Nonorthogonal tRNA\textsubscript{cys}\textsubscript{Amber} for protein and nascent chain labeling

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ABSTRACT

In vitro-transcribed suppressor tRNAs are commonly used in site-specific fluorescence labeling for protein and ribosome-bound nascent chains (RNCs) studies. Here, we describe the production of nonorthogonal \textit{Bacillus subtilis} tRNA\textsubscript{cys}\textsubscript{Amber} from \textit{Escherichia coli}, a process that is superior to in vitro transcription in terms of yield, ease of manipulation, and tRNA stability. As cysteinyl-tRNA synthetase was previously shown to aminoacylate tRNA\textsubscript{cys}\textsubscript{Amber} with lower efficiency, multiple tRNA synthetase mutants were designed to optimize aminoacylation. Aminoacylated tRNA was conjugated to a fluorophore to produce BODIPY FL-cysteinyl-tRNA\textsubscript{cys}\textsubscript{Amber}, which was used to generate ribosome-bound nascent chains of different lengths with the fluorophore incorporated at various predetermined sites. This tRNA tool may be beneficial in the site-specific labeling of full-length proteins as well as RNCs for biophysical and biological research.

Keywords: aminoacylation; fluorescence labeling; ribosome-bound nascent chain; suppressor tRNA; time-resolved fluorescence anisotropy

INTRODUCTION

The ability to label a specific protein at a defined location is a key requirement for fluorescence spectroscopy, and it is achieved through various established protocols in the field of protein chemistry. Fluorescence spectroscopy of ribosome-bound nascent chains (RNCs) has recently been used to increase our understanding of aspects of protein biogenesis, such as protein trafficking (Flanagan et al. 2003; Woolhead et al. 2004; Holtkamp et al. 2012), chaperone recognition (Lin et al. 2012), and cotranslational protein folding (Ellis et al. 2008; Knight et al. 2013). Unfortunately, the presence of the ribosome hampers standard labeling of RNCs, necessitating the use of alternative strategies. With the exception of a recently applied biarsenical fluorescein derivative (FIAsH) (Lampropoulou et al. 2014), fluorophores are introduced into RNCs during translation through the use of either (i) fluorescent unnatural amino acids (Saraogi et al. 2011) or (ii) tRNA charged with a standard amino acid, which is subsequently modified with a fluorophore (Flanagan et al. 2003; Woolhead et al. 2004; Ellis et al. 2008; Lin et al. 2012).

Although the aforementioned techniques can label newly formed chains selectively, they are limited in different aspects. While the expansion of the genetic code with fluorescent amino acids and orthogonal tRNA is an efficient labeling system, it can only be performed with an environment-sensitive fluorophore [l-(7-hydroxycoumarin-4-yl) ethylglycine] (Wang et al. 2006), which hinders interpretation of the derived biophysical data (e.g., FRET distances). In contrast, aminoacylated suppressor tRNA (either lysine-specific [Flanagan et al. 2003] or cysteine-specific [Gubbens et al. 2010]) can be modified with various fluorophores, and these can be incorporated into the protein through cognate stop-codon recognition. However, artificial preparation of suppressor tRNA is costly, as it must be performed through in vitro transcription. Lastly, while isolation of tRNA from cells can be efficient, it is generally restricted to tRNA\textsubscript{lys} and tRNA\textsubscript{fMet}. In the case of the former, site-specific incorporation of a single fluorophore is largely restricted to rare proteins with a single lysine (Lin et al. 2012), because lysine residues are highly abundant in proteins. The use of tRNA\textsubscript{fMet} is also limited, as it allows only N-terminal protein labeling (Ellis et al. 2008; Knight et al. 2013).

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To overcome the above limitations, we developed a method of producing suppressor tRNA through overexpression in *Escherichia coli*. This system allows (i) site-specific labeling and (ii) incorporation of an environment-insensitive fluorophore, and offers the additional advantages of (iii) involving simple purification procedures and (iv) being low-cost. Multiple aminoacyl-tRNA synthetase mutants were designed to increase the aminoacylation efficiency, as well as the overall yield, based on the available crystal structure (Hauenstein et al. 2004). The performance of the engineered tRNA is evaluated in the *E. coli* cell-free system, which is used to produce single-labeled RNCs with the fluorophore incorporated at different sites.

**RESULTS AND DISCUSSION**

**Suppressor tRNA design and production**

Our objective was to develop a procedure that would utilize the cellular machinery of *E. coli* that would prepare suppressor tRNA in ample amounts. In addition, the suppressor tRNA needed to be purified from the endogenous *E. coli* RNA with minimum manipulation. Therefore, to simplify the purification of target tRNA from the small RNA (sRNA) pool, *Bacillus subtilis* (Bsu) tRNA was overexpressed in *E. coli*; the distinct sequence differences between *E. coli* and Bsu tRNA enabled the latter to be easily isolated using an anti-Bsu oligonucleotide probe (Fig. 1A; Supplemental Fig. 1). Moreover, Bsu tRNA<sup>59</sup> can be readily aminoacylated by the *E. coli* cysteinyl-tRNA synthetase candidate for large-scale aminoacylation, we also prepared tRNA<sup>59,UGC</sup> (wild type) as a control. To determine the most suitable cysteinyl-tRNA synthetase mutant for further preparation of cysteinyl-tRNA<sub>cys</sub> for charging *Bsu* tRNA<sup>UGC</sup>, *Bsu* tRNA<sup>59,Amber</sup> were calculated by using aminoacylation assays to examine the incorporation of 35S-L-cysteine in tRNA precipitated with trichloroacetate (TCA). Our goal was to identify an enzyme with an increased reaction rate toward *Bsu* tRNA<sup>59,Amber</sup>; such an enzyme may have a profound influence on overall yields, as the equilibrium of the aminoacylation reaction is based on charging and hydrolysis of the ester bond (Bonnet and Ebel 1972). Compared with the wild-type enzyme, the D436S mutant exhibited a fivefold increase in the aminoacylation efficiency for charging *Bsu* tRNA<sup>59,Amber</sup>. In addition, a 16-fold decrease in the aminoacylation efficiency of charging *Bsu* tRNA<sup>59,UGC</sup> was observed (Table 2), which may benefit the production of *Bsu* cysteinyl-tRNA<sup>59,Amber</sup> in the event of contamination with tRNA<sup>59,UGC</sup>. The aminoacylation plateau for D436S mutant was ~25% of theoretical aminoacylation yield (assuming 1 A<sub>260</sub> unit is 1600 pmol of tRNA) toward *Bsu* tRNA<sup>59,Amber</sup> while the wild-type enzyme did not reach the plateau during the assay (30 min). Therefore, the D436S mutant was used for further preparation of cysteinyl-tRNA<sup>59,Amber</sup> in this study. The aminoacylated tRNA was conjugated with BODIPY FL maleimide (Fig. 1C) and the overall efficiency was estimated using the absorbance at 260 and 504 nm, expressed as the fraction of tRNA labeled with BODIPY FL (Table 3).

