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In vitro-Constructed Ribosomes Enable Multi-site Incorporation of Noncanonical Amino Acids into Proteins

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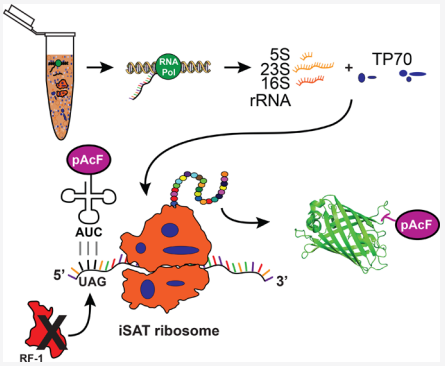
ABSTRACT: Efforts to expand the scope of ribosome-mediated polymerization to incorporate noncanonical amino acids (ncAAs) into peptides and proteins hold promise for creating new classes of enzymes, therapeutics, and materials. Recently, the integrated synthesis, assembly, and translation (iSAT) system was established to construct functional ribosomes in cell-free systems. However, the iSAT system has not been shown to be compatible with genetic code expansion. Here, to address this gap, we develop an iSAT platform capable of manufacturing pure proteins with site-specifically incorporated ncAAs. We first establish an iSAT platform based on extracts from genomically recoded *Escherichia coli* lacking release factor 1 (RF-1). This permits complete reassignment of the amber codon translation function. Next, we optimize orthogonal translation system components to demonstrate the benefits of genomic RF-1 deletion on incorporation of ncAAs into proteins. Using our optimized platform, we demonstrate high-level, multi-site incorporation of *p*-acetyl-phenylalanine (pAcF) and *p*-azido-phenylalanine into superfolder green fluorescent protein (sfGFP). Mass spectrometry analysis confirms the high accuracy of incorporation for pAcF at one, two, and five amber sites in sfGFP. The iSAT system updated for ncAA incorporation sets the stage for investigating ribosomal mutations to better understand the fundamental basis of protein synthesis, manufacturing proteins with new properties, and engineering ribosomes for novel polymerization chemistries.



Proteins make up an integral class of biomolecules used to fulfill structural, functional, and enzymatic processes required to support life. These sequence-defined polypeptides are primarily composed of 20 canonical amino acid building blocks. This limited set of amino acids constrains the chemical and physical properties of proteins. To address this limitation, the genetic code can be expanded to incorporate noncanonical amino acids (ncAAs) into polypeptides, creating new protein functions and properties.[1−8]

Expanding the genetic code involves engineered translation machinery and codon reassignment. The engineered translation machinery includes (i) aminoacyl-transfer RNA (tRNA) substrates, typically produced by an orthogonal aminoacyl-tRNA synthetase (o-aaRS) specifically acylating a ncAA to its cognate orthogonal tRNA (o-tRNA), that can decode an unassigned codon, (ii) delivery of ncAA−tRNA substrates by elongation factor Tu (EF-Tu) to the ribosome, and (ii) compatible ribosomes. The engineered translation machinery is "orthogonal" in that it does not recognize the 20 canonical amino acids or cross-react with native components of molecular translation. Most commonly, the amber stop codon (UAG) is reassigned to a ncAA in an approach called amber suppression.[1]

To date, >200 ncAAs have been incorporated into proteins using amber suppression, enabling a wave of new applications in molecular imaging,[9] post-translational modifications and their mimics,[10−14] fluorescent probes,[15−17] modified human therapeutics,[18] antibody−drug conjugates,[19−21] macrocyclic and peptidomimetic drugs,[22−25] protein polymers,[26,27] and genetically encoded materials.[28−30] Such advances have been made in both cellular and cell-free gene expression systems. For example, in cells, a genomically recoded *Escherichia coli* strain was developed (C321.ΔA) that was used to manufacture protein polymers with multiple, identical ncAAs in high yields.[2] In cell-free systems, derivatives of this same strain were used to create a one-pot platform for synthesizing proteins bearing ncAAs with high yields (>2.5 g/L).[31] Advantages of cell-free systems include the fact that they avoid cell viability constraints and enable direct access to the reaction conditions, which allows precise control of orthogonal translation system components and their substrates necessary for high-level ncAA incorporation.[7]

The *in vitro* synthesis, assembly, and translation (iSAT) system is a cell-free system that enables efficient one-step co-activation of ribosomal RNA (rRNA) transcription, assembly of transcribed rRNA with native ribosomal proteins into *E. coli*

