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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 ± 20 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

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two proteins, chloramphenicol acetyltransferase (CAT) and superfolder 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

standard KC6 extract was included as a negative control, since it contains the *E. coli* tRNAs but not the o-tRNA. The gel was run at 180 V for 75 min with TBE buffer and subsequently stained with ethidium bromide. The nucleic acids were then transferred onto a Nylon+ membrane using the XCell II Blot module (Invitrogen) as per the manufacturer protocol. The nucleic acids were photo-crosslinked to the membrane with UV light, and the membrane was washed first with a 1× PBS solution containing 0.1% w/v SDS, then with a 1× PBS solution containing 0.5% w/v casein. The hybridization probe was then denatured at 95 °C for 5 min and was incubated with the membrane in 6× SSC buffer (Invitrogen) overnight in a 55 °C water bath. The Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology, Rockford, IL) was used to detect the o-tRNA according to the manufacturer instructions, except that the membrane was incubated twice in blocking buffer at 52 °C for 20 min per wash after the hybridization step.

3. Results

3.1. Determination of the orthogonal tRNA concentration in the orthogonal cell extract

Three components are required for site-specific incorporation of non-natural amino acids: the non-natural amino acid (nnAA), the orthogonal tRNA (o-tRNA), and the non-natural aminoacyl-tRNA synthetase (nnAARS). In the standard CFPS platform, the o-tRNA is supplied in the cell extract (the orthogonal extract), while the nnAA and the purified nnAARS are added separately to the reaction mixture [12]. Since the modified protein yields are almost always lower than those of their native counterparts, we hypothesized that the nnAA incorporation pathway is much less active than that for the natural amino acids. At 2 mM, pAzF is provided in vast stoichiometric excess since only one nnAA is incorporated into each protein, and only 1–40 μM of protein is produced in a typical CFPS reaction. Typically, 250–350 μg/ml (7–10 μM) of the *p*-azidophenylalanyl-tRNA synthetase (pAzFRS) is required in the CFPS reaction to ensure that this enzyme is not limiting, suggesting very slow synthetase turnover. While the o-tRNA is constitutively expressed in the cell extract strain during fermentation, its concentration (and its sufficiency) in the cell-free reaction was unknown [12].

In order to measure the o-tRNA concentration in the orthogonal extract, we first synthesized the o-tRNA *in vitro*. Since the o-tRNA sequence has a 5′ pyrimidine [16] and T7 RNA polymerase efficiently transcribes only RNAs that begin with 5′ purines [17,18], we could not use conventional runoff transcription [19]. We therefore adapted the approach of Fechter et al. [20] by inserting a *cis*-acting hammerhead ribozyme coding sequence between the consensus T7 RNAP promoter and the tRNA template and used this "transzyme" method to produce the orthogonal tRNA *in vitro* (Fig. S2 A and B). The orthogonal tRNA was subsequently purified using either polyacrylamide gel electrophoresis (Fig. S2 C) or size exclusion chromatography (Fig. S2 D), and its biologic activity was confirmed by CFPS of modified CAT in three sets of reactions. 269 ± 40 μg/ml and 290 ± 26 μg/ml of soluble, full-length CAT109-pAzF accumulated in PANOx SP reactions that were supplemented with the purified o-tRNA and were incubated either with the orthogonal or with the standard KC6 extract, respectively (Fig. S2 E and F). 450 ± 20 μg/ml of soluble, full-length CAT109pAzF accumulated in Glu NMP reactions supplemented with the purified o-tRNA (Fig. S3).

A Northern blot protocol was then devised to selectively label the o-tRNA that was separated in cell extract samples using electrophoresis. The purified o-tRNA was used as a calibration standard to estimate the amount of o-tRNA in the orthogonal extract. Different concentrations of the pure o-tRNA were run on a denaturing polyacrylamide gel along with different dilutions of the orthogonal extract. A Northern blot was obtained from this gel, and the RNA bands on the blot membrane were quantified by densitometry (Fig. 1). From three extract dilutions, we estimated that the o-tRNA concentration in the orthogonal extract is 49 ± 7 μg/ml. At 28% v/v cell extract, this corresponds to 14 ± 2 μg/ml (0.6 ± 0.1 μM) o-tRNA in the CFPS solution.

