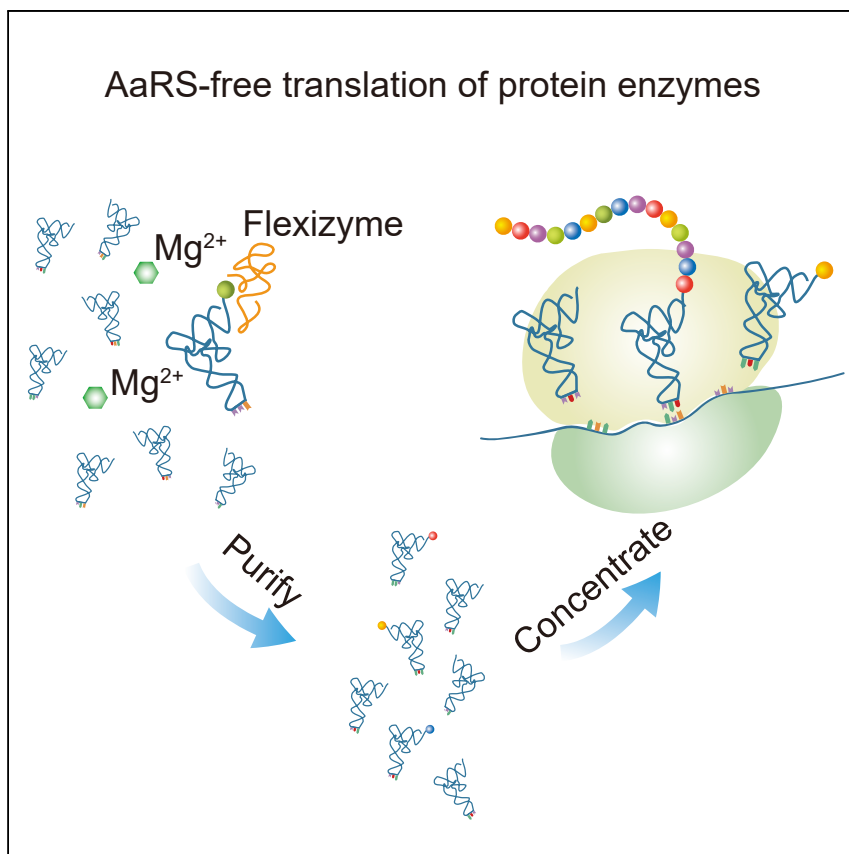


Article

Translating protein enzymes without aminoacyl-tRNA synthetases



Toward establishing a highly simplified translation apparatus without aaRS, Zhu and colleagues demonstrate the aaRS-free translation of protein enzymes using solely flexizyme-charged tRNAs. This work may greatly simplify the effort needed to realize mirror-image translation by reducing the requirement to chemically synthesize dozens of large aaRS proteins.

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HIGHLIGHTS

Improving the yield of aaRS-free translation using solely flexizyme-charged tRNAs

Translating multiple protein enzymes of distinct functions in the absence of aaRS

Producing an active aaRS (TrpRS) under aaRS-free conditions

Realizing mirror-image tRNA charging with D-amino acids using L-flexizyme



Article

Translating protein enzymes without aminoacyl-tRNA synthetases

Ji Chen,¹ Mengyin Chen,¹ and Ting F. Zhu^{1,2,*}

SUMMARY

Protein translation from the ribosome seemingly requires dozens of sophisticated aminoacyl-tRNA synthetases (aaRSs). Despite the discovery of tRNA-aminoacylating ribozymes, such as the flexizyme, synthesizing protein enzymes from highly simplified translation systems in the absence of aaRS remains undemonstrated. Here, we show the translation of multiple protein enzymes of distinct functions using solely flexizyme-charged tRNAs, through improving the translation yield by concentrating cation-depleted tRNAs. Notably, we used the aaRS-free translation system to produce an active aaRS (TrpRS), which in turn catalyzed the charging of more tRNAs. Moreover, toward realizing mirror-image translation, we performed the mirror-image tRNA charging with D-amino acids by a synthetic L-flexizyme. Our work demonstrates the feasibility of translating protein enzymes from a highly simplified translation apparatus without aaRS and drastically reduces the requirement to chemically synthesize dozens of large aaRS proteins for realizing mirror-image translation.

INTRODUCTION

The emergence of protein enzymes is key to the transition from RNA-based life to modern biology.^{1–3} The discovery of tRNA-aminoacylation ribozymes suggests the possibility of synthesizing protein enzymes from highly simplified translation systems with tRNAs charged by ribozymes.^{4–8} Additionally, other systems using pre-charged tRNAs prepared by aaRS, urzyme, and chemical acylation have also been reported.^{9–13} Among them, the highly robust and versatile tRNA-aminoacylating ribozyme, flexizyme, discovered through *in vitro* selection has been shown capable of charging a wide variety of amino acids to tRNAs.⁸ Using tRNAs charged by flexizyme and aaRS, incorporation of multiple unnatural amino acids into translated peptides was achieved, enabling the practical selection of peptide drugs.^{14–16} However, in part due to the low translation yield, when using solely flexizyme-charged tRNAs in the absence of aaRS (hereinafter referred to as “aaRS-free”), only short peptides were translated,¹⁷ whereas the ribosomal production of full-length, functional protein enzymes with all 20 proteinogenic amino acids under aaRS-free conditions has remained undemonstrated thus far.

