies were picked and sequenced. Two consensus mutations, F21TAG and N86TAG, were observed when cells were grown in the presence of 1 mM p-benzoylphenylalanine (pBzF) and 1 mM O-t-butyl tyrosine (OtBuY), respectively. The F21TAG *metA* mutant showed markedly higher endpoint growth compared to WT in the presence of pBzF when retransformed into *metA* cells (Figure 1).

We next expressed and purified the N-terminal 6×His tagged WT, N86OtBuY, and F21pBzF variants of *metA*, referred to hereafter as WT *metA*, N86OtBuY *metA*, and F21pBzF *metA*. The yields of the WT protein were typically 8–9 mg/L and those of the mutants were 5–6 mg/L. Temperature dependent protein unfolding was then measured using circular dichroism (CD) spectroscopy. The F21pBzF mutant showed a remarkable 21°C degree increase in melting temperature (T_m) compared to the WT protein (53°C for WT vs 74°C for the F21pBzF mutant) (Figure 2), while N86OtBuY *metA* showed a modest 6°C increase (Figure S1). Given the substantially enhanced stability of F21pBzF *metA*, we focused on this mutant for further study. The WT *metA* and F21pBzF *metA* were then co-expressed bicistronically to generate a mixture of homo and heterodimers. CD melting curves for this mixture had an expected bimodality, with a lower T_m curve matching the WT protein at ~51°C and a higher T_m curve at ~65°C – likely corresponding to a WT/mutant heterodimer (and mutant homodimer in much lower abundance, consistent with the typically lower expression yields of proteins containing a ncAA) (Figure S2).

Based on our homology model, *metA* is predicted to be a homodimer which we verified by size exclusion elution chromatography of the purified recombinant protein (Figure S3) and dynamic light scattering. Phe 21 is predicted to lie in a highly flexible N-terminal domain region that interacts with the other monomer of the homodimer in a domain swapping mode (Figure 3). This observation suggests that pBzF 21 may stabilize the dimeric state of *metA*. Interestingly, many of the other colonies which grew at elevated temperatures but did not converge on a consensus sequence had mutations in the same N-terminal domain, indicating it may be a generally important area for *metA* stability. When *metA E. coli* was re-transformed with the F21TAG *metA* mutant and the cognate tRNA/aaRS pair (as pEVOL-pBzF) in media containing 1 mM pBzF, bacteria appeared to be both be able to grow faster than wildtype (WT) versions of *metA* and saturate at (>60%) higher OD in side by side cultures (Figure S4).

The benzophenone side chain of pBzF can form pi interactions, hydrogen bonds or covalent interactions with neighboring residues. The latter can be formed through electrophilic addition of nucleophilic amino acid side chains to the ketone. Given the large increase in T_m associated with this mutation, we explored that possibility that the aryl keto group is forming a covalent bond with a Lys, Cys or Ser residue. As denaturing SDS-PAGE gels of our protein and mass spectrometry consistently show only the monomeric mass (Figure S8), this bond is likely highly reversible. On the basis of the predicted location of Phe 21 in *E. coli metA*, a small proximity-based list of candidate residues was generated that could form a covalent adduct with the benzophenone side chain. Among these, mutagenesis of Cys 90 to Ser showed a complete reversion of the melting temperature from 74°C to 53°C (while the same mutation is actually slightly stabilizing in the case of the WT protein), consistent with the reduced nucleophilicity of serine compared to cysteine. Covalent modification of Cys 90

with iodoacetamide (IA) similarly reduced the T_m for F21pBzF *metA* to 53°C while not significantly affecting the T_m of the WT protein. These results suggest that the increased thermostability afforded by the benzophenone side chain is due to its interaction with Cys 90, likely in the form of a hemithioether (Figure 2).

