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Using *E. coli*-based cell-free protein synthesis to evaluate the kinetic performance of an orthogonal tRNA and aminoacyl-tRNA synthetase pair

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ABSTRACT

Even though the orthogonal tRNA and aminoacyl-tRNA synthetase pairs derived from the archaeon *Methanocaldococcus jannaschii* have been used for many years for site-specific incorporation of non-natural amino acids (nnAAs) in *Escherichia coli*, their kinetic parameters have not been evaluated. Here we use a cell-free protein synthesis (CFPS) system to control the concentrations of the orthogonal components in order to evaluate their performance while supporting synthesis of modified proteins (i.e. proteins with nnAAs). Titration experiments and estimates of turnover numbers suggest that the orthogonal synthetase is a very slow catalyst when compared to the native *E. coli* synthetases. The estimated k_{cat} for the orthogonal synthetase specific to the nnAA *p*-propargyloxyphenylalanine (pPaF) is 5.4×10^{-5} s^{-1}. Thus, this catalyst may be the limiting factor for nnAA incorporation when using this approach. These titration experiments also resulted in the highest reported cell-free accumulation of two different modified proteins (450 μg/ml CAT109pAzF and 428 ± 2 μg/ml sfGFP23pPaF) using the standard KC6 cell extract and either the PANOx SP or the inexpensive Glu NMP cell-free recipe.

1. Introduction

Site-specific incorporation methods for non-natural amino acids (nnAAs) were developed to enable precise post-translational modification of proteins and to broaden their chemical diversity [1]. *In situ* biologic acylation of the amber suppressor tRNA was favored over exogenous chemical acylation, since the latter suffers from low modified protein yields (~50 μg/ml) [2], laborious tRNA aminoacylation, and the absence of an efficient method for introducing the aminoacylated tRNA into intact cells [3]. Originally developed by the Schultz laboratory, *in situ* biologic acylation uses orthogonal components derived from the archaebacterium *Methanocaldococcus jannaschii* and mimics the incorporation of natural amino acids into proteins: First, the evolved orthogonal aminoacyl-tRNA synthetase catalyzes the formation of an ester bond between the 3′ terminus of the orthogonal tRNA and the nnAA. Second, the aminoacylated orthogonal tRNA forms a ternary complex with the elongation factor Ef-Tu and GTP. Finally, the ternary complex enters the ribosome, outcompetes the endogenous Release Factor 1 (RF1) which naturally terminates translation at the amber (UAG) stop codon, and inserts the nnAA into the nascent polypeptide chain. This method has enabled the incorporation of more than 30 nnAAs at the amber stop codon *in vivo* [4]. Of these >30 nnAAs, our laboratory has been particularly interested in two, namely *p*-azido-L-phenylalanine (pAzF) (1) and *p*-propargyloxy-L-phenylalanine (pPaF) (2, Fig. S1) because these nnAAs (and the proteins that contain them) can be directly coupled via the bioorthogonal copper(I)-catalyzed azide–alkyne cycloaddition reaction.

Since the landmark experiment of Nirenberg and Matthaei (1961) and the demonstration of the continuous exchange method of Spirin and colleagues [5,6], cell-free protein synthesis (CFPS) using *Escherichia coli* extracts has been vastly improved. The stabilization of amino acids [7,8], a natural chemical environment [9], and the activation of central metabolism [10] enabled the cell-free production of a wide variety of proteins at high concentrations. CFPS was also shown to produce modified (i.e. nnAA-containing) proteins at high concentrations (150–930 μg/ml) and suppression efficiencies (25–96%) (which is defined as the ratio of the modified protein yield to the yield of its natural counterpart) [11,12]. A crucial factor in the development of modified protein production was the open nature of the cell-free platform, which allows the experimenter to precisely control the reagent concentrations in the protein translation environment.

Despite the fact that the *M. jannaschii*-derived orthogonal components have been widely used for the production of modified proteins, their kinetic parameters have not been investigated. Taking advantage of the open nature of the CFPS platform, we present here a systematic assessment of site-specific nnAA incorporation into two proteins, chloramphenicol acetyltransferase (CAT) and

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super-folder green fluorescent protein (sfGFP), which are synthesized at lower suppression efficiencies and modified yields than dihydrofolate reductase (DHFR) [12]. A Northern blot protocol was developed to measure the o-tRNA concentration in the orthogonal cell extract (i.e. a cell extract that contains the o-tRNA), an *in vitro* transcription method was adapted to exogenously produce the o-tRNA, and the orthogonal macromolecules were carefully titrated into CFPS reactions to determine their limiting concentrations. These methods were then used to assess the turnover number of the orthogonal synthetase and to compare the performance of the orthogonal macromolecules to that of their endogenous counterparts.

2. Materials and methods

Preparation of the plasmids, the cell extract, the purified o-tRNA, the orthogonal synthetases, and the bulk nucleic acid solution are explained in detail in the Supplementary Information.

2.1. Cell-free protein synthesis (CFPS)

The PANOx SP (PEP, Amino Acids, NAD, Oxalic Acid, Spermidine and Putrescine) [9] or the Glu (Glutamate) NMP recipes [10] were used in the cell-free reactions, with several changes in the reagents and reagent concentrations as indicated below. All of the chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated.