One of the main reasons for the low yield of aaRS-free translation is that, compared with tRNA aminoacylation by aaRS,^{18,19} the flexizyme charging of tRNAs lacks recycling.⁸ In addition, the use of *in vitro* transcribed, unmodified tRNAs for aaRS-free charging also contributes to the low translation yield.^{20,21} Here, we set out to test the ability of aaRS-free systems to translate protein enzymes with all 20 proteinogenic amino acids using tRNAs charged by flexizyme. Our results show that through improving the translation yield by concentrating the flexizyme-charged,

The bigger picture

The requirement of dozens of large aminoacyl-tRNA synthetases (aaRSs) for tRNA charging presents a major hurdle in building synthetic ribosome translation systems including the mirror-image translation apparatus. The discovery of tRNA-charging ribozymes, such as the flexizyme, opens the possibility of circumventing the laborious synthesis of the aaRS proteins. Here, through improving the translation yield by concentrating cation-depleted tRNAs, we show that a highly simplified translation system using solely flexizyme-charged tRNAs is capable of producing protein enzymes of up to 334 aa with all 20 proteinogenic amino acids. Furthermore, we demonstrate the mirror-image tRNA charging with D-amino acids using a synthetic L-flexizyme, suggesting the feasibility of realizing mirror-image translation in a similar aaRS-free translation system.

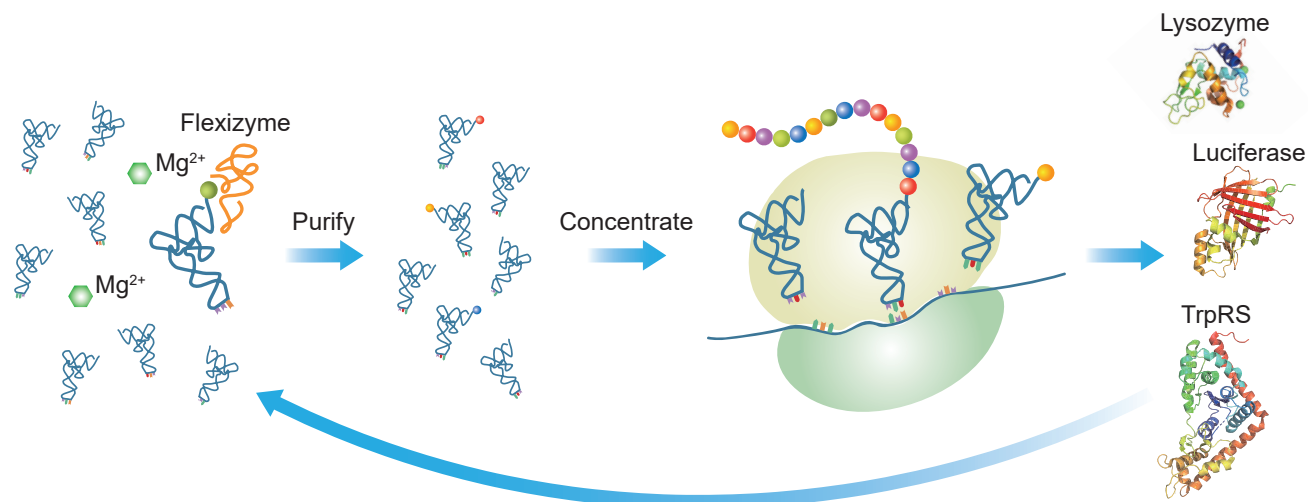


Figure 1. Schematic overview of aaRS-free translation of protein enzymes solely with flexizyme-charged tRNAs

The tRNAs are charged by flexizyme, generating a population of tRNAs charged with all 20 proteinogenic amino acids for the translation of protein enzymes. The charged tRNAs are purified by HPLC to reduce Mg^{2+} contamination and concentrated for aaRS-free translation. The aaRS (e.g., TrpRS) translated by the aaRS-free system can, in turn, catalyze the charging of more tRNAs. PDB sources: 1DPX (lysozyme), 5B0U (luciferase), 5V0I (TrpRS).

cation-depleted tRNAs, multiple protein enzymes of distinct functions, such as the lysozyme, luciferase, and even aaRS itself can be translated (Figure 1). Moreover, we carried out the charging of mirror-image (L-) tRNAs with mirror-image (D-) amino acids by a synthetic mirror-image (L-) flexizyme, which will enable the future realization of a highly simplified mirror-image translation apparatus.