In addition, the high enrichment of *Bsu* tRNA<sup>59,Amber</sup> in sRNA raised the possibility that the purification procedure may be simplified. Specifically, we investigated the effects of omitting the oligonucleotide probe capture step. As such, the oligonucleotide-captured tRNA was aminoacylated and conjugated to BODIPY FL (henceforth referred to as purified† tRNA, Table 4), or the sRNA pool was directly aminoacylated and reacted with a fluorophore (henceforth referred to as crude‡ tRNA, Table 4). It should be noted that

**Cysteinyl-tRNA synthetase mutants and aminoacylation**

The isolated tRNA was aminoacylated with cysteine using our engineered cysteinyl-tRNA synthetase mutants (Hauenstein et al. 2004). These mutations are designed to compensate for the dramatic decrease in aminoacylation efficiency for tRNAs (e.g., tRNA<sup>59,Amber</sup>) engineered in the anticodon loop (Komatsoulis and Abelson 1993), which includes the changes in the base size and the pairing of hydrogen bond donor/acceptor, as compared to tRNA<sup>59,UGC</sup>. For example, 6-O in G34 is a hydrogen bond acceptor for cysteine and ATP, which suggests that the mutations do not influence the catalytic activity of the enzyme (Table 2). The reaction rates of cysteinyl-tRNA synthetase mutants acting on *Bsu* tRNA<sup>59,UGC</sup> and *Bsu* tRNA<sup>59,Amber</sup> were calculated by increased production of the desired tRNA.
the crude‡ tRNA may also contain BODIPY FL-cysteinyl-tRNAcysUGC (from E. coli) in addition to Bsu BODIPY FL-cysteinyl-tRNAcysAmber, which may result in nonspecific labeling of synthesized protein on cysteine codons during translation. Based on the absorbance measurement at 260 and 504 nm (Table 3), ~20%–25% of purified† tRNA was labeled. In comparison, crude‡ tRNA labeling yields typically reached 18%–21%, suggesting ~70% of sRNA is composed of overexpressed Bsu tRNAcysAmber. When crude‡ tRNA was prepared with wild-type cysteinyl-tRNA synthetase, the overall labeling yields were lower (12%–15%), suggesting either a lower aminocacylation plateau for tRNAcysAmber or the aminocacylation did not reach equilibrium during the experiment.

### Stability of overexpressed and in vitro-transcribed tRNA

Previous studies have shown that cysteinyl-tRNA possesses an apparently low half-life (Gubbens et al. 2010). Therefore, the stability of BODIPY FL-cysteinyl-tRNAcysAmber in cell extract was characterized. The apparent half-lives of overexpressed and in vitro-transcribed BODIPY FL-cysteinyl-tRNAcysAmber are ~40 and 25 min, respectively (Fig. 2), which are sufficient for the production of RNCs under common experimental conditions. Therefore, although the purification of overexpressed-tRNA may be more labor intensive than the purification of tRNA transcribed in vitro (Table 4; Table 1: Yields of sRNA under the indicated induction conditions for tRNA overexpression).

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>sRNA yield (mg/g of cells)</th>
</tr>
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<tbody>
<tr>
<td>30°C, 5 h</td>
<td>1.5–2</td>
</tr>
<tr>
<td>42°C, 5 h</td>
<td>3–4</td>
</tr>
<tr>
<td>42°C, 1 h + 42°C, 1 mM IPTG, 4 h</td>
<td>5–6</td>
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</tbody>
</table>

*Initial growth: 30°C until cell OD600 ~0.6.

**Range of three independent experiments.**
Lukavsky and Puglisi 2004; Easton et al. 2010; Koubek et al. 2013), the former is advantageous in its stability.

**Specificity of Bsu BODIPY FL-cysteinyl-tRNA<sub>Amber</sub>**

We determined the specificity of BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> prepared as either crude tRNA or purified tRNA using a model protein, Entner–Doudoroff aldolase (Eda, 213 amino acids, M<sub>w</sub> ~22 kDa, PDB: 1FQO) (Wymer et al. 2001). An internal nonsense mutation was introduced into the coding sequence of eda, which produces BODIPY FL-labeled full-length protein and stalled RNCs (Fig. 3A–C) if the prepared tRNA works as intended. The commercial FluoroTect (BODIPY FL-lysyl-tRNA<sub>lys</sub>) was used if the prepared tRNA works as intended. The incorporation of 35S-L-cysteine in tRNA precipitated with trichloroacetate (TCA) and expressed as the first-order reaction rate.

