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An amino acid depleted cell-free protein synthesis system for the incorporation of non-canonical amino acid analogs into proteins

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ABSTRACT

Residue-specific incorporation of non-canonical amino acids into proteins is usually performed *in vivo* using amino acid auxotrophic strains and replacing the natural amino acid with an unnatural amino acid analog. Herein, we present an efficient amino acid depleted cell-free protein synthesis system that can be used to study residue-specific replacement of a natural amino acid by an unnatural amino acid analog. This system combines a simple methodology and high protein expression titers with a high-efficiency analog substitution into a target protein. To demonstrate the productivity and efficacy of a cell-free synthesis system for residue-specific incorporation of unnatural amino acids *in vitro*, we use this system to show that 5-fluorotryptophan and 6-fluorotryptophan substituted streptavidin retain the ability to bind biotin despite protein-wide replacement of a natural amino acid for the amino acid analog. We envisage this amino acid depleted cell-free synthesis system being an economical and convenient format for the high-throughput screening of a myriad of amino acid analogs with a variety of protein targets for the study and functional characterization of proteins substituted with unnatural amino acids when compared to the currently employed *in vivo* methodologies.

1. Introduction

Modern, high-throughput protein production and screening methodologies have proven to be useful for drug development, structure determination, and protein engineering. In particular, the cell-free synthesis of proteins offers an attractive alternative to traditional *in vivo* protein expression methodologies (Forstner et al., 2007; Rosenblum and Cooperman, 2014; Sawasaki et al., 2002; Tao and Zhu, 2006). Cell-free protein synthesis (CFPS) allows the researcher to have greater control over expression conditions and can also lead to the expression of proteins *in vitro* that are otherwise difficult to express *in vivo*. CFPS systems have been optimized for the expression of a variety of proteins, including membrane proteins (Isaksson et al., 2012; Schwarz et al., 2007), post-translationally modified proteins (Ezure et al., 2010; Sawasaki et al., 2002), and proteins containing disulfide bonds (Kim and Swartz, 2004). CFPS systems have also become increasingly cost-effective and productive in comparison to traditional *in vivo* expression systems (Calhoun and Swartz, 2005; Carlson et al., 2012; Caschera and Noireaux, 2013; Kim and Choi, 2001; Yang et al., 2012; Zawada et al., 2011).

The utility of CFPS can be further expanded by the inclusion of chemically and functionally distinct unnatural amino acids in addition to the canonical 20 amino acids that make up proteins in nature. While hundreds of different non-canonical amino acids have been translationally incorporated into proteins, much of this work has primarily been carried out *in vivo* (reviewed in (Liu and Schultz, 2010)). Recent advancements in CFPS systems have made *in vitro* production of proteins containing unnatural amino acids via site-specific incorporation an attractive alternative to *in vivo* methods (Albayrak and Swartz, 2013a, 2013b; Arthur et al., 2013; Bundy and Swartz, 2010; Goerke and Swartz, 2009; Hong et al., 2014; Loscha et al., 2012; Ozawa et al., 2012). These studies have focused on the site-specific incorporation of unnatural amino acids via the use of engineered translation system components, and the orthogonal suppression of stop-codons (Liu and Schultz, 2010) or

Abbreviations: CFPS, Cell-free protein synthesis.

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4-base codons (Sisido et al., 2005) in a limited number of sites in a protein. While this technique has its utility, the complete substitution of a natural amino acid with an amino acid analog (residue-specific incorporation) can confer interesting structural and functional properties to proteins, from greater stability to thermal denaturation (Tang et al., 2001) to altered fluorescent properties in fluorescent proteins (Bae et al., 2003; Yoo et al., 2007) and stands as a complementary methodology to site-specific incorporation techniques for making protein based polymers containing unnatural amino acids. In some cases, the simple replacement of a canonical amino acid with an amino acid analog is sufficient to confer these properties (Bae et al., 2003; Tang et al., 2001), while in others, additional protein evolution was needed for the unnatural amino acid-substituted protein to regain function (Montclare and Tirrell, 2006; Yoo et al., 2007).

Residue-specific incorporation is usually performed with analogs that are compatible with the cellular translation machinery; for example, replacing all methionines with selenomethionines (Budisa et al., 1995; Kigawa et al., 2002), or replacing all tryptophans with fluorinated tryptophan analogs (Bacher and Ellington, 2001; Mat et al., 2010). Residue-specific incorporation methods can be challenging because even a small amount of the contaminating natural amino acid can lead to the production of low levels of wild-type protein or incomplete analog substitution which can confound the characterization of the analog-substituted protein. In contrast, site-specific incorporation typically uses engineered tRNA synthetase-tRNA pairs which are orthogonal to the cellular synthetases and tRNAs and are specific for an unnatural amino acid (in theory, (Nehring et al., 2012)) that is generally inserted across from a single, engineered stop codon.

In vivo methods for unnatural amino acid incorporation are of limited utility when a given amino acid analog is toxic or cannot be taken up by the cell. Unnatural amino acid analogs can be toxic to cells to varying degrees depending on their compatibility with the cellular proteome. This usually results in lower titers of the analog-substituted protein compared to the wild-type protein, due to the cytotoxic effects of the unnatural amino acid analog itself, as well as reduced (less than 100%) substitution of the amino acid analog for the natural amino acid (See for example (Montclare et al., 2009; Budisa et al., 1995, 2004)).

