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Cell-free unnatural amino acid incorporation with alternative energy systems and linear expression templates

Research Paper

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Site-specific incorporation of unnatural amino acids (uAAs) during protein synthesis expands the proteomic code through the addition of unique residue chemistry. This field provides a unique tool to improve pharmacokinetics, cancer treatments, vaccine development, proteomics and protein engineering. The limited ability to predict the characteristics of proteins with uAA-incorporation creates a need for a low cost system with the potential for rapid screening. *Escherichia coli*-based cell-free protein synthesis is a compelling platform for uAA incorporation due to the open and accessible nature of the reaction environment. However, typical cell-free systems can be expensive due to the high cost of energizing reagents. By employing alternative energy sources, we reduce the cost of uAA-incorporation in CFPS by 55%. While alternative energy systems reduce cost, the time investment to develop gene libraries can remain cumbersome. Cell-free systems allow the direct use of PCR products known as linear expression templates, thus alleviating tedious plasmid library preparations steps. We report the specific costs of CFPS with uAA incorporation, demonstrate that LETs are suitable expression templates with uAA-incorporation, and consider the substantial reduction in labor intensity using LET-based expression for CFPS uAA incorporation.

Introduction

The 23 proteomic amino acids have provided for rich biological diversity on earth [1]. Yet the narrow range of chemistries provided by these residues can frequently pose a challenge to the biochemist's quest for site-specific modifications to protein. The site-specific incorporation of unnatural amino acids (uAAs) in proteins unlocks the potential for unique residues. This rapidly growing field provides a unique tool that has already been applied toward improving pharmacokinetics, cancer treatments, vaccine development, proteomics and protein engineering [2–11]. In short, the ability to site-specifically incorporate uAAs is a strong platform to expand the chemistry of life [8,12–14]. Although decades of work have been devoted to this area of research, major strides have recently been made toward simple, productive, and readily transferable methods of site-specific uAA-incorporation [15–19]. Most notably, Shultz and coworkers have developed several evolved aminoacyl tRNA-synthetase/tRNA pairs that act orthogonally to native synthetase/tRNA pairs, allowing for high fidelity protein synthesis without interactions between native and evolved synthetase/tRNA pairs. These evolved synthetases incorporate the uAAs site-specifically at Amber codons [8,14,20]. Over 70 uAAs have been incorporated with high specificity using this system [8]. This study employs an evolved synthetase/tRNA pair from *Methanocaldococcus jannaschii* that incorporates the uAA p-propargyloxyphenylalanine [21].

A primary challenge with any uAA-incorporation system is accurately predicting the changes in protein behavior due to the novel residue chemistry provided by an uAA. *In silico* predictions methods are limited and many of the most accurate protein folding predictions algorithms are based heavily on homology, making them less useful when considering novel residue characteristics [22]. Thus the physical screening of many sites may be necessary to find stable, efficacious sites for uAA incorporation and high-throughput screening options would be desirable.

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While *in vivo* screening for effective uAA incorporation sites can be successfully employed for high margin applications such as pharmaceuticals, a less expensive screen is desirable for wide spread use of the technology in lower margin applications, such as industrial biocatalysis. In addition, rapid screening would be propitious for applications screening many sites. To address this need, we propose a cell-free approach to site-specific incorporation of uAAs. Cell-free protein synthesis (CFPS) efficiently harnesses the innate ability of the cellular machinery to transcribe and translate while simultaneously allowing superior control over the synthesis environment compared to *in vivo* techniques [23–25]. A cell-free environment provides pronounced advantages for general protein production and uAA-incorporation, straightforward isotope or other labeling, simplified purification, direct manipulation of reactant concentrations, and high yielding production of toxic proteins or using toxic reactants [26–30].

The attributes of CFPS lend this system to uAA-incorporation screening applications. However, the cost of energizing traditional cell-free systems can be inhibitory for price-sensitive applications such as bulk biocatalyst production, as the energizing reagents often constitute the bulk of the system expense [31]. Traditionally, compounds with high energy phosphate bonds such as phosphoenolpyruvate [32] or creatine phosphate [33] have been used to energize CFPS resulting in a high energy cost per mg protein produced. Alternative energy sources such as glycolytic intermediates have reduced the cost of energizing CFPS and paved a way to make cell-free systems more commercially viable [17,34]. However, these cost-reduction methods have not yet been reported in connection with uAA-incorporation. Here, we report the impact these energy systems have on uAA-incorporation in CFPS.