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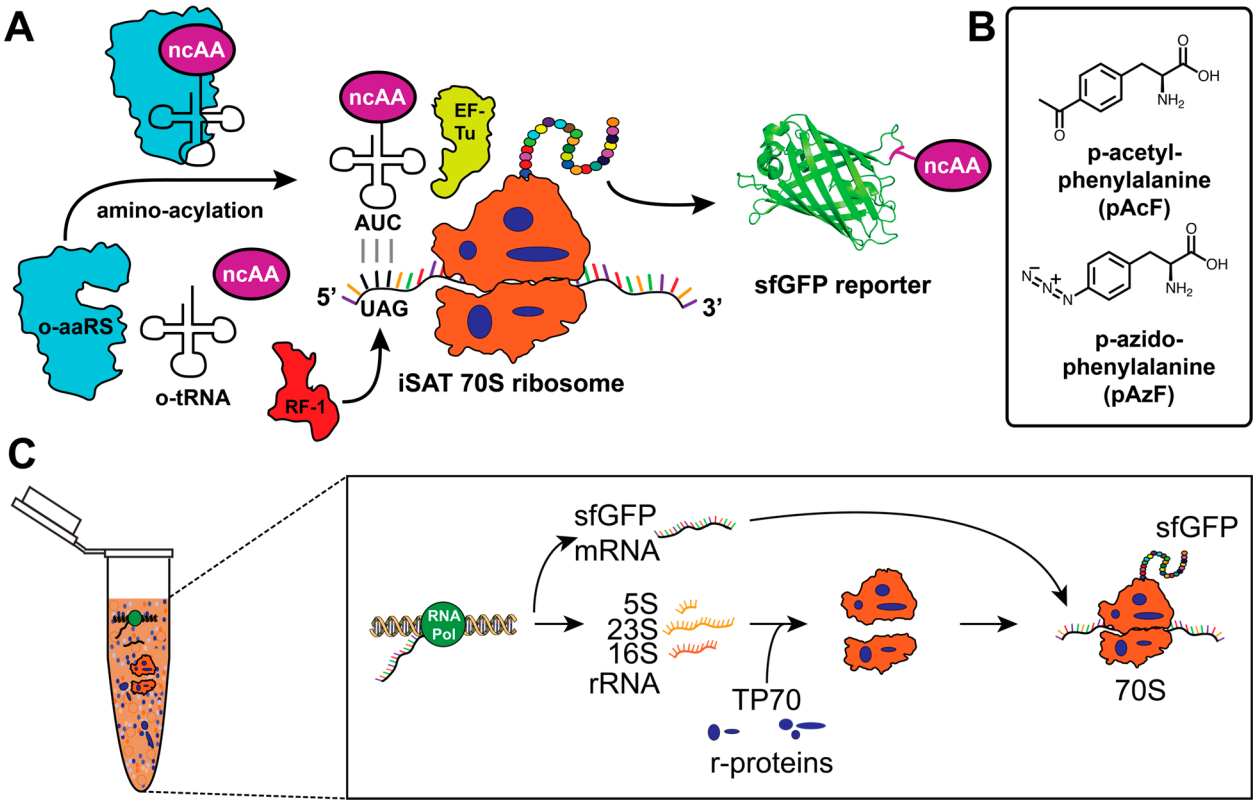

Figure 1. Overview of noncanonical amino acid (ncAA) incorporation using *in vitro* synthesis, assembly, and translation (iSAT)-assembled ribosomes *in vitro*. (A) ncAA incorporation using amber suppression. The amber stop codon UAG is reassigned to a sense codon that encodes the ncAA. This is achieved using an orthogonal aminoacyl-tRNA synthetase (o-aaRS) that aminoacylates its cognate orthogonal tRNA (o-tRNA) with the ncAA, after which the ncAA-bearing o-tRNA binds to the amber codon and delivers the ncAA to the iSAT-assembled 70S ribosome engaged in translation. In strains featuring *prfA*, release factor 1 (RF-1) competes for binding at the UAG site, which can prematurely terminate the translated product at that position instead of incorporating a ncAA. (B) Noncanonical amino acids used in this study. Two ncAAs, *p*-acetyl-phenylalanine (pAcF) and *p*-azido-phenylalanine (pAzF), were tested for incorporation into proteins via iSAT. (C) iSAT method of *in vitro* ribosome construction. A DNA template encoding the rRNA operon and a reporter gene are mixed with purified ribosomal (r-) proteins (TP70), energy and building block substrates, salts, buffers, and T7 RNA polymerase (RNAP) in a cell-free, ribosome-free S150 crude extract. Fifteen microliter scale reactions were performed in microcentrifuge tubes at 37 °C. T7 RNAP transcribes 23S, 16S, and 5S rRNA from the DNA template, as well as the superfolder green fluorescent protein (sfGFP) reporter mRNA. As rRNA is being transcribed, TP70 r-proteins bind and assemble into 50S and 30S subunits, which then bind to mRNA, forming the 70S ribosome (70S) and translating the active sfGFP protein as a reporter of ribosome activity.

ribosomes, and synthesis of functional protein by these ribosomes in a ribosome-free S150 crude extract.[32] The iSAT system generates highly active *E. coli* ribosomes, including modified ribosomes containing 23S rRNA mutations.[32−37] Upon combination of iSAT with ribosome display, *in vitro*-directed evolution of the ribosome is possible.[37] The small subunit of the ribosome can also be engineered,[38] and efforts are also underway to enable mirror image polymerization.[39,40] While the iSAT system is poised to facilitate new applications in genetic code reprogramming, the system has not yet been established to be compatible with orthogonal ncAA incorporation machinery (e.g., o-tRNAs and o-aaRSs) that recognize only ncAAs and enable site-specific incorporation of ncAAs into proteins.