In contrast, CFPS is not limited by the cytotoxic effects of the amino acid analogs to the expression strains or the amino acid transport limitations of the amino acid analogs themselves (Budisa and Pal, 2004). Since cytotoxic effects are irrelevant in CFPS systems, discrepancies between analog-substituted protein titers and wild-type protein titers will reflect the suitability of an amino acid analog as a substrate for translational machinery. Additionally, as long as the expressed protein does not interfere with transcription or translation, CFPS can also be used to produce toxic proteins substituted with unnatural amino acid analogs. The greater control over the reaction components afforded by CFPS should make higher substitution efficiencies possible. In the work reported herein, we describe the production of an amino acid depleted *Escherichia coli* cell-free lysate for the global replacement of a canonical amino acid (tryptophan) with synthetic amino acid analogs. Tryptophan is present in the lowest amount of all the amino acids in the *E. coli* proteome (~1%, (Sharp and Li, 1987)) and we postulated that it might therefore be the easiest to deplete from an *E. coli* cell-free lysate. We then used this tryptophan-depleted CFPS system to express streptavidins modified with amino acid analogs, and evaluated the impact of complete analog substitution on protein function. Using this system, we obtained 100% analog incorporation efficiency, which represents a significant improvement over previously reported residue-specific incorporation methods done *in vivo* (Budisa et al., 2004, 1995; Montclare et al., 2009; Pratt and Ho, 1975). We believe that this methodology represents a significant improvement over commercially available cell-free lysates and traditional cell-free lysate preparation methods for the residue-specific incorporation of amino acid analogs into proteins *in vitro*, and also represents an efficient and economical alternative to recombinant translation systems such as the PURE system for the incorporation of amino acid analogs into proteins.

2. Materials and methods

2.1. Materials

E. coli strain BL21(DE3) was obtained from Life Technologies (Carlsbad, CA). T7 RNA polymerase was obtained from Epicentre Biotechnologies (Madison, WI). All chemicals and reagents were either molecular biology or ACS grade and obtained from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Rockford, IL). All primers and oligonucleotides were purchased from IDT (Coralville, IA). Solutions were made in sterile, deionized water in triple-rinsed glassware.

2.2. Preparation of S30 lysate

Medium G was prepared as described previously (Wong, 1983) and supplemented with 100 mM glucose, 22 mM NaH_2PO_4 and 40 mM Na_2HPO_4 to give Medium G-PG. 1 L of the medium was further supplemented with 20 mg of tryptophan, inoculated with a 1:100 dilution of an overnight culture of *E. coli* BL21(DE3)ΔtrpC (see Supplementary materials and methods for strain construction) and grown to mid-log at 37 °C with shaking. The cells were then spun down at 6000 × *g* at 16 °C and washed once with phosphate buffered saline and then resuspended in 1 L of Medium G-PG lacking tryptophan, and incubated at 37 °C with shaking for 50 min. The cells were then spun down and washed twice with S30 Buffer A (10 mM Tris-acetate buffer (pH 8.2), 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT, and 0.5 mL/L 2-mercaptoethanol). Following the final wash step the cell pellet was flash frozen in either liquid nitrogen or a dry ice-ethanol bath and stored at −80 °C for no more than 2 days.

The frozen cells were thawed on ice and resuspended in 1 mL of S30 Buffer B (Buffer A without 2-mercaptoethanol) per gram of bacterial cell pellet. The resuspended cells were then further lysed using a single pass through a French pressure cell press (Thermo Fisher, Rockford, IL) at 3000 psi. The bacterial cell lysate was collected on ice and then centrifuged at 30,000 × *g* for 30 min at 4 °C. The cleared cell lysate was decanted into a clean centrifuge tube and centrifuged again at 30,000 × *g* for 30 min at 4 °C. The resulting supernatant was incubated at 37 °C with shaking for 80 min to clear bound endogenous mRNA from transcription and translation enzymes and ribosomes. Following this incubation period the lysate was dialyzed in Slide-A-Lyser (Pierce) dialysis cassettes with a 7000 Da MWCO. The buffer was exchanged three times, at 4 °C against 75–100 volumes S30 Buffer B. The first buffer exchange was performed after 8 h, followed by two shorter exchanges of 45 min each. Subsequent to this, the bacterial cell lysate was carefully removed from the dialysis cassette and centrifuged at 4000 × *g* for 10 min at 4 °C. The resulting S30 lysate was aliquotted into chilled tubes on ice and flash frozen in liquid nitrogen or an ethanol/dry ice bath and stored at −80 °C till further use.

2.3. Cell-free protein synthesis reactions

Each 50 μL CFPS reaction contained 17.5 μL of S30 lysate, 2.5 μL of Ampliscribe T7 RNA polymerase (Epicenter Biotechnologies), between 50 and 250 ng template DNA, 55 mM Hepes-KOH (pH 7.5) containing 1.7 mM DTT, 1.2 mM ATP (pH 7), 0.8 mM each of CTP,