RESULTS

Improving the yield of aaRS-free translation

We first analyzed the aaRS-free translation system to address the apparent low yield issue.¹⁷ Our rationale was that the yield of aaRS-free translation might be improved by increasing the concentrations of flexizyme-charged tRNAs to compensate for the lack of tRNA recycling. Earlier studies showed that adding excessive tRNAs in *Escherichia coli* (*E. coli*) translation systems with aaRS inhibited the translation,^{22,23} which was attributed to uncharged tRNAs competing out the charged tRNAs by occupying the ribosomal A-site²² or cation imbalance from the addition of large amounts of tRNAs.²³ However, all of these experiments were performed in the presence of aaRS, and thus the exact charging yields were not determined, making it difficult to differentiate between the influence of inefficient charging and altered cation (e.g., Mg^{2+}) concentrations.

To evaluate the effect of tRNA concentrations and charging yields on aaRS-free translation, we performed the aaRS-free translation of a short peptide using fluorescein (FAM)-labeled phenylalanine (Fph-tRNA^{fMet}) to facilitate the quantification of translation yields¹⁷ (Figures 2A, 2B, S1, and S2). The mRNA template was decoded by tRNA^{fMet}, tRNA^{Lys}, tRNA^{Tyr}, and tRNA^{Asp}, among which tRNA^{fMet} was charged with Fph-tRNA^{fMet} by the 45-nt enhanced flexizyme,¹⁷ and the others were charged with their cognate amino acids by the 46-nt dinitro-flexizyme.⁸ Unmodified tRNAs transcribed *in vitro* by the T7 RNA polymerase were used, as they have been shown to operate in ribosomal peptide synthesis assays.²⁴ The individual charging yield for each tRNA was determined by polyacrylamide gel electrophoresis under acidic conditions (acid PAGE), which was used to deduce the weighted average (overall)

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<https://doi.org/10.1016/j.chempr.2021.01.017>