Here, to address this gap, we update the iSAT platform to demonstrate the robust incorporation of the ncAAs *p*-acetyl-phenylalanine (pAcF) and *p*-azido-phenylalanine (pAzF) (Figure 1) at up to five amber codon sites in a reporter superfolder green fluorescent protein (sfGFP), with high suppression efficiency and accuracy. This was accomplished through use of *E. coli* chassis strains that were genomically modified for enhanced ncAA incorporation (i.e., ΔprfA) for S150 ribosome-free extract production, overexpression of o-tRNA within the chassis organism, and optimization of orthogonal translation system components *in vitro*. This demonstration of compatibility with orthogonal translation machinery establishes iSAT as a robust platform capable of ribosome repurposing *in vitro* for ncAA incorporation, setting the stage for engineering ribosomes to polymerize novel chemistries.[6]

MATERIALS AND EXPERIMENTAL DETAILS

Generation of *E. coli* Strains and Plasmids. The strains and plasmids used in this study are listed in Table 1. To generate MRE600/pEVOL, MRE600 cells were made electro-

Table 1. Strains Used in This Study

name	genotype	RF-1 present?	pEVOL present?
MRE600	Δrna	yes	no
C321.ΔA.705	ΔprfA ΔendA Δgor Δrne	no	no
MRE600/pEVOL	Δrna	yes	yes
C321.ΔA.705/pEVOL	ΔprfA ΔendA Δgor Δrne	no	yes

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competent by first growing cells to an OD_{600} of 0.6 in LB-Lennox medium at 37 °C, then rapidly chilling them on ice, and washing them three times with 20% glycerol. Cells were resuspended in 20% glycerol at an OD_{600} of 50 and flash-frozen at −80 °C for storage using liquid nitrogen. Ten nanograms of the pEVOL plasmid was transformed into electrocompetent MRE600 cells at 4 °C using electroporation, and bacterial colonies harboring the plasmid were selected for on LB-agar plates containing 30 μg mL^{-1} chloramphenicol.

Strain Culture and Harvest. *E. coli* cell strains for S150 extract and TP70 preparations were grown in 1 L of 2×YPTG Tunair shake flasks. Strains with the MRE600 background were grown at 37 °C, and those with the C321.ΔA.705 background were grown at 34 °C. Cell strains containing the pEVOL plasmid, regardless of the strain background, were induced with 0.02% arabinose at OD_{600} values of 0.6−0.8 to express OTS components off the plasmid. All cell strains were harvested at OD_{600} values of 3.0−3.3, and the resulting cell pellets washed twice in S150 lysis buffer [20 mM Tris-chloride (pH 7.2 at 4 °C), 100 mM ammonium chloride, 10 mM magnesium chloride, 0.5 mM EDTA, and 2 mM dithiothreitol] and flash-frozen in liquid nitrogen. Pellets were stored at −80 °C until they were used for extract preparation.

Preparation of S150 Extracts. S150 crude cell-free extracts were prepared from each cell strain as previously reported.[33,35] Protein concentrations of each S150 extract were measured using the Bradford assay with bovine serum albumin (BSA) as a standard.

Total Protein of the 70S Ribosome (TP70) Preparation. TP70 was prepared from ribosomes isolated from the MRE600 crude cell extract in the process of producing the MRE600 S150 ribosome-free extract as previously described.[33−35]

Preparation of Orthogonal Translation System Components. Orthogonal translation system (OTS) parts were prepared as previously reported.[41−43] Briefly, for the ncAAs pAcF and pAzF, 100 mM aliquots of each ncAA were prepared in a 0.1 N sodium hydroxide solution. The linear DNA template of the orthogonal tRNA transzyme (o-tz-tRNA) was amplified from the pY71-o-tz-tRNA plasmid backbone by polymerase chain reaction using primers T7tRNA500-f (5′-CCGAAGGTAACTGGCTTCAGCAGAG-3′) and T7tRNAopt-r (5′-TGGTCCGGCGGAGGGGATTT-GAACCCCTG-3′). His-tagged pAzF and pAcF o-tRNA synthetases were overexpressed in NiCO(DE3) cells for 3 h postinduction at an OD_{600} of 0.6 with 0.3 mM IPTG. Cells were lysed using sonication and synthetases purified from the lysate using Ni-NTA agarose resin (Qiagen), dialyzed into aaRS storage buffer [15 mM Tris-acetate (pH 8.2), 21 mM magnesium acetate, 90 mM potassium acetate, 200 mM potassium glutamate, and 20% (w/v) glycerol] overnight, and concentrated to 10 mg mL^{-1}.

iSAT Batch Reactions. iSAT reactions were performed at 37 °C at a 15 μL scale as previously reported,[32,33,35] with the addition of a crowding agent, 2% PEG-2000, and a reducing agent, 2 mM dithiobutylamine, to improve iSAT activity.[34] iSAT reactions for S150 extracts from each cell strain were optimized for concentrations of magnesium glutamate, DNA templates, TP70, and orthogonal translation system components when necessary to maximize reaction productivity and minimize consumption of parts.

sfGFP Quantification. sfGFP quantification was performed as previously reported,[33−35,44] using measurements of relative fluorescence units (RFU) from a model CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA) and a BioTek (Winooski, VT) Synergy 2 plate reader. RFU values were converted to molar concentrations using a linear standard curve made in house by expressing [^{14}C]-leucine-labeled sfGFP in *E. coli* PANOx CFPS reactions and relating RFUs to the trichloroacetic acid precipitable soluble protein yield.

Full-Length sfGFP Purification. All synthesized sfGFP variants [wild type (wt), T216TAG, N212TAG/T216TAG, and 5TAG] were purified via iSAT reactions as previously described[41−43] using Gravity flow Strep-Tactin Sepharose columns (IBA Lifesciences) and then concentrated using 10 kDa molecular weight cutoff Microcon centrifugal filters (EMD Millipore).