3.2. Titration of orthogonal components to identify non-limiting concentrations

To determine non-limiting concentrations of the orthogonal macromolecules, the o-tRNA and pPaFRS (the synthetase that specifically recognizes pPaF) were separately titrated into CFPS reactions using the standard KC6 extract (which does not contain o-tRNA). For this study, the accumulation of modified sfGFP containing pPaF at position 23 (sfGFP23pPaF) was measured (Fig. 2), since yields are higher than for CAT and sfGFP therefore serves as a more sensitive reporter (Fig. S4). In these experiments, the two components that are not titrated are provided in excess. For example, in the o-tRNA titration experiments, 14 μM of pPaFRS and 4 mM of pPaF were added to the CFPS reaction mixtures (Fig. 2A). In the pPaFRS titration experiments, 1.5 μM o-tRNA and 4 mM pPaF were supplied in each reaction solution (Fig. 2B). These experiments indicated that the non-limiting orthogonal component concentrations were 1.5 μM for the o-tRNA and 9 μM for the pPaFRS enzyme (Fig. 2). Furthermore, ~160 μg/ml sfGFP23pPaF accumulated in CFPS mixtures that contain either the orthogonal extract containing 0.6 ± 0.1 μM o-tRNA or the standard extract and 0.5 μM of *in vitro*-synthesized o-tRNA. Assuming that the o-tRNA is the limiting factor in both cases, these results suggest that *in vitro*- and *in vivo*-produced o-tRNA are about equally competent for nnAA insertion.

Identifying the non-limiting concentrations of the orthogonal macromolecules allowed us to compare their performance to that of the native components. There are ~125,000 tRNAs per cell [21], which corresponds to ~8 μM in the CFPS solution [22]. Assuming that the concentration of each of the 46 tRNAs [23] is the same (an admittedly incorrect, but simplifying assumption), then each *E. coli* tRNA is present at ~0.2 μM in the CFPS reaction, and approximately a 8-fold higher concentration of the o-tRNA is needed. On the other hand, there are 400–1200 molecules of each of the *E. coli* aminoacyl-tRNA synthetases (aaRSs) per cell [24], which is equivalent to 25–76 nM in the CFPS solution. In order to effectively incorporate one nnAA per protein in the cell-free reaction, the nnAARS needs to be added at 120- to 360-fold higher concentrations than the endogenous aaRSs (i.e. at stoichiometric rather than catalytic concentrations). Even though the modified protein yields were improved by increasing o-tRNA concentrations, it therefore appears that the orthogonal synthetase is the more ineffective orthogonal component when compared to the native *E. coli* components.

An estimate of synthetase turnover numbers ($k_{cat,AARS}$) supports this conclusion. In the Glu NMP system, the product protein is synthesized at a steady rate during the CFPS reaction [10]. We can therefore assume that modified CAT is produced at a rate of ~45 μg/ml-h in the Glu NMP reaction (Fig. S3). This number corresponds to a synthesis rate of 0.49 nM/s, or a tyrosine incorporation rate of 5.4 nM/s, since there are eleven tyrosines in CAT. Dividing this number by the concentration range of each of the endogenous synthetases (25–76 nM), we estimate a k_{cat} of 0.07–0.22 s^{-1} for the endogenous tyrosyl-tRNA synthetase (TyrRS). This number is lower than that reported for tyrosyl-tRNA turnover (1.9 s^{-1}) in *E. coli* [24]. However, when this calculation is repeated using the optimal concentration of 9 μM for pPaFRS, we obtain a k_{cat} of 5.4×10^{-5} s^{-1},