Hemithioethers are usually intermediates in thioether formation and the formation of hemithioethers is readily reversible, with previous studies having shown that ketone to hemithioether equilibria can vary by 4 orders of magnitude or more, depending on conditions^{22–23}. Here, it is possible that a hemithioether is stabilized by the surrounding protein architecture. Mutation of Phe 21 to p-fluoroacetyl-phenylalanine or p-acetylphenylalanine afforded T_m s of 60°C and 53°C, respectively, which may reflect the reduced stability of the thiol adducts or additional stabilizing interactions with the additional aryl ring of pBzF (Figure S5). Because hemithioethers may be further reacted with an additional thiol to form a stable thioether, it may also be possible to trap the F21pBzF *metA* covalent dimer irreversibly by addition of β -mercaptoethanol (BME). Indeed, addition of BME trapped a fraction of the mutant in its homodimeric form as evidenced by SDS-PAGE, while Mass spectrometry of the trapped complex confirmed the covalent addition of a single BME (Figure S6).

The putative crosslink between pBzF 21 and Cys 90 was further confirmed using ^{13}C NMR, in which a ^{13}C labeled version of pBzF was synthesized and incorporated into *metA*. Proton-carbon heteronuclear multiple bond correlation (HMBC) NMR on this sample shows three distinct non-carbonyl aromatic crosspeaks that likely correspond to the hemithioether and hydrated forms of pBzF (Figure 3, S7) in the region of 120–135 ppm on the ^{13}C axis while the 195 ppm signal due to the sp^2 carbonyl group was not observed²⁴. We further attempted to obtain a crystal structure of *metA*, but thus far have been unsuccessful in obtaining crystals for the thermostable form of this protein.

While there have been extensive protein engineering efforts to increase melting temperatures, most result in small improvements on the order of 5–10°C^{25–28}. Two notable previous examples include a 25°C melting temperature increase in GH11 xylanase – which involved seven mutations that resulted in subtle perturbations to the protein's overall structure, and a 26°C melting temperature increase in Leishmania triosephosphate isomerase, in which a single glutamate was mutated to glutamine which restored a hydrogen bonding network within the protein that had been naturally lost^{29–30}. In our case, a single novel point mutation was selected that was able to increase the melting temperature of *metA* by over 20°C by stabilizing the protein's native dimer configuration. In doing so, we underscore how an expanded chemical space gives natural systems unique avenues for evolving enhanced properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Screening for high thermal stability mutants of *metA*.

(a) An amber stop codon scanning library of *metA* was co-transformed with an orthogonal amber suppressor tRNA/aaRS pair into JW3973-1 *E. coli*. Transformants were incubated in minimal media liquid culture supplemented with 1 mM of the cognate ncAA at 44°C for 20–40 hours. Cells were then plated and colonies were sequenced. The consensus clone F21pBzF *metA* was retransformed into JW3973-1 and shows an increased growth rate compared to JW3973-1 transformed with WT *metA*. (b) ncAAs used in this screen.

Figure 2. Thermal denaturation of *metA*.

(a) Purified recombinant WT *metA* (green) and F21pBzF *metA* (blue) were analyzed by CD spectrometry at 223 nm in 200 mM sodium phosphate, pH 7.4. Fraction unfolded is represented as normalized ellipticity. F21pBzF *metA* shows a 21°C increase in melting temperature compared to its WT counterpart. (b,c) Mutation of Cys 90 to Ser (b) or Cys 90 alkylation by IA (c) completely reverts the T_m increase of the F21pBzF mutant to WT *metA* or near WT *metA* temperatures.

Figure 3. Expected UAA interaction.

(a) A homology model of *E. coli metA* was generated based on *metA* from *B. cereus* (pdb 2VDJ). The protein is predicted to form a homodimer. The mutant residue, Phe 21 (red), is expected to crosslink with Cys 90 (magenta) of the other monomer. (b) HMBC spectra of ^{13}C labeled F21pBzF *metA* shows non-carbonyl crosspeaks consistent with hemithioacetal formation. The 195 ppm carbonyl signal of pBzF was not observed.