Mass Spectrometry. Top-down mass spectrometry analysis was performed for purified wt sfGFP, sfGFP-T216TAG, sfGFP-N212TAG/T216TAG, and sfGFP-5TAG as previously reported.[41−43]

RESULTS AND DISCUSSION

Selection and Generation of *E. coli* Strains Suitable for ncAA Incorporation in iSAT. We aimed to develop an optimized *in vitro* platform for building ribosomes that could manufacture proteins with site-specifically incorporated ncAAs. The goal was not to engineer the ribosome to be better than a natural ribosome at incorporating specific ncAAs, but rather to demonstrate that iSAT and existing orthogonal translation systems were able to cooperate in producing protein(s) with multiple ncAAs.

The iSAT platform leverages a ribosome-free S150 crude extract to enable the efficient transcription of template-derived rRNA. iSAT co-activates rRNA synthesis and processing, ribosome assembly, and translation in a one-pot reaction. Historically, the *E. coli* strain used for generating cell-free, ribosome-free, S150 extracts used to perform iSAT is MRE600[32] due to the rRNA-stabilizing influence of ribonuclease A (Δrna) deletion. However, we hypothesized that strain MRE600 may be a suboptimal chassis for enabling ncAA incorporation by amber suppression in iSAT due to the presence of active ribosomal release factor 1 (RF-1, *prfA*). RF-1 can outcompete ncAA-loaded suppressor tRNAs at amber codons intended to encode ncAAs to instead terminate translation.[45]

To assess ncAA compatibility with iSAT, we used a genomically recoded chassis organism based on an *E. coli* strain lacking RF-1, called C321.ΔA, to generate S150 extracts for iSAT use. C321.ΔA is unique because all occurrences of the amber stop codon (TAG) in the lysate source strain have been genomically recoded to the TAA codon, which permits the deletion of RF-1 and reassignment of the amber codon translation function for a defined ncAA.[46] This alleviates poor protein expression yields and inefficient incorporation of multiple identical ncAAs by amber suppression that otherwise arises from RF-1 competition.[31,47] Cell-free protein synthesis systems derived from this genomically recoded strain have been previously engineered to remove nucleases (e.g., C321.ΔA.705), enabling high-level (more than grams per liter) expression of pure proteins harboring tens of ncAAs (Table 1).[41,42] Here, we set out to test the nuclease deficient, RF-1 deletion strain C321.ΔA.705 in iSAT.

To test iSAT for both MRE600- and C321.ΔA.705-based extracts for ncAA incorporation, we first transformed each organism with a pEVOL plasmid that expresses both the o-

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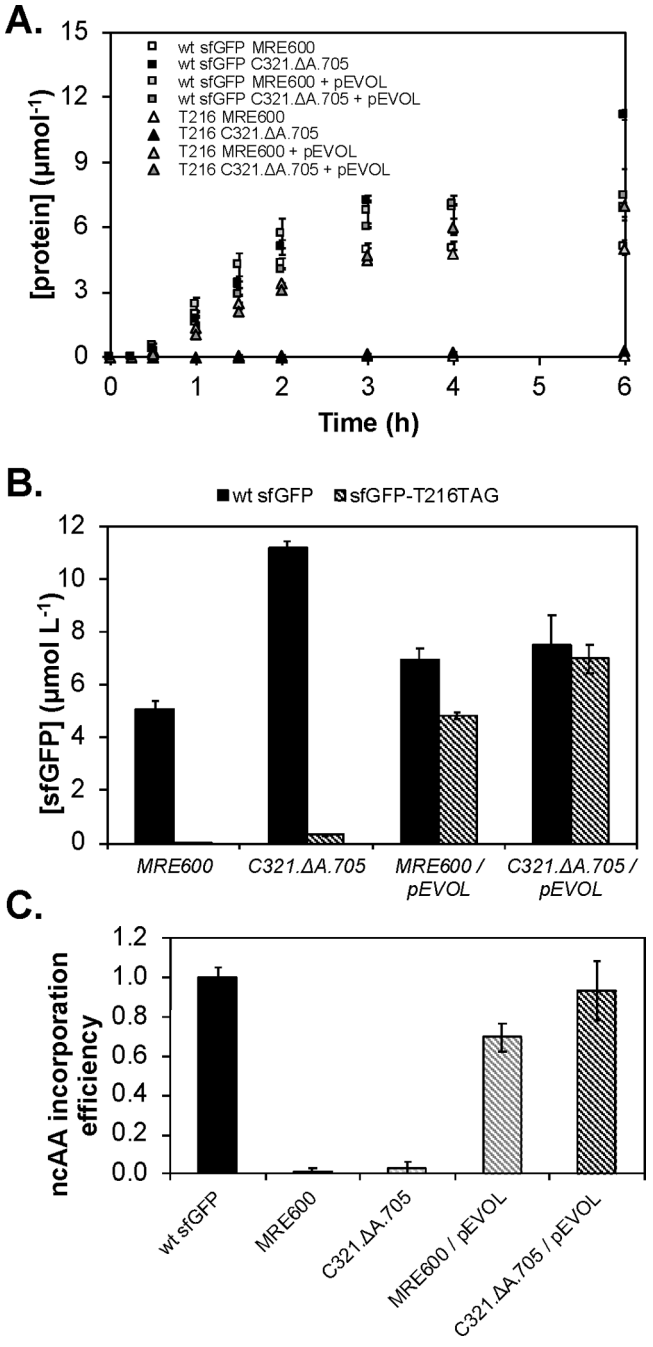