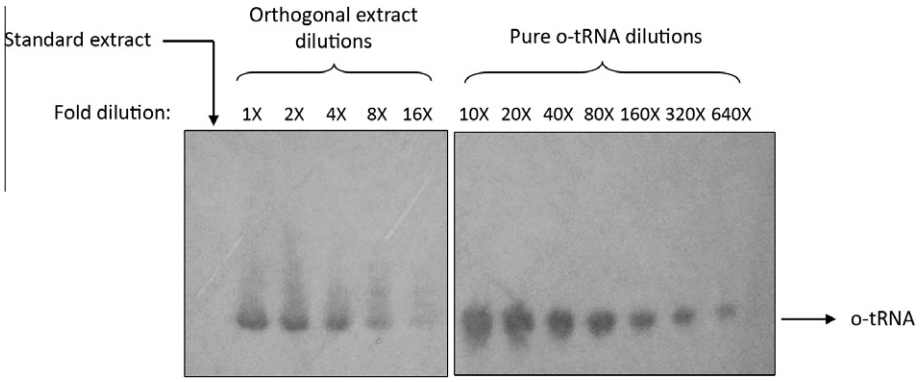

Fig. 1. Northern blot analysis of the orthogonal tRNA. Indicated dilutions of the orthogonal extract and the gel-purified *in vitro*-synthesized o-tRNA (initial concentration = 940 ± 20 μg/ml) were analyzed to estimate the o-tRNA concentration in the extract. The standard KC6 cell extract was run as a negative control; absence of a band in that lane shows that none of the endogenous *E. coli* tRNAs were labeled. From three extract dilutions, the o-tRNA concentration in the orthogonal extract was estimated by densitometry to be 49 ± 7 μg/ml (2.2 ± 0.3 μM).

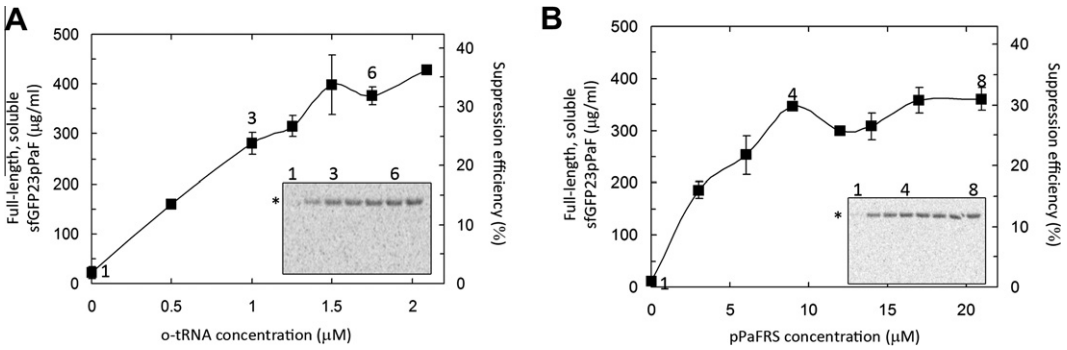

Fig. 2. Titration of (A) o-tRNA and (B) pPaFRS into cell-free reactions using standard KC6 extract. SEC-purified o-tRNA was used in these titration experiments. The reactions were incubated at 30 °C for 10 h and the full-length sfGFP23pPaF yields were calculated from scintillation counting and densitometry. The autoradiograms in the inner panels show bands of full-length modified protein, which are denoted by an asterisk (*). Suppression efficiency is the ratio of the yield of full-length sfGFP23pPaF to that of natural sfGFP synthesized at the same reaction conditions. Error bars indicate ±1 standard deviation for 3 reactions.

more than three orders of magnitude lower than the estimated turnover number for *E. coli* TyrRS.