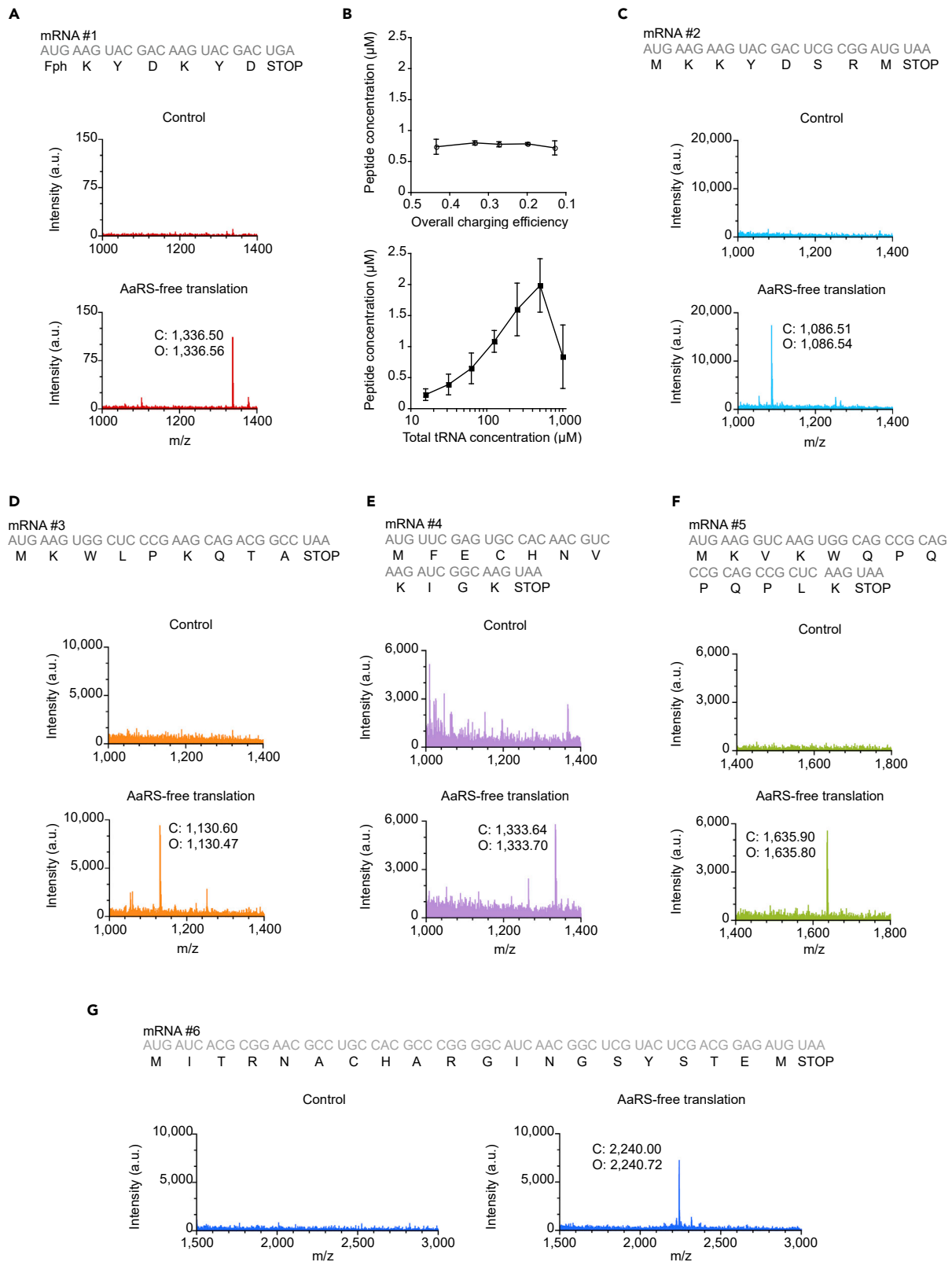


Figure 2. AaRS-free translation of short peptides

(A) MALDI-TOF-MS analysis of translated short peptides from mRNA #1

(B) AaRS-free translation yield of short peptides, analyzed by Tricine-SDS-PAGE (Figures S2A and S2B): upper, uncharged tRNA concentrations ranged from 160–540 μM while the flexizyme-charged tRNA concentration remained at 70 μM , resulting in charging yields ranging from 44%–13%; lower, total tRNA concentrations ranged from 16–1,003 μM while the charging yield remained at 56%. Error bar, standard deviations from three independent experiments.

(C–G) MALDI-TOF-MS analysis of translated short peptides from mRNA #2 to mRNA #6. Control: translation with 55–135 μM of total uncharged tRNAs and 100 μM of each amino acid (for mRNA #1, 5 μM of flexizyme-charged FAM-labeled Fph-tRNA^{fMet} was added to both the control and aaRS-free translation experiments). AaRS-free translation: aaRS-free translation with 170–414 μM of total flexizyme-charged tRNAs (Table S1). a.u., arbitrary units; C, O, calculated and observed *m/z* values, respectively.

charging yield of the translation system (Table S1). We first performed tRNA titration by adding charged total tRNAs with an overall charging yield of $\sim 44\%$ (mixed Fph-tRNA^{fMet}: Lys-tRNA^{Lys}: Tyr-tRNA^{Tyr}: Asp-tRNA^{Asp} at 1:2:2:2 molar ratio) and total tRNA concentrations from 20–644 μM in the final translation system, and discovered that the translation yield reached the highest level when the total tRNA concentration was at ~ 160 μM , and without plateauing, the translation yield decreased upon further increases of tRNA concentrations (Figure S1A). We attributed the observed inhibition not to the flexizyme buildup in the translation system, because in a control experiment, the addition of purified dinitro-flexizyme to a fixed amount of total tRNAs did not inhibit the translation (Figure S1B). Next, we added 90–470 μM of uncharged tRNAs to the aaRS-free translation system in the presence of 70 μM charged tRNAs, with the overall charging yield decreased from 44% to 13%, but the overall translation yield remained largely unaffected (Figure 2B).

We reasoned that another factor that could be responsible for the observed translation inhibition was the increased cation concentration due to Mg^{2+} carryover from the flexizyme-charged tRNAs. To test this theory, we added exogenous flexizyme and tRNAs with MgCl_2 to the aaRS-free translation system and discovered that the translation was indeed inhibited by increased MgCl_2 carryover (Figures S1C and S1D). Enlightened by this result, we used the high-performance liquid chromatography (HPLC) equipped with a C18 column to purify the flexizyme-charged tRNAs to reduce the Mg^{2+} concentrations (except Fph-tRNA^{fMet}, which was treated by ultrafiltration instead to minimize fluorescence quenching). This process also removed most of the flexizyme and modestly improved the overall charging yield from 44% to 56% (Figure S3). We then added the purified, flexizyme-charged tRNAs to the aaRS-free translation system and discovered that the translation yield was significantly improved by 5-fold as a result of concentrating the flexizyme-charged tRNAs. An additional 2-fold improvement was observed upon reducing the Mg^{2+} contamination by HPLC, resulting in a ~ 10 -fold overall improvement of translation yield (Figures 2B and S1A), with the optimal total tRNA concentration shifted from 160 to 500 μM . However, when tRNA concentrations further increased from 500 to 1,000 μM , the overall translation yield reduced by about 50%, potentially resulting from the Mg^{2+} associated with Fph-tRNA^{fMet}. Moreover, similar titration assays with high concentrations of uncharged tRNAs in the presence of aaRS did not lead to improvement of translation yield (Figure S4), suggesting that the improvement of aaRS-free translation yield likely resulted from the increased concentrations of flexizyme-charged tRNAs per se.

AaRS-free translation of short peptides

Having discovered that concentrating the flexizyme-charged, cation-depleted tRNAs improved the yield of aaRS-free translation, we sought to test the aaRS-free translation on multiple short peptides and determine the translation fidelity under high flexizyme-charged tRNA concentrations. We obtained a minimal set of 21 *E.*

coli tRNAs through *in vitro* transcription by the T7 RNA polymerase, including one tRNA (tRNA^{fMet}) for translation initiation and 20 others for translation elongation (Table S2). Each tRNA was separately charged by flexizyme with charging yields ranging from 20%–60% (Figure S5A). The flexizyme-charged tRNAs were mixed at a molar ratio according to the abundance of their cognate codons on the mRNA before being added to the aaRS-free translation system to a final concentration ranging from 170–520 μM (Table S1). We designed and *in vitro* transcribed five distinct mRNA sequences that allowed Watson-Crick base pairing to the anticodon of flexizyme-charged tRNAs, and the aaRS-free translated short peptides were evaluated by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) to examine the translation fidelity (Figures 2C–2G).