The enzymes were trapped on a nitrocellulose filter, and the amount of radioactive [35S]-cysteinyl-adenylate bound to the active sites was measured. Reaction rates of the prepared cysteinyl-tRNA synthetase mutants toward Bsu tRNA<sub>UGC</sub> and Bsu tRNA<sub>Amber</sub> were calculated by using aminoacylation assays to examine the incorporation of 35S-L-cysteine in tRNA precipitated with trichloroacetate (TCA) and expressed as the first-order reaction rate.

**TABLE 2. Site-burst activity assays were performed to confirm that cysteinyl-adenylate is readily formed by all of the examined mutant cysteinyl-tRNA synthetases**

| Mutant   | C<sub>280</sub> (µM)<sup>a</sup> | C<sub>burst</sub> (µM)<sup>b</sup> | Enzyme active<sup>c</sup> (%) | Reaction rate (min<sup>−1</sup>)
<table>
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<tbody>
<tr>
<td>Wild type</td>
<td>64</td>
<td>16</td>
<td>25</td>
<td>&gt;100&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>R427K</td>
<td>81</td>
<td>27</td>
<td>33</td>
<td>&gt;100&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>R427E</td>
<td>58</td>
<td>12</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>D436S</td>
<td>87</td>
<td>17</td>
<td>20</td>
<td>5.8</td>
</tr>
<tr>
<td>R439E</td>
<td>95</td>
<td>27</td>
<td>29</td>
<td>1.2</td>
</tr>
<tr>
<td>R427E/D436S</td>
<td>100</td>
<td>27</td>
<td>27</td>
<td>1.3</td>
</tr>
<tr>
<td>R427E/D436N</td>
<td>120</td>
<td>26</td>
<td>21</td>
<td>6.0</td>
</tr>
<tr>
<td>R427K/D436Q</td>
<td>96</td>
<td>18</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>R427E/D436e</td>
<td>80</td>
<td>26</td>
<td>33</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

The enzymes were trapped on a nitrocellulose filter, and the amount of radioactive [35S]-cysteinyl-adenylate bound to the active sites was measured. Reaction rates of the prepared cysteinyl-tRNA synthetase mutants toward Bsu tRNA<sub>UGC</sub> and Bsu tRNA<sub>Amber</sub> were calculated by using aminoacylation assays to examine the incorporation of 35S-L-cysteine in tRNA precipitated with trichloroacetate (TCA) and expressed as the first-order reaction rate.

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**TABLE 3. Yields of producing BODIPY FL labeled tRNA**

<table>
<thead>
<tr>
<th>tRNA&lt;sup&gt;53&lt;/sup&gt;Amber</th>
<th>CysRS</th>
<th>BODIPY FL</th>
<th>Gel filtration</th>
<th>Yield&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA&lt;sup&gt;53&lt;/sup&gt;Amber</td>
<td>D436S</td>
<td>+</td>
<td>+</td>
<td>20–25</td>
</tr>
<tr>
<td>sRNA D436S</td>
<td>+</td>
<td>+</td>
<td>18–21</td>
<td></td>
</tr>
<tr>
<td>sRNA WT</td>
<td>+</td>
<td>+</td>
<td>12–15</td>
<td></td>
</tr>
<tr>
<td>sRNA D436S</td>
<td>−</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as fraction of BODIPY FL labeled tRNA (measured by absorbance at 504 nm) and total tRNA, considering 1 A<sub>260</sub> contains 1600 pmol of tRNA.

FL-labeled Eda was observed when a wild-type construct was used, while no protein was detected when expressed from a control containing D55<sup>+</sup> ([*]Amber mutation) (Fig. 3B, lanes 1,2). In contrast, no BODIPY FL-labeled Eda was observed when purified tRNA was used (Fig. 3B, lane 5). The addition of the pET21a-eda(D55<sup>+</sup>) vector into extracts containing purified tRNA resulted in the production of BODIPY FL-labeled protein, which suggests high reactivity and specificity of BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> (Fig. 3B, lane 6). Crude tRNA also suppressed the Amber stop-codon in pET21a-eda(D55<sup>+</sup>) and produced fluorophore-attached Eda (Fig. 3B, lane 4). Most notably, labeled Eda was not observed when the wild-type construct and crude tRNA were applied (Fig. 3B, lane 3). A similar result was also observed when Eda<sub>72</sub> RNC was produced (Fig. 3C). We surmise that these phenomena may result from the low tRNA<sub>Amber</sub> abundance in the sRNA pool or in the cells (Ikemura 1981), a low abundance of UGC/UGU codons in the genes (von Ehrenstein 1967; Ikemura 1981), competition between endogenous cysteinyl-tRNA<sub>Amber</sub> and BODIPY
FL-cysteinyl-tRNA\textsubscript{cys}{\textsuperscript{UGC}}, or the lower aminoacylation efficiency of tRNA\textsubscript{cys}{\textsuperscript{UGC}} by the cysteinyl-tRNA synthetase D436S mutant as compared to the wild-type enzyme. Our results suggest that crude\textsuperscript{‡} tRNA can be used to directly label proteins and nascent chains without the interference from nonspecific labeling.