Figure 2. Comparing protein synthesis from iSAT reactions of wild-type (wt) sfGFP and sfGFP-T216TAG in S150 extract source strains. Standard batch iSAT reactions were performed at 37 °C with a plasmid template containing either wt sfGFP or sfGFP with position 216 mutated from threonine to the TAG codon (sfGFP-T216TAG). For sfGFP-T216TAG synthesis, orthogonal translation system components were also added to iSAT reaction mixtures to facilitate ncAA incorporation. Values in panels A and B represent standard deviations, and error bars represent one standard deviation from the mean, with $n \geq 3$ for n reactions. Error bars in panel C represent the propagated error. (A) Reaction kinetics of protein synthesis activity for wt sfGFP and sfGFP-T216TAG in selected strain backgrounds for S150 extracts. For all strain backgrounds, protein synthesis activity for both wt sfGFP and sfGFP-T216TAG generally proceeds at a linear rate of synthesis up to 2 h after the start of iSAT reactions before plateauing by 6 h. Exceptions were the production of sfGFP-T216TAG using MRE600 and C321.ΔA.705 S150 extracts, which was too low to notice a kinetic pattern, and wt sfGFP production using the C321.ΔA.705 S150 extract, which showed some continued protein synthesis activity up to 6 h. (B) Comparing sfGFP synthesis in S150 extract source strains at 6 h. Concentrations of wt sfGFP (solid) and sfGFP-T216TAG (striped) were measured 6 h after the start of iSAT reactions. (C) Suppression efficiency of ncAA incorporation in iSAT using different S150 extract source strains. The ncAA incorporation efficiency was normalized to the ratio of average sfGFP-T216TAG yield over wt sfGFP yield for iSAT reactions using each type of S150 extract. The value of 1.0 represents ncAA incorporation that is as efficient as wt protein synthesis.

aaRS and o-tRNA off constitutive and arabinose-inducible promoters.[48] pEVOL plasmids encoding the *Methanocaldococcus jannaschii* o-aaRS/o-tRNA pair catalyzing incorporation of the ncAAs pAcF and pAzF were used, yielding strains MRE600/pEVOL and C321.ΔA.705/pEVOL. MRE600 and C321.ΔA.705 cells with and without the pEVOL plasmid were grown to OD_{600} values of ∼3.0−3.3 before being harvested for S150 extract preparation (Figure S1). pEVOL-bearing strains were additionally induced before the exponential phase with arabinose to activate expression of orthogonal translation components and enrich the subsequent S150 extracts with the necessary component parts for ncAA incorporation. S150 extracts for each strain were prepared as previously reported.[32,33,35]

Comparing and Optimizing ncAA Incorporation in S150 Extract Types. After preparation of S150 extracts, we investigated iSAT activity in S150 extracts from each of the four strains, using sfGFP as the model reporter protein. Standard batch iSAT reactions synthesizing wt sfGFP or a mutant variant with an amber codon replacing threonine at position 216 (sfGFP-T216TAG) were performed using the selected extract types, and sfGFP levels were measured by fluorescence over time. To normalize across strains, we chose to compare iSAT activity using the optimal magnesium concentration of each strain type (10 mM for MRE600 and MRE600/pEVOL and 14 mM for C321.ΔA.705 and C321.ΔA.705/pEVOL) at the set total protein concentration (3.4 mg mL^{-1}) for all standard batch reactions (Table S1 and Figures S2 and S3).

iSAT activity among the extract types during wt sfGFP synthesis exhibited similar reaction kinetics as previously reported,[35] proceeding at a linear rate of synthesis up to 2 h after initiation before plateauing by around 6 h (Figure 2A). On the basis of these observations, we quantified reaction yields 6 h after the start of the reaction by using fluorescence readouts and a standard curve created by [^{14}C]-leucine incorporation. At 6 h, wt sfGFP yields of 5.1 ± 0.34, 11 ± 0.24, 6.9 ± 0.48, and 7.5 ± 1.2 μmol L^{-1}, were measured from S150 extracts derived from MRE600, C321.ΔA.705, MRE600/pEVOL, and C321.ΔA.705/pEVOL, respectively (Table 2 and Figure 2B).