The MALDI-TOF MS results showed that all 21 flexizyme-charged tRNAs accurately decoded the mRNAs with up to ~ 200 -fold molar excess over the ribosome (e.g., 414 μM tRNAs: 2 μM ribosome, with mRNA #5). In the control experiments with uncharged tRNAs and free amino acids (Figures 2C–2G), no peptide products were detected, thus minimizing contamination concerns of aaRS and charged tRNAs from ribosome preparation. Notably, we encoded a short message “MITR-NACHARGINGSYSTEM” into mRNA #6 (Figure 2G) and successfully translated the full-length information-carrying peptide. However, when we increased the total tRNA concentration to 520 μM , an additional +12 Da peak was detected (Figure S6), potentially due to mRNA misdecoding resulting from the high tRNA concentration and use of unmodified tRNAs for translation.

AaRS-free translation of protein enzymes

Encouraged by the successful translation of short peptides, we carried out the aaRS-free translation of protein enzymes solely with *in vitro* transcribed, unmodified tRNAs charged by flexizyme. We first chose two small enzymes, the 130-aa chicken lysozyme and the 169-aa *Gaussia* luciferase, as models. Neither of the enzymes is native to *E. coli* and thus minimizes contamination concerns from ribosome preparation. A subset (underlined amino acids in Figures S7A and S7B) of the 21 flexizyme-charged tRNAs were purified by HPLC to reduce Mg^{2+} carryover, and the individual charging yields after HPLC purification were determined by acid PAGE (Figure S5B), resulting in an overall charging yield of $\sim 40\%$. The total tRNA concentrations of ~ 330 μM for chicken lysozyme and ~ 430 μM for *Gaussia* luciferase (Table S1) were approximately ~ 10 - to 20-fold higher than those used in other *in vitro* translation systems.^{17,20,24}

We first tested the aaRS-free translation of the full-length proteins using the FAM-labeled Fph-tRNA^{fMet} reporter. Analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the fluorescently labeled product bands were consistent with the molecular weight of the chicken lysozyme and *Gaussia* luciferase (14.8 and 18.7 kDa, respectively), and the mobility of the product bands was also similar to that of commercial chicken lysozyme and recombinant *Gaussia* luciferase, respectively (Figures 3A, 3C, and S8A–S8D). In comparison, the product bands were absent in the control experiments lacking DNA templates, or with uncharged tRNAs and free amino acids, respectively. These results suggested that aaRS-free translation was sufficiently processive to accomplish the synthesis of full-length proteins before the flexizyme-charged tRNAs were hydrolyzed. Attempts to characterize the aaRS-free translated proteins from the excised product bands using liquid chromatography-tandem mass spectrometry (LC-MS/MS) were unsuccessful due to ribosomal protein contamination (however, this was addressed by translating a larger protein with a molecular weight more different from those of the ribosomal proteins, as described below). Next, we replaced the FAM-labeled Fph-tRNA^{fMet} with

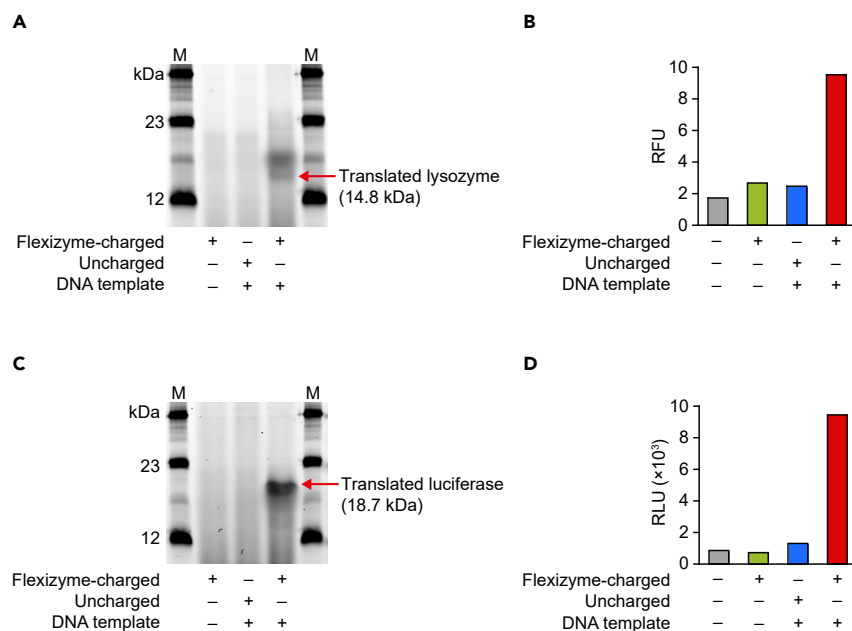


Figure 3. AaRS-free translation of protein enzymes

(A) AaRS-free translation of N-terminal FAM-labeled chicken lysozyme, analyzed by 15% SDS-PAGE, scanned by Typhoon FLA 9500 under Cy2 mode. M, Benchmark fluorescent protein standard.