The suppression efficiency for the full-length protein was quantified by Western blot analysis of the translated products. We observed that in the presence of crude\textsuperscript{‡} tRNA, eda(D55\textsuperscript{*}) could be translated with 80\% ± 20\% efficiency compared to Eda (wild type) (Fig. 4A; lanes 2,3,7,8 in Fig. 4B). In addition, cysteinyl-tRNA\textsubscript{cys}{\textsuperscript{Amber}} could also suppress Amber stop codons with slightly lower efficiency (50\% ± 5\% compared to the wild type, Fig. 4A; lane 4 in Fig. 4B), as described in a previously published report (Gubbens et al. 2010). Most important, only traces of full-length proteins were detected when tRNA\textsubscript{cys}{\textsuperscript{Amber}} (nonaminoacylated) was directly applied into the reaction (0.5\% ± 0.2\% compared to the wild type, Fig. 4A; lanes 5,9 in Fig. 4B). The low suppression efficiency of uncharged tRNA\textsubscript{cys}{\textsuperscript{Amber}} in the aforementioned cases may be the consequence of the weak

**FIGURE 2.** Stability of BODIPY FL-cysteinyl-tRNA\textsubscript{cys}{\textsuperscript{Amber}} in cell extracts following generation by overexpression or in vitro transcription. (A) A typical NaOAc-Urea gel showing decreases of fluorescently labeled cysteinyl-tRNA over time (in minutes). (B) Signals are evaluated as relative fluorescent decrease of a given sample. An averaged signal is fitted with a single exponential decay curve. Error bars represent standard deviation at a given time point (n = 4).

**FIGURE 3.** (A) Suppression of the Amber stop codon by BODIPY FL-cysteinyl-tRNA\textsubscript{cys}{\textsuperscript{Amber}}. RNase H-cleaved mRNA results in stalled RNCs of the desired size. (B) BODIPY FL-labeled full-length protein was produced by Amber stop-codon suppression with either crude\textsuperscript{‡} or purified\textsuperscript{†} tRNA. (C) Production of BODIPY FL-labeled Eda\textsubscript{72} RNC. The presence of RNCs was confirmed by puromycin assay. (D) A typical Tris–acetate gel of fluorescently labeled RNCs with different chain lengths (namely, Eda\textsubscript{72}, Eda\textsubscript{111}, Eda\textsubscript{161}, and Eda\textsubscript{FL}).
aminoacylation efficiency of the E. coli cell extract toward the uncharged tRNAcys\textsuperscript{Amber}. Therefore, the application of crude\textsuperscript{‡} tRNA probably leads to the high extent of BODIPY FL labeling. The observed amber suppression efficiencies were similar to those previously reported for E. coli extract, e.g., 50% yield of wild-type protein was reached with 2.5 μM tRNA\textsuperscript{Ser}\textsuperscript{Amber} (Agafonov et al. 2005), 89% yield of the wild-type was reported with the use of 2.5 μM tRNA\textsuperscript{Amber} and aptamer against E. coli release factor 1 (RF1) (Sando et al. 2007); the use of the orthogonal Amber suppression system led to 50%–120% wild-type expression levels as well (Smolskaya et al. 2013).

To estimate the absolute amount of labeled proteins after translation, we performed a medium-scale cell-free reaction (200–300 μL) and isolated the products using C-terminally fused His-tag. The amount of BODIPY FL Eda was measured by the absorbance of BODIPY FL at 504 nm [ε ∼ 0.08 (cm × μM)\textsuperscript{-1}] in the fraction eluted from the Ni\textsuperscript{2+} column with 500 mM imidazole. Only samples with BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber} (3 μM) and pET21a-\textit{eda}(D55\textsuperscript{∗}) (Fig. 4C) absorbed at 504 nm with translational yields 3.4 ± 1.9 pmol of BODIPY FL-Eda in 10 μL original reaction, suggesting >10% of BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber} is used for the production of BODIPY FL-Eda. Lack of BODIPY FL signal in the negative control excluded the possibility of contamination from the free BODIPY FL or BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber}. In fact, the produced BODIPY FL-Eda should be even more than 3.4 ± 1.9 pmol as the labeled protein could be lost during the purification. In addition, 30%–40% proteins were found in the pellet fraction after translation (Fig. 4D).

**FIGURE 4.** (A) Comparison of expression yield of Eda wild-type (Eda WT) with the yield of Eda D55\textsuperscript{∗} in the presence of crude\textsuperscript{‡} tRNA [Eda (D55\textsuperscript{∗}) + crude\textsuperscript{‡} tRNA], cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber} [Eda (D55\textsuperscript{∗}) + cys-tRNA\textsuperscript{cys}\textsuperscript{Amber}], and uncharged tRNA\textsuperscript{cys}\textsuperscript{Amber} [Eda (D55\textsuperscript{∗}) + sRNA]. (B) Western blot of translational reaction with different components (lanes 1–6) and Ni\textsuperscript{2+} column-purified proteins (fraction eluted with 500 mM imidazole, lanes 7–9). Three times higher volume was loaded to lane 9 compared with lanes 7 and 8. Proteins were detected with anti-Flag primary antibodies. (C) Absorbance measurement of 500 mM imidazole-eluted fractions from 300 μL cell-free reaction containing 3 μM BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber} without vector (negative control) or with pET21a-Flag-eda(D55\textsuperscript{∗}). The volume of each fraction was adjusted to 100 μL. (D) Western blot of supernatant and pellet fraction using translational reaction containing pET21a-Flag-eda(D55\textsuperscript{∗}) and BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber}. Protein was detected with anti-Flag primary antibodies.

**Production of various RNCs with Bsu BODIPY FL-cysteinyl-tRNA\textsuperscript{cys}\textsuperscript{Amber}**

Different fluorescence spectroscopies of RNCs have been applied for the protein biogenesis studies. In fact, single fluorophores attached to RNCs already provide useful information due to the anisotropic sensitivity of the probe. Fluorescence depolarization is a complex process that may be described with multiexponential decay (Lakowicz 2006) due to the different types of depolarization events experienced by the fluorophore (Szabo 1984).