After testing wt sfGFP expression, we assessed sfGFP-T216TAG synthesis in the presence or absence of orthogonal translation system components, based on our previous cell-free gene expression work in S30 extracts.[42] Specifically, we added 10 μg/mL linear DNA encoding optimized orthogonal tRNA in the form of a transzyme (o-tRNA) for *in situ* tRNA synthesis. Orthogonal pAcFRS was overexpressed, purified as previously described, and added at a concentration of 0.1 mg mL^{-1}. The ncAA, here pAcF, was supplied at a concentration

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Table 2. Background Cell-Free Protein Expression Activity of S150 Extracts in the Absence of the rRNA Operon Plasmid Template[a]

	MRE600	C321.ΔA.705	MRE600/pEVOL	C321.ΔA.705/pEVOL
background (−) pT7rrnb	0.03 ± 0.03 μmol L^{-1}	0.04 ± 0.03 μmol L^{-1}	0.08 ± 0.05 μmol L^{-1}	0.04 ± 0.03 μmol L^{-1}
activity (+) pT7rrnb	5.1 ± 0.34 μmol L^{-1}	11 ± 0.24 μmol L^{-1}	6.9 ± 0.48 μmol L^{-1}	7.5 ± 1.2 μmol L^{-1}
background:activity ratio	0.59%	0.36%	1.2%	0.53%

[a]Standard iSAT batch reactions for each S150 extract were performed in the absence (−) and presence (+) of the pT7rrnb rRNA operon plasmid. Wild-type sfGFP synthesis yields were measured after 6 h for each condition, and background:activity ratios were determined by dividing the average sfGFP values obtained from (−) pT7rrnb experiments to those from (+) pT7rrnb. Values represent averages, and error bars represent one standard deviation from the mean, with $n \geq 3$ for n reactions.

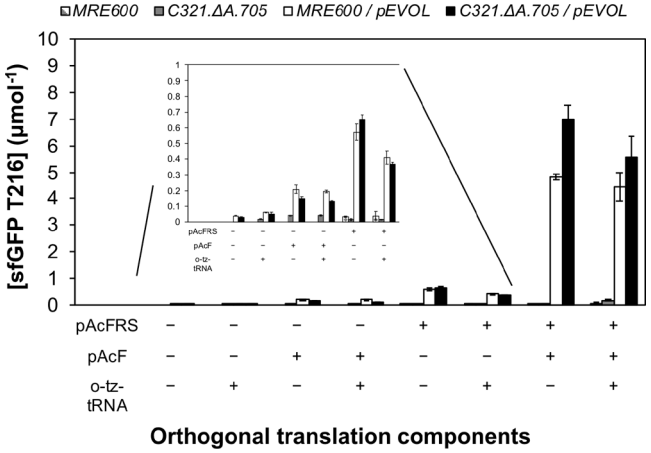

Figure 3. Orthogonal translation system components are necessary for activating ncAA incorporation in iSAT. Standard batch reactions for each type of the S150 extract synthesizing sfGFP-T216TAG were performed at 37 °C, and yields measured after 6 h. For each condition, 0.1 mg mL^{-1} pAcFRS, 2 mM pAcF, and/or 10 ng μL^{-1} o-tz-tRNA DNA template was added to (+) or omitted from (−) the reaction mixtures. The inset shows magnified data for six of eight conditions with lower levels of expression. Values represent averages, and error bars represent one standard deviation from the mean, with $n \geq 3$ for n reactions.

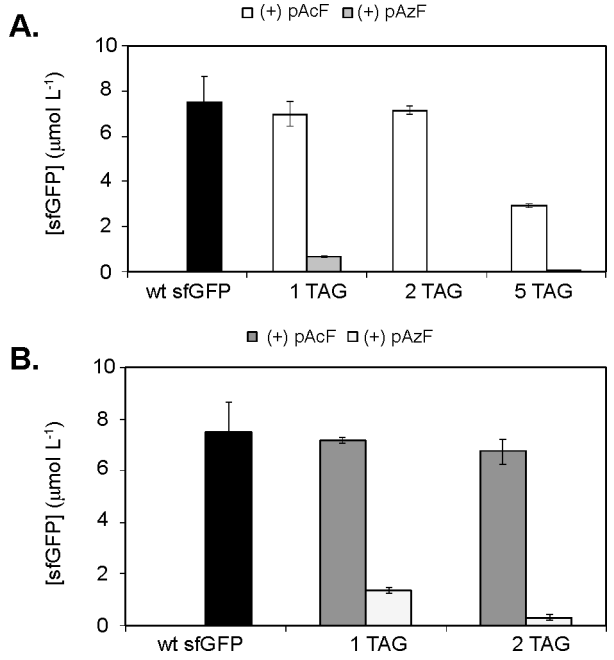

Figure 4. Incorporation of multiple ncAAs into sfGFP using iSAT. (A) C321.ΔA.705/pEVOL-derived S150 extract that can catalyze site-specific incorporation of one, two, and five ncAAs into sfGFP. Standard batch reactions were performed using C321.ΔA.705/pEVOL S150 extracts to synthesize sfGFP containing zero, one, two, and five amber codon sites in the presence (+) or absence (−) of 2 mM pAcF. Yields of each sfGFP variant were measured after 6 h by fluorescence. (B) C321.ΔA.705/pEVOL S150 extract can incorporate the noncanonical amino acid *p*-azido-phenylalanine into the sfGFP reporter protein. Standard batch reactions were performed with the addition of 0.1 mg mL^{-1} *p*-azido-phenylalanine (pAzF) orthogonal synthetase in the presence (+) or absence (−) of 2 mM pAzF. Reporter protein synthesis yields for wt sfGFP, sfGFP-T216TAG, and sfGFP- 2TAG were measured after 6 h by fluorescence. Values represent averages, and error bars represent one standard deviation from the mean, with $n \geq 3$ for n reactions.