4. Discussion

The accessibility of and control over the cell-free reaction environment enabled us to conduct a careful analysis of the orthogonal macromolecules required for site-specific incorporation of nnAAs. Exact concentrations of the orthogonal components were added directly into the CFPS solution to conduct experiments that would have been impossible *in vivo*. Titration experiments and estimates of synthetase turnover numbers suggest that the orthogonal aminoacyl-tRNA synthetase is a very slow orthogonal catalyst when compared to the native *E. coli* aminoacyl-tRNA synthetase.

Therefore, we believe that future efforts for improving site-specific incorporation of non-natural amino acids should focus on evolving the nnAARSs for higher catalytic rates (higher $k_{cat,nnAARS}$) and for higher affinity for the o-tRNA (lower $K_{M,o\text{-}tRNA}$). The aminoacylation reaction for nnAA incorporation has three substrates: the orthogonal tRNA (o-tRNA), the nnAA and ATP. The nnAA (2–4 mM) and free ATP (~200 μM) [10] are at much higher concentrations than the o-tRNA (~1 μM, Fig. 2A). If we assume that these nnAA and ATP concentrations are much higher than the Michaelis constants of the orthogonal synthetase for these substrates (these values were not reported for the evolved orthogonal synthetases pAzFRS and pPaFRS), then aminoacylation can be treated as an enzymatic reaction with one substrate:

$$\frac{d(nnAA - o\text{-}tRNA)}{dt} = \frac{k_{cat,nnAARS}[nnAARS]_{Total}[o\text{-}tRNA]}{K_{M,o\text{-}tRNA} + [o\text{-}tRNA]}, \quad (1)$$

where $k_{cat,nnAARS}$ is the turnover rate of the orthogonal aminoacyl-tRNA synthetase, and $K_{M,o\text{-}tRNA}$ is the Michaelis constant of the enzyme for the orthogonal tRNA. A more effective orthogonal synthetase would increase the rate of aminoacylation (d(nnAA – o-tRNA)/dt), and therefore the rate of site-specific incorporation of nnAAs in CFPS. High o-tRNA concentrations would probably not be required with a more active nnAARS. In fact, an engineered synthetase (oMeYRS) was recently demonstrated to incorporate the nnAA *o*-methyltyrosine 10-fold more efficiently than the original oMeYRS *in vivo* [25].

In the experiments evaluating the orthogonal components, we developed methods for measuring o-tRNA concentrations in the orthogonal cell extract and for producing and supplying this reagent separately. In the course of this work, 450 ± 20 μg/ml of CAT109pAzF (suppression efficiency = 47%) and 428 ± 2 μg/ml of sfGFP23pPaF (suppression efficiency = 36%) were obtained by adding excess purified o-tRNA and orthogonal synthetase to the standard KC6 cell extract in the Glu NMP and PANOx SP CFPS platforms, respectively. These numbers represent the best modified protein yields for these two nnAAs. The highest previously reported cell-free yields for these proteins were 400 ± 11 μg/ml CAT109pAzF [12] and 271 ± 19 μg/ml sfGFP216pPaF [11], which were achieved by the addition of either purified natural vesicles to increase ATP supply or a concentrated solution of nucleic acids, respectively. Purified natural vesicles are laborious to prepare [26], and adding

a concentrated solution of nucleic acids was not as productive as the addition of purified orthogonal components (Fig. S3). 0.5 mg of tRNA transcript can be obtained from a 1-ml *in vitro* transcription reaction upon optimization [20], which is enough for 15 ml of CFPS reactions (at 1.5 μM o-tRNA). The o-tRNA preparation can easily be scaled up if more o-tRNA is needed. In addition, o-tRNA production is decoupled from cell extract preparation, thereby allowing the use of more productive standard KC6 extracts (Fig. S4). Finally, since the same o-tRNA can be used for incorporation of more than 30 nnAAs [27] by adding the aminoacyl-tRNA synthetase specific to the desired nnAA, this improved and modular CFPS platform can be used to incorporate any of these nnAAs effectively into a protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.108.

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