For a fluorophore attached to a macromolecule where the fluorophore can undergo segmental motions due to a
nonrigid bond and the macromolecule moves independently, anisotropy ($r$) of fluorescence in time ($t$) equals

$$r(t) = r_0(f_0 e^{-t/\theta_f} + f_s e^{-t/\theta_s}),$$  \hspace{1cm} (1)

where $r_0$ is the fundamental anisotropy (anisotropy of a frozen solution), $\theta_f$ is a rotational correlation time for the hindered fluorophore’s segmental motion, and $\theta_s$ is the rotational correlation time of the macromolecule itself. $f_0$ is the amplitude of the segmental motion and $f_s$ is the amplitude of global motion while

$$f_0 + f_s = 1. \hspace{1cm} (2)$$

This implies that if $f_0 = 1$, then the fluorophore is rigidly bound to the macromolecule, while if $f_0 = 1$, the fluorophore moves without any restriction imposed by the bond. Therefore, the amplitudes describe the freedom of movement of the fluorophore to the macromolecule. In the case of three independent motions (given for example by various different nonrigid bonds between the fluorophore and the macromolecule), anisotropy decay can be expressed as

$$r(t) = r_0(f_0 e^{-t/\theta_f} + f_1 e^{-t/\theta_1} + f_s e^{-t/\theta_s}),$$ \hspace{1cm} (3)

where $f_0$ and $f_1$ represent different segmental motions, index $f_0$ is given to unrestricted motion, and

$$f_0 + f_1 + f_s = 1. \hspace{1cm} (4)$$

Therefore, $f_0$ can still be interpreted as the extent of the fluorophore segmental motion.

Time-resolved fluorescence anisotropy has been applied in the characterization of N-terminally labeled RNCs. These studies revealed the presence of partially folded nascent chains (Ellis et al. 2008), the possible conformation inside the ribosomal tunnel (Ellis et al. 2009), and nascent chain adhesion to the ribosomal surface (Knight et al. 2013). Their conclusions were based on the presence of slow ribosome rotation ($f_0$), tumbling of the partially folded nascent chain ($f_1$), or fast local rotation of the fluorophore ($f_0$) that the fluorophore experiences (Weinreis et al. 2010). In addition, MD simulations have shown that only the environment near the probe can be investigated (Schröder et al. 2005). Using site-specific labeling together with time-resolved anisotropy may reveal information on the nascent chain dynamics in the vicinity of the probe and shed light on protein biogenesis. Such studies would require relatively large amounts of fluorophore-attached tRNA due to the necessary systematic approach that may need to be applied (probing various sites and various chain lengths), which can be easily fulfilled by our method. Therefore, we would like to demonstrate the performance of Bsu BODIPY FL-cysteinyl-tRNA<sup>59</sup>Amber by preparing various chain lengths of Eda RNCs with BODIPY FL at different sites.

We prepared Eda RNCs with four different chain lengths (Eda<sub>72</sub>, Eda<sub>11</sub>, Eda<sub>161</sub>, and Eda<sub>full length</sub> (FL); Fig. 3D) labeled at four different positions at the N terminus, namely, K5, T14, L36, and D55 (Fig. 5A). We confirmed the production of fluorophore-labeled Eda(D55<sup>∗</sup>) RNC with four different chain lengths, as revealed through puromycin-release assays on a fluorescence gel (Fig. 3D). The successful incorporation of the fluorophore at different positions in the 72 amino acid long Eda RNCs [denoted as Eda(K5<sup>∗</sup>)<sub>72</sub>, Eda(T14<sup>∗</sup>)<sub>72</sub>, Eda(L36<sup>∗</sup>)<sub>72</sub>, and Eda(D55<sup>∗</sup>)<sub>72</sub>] was demonstrated using time-resolved fluorescence anisotropy. Various anisotropy decays were expected for fluorophores labeled at different sites. For example, the fluorophore movement in Eda(D55<sup>∗</sup>)<sub>72</sub> should have been highly restricted as the probe was still inside the ribosomal tunnel (Fig. 5B) and only a limited amount of configurations were available for the fluorophore after excitation. In contrast, Eda(K5<sup>∗</sup>)<sub>72</sub> was expected to be away from the ribosomal tunnel exit (Fig. 5B), which allowed a higher extent of local probe movement and thus smaller confinement. The fluorophore confinements of Eda(T14<sup>∗</sup>)<sub>72</sub> and Eda(L36<sup>∗</sup>)<sub>72</sub> were expected within the range given by Eda(K5<sup>∗</sup>)<sub>72</sub> and Eda(D55<sup>∗</sup>)<sub>72</sub>.

The deconvoluted amplitude of slow movement ($f_0$) for Eda(K5<sup>∗</sup>)<sub>72</sub> was slightly smaller than for Eda(T14<sup>∗</sup>)<sub>72</sub> (Table 5) but significantly smaller than Eda(L36<sup>∗</sup>)<sub>72</sub> and Eda(D55<sup>∗</sup>)<sub>72</sub>. The amplitude for the slow movement in Eda(L36<sup>∗</sup>)<sub>72</sub> was smaller than Eda(D55<sup>∗</sup>)<sub>72</sub>, as expected. The high constraint in Eda(L36<sup>∗</sup>)<sub>72</sub> (0.78 ± 0.02) was probably given by the fluorophore position near the ribosome tunnel exit, i.e., 36 amino acids from the peptidyl transfer center (Lu and Deutsch 2005). The difference in the recovered anisotropies between Eda(K5<sup>∗</sup>) and Eda(T14<sup>∗</sup>), as well as Eda(L36<sup>∗</sup>) and Eda(D55<sup>∗</sup>), became more prominent with increasing chain length (data not shown). In short, both the fluorescence gel analysis and the anisotropy decay of Eda<sub>72</sub> suggested the successful site-specific labeling on the predetermined sites.