of 2 mM in each CFPS reaction. Total protein yields were quantified by sfGFP fluorescence. Extracts created from cell strains possessing the pEVOL orthogonal translation system expression system synthesized more sfGFP-T216TAG than those from strains without pEVOL (Figure 2B). By normalizing the efficiency of ncAA incorporation as the ratio of sfGFP-T216TAG synthesized to wt sfGFP synthesized, we observed that, as expected, S150 extracts made from C321.ΔA.705/pEVOL outperformed the other extracts tested, incorporating pAcF at a suppression efficiency of 93% to yield 7.0 ± 0.55 μmol L^{-1} sfGFP-T216TAG (Figure 2C). The MRE600/pEVOL S150 extract performed second best with a 70% suppression efficiency, yielding 4.8 ± 0.12 μmol L^{-1} sfGFP-T216TAG. For extracts made from strains without the pEVOL system, the ncAA suppression efficiency was <3%, at 0.06 ± 0.007 μmol L^{-1} sfGFP-T216TAG for the MRE600 S150 extract and 0.34 ± 0.02 μmol L^{-1} for C321.ΔA.705. We also synthesized a second sfGFP variant with a single amber codon at position E132, and yields were similar to those of the T216 variant (Figure S4).

We next carried out a series of optimization reactions to enhance ncAA incorporation in iSAT reactions by adjusting concentrations of the exogenously supplied orthogonal translation system components. We first tested sfGFP-T216TAG synthesis in all S150 extract types in the presence (+) or absence (−) of individual orthogonal translation system components, specifically, orthogonal pAcF synthetase (pAcFRS), pAcF, and orthogonal pAcF tRNA transzyme DNA template (o-tz-tRNA) (Figure 3). We used a transzyme DNA template instead of adding purified orthogonal tRNA because previous studies have shown that o-tRNA transcribed in crude cell-free extracts acquires post-transcriptional processing and is more active.[42,49] It is noteworthy that addition of purified pAcFRS seems to be required for high-level sfGFP-T216TAG synthesis regardless of the extract chassis background. This was expected from strains not possessing pEVOL (MRE600 and C321.ΔA.705) as they did not express any orthogonal translation system components

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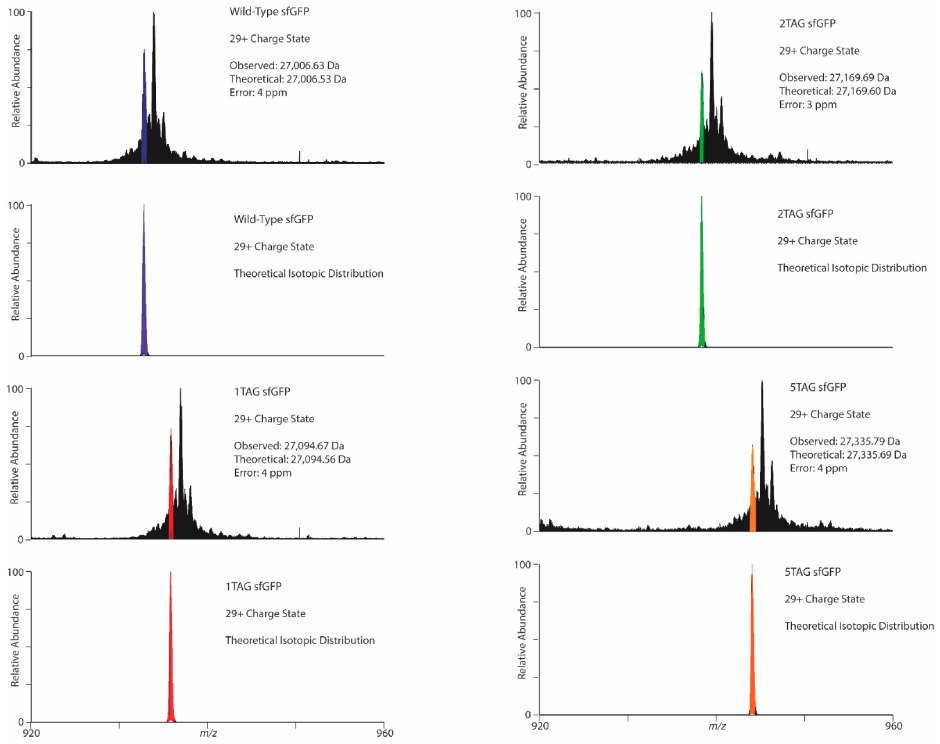

Figure 5. Mass spectrometry analysis demonstrates complete, site-specific incorporation of ncAAs in the iSAT system. Spectra of the +29 charge state of wild-type sfGFP, sfGFP-T216TAG, sfGFP-2TAG, and sfGFP-5TAG demonstrating site-specific incorporation of pAcF. The major peaks, colored, in each spectrum correspond to the theoretical peaks for each species. Peaks to the right of major peaks are a result of protein oxidation, which is a common electrochemical reaction that occurs during electrospray ionization. Water loss events from sfGFP were detected at minor levels left of the major peaks.

during cell growth, but not for MRE600/pEVOL and C321.ΔA.705/pEVOL, which express pAcFRS constitutively off the vector. This result suggested that endogenous concentrations of pAcFRS in the strain were not sufficient for enabling robust ncAA incorporation in iSAT. In addition, we observed that in the absence of the ncAA pAcF but in the presence of pAcFRS, some background read-through was observed: 0.3−0.7 μmol L^{-1} sfGFP was synthesized in RF1 deletion strains despite the omission of the ncAA.