While alternative energy systems can reduce the overall cost of CFPS for uAA-incorporation, there remains the time-intensive aspect of producing mutant libraries of plasmid DNA. The use of PCR-synthesized linear DNA is an attractive alternative to using plasmid DNA *in vitro* due to its short preparation time, ease of production, and high-throughput synthesis capacity [35–40]. Using LETs with CFPS eliminates the need for DNA cloning [35], enables rapid high-throughput screening [37–39] and facilitates high-throughput genomic analysis [36,40]. We report for the first time the use of LETs in connection with batch CFPS for uAA-incorporation and in combination with an alternative energy system. The potential cost- and labor-reductions of uAA-incorporation with CFPS using LETs prepares the way for large scale cost-effective protein screening, conceivably opening the doors to many promising technologies that might be otherwise inhibited by cost.

Materials and methods

Preparation of cell extract and E. coli*-orthogonal tRNA synthetase*

Cell extract for this work was prepared using *Escherichia coli* strain BL21 Star™ (DE3) cells (Invitrogen, Carlsbad, CA) harboring the pEVOL-*pPrF* plasmid [21]. Cells were grown in shake flask fermentations as previously reported [15]. Fermentations were induced at 0.6 OD_{600} with 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.02% (w/v) L-arabinose to express T7 RNA polymerase and the *E. coli*-orthogonal tRNA synthetase, respectively. Cells were harvested at late exponential phase (OD_{600} 4–5.4), lysed with an Emusiflex B-15 French Press (Avestin, ON, Canada) and further prepared as previously described [15].

To further supplement the CFPS reactions, the *E. coli*-orthogonal synthetase was produced *in vivo* and purified, as previously described [11]. The *E. coli*-orthogonal tRNA synthetase was overexpressed from the plasmid pEVOL-*pPrF* harbored in BL21 Star™ (DE3) in shake flasks containing 1 L of 2xYT media at 37°C and 280 rpm. Expression was induced with 1 mM IPTG at 0.5 OD_{600} and incubated overnight. Cells were lysed by Emusiflex B-15 French Press (Avestin, ON, Canada) and the synthetase was purified using HisTrap™ HP columns (GE Healthcare, WI).

Preparation of linear expression templates (LETs)

Linear expression templates (LETs) were generated from pY71-sfGFP using two step PCR as previously described [35]. In the first PCR the gene of interest was amplified using gene specific primers. The amplified genes were then advanced to second PCR with ultramers to include the ribosome binding site, T7 promoter and T7 terminator with sequences based on those optimized by Ahn and coworkers [41]. The final PCR product was purified using QIAquick® PCR purification kit following the manufacturer's instruction (Qiagen, Valencia, CA). Plasmid and LETs were used immediately or stored in ddH_2O at −20°C until use. The primer sequences used for generating LETs are tabulated in Supplementary Table S1.

CFPS reactions and protein yield determination

Phosphoenolpyruvate (PEP), *E. coli* tRNA mixture, creatine phosphate (CP), and creatine kinase (CK) were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and L-[U-^{14}C] Leucine was purchased from PerkinElmer Inc. (Waltham, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). *para*-Propargyloxyphenylalanine (pPa) was synthesized and characterized as described previously [12]. Energy systems based on the following were used in this work: (1) PANOxSP [42], (2) simplified PANOx (PANOx*) [43], (3) creatine phosphate/kinase (CP/CK) [44], glucose [34], (4) fructose 1,6-bisphosphate (F1,6BP) [17], and (5) glutamate [45]. Detailed initial reaction compositions are described in Supplementary Table S2. Plasmid based reactions were performed with plasmid pY71-sfGFP encoding for a superfolder derivative of green fluorescent protein (GFP) and pY71-sfGFP-T216uAA encoding for pPa incorporation at residue 216 (pPaGFP). The GFP gene was derived from accession number 2B3P_A and the sequence for the expression vector pY71 and the GFP used in this work have been detailed previously [12]. Protein yield was determined using a linearly correlated calibration curve between fluorescence measurement and protein concentration as described previously [15].

Nuclease inhibition

On the basis of the findings of Amundsen and coworkers, small molecules CID 697851 (IC_{50} of 33 μM, ChemBridge, San Diego) and CID 1517823 (IC_{50} of 5.1 μM, Vitas-M Laboratory, Netherlands) from chemical class cyanothiophene and pyrimidopyridone, respectively, were selected as the experimental nuclease inhibitors [46]. The compounds were first dissolved in DMSO and appropriate dilutions for CFPS reactions were made

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