Overall, we believe that the production of tRNA<sup>59</sup>Amber by overexpression will be of broad utility for protein-related research. This material can be produced in large quantities (Table 4), and only minimal manipulation is required to prepare ample amounts of BODIPY FL-cysteinyl-tRNA<sup>59</sup>Amber for the incorporation of fluorophores at any given site in either proteins or RNCs. These properties promise to be invaluable for studies that require large quantities of fluorophore-labeled proteins/RNCs.

**MATERIALS AND METHODS**

**Preparation of the cysteinyl-tRNA synthetase**

Cysteinyl-tRNA synthetase (CysRS) construct with the C-terminal His-tag was prepared from *E. coli* DH5α genomic DNA using primers CysS F and CysS R. The construct was modified using site-directed mutagenesis to generate mutants R427K, R427E, D436S, R439E, R427E/D436S, R427E/D436N, R427K/D436Q, and R427E/D436E with listed F/R oligonucleotides (Supplemental Table 1). All CysRS construct were transformed into *E. coli* BL21 (DE3) strain and cells (200 mL culture) were grown to OD ~0.6 when IPTG
was added to final 1 mM concentration. Cells were harvested after 4 h of incubation and disrupted by French-press in Lysis buffer (20 mM HEPES-KOH, 250 mM NaCl, pH = 8.0). Cell debris was separated from supernatant by centrifugation for 30 min at 15,000 g and supernatant was loaded onto 3 mL Ni-column preequilibrated with Lysis buffer. Column was washed with 12 and 9 mL of Lysis buffer with 10 and 50 mM imidazole, respectively. Cysteinyl-tRNA synthetase was eluted with Lysis buffer containing 200 mM imidazole. Eluted fraction was dialyzed against 20 mM HEPES·KOH, 20 mM NaCl (pH = 7.5) then concentrated using Amicon centrifugation cells (NWCO = 3000). After the addition of 1 mM dithiothreitol (DTT) and 50% glycerol (v/v), the enzyme was stored in −20°C.

Active site titration assay

The active site titration assay of the cysteinyl-tRNA synthetase mutants was performed at 37°C in 30 µL reactions containing 50 mM HEPES-KOH (pH = 7.5), 20 mM KCl, 10 mM MgCl₂, 5 mM DTT, 2 mM ATP, and 50 µM L-cysteine with 35S-L-cysteine (specific activity 500 µCi/mL). Reaction was started by addition of 10 mM CysRS and aliquots of 8 µL are sampled at 2, 5, and 10 min. The aliquot was filtered through nitrocellulose filter, washed extensively with 95% ethanol, and analyzed by scintillation counting. The maximum amount of detected 35S-cysteine was corresponding to the cysteinyl-adenylate bound to the active cysteinyl-tRNA synthetase.

Overexpression of Bsu tRNA and isolation of small RNA pool

The construct was originated from pUC19 plasmid in which T7 RNA polymerase promoter was added between restriction sites EcoRI and KpnI using oligonucleotides T7P F and T7P R. Bsu tRNA gene was inserted into the construct between restriction sites KpnI and PstI using insert formed from overlapping oligonucleotides filled with Klenow fragment Bsu WT F and Bsu R for tRNA⁵⁰UGC or nucleotides Bsu Amber F and Bsu R. These inserts also included several flanking nucleotides to ensure proper primary transcript processing (Kirsebom 2007). T7 RNA polymerase terminator was inserted in single PstI restriction sites by nucleotides T7T F and T7T R. Both constructs (pUC19-T7P-Bsu tRNA[WT]-T7T and pUC19-T7P-Bsu tRNA[Amber]-T7T) were then transformed into the production strain E. coli (BL21 [DE3]).

Induction conditions were screened in 200 mL cultures by varying temperature and addition of IPTG. Cells were incubated overnight at 30°C after which the temperature is increased to 42°C. After 1 h incubation at 42°C, IPTG was added to the final 1 mM concentration. After four additional hours, cells were harvested by centrifugation, and wet cell pellet was weighted. The culture was resuspended in cold extraction buffer (20 mM Tris, 10 mM MgCl₂, 1% SDS and 1 mM EDTA, pH = 7.0) and RNA was extracted by acidic phenol:chloroform (pH = 4). After centrifugation at 15,000g for 10 min, the upper aqueous layer was collected and reextracted with phenol:chloroform followed by RNA precipitation in ethanol.

**TABLE 5.** Anisotropy decay fitting results for full-length Eda labeled at different sites bound to ribosome

<table>
<thead>
<tr>
<th>Sample</th>
<th>f₁ (°)</th>
<th>θ₁ (°)</th>
<th>f₁ (°)</th>
<th>θ₁ (°)</th>
<th>f₁ (°)</th>
<th>θ₁ (°)</th>
<th>χ²&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eda(K5*)&lt;sub&gt;72&lt;/sub&gt;</td>
<td>0.58 ± 0.02</td>
<td>1000</td>
<td>0.16 ± 0.01</td>
<td>4 ± 1</td>
<td>0.26 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Eda(T14*)&lt;sub&gt;72&lt;/sub&gt;</td>
<td>0.62 ± 0.02</td>
<td>1000</td>
<td>0.15 ± 0.01</td>
<td>3 ± 1</td>
<td>0.23 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>0.41</td>
</tr>
<tr>
<td>Eda(L36*)&lt;sub&gt;72&lt;/sub&gt;</td>
<td>0.78 ± 0.02</td>
<td>1000</td>
<td>0.11 ± 0.01</td>
<td>4 ± 1</td>
<td>0.11 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.26</td>
</tr>
<tr>
<td>Eda(D55*)&lt;sub&gt;72&lt;/sub&gt;</td>
<td>0.84 ± 0.01</td>
<td>1000</td>
<td>0.08 ± 0.01</td>
<td>4 ± 1</td>
<td>0.09 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Fundamental anisotropy fixed at 0.37 (Karolin et al. 1994).<sup>a</sup>

<sup>a</sup>Set at 1000 nsec.