On the basis of these results, we selected C321.ΔA.705/pEVOL as the chassis for ncAA incorporation in iSAT and sought to optimize orthogonal translation system component concentrations to maximize sfGFP yields while minimizing background read-through. From this investigation, the optimal component concentrations were determined to be ∼1−2 mM pAcF, 0.1 μmol L^{-1} pAcFRS, and 0 ng μL^{-1} o-tz-tRNA, which when used together yielded 7.0 μmol L^{-1} sfGFP-T216TAG (Figure S5).

Expanding ncAA Incorporation to Multiple Sites and Different ncAAs in iSAT. We then tested the efficacy of iSAT in incorporating multiple identical and/or different ncAAs using the S150 extract derived from C321.ΔA.705/pEVOL. To achieve this, iSAT was used to synthesize wt sfGFP, sfGFP-T216TAG, sfGFP with two amber codons at positions N212 and T216 (sfGFP-2TAG), and sfGFP with five amber codons at positions D36, K101, E132, D190, and E213 (sfGFP-5TAG). We observed synthesis of 7.1 μmol L^{-1} sfGFP-2TAG, which is comparable to the level of 7.0 μmol L^{-1} observed with sfGFP-T216TAG. As seen previously,[47] this particular 5TAG construct results in misfolding of sfGFP (Figure 4A). While only small improvements in protein synthesis yields of sfGFP-T216TAG were observed when comparing extracts in C321.ΔA.705/pEVOL versus MRE600/pEVOL (Figure 3), protein synthesis yields were >2-fold higher for sfGFP-2TAG and sfGFP-5TAG (Figure S6). This increase in the level of protein synthesis for multiple suppression events when comparing C321.ΔA.705/pEVOL versus MRE600/pEVOL iSAT reactions was expected on the basis of refs 31 and 47. This also highlights the superiority of C321.ΔA.705/pEVOL extracts as compared to those from MRE600/pEVOL for cell-free reactions that link iSAT and genetic code expansion. To demonstrate the system's capacity for incorporation of different ncAAs, we also performed incorporation of the ncAA pAzF at up to two amber codons in sfGFP (Figure 4B).

To confirm incorporation of ncAAs at all encoded positions, we purified wt sfGFP, sfGFP-T216TAG, sfGFP-2TAG, and sfGFP-5TAG from iSAT reactions incorporating pAcF (Figure S7) and analyzed their protein composition using top-down liquid chromatography-mass spectrometry (LC-TDMS). Intact masses for all sfGFP variants were determined, demonstrating >90% pAcF incorporation at one, two, and five sites with a <4 ppm difference between theoretical and experimental masses (Figure 5). Note that because the mass of pAcF is higher than those of all naturally occurring amino acids, any mis-incorporations would occur at a mass lower than those of the recoded sfGFP-T216TAG, sfGFP-2TAG, and sfGFP-5TAG peaks. The absence of significant peaks in these regions confirmed successful, efficient, and pure ncAA incorporation into sfGFP using iSAT.

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CONCLUSIONS

We demonstrate that the iSAT platform is capable of site-specific incorporation of the ncAAs pAcF and pAzF at multiple sites into sfGFP. These results were achieved by selecting an *E. coli* chassis strain for the S150 ribosome-free extract that was genomically modified for enhanced ncAA incorporation (i.e., ΔprfA), overexpressing o-tRNA and other orthogonal translation system machinery within the strain, and optimizing orthogonal translation system components *in vitro*. Using our optimized S150 platform derived from strain C321.ΔA.705/pEVOL, we demonstrate synthesis of 7.0 ± 0.55 μmol L^{-1} sfGFP-T216TAG at an ∼93% suppression efficiency and a high incorporation efficiency.

Activating ncAA incorporation in iSAT enables a number of applications. First, iSAT's ncAA compatibility can be expanded through application of other orthogonal translation systems. Here we demonstrated incorporation of pAcF and pAzF using an engineered *M. jannaschii* o-aaRS/tRNA system, but for other ncAAs, different orthogonal translation systems can be implemented. Second, the iSAT platform is now poised for follow-on efforts in genetic code reprogramming. While site-specific incorporation of noncanonical α-amino acids into peptides and proteins works well for finely tuned systems, there is poor compatibility with the natural ribosome for numerous classes of other noncanonical monomers (e.g., backbone-extended δ, γ, ε, etc., amino acids), leading to inefficiencies. iSAT can in the future be combined with ribosome display, in a platform call RISE,[37] to engineer the ribosome for altered chemical properties. Utilization of iSAT with flexizyme-aminoacylated tRNAs would be necessary for the incorporation of amino acids without aaRSs.[27,50−57]

Looking forward, we anticipate that by decoupling cell survival from ribosomal function, the cell-free, ribosome-free iSAT platform described here will facilitate an improved understanding of molecular translation and enable efforts to repurpose and engineer ribosomes for synthetic biology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00829.

Figures S1−S7 and Table S1 (PDF)

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Author Contributions

M.C.J. and Y.L. conceived the study. Y.L. designed and performed experiments and analysis. R.G.D. and P.M.T. performed mass spectrometry experiments and analysis. Y.L. and M.C.J. wrote the manuscript. M.C.J. and N.L.K. played a supervisory role.

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Notes

The authors declare no competing financial interest.

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