(B) Enzymatic assay of crude aaRS-free translated chicken lysozyme, with fluorescently labeled bacterial (*Micrococcus lysodeikticus*) cell wall materials as substrates. RFU, relative fluorescence unit.

(C) AaRS-free translation of N-terminal FAM-labeled *Gaussia* luciferase, analyzed by 15% SDS-PAGE, scanned by Typhoon FLA 9500 under Cy2 mode. M, Benchmark fluorescent protein standard.

(D) Enzymatic assay of crude aaRS-free translated *Gaussia* luciferase, with coelenterazine as substrate. RLU, relative luminescence unit.

unlabeled Met-tRNA^{fMet} for translation initiation and performed enzymatic assays to test whether the translated proteins can fold correctly *in vitro* and possess their corresponding catalytic activities. The results showed that after incubation for up to 24 h in the folding buffers (see [Experimental procedures](#)), the aaRS-free translated enzymes carried out the catalysis of their corresponding substrates: the chicken lysozyme released FAM-labeled cell debris and the *Gaussia* luciferase emitted bioluminescence, respectively ([Figures 3B](#) and [3D](#)), whereas the control experiments lacking DNA templates, or with uncharged tRNAs and free amino acids, did not generate detectable signals, thus minimizing contamination concerns of auto-fluorescence or contaminating luminescence from the aaRS-free translation system. Comparing the emitted bioluminescence of the aaRS-free translated *Gaussia* luciferase with known standards of recombinant luciferase suggested a translation yield of ~ 25 nM ([Figure S9](#)), which was ~ 80 -fold lower than the maximal yield of the aaRS-free translation of a 7-aa peptide ([Figure 2B](#)), likely as a result of the lower availability of flexizyme-charged tRNAs for each translated codon, as well as the limited folding efficiency of the *Gaussia* luciferase with multiple disulfide bonds.²⁵

AaRS-free translation of aaRS

Next, we sought to explore the possibility for the aaRS-free translation system to produce functional aaRS itself, an important step in establishing a proposed self-reproducing translation apparatus.^{26,27} Here, we used the 334-aa *E. coli* TrpRS as a model. A

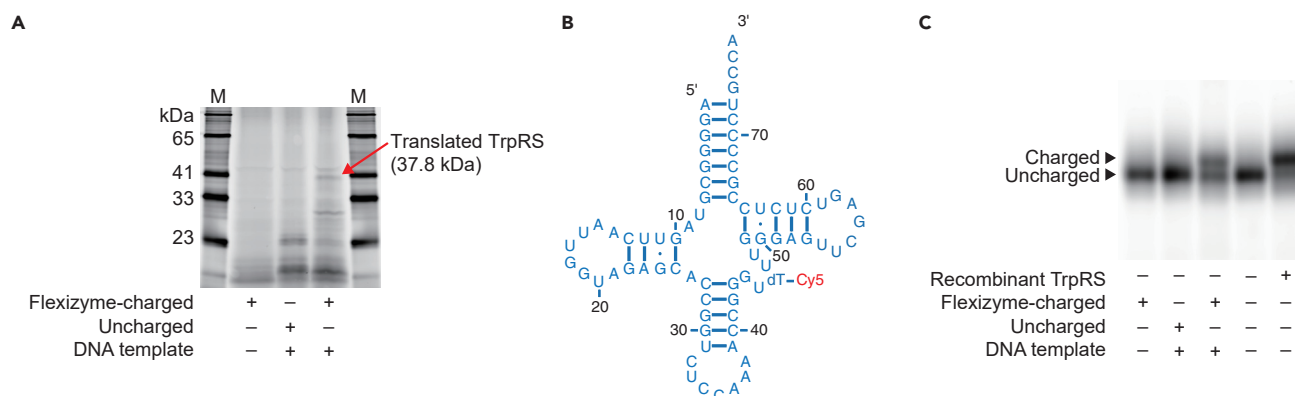


Figure 4. AaRS-free translation of TrpRS

(A) AaRS-free translation of N-terminal FAM-labeled *E. coli* TrpRS, analyzed by 15% SDS-PAGE, scanned by Typhoon FLA 9500 under Cy2 mode. M, Benchmark fluorescent protein standard.

(B) Sequence and secondary structure of internally Cy5-labeled tRNA^{Trp}.

(C) Enzymatic assay of crude aaRS-free translated TrpRS, with Cy5-tRNA^{Trp} as substrate, analyzed by 8% acid PAGE, and scanned by Typhoon FLA 9500 under Cy5 mode.

large portion (14 out of 21 in total) of the *in vitro* transcribed flexizyme-charged tRNAs were purified by HPLC to reduce Mg^{2+} carryover (underlined amino acids in Figure S7C), resulting in an overall charging yield of $\sim 42\%$ and total tRNA concentration of $\sim 170 \mu M$ (Table S1). Again, we used the FAM-labeled Fph-tRNA^{fMet} reporter to test the translation of the full-length protein, and a product band indicative of the 334-aa *E. coli* TrpRS (37.8 kDa) was observed by SDS-PAGE (the mobility of the fluorescently labeled protein band was similar to that of recombinant TrpRS, Figures S8E and S8F), whereas this band was absent in the control experiments lacking DNA templates, or with uncharged tRNAs and free amino acids (Figure 4A). We also observed several faster migrating bands, which likely correspond to the truncated TrpRS translation products and unused Fph-tRNA^{fMet} (Figure S8G). To further confirm the aaRS-free translation of TrpRS, we analyzed the protein content from the excised product band using LC-MS/MS and identified four non-overlapping peptide segments from *E. coli* TrpRS, resulting in a sequence coverage of $\sim 16\%$ (Table S3). In comparison, no peptides corresponding to *E. coli* TrpRS were detected in the control experiment with uncharged tRNAs and free amino acids, suggesting that the detected TrpRS was not from endogenous aaRS contamination.