<sup>b</sup>Worst χ² of three independent experiments is presented.
Recovered RNA pellet was dissolved in 300 mM NaOAc (pH = 5.5) after which 0.54 volumes of isopropanol was gradually added into the solution. This mixture is incubated for 1 h at 20°C followed by centrifugation at 12,000g for 5 min at 20°C. The supernatant, which contains small RNA, was collected while the redissolved RNA pellet in 300 mM NaOAc was fractionated again with 0.54 volumes of isopropanol. After centrifugation supernatants were pooled and the isopropanol concentration was increased to 50% (v/v). RNA was precipitated overnight in −20°C and the recovered pellet was dissolved in 6× NHE buffer (1.2 M NaCl, 30 mM HEPES-KOH, 15 mM EDTA, 0.5 mM DTT, pH = 7.5) for anti-Bsu probe isolation or in refolding buffer (50 mM HEPES-KOH, pH = 7.5) for direct aminocacylation.

Isolation of Bsu tRNA

Biotinylated anti-Bsu probe was immobilized on streptavidin sepharose beads (GE Healthcare Life Sciences), which were trapped in pipette tip. Bsu tRNA<sub>Amber</sub> was isolated from the sRNA pool using a method described previously (Miyaochi et al. 2007). Briefly, 30 nmol of anti-Bsu oligonucleotide was immobilized on streptavidin sepharose beads in binding buffer (400 mM NaCl, 10 mM HEPES-KOH, 5 mM EDTA, pH = 7.5) at room temperature and the beads were trapped within a pipette tip by quartz wool. tRNA was bound to the oligonucleotide by repeated pipetting of the sRNA dissolved in 6× NHE buffer at 65°C. The streptavidin beads within the pipette tip were washed in 0.5× NHE buffer (100 mM NaCl, 2.5 mM HEPES-KOH, 1.25 mM EDTA, 0.5 mM DTT, pH = 7.5) at 42°C and eluted into 0.1× NHE buffer (20 mM NaCl, 0.5 mM HEPES-KOH, 0.25 mM EDTA, 0.5 mM DTT, pH = 7.5).

In vitro tRNA transcription

Bsu tRNA<sub>Amber</sub> was transcribed from using in house prepared T7 RNA polymerase (a generous gift from Professor Ya-Ming Hou, Thomas Jefferson University) from filled oligonucleotides Bsu T7 F and Bsu T7 R with Klenow fragment using methods described previously (Koubek et al. 2013). Transcribed tRNA was purified on Mono Q Fast Performance Liquid Chromatography (FPLC) (Koubek et al. 2013). Isolated tRNA and different sRNA preparations were analyzed on 12% TBE-Urea gel using 2 µg of each sample.

Aminocacylation reaction

Transcribed or isolated tRNA was heated to 65°C in the refolding buffer for 10 min when MgCl<sub>2</sub> is added to final concentration 15 mM. tRNA was allowed to slowly cool down to room temperature and stored in −20°C.

Aminocacylation analysis of cysteinyl-tRNA synthetase mutants was performed at 37°C in 30 µL reactions containing 50 mM HEPES-KOH (pH = 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 2 mM ATP, 50 µM CysRS, and 5 µM L-cysteine (specific activity 500 µCi/mL) and 4 µM tRNA (estimated by absorbance at 260 nm, considering 1 µg/µL has 40 µM concentration). Reaction was started by addition of 30 nM CysRS and aliquots of 5 µL were sampled every 5 min. After each reaction, aliquot was alkylated using 10 µL of 0.36 M iodoacetic acid, 0.15 M NaOAc (pH = 5.0) in formamide at 37°C for 30 min, cysteinyl-tRNA was precipitated with 10% trichloroacetic acid (TCA). The precipitate, which was filtered through 3 mm filter and washed extensively with ice-cold 10% TCA and 95% ethanol, was analyzed by scintillation counting. The synthetase efficiency was calculated as the first-order reaction constant (in minutes<sup>−1</sup>).

Preparation of BODIPY FL-cysteinyl-tRNA

For large-scale BODIPY FL-cysteinyl-tRNA preparation, 2.5 mg of tRNA<sub>Amber</sub> was reacted with 100 µM L-cysteine in 50 mM HEPES-KOH (pH 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 2 mM ATP, 50 units of pyrophosphatase (prepared as described previously) (Koubek et al. 2013) in 6 mL reaction with 250 nM CysRS D436S at 37°C for 30 min. The reaction was extracted with acidic phenol:chloroform and cysteinyl-tRNA was precipitated in ethanol. After recovering the aminocacylated tRNA, the pellet was redissolved in 1.6 mL of 50 mM Tris-HCl (pH = 7.0). Four hundred microliters of 10 mM BODIPY FL maleimide in DMSO was added into the mixture for 1 h at 20°C. After the reaction is stopped by addition of 500 µL of 3 M NaOAc (pH = 5.5), formed BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> was purified from unreacted dye on PD-10 desalting column (GE Healthcare Life Sciences) reequilibrated with 0.3 M NaOAc (pH = 5.5). Ethanol precipitated BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> was redissolved in refolding buffer (50 mM HEPES-KOH, pH = 7.5) and stored in −80°C.

Testing of BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> stability

Two micromoles of BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> (either overexpressed or transcribed in vitro) was incubated in 37°C with cell extract at 37°C and 2 µL aliquots after 0, 5, 10, 15, 20, 25, and 60 min and were immediately mixed with NaOAc-Urea Loading buffer and stored on ice. After NaOAc-Urea electrophoresis at 4°C, remaining BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> was visualized by fluorescent scanning at Typhoon using 488-nm laser and 520-nm band pass filter. The data were evaluated as a relative decrease of BODIPY FL-cysteinyl-tRNA<sub>Amber</sub> in each parallel with ImageJ as a single exponential decay.