20 or 25 μl PANOx SP reaction solutions contained, unless otherwise noted: 10 mM ammonium glutamate, 175 mM potassium glutamate, 1.2 mM ATP, 0.86 mM each of CTP, GTP and UTP, 34 μg/ml folinic acid, 170.9 μg/ml *E. coli* tRNAs (Roche Applied Science, Penzberg, Germany), 33 mM phosphoenolpyruvate (PEP) (Roche Applied Science), 1.5 mM spermidine, 1 mM putrescine, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A, 2.7 mM sodium oxalate, 2 mM each of the 20 amino acids, 5 μM *L*-[^{14}C(U)]-leucine (Amersham Pharmacia, Uppsala, Sweden), 2 mM *p*-azido-L-phenylalanine (pAzF, Chem-Impex International, Wood Dale, IL) or 4 mM *p*-propargyloxy-L-phenylalanine (pPaF, synthesized according to reference [13]), 0.2–0.35 mg/ml pAzFRS or 0.5 mg/ml pPaFRS (prepared as described above), 100 μg/ml T7 RNA polymerase (RNAP), 6 nM plasmid and either 0.24 volume of standard KC6 extract or 0.28 volume of orthogonal extract. The magnesium glutamate concentration was optimized for each cell extract lot; 12 and 20 mM of this reagent was added to reactions with the standard extract and the orthogonal extract, respectively.

The glutamate salts were diluted from a 10-fold concentrated (10×) solution. Similarly, a 10× NTP Master Mix solution contained the four NTPs, folinic acid and the *E. coli* tRNAs and was adjusted to pH 7.3 with potassium hydroxide. The natural amino acids were diluted from a stock solution, which contained a 50 mM concentration of each amino acid. This stock solution was prepared by adding the amino acids in the following order (given in their one-letter code): R, V, W, F, I, L, C, M, A, N, D, E, G, Q, H, K, P, S, T, Y. During the preparation, it was ensured that each amino acid was dissolved before addition of the next, except tyrosine, which is added last and remains suspended in the solution. Different from the PANOx SP system, the Glu NMP reactions contained 130 mM potassium glutamate instead of 175 mM, 4 mM potassium oxalate instead of 2.7 mM, NMPs instead of NTPs (at the same concentrations as in the PANOx SP recipe), and 100 mM dibasic potassium phosphate. The reactions were incubated at 30 °C for 10 h. CFPS reactions containing pAzF were prepared in a dark room and the tubes containing the reaction solutions were wrapped in aluminum foil to prevent photodissociation of the aromatic azide [14].

2.2. Protein quantification and suppression efficiency calculations

Proteins radioactively labeled by *L*-[^{14}C(U)]-leucine incorporation were quantified as follows. A 4 μl sample from each CFPS reaction solution was spotted on two of three Whatman MM filter papers (Whatman, Springfield Mill, United Kingdom). One of these papers was used to measure the total synthesized protein concentration and the other to measure total system radioactivity. The remaining cell-free solution was centrifuged at 20,800*g* and 4 °C for 15 min. 4 μl of the supernatant was spotted onto the third filter paper. The papers were dried either overnight at room temperature or for 1 h under an incandescent light bulb. The paper containing the soluble protein and one of the two papers containing the total protein were submerged in ice-cold 5% w/v trichloroacetic acid (TCA) for 15 min to precipitate the proteins [15]. This incubation was repeated twice with fresh 5% TCA for a total of three times. The TCA-washed papers were rinsed with deionized water and dried either overnight at room temperature or for 1 h under the light bulb. A Beckman LS 3801 liquid scintillation counter (Beckman Coulter, Brea, CA) was used to measure the radioactivity on the filter papers. In each experiment, a separate set of "blank" cell-free reactions was incubated to determine background radioactivity; these reactions contained all of the reagents except the protein template DNA. The background radioactivity was subtracted from the protein radioactivity measurements to obtain true protein concentrations. The leucine content and the molecular weight of the protein were used to convert radioactivity measurements to protein concentrations. Full-length protein amounts were determined by densitometry analysis of soluble protein bands on SDS–PAGE gel autoradiograms.

CFPS samples containing CAT were diluted in a sample buffer containing lithium dodecyl sulfate (LDS) at pH 8.4 (Invitrogen, Carlsbad, CA), and denatured with 50 mM DTT at 72 °C for 10 min. sfGFP samples were incubated in the same solution except with 125 mM DTT at 95 °C for 15 min to ensure complete denaturation. The solutions were then loaded onto 10% Bis–Tris SDS–PAGE gels, and the gels were subsequently run with the MES SDS running buffer at pH 7.3 (Invitrogen). The gels were subsequently dried and exposed overnight to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA). The screens were scanned using the Typhoon imaging system (GE Healthcare, Uppsala, Sweden) and the band intensities on the autoradiogram were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

The suppression efficiency is defined as the ratio of the yield of full-length modified protein to that of its natural counterpart. In the experiments with reported suppression efficiencies, the natural protein was synthesized under the same cell-free reaction conditions, and the full-length and soluble protein yields were measured using scintillation counting and densitometry.

2.3. Northern blot analysis of the orthogonal tRNA

A single-stranded, biotinylated DNA oligonucleotide probe (with the sequence 5′ biotin – TGGTCCGGCGGGCCGGATTTG-3′) was designed to hybridize to the 3′ end of the o-tRNA. In this detection protocol, streptavidin-conjugated horseradish peroxidase binds the biotinylated probe. The reaction of the chemiluminescent substrate luminol with the horseradish peroxidase produces light, which is captured on an X-ray film to quantify the tRNA using densitometry.

Different dilutions of the orthogonal extract and the gel-purified o-tRNA solution were denatured at 95 °C for 5 min and diluted in a TBE-Urea sample buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, 3.5 M urea, 6% Ficoll™ Type 400, 0.005% bromophenol blue, 0.025% xylene cyanol; Invitrogen) prior to loading onto a 15% denaturing polyacrylamide gel (TBE-Urea gel, Invitrogen). The

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