To further examine the tRNA-aminoacylating activity of the aaRS-free translated TrpRS, we designed and synthesized an internally Cy5-labeled tRNA substrate (Cy5-tRNA^{Trp}, Figure 4B), allowing the *in situ* detection of the charged Cy5-tRNA^{Trp} without interference from other charged tRNA species. Using Met-tRNA^{fMet} for translation initiation, and after the TrpRS was translated, we added Cy5-tRNA^{Trp} along with tryptophan and adenosine triphosphate (ATP) to the aaRS-free translation system. The aaRS-free translated TrpRS successfully charged tryptophan onto Cy5-tRNA^{Trp}, whereas in the control experiments lacking DNA templates, or with uncharged tRNAs and free amino acids, no Cy5-tRNA^{Trp} charging was observed (Figure 4C), suggesting that the observed Cy5-tRNA^{Trp} charging activity was unlikely due to endogenous aaRS contamination from ribosome preparation or flexizyme charging.

AaRS-free charging of mirror-image tRNAs

After the realization of mirror-image genetic replication, transcription, and reverse transcription,^{28,29} the next major step in establishing the mirror-image central

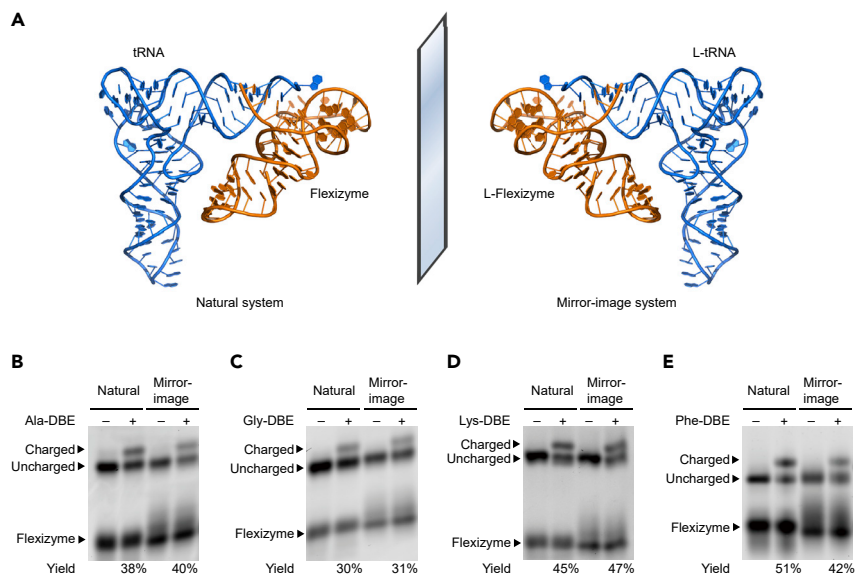


Figure 5. AaRS-free charging of mirror-image tRNAs

(A) D-tRNA charging catalyzed by D-flexizyme, and its mirror-image version, mirror-image tRNA charging catalyzed by L-flexizyme. PDB sources: 1EHZ (tRNA), 3CUL (flexizyme).
 (B) L-flexizyme charging of D-alanine onto enzymatically transcribed mirror-image tRNA^{Ala}, with the natural version performed for comparison.
 (C) L-flexizyme charging of glycine onto enzymatically transcribed mirror-image tRNA^{Gly}, with the natural version performed for comparison.
 (D) L-flexizyme charging of D-lysine onto enzymatically transcribed mirror-image tRNA^{Lys}, with the natural version performed for comparison.
 (E) L-flexizyme charging of D-phenylalanine onto enzymatically transcribed mirror-image tRNA^{Phe}, with the natural version performed for comparison.

dogma of molecular biology is to build a mirror-image ribosome translation machine.^{28–32} However, the total chemical synthesis of the dozens of mirror-image aaRS proteins, which typically range from 300–1,000 aa in size, poses a significant challenge. We reasoned that a mirror-image version of the highly simplified aaRS-free translation apparatus capable of producing protein enzymes could circumvent the laborious synthesis of dozens of the mirror-image aaRS proteins, despite the challenges to chemically synthesize the mirror-image ribosomal proteins³² and translation factors.