Preparation of cell-free system

E. coli cell-free system was prepared from BL21 (DE3) as S12 extract as described previously (Kim et al. 2006), with the exception of buffers A and B which contain 60 mM KOAc instead of 60 mM potassium glutamate.

Preparation of template DNA for cell-free reaction

Entner–Douderoff aldolase construct was prepared from BL21 (DE3) genomic DNA by PCR using primers eda F and eda R and inserted into pET21a between the restriction sites NdeI and XhoI. This construct was modified with several mutations in the N-terminal coding region by replacing standard amino acids with Amber codon, namely, K5*, T14*, L36*, and D55*, by site-directed mutagenesis using respective primers (Supplemental Table 1). Flag tag was added at the 5′ end of the gene using annealed phosphorylated oligonucleotides Flag F and Flag R in NdeI cleaved pET21a-edα or pET21a-edα (D55*).

All constructs were isolated using Plasmid MidiPrep Purification Kit (Genemark). Their concentration was adjusted to 1 µg/mL.
Production of full-length Eda

For the production of BODIPY FL labeled full-length protein, a cell-free reaction was carried out in 10 µL containing polyin buffer (57 mM HEPES.KOH (pH = 8.0), 2 mM ATP, 1 mM GTP, CTP, and UTP, 2 mM DTT, 0.17 mg/mL E. coli tRNA mixture, 0.64 mM cAMP, 90 mM potassium glutamate, 34 µg/mL folic acid, 1.5 mM of each amino acid, 67 mM creatine phosphate, 2% PEG (8000), 10 µg/mL creatine kinase), 40 µg/mL of template DNA, and 1–3 µM of BODIPY FL-cysteinyl-tRNA\(^{\text{Amber}}\) or 0.1 µL of FluoroTect. After 2 h reaction in 37°C, the mixture was precipitated with 7× volume acetone, pelleted down, resuspended in 15 µL of 1× SDS PAGE loading buffer, heated up at 95°C for 2 min and resolved on Tris-Glycine SDS PAGE and detected either by Typhoon fluorescence scanning or by Western blot using anti-Flag antibodies as described previously (Huang et al. 2013).

For medium-scale labeling of purified proteins, a 200–300 µL reaction was prepared in a similar manner. After 1 h incubation, precipitated proteins were separated by centrifugation and the supernatant was mixed with 1.2 mL of binding buffer (30 mM HEPES.KOH, 250 mM NaCl, pH = 8.0). The mixture was incubated with Ni2+ Sepharose (200 µL, GE Healthcare), the resin was collected by centrifugation, washed 3× with 500 µL of binding buffer with 10 mM imidazole, once with 500 µL of binding buffer with 50 mM imidazole and eluted 2× with 250 µL of binding buffer containing 500 mM imidazole. The collected fractions were pooled and concentrated to 100 µL.

Production of RNCs

BODIPY FL-labeled RNCs were prepared in 50 µL reactions containing polyin buffer, 18 µL of BL21 (DE3) cell extract DNA [pET21a-eda(K5*)], pET21a-eda(T14*), pET21a-eda(L36*), or pET21a-eda(D55*)], 6 µg anti-ssrA oligonucleotide, 2 units RNase H (NEB), 6 µg anti-edA oligonucleotide (G72, L111, T161, or FL). After incubation at 37°C for 30 min, reactions were centrifuged at 16,000g for 10 min to remove any aggregates. Supernatant was loaded onto 200 µL 1.1 M sucrose cushion [1.1 M sucrose, 20 mM Tris-HCl, 10 mM Mg(OAc)\(_2\), 0.5 mM EDTA, 500 mM NH\(_4\)Cl, 1 mM DTT, pH = 7.0] and RNCs were pelleted by centrifugation on TLA 120.1 rotor at 200,000 g for 1 h. After the supernatant was carefully removed, the pellet was washed once in sucrose cushion and once in resuspension buffer [10 mM Tris-HCl, 10 mM Mg(OAc)\(_2\), 0.5 mM EDTA, 60 mM NH\(_4\)Cl, 1 mM DTT, pH = 7.0]. Pure RNCs were dissolved in the resuspension buffer by vigorous shaking for 1 h at 4°C. After any possible aggregates were removed by centrifugation at 16,000g for 10 min, the supernatant containing RNC was collected for further analysis.

The integrity of RNCs was tested by puromycin assay—a portion of RNCs was incubated with 1 mM puromycin for 30 min at 37°C. Peptidyl-tRNA and puromycin-released peptides were analyzed by Tris–acetate SDS PAGE (Kirkdhofer et al. 2007) and Typhoon fluorescence scanning.

Time-resolved fluorescence anisotropy measurement

The measurement was taken on a multifrequency phase and modulation fluorimeter (Chronos; ISS) equipped with a diode laser (473 nm), calcite prism polarizers, excitation filter Semrock Brightline 470/22, and emission filter Semrock Brightline 536/40. Anisotropic decay was measured by multifrequency phase and modulation technique at 10–250-MHz modulation frequencies at 25°C. Signal was corrected for independently measured G factor, each replica was measured three times, and the signals of these measurements were averaged. Fluorescent lifetime was determined from independent measurement at magic polarizer conditions with fluorescein as the lifetime standard (τ = 4.0 nsec).

Modulated anisotropy decay data were fitted with the included software package, one rotational correlation time set at 1000 nsec, and the acceptance of the fitted model was evaluated by decreasing χ\(^2\) by at least factor 2.5 as described previously (Ellis et al. 2008).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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