In a proof of concept experiment to test the charging of mirror-image (L-) tRNAs with mirror-image (D-) amino acids by a synthetic mirror-image (L-) flexizyme (Figure 5A), we applied our previously established mirror-image gene transcription system based on the mirror-image version of a designed mutant of the *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4)^{29,33,34} to transcribe the mirror-image tRNAs (Figure S10A). To avoid the high cost of synthesizing 21 different L-RNA primers, we applied a universal primer for the transcription of mirror-image tRNAs (Figure S10B). The universal primer was modified near the 3' end by phosphorothioate so that the fully extended primers were efficiently cleaved by I₂ via a previously reported mechanism,^{35,36} generating full-length mirror-image tRNAs. The I₂-mediated cleavage generates RNA with hydroxyl-terminated 5'-end, as verified by MALDI-TOF MS (Figures S11A–S11C). The transcribed mirror-image tRNAs were, as expected, resistant to natural RNase A digestion and unable to be charged by natural aaRS (Figures S12A and S12B). We also showed that the enzymatically transcribed 76-nt

mirror-image tRNA was of much higher purity than that prepared by chemical synthesis (Figure S10C) and thus is more suited for L-flexizyme charging and future mirror-image translation. Next, we successfully applied a chemically synthesized 46-nt L-flexizyme (dinitro-flexizyme) to charge four representative D-amino acids (lysine, alanine, glycine, and phenylalanine) that belong to different amino acid categories (polar [Lys], nonpolar [Ala], achiral [Gly], and aromatic [Phe], respectively) to their cognate mirror-image tRNAs, with similar efficiencies comparable to those of the natural system (Figures 5B–5E).

DISCUSSION

We have demonstrated that the aaRS-free translation of protein enzymes with an exclusive set of flexizyme-charged tRNAs. Our finding that neither aaRS-catalyzed tRNA charging nor tRNA recycling is required for sufficiently processive and faithful ribosome translation of protein enzymes, possessing more structural motifs and hence more catalytic functions than short peptides, suggests the possible emergence of protein enzymes from prebiotic versions of highly simplified aaRS-free translation systems. Notably, the average size of modern natural proteins is ~270–470 aa,³⁷ and the aaRS-free translation of proteins as large as TrpRS suggests the likelihood of producing other important protein enzymes to further improve the translation efficiency and fidelity.²¹ Our discovery that high concentrations of flexizyme-charged tRNAs significantly improves the yield of aaRS-free translation may also shed light on the possible conditions for the emergence of protein enzymes on prebiotic Earth, where abundant feedstocks of ribozyme-charged tRNAs might be important for primitive translation systems to operate efficiently. However, the question remains as to whether ribosome translation could function without any complex protein machinery (e.g., other ribosomal proteins and translation factors), and continuous efforts to search for a “protein-free” translation apparatus could help solve this mystery.

One of the limitations of the current aaRS-free translation system is that the charging of tRNAs must be decoupled from translation, in that they were pre-charged before being added to the translation system, since the flexizyme is a non-specific catalyst that charges various amino acids to tRNAs.⁸ This feature will ultimately limit the recycling of tRNAs like the multiple-turnover charging mechanisms (such as aaRS) do. Future developments of amino-acid-specific and tRNA-specific aminoacylating ribozymes^{38–41} may help realize the charging of tRNAs *in situ* with multiple turnovers. In addition, since the reaction conditions, such as Mg²⁺ concentration, are very different between the flexizyme charging and ribosome translation systems, for co-translational tRNA recycling and recharging, aminoacylating ribozymes that function at lower Mg²⁺ concentrations should be sought. On the other hand, the methodology of reducing cation (e.g., Mg²⁺) contamination by purification and using high concentrations of flexizyme-charged tRNAs, which we show to have significantly improved the yield of aaRS-free translation, can be applied to other *in vitro* translation systems using pre-charged tRNAs (with or without aaRS) for producing peptides or proteins from all or partial unnatural amino acids, enabling practical applications in many fields of synthetic biology and drug discovery.^{16,42}

Finally, the realization of aaRS-free translation of protein enzymes will help us establish a highly simplified translation apparatus without any aaRS, as a more feasible model for realizing mirror-image translation, since all the aaRS proteins combined represent nearly 30% (~1.4 MDa) in molecular weight of the *E. coli* translation

apparatus, including the ribosome, translation factors, aaRSs, and tRNAs (with a total molecular weight of ~ 4.9 MDa). It will also help to address the problem of using unmodified mirror-image tRNAs for translation, since certain tRNA modifications appeared important for efficient aaRS charging.²⁰ Moreover, the translation of the small 169-aa *Gaussia* luciferase also provides a sensitive and chiral-specific assay for testing mirror-image translation. Undoubtedly, the requirement of chemically synthesizing the mirror-image versions of ribosomal proteins and translation factors still poses significant challenges. Although some of them are considered non-essential and can be omitted under certain circumstances,^{43–45} the translation yield and fidelity might also be compromised.¹⁹ Ultimately, with all the essential proteins chemically synthesized and folded, a highly simplified aaRS-free translation system will be assembled into a functional mirror-image translation apparatus. With continuous supplies of flexizyme-charged mirror-image tRNAs, it will be capable of synthesizing various D-proteins, including most of the essential ribosomal proteins (most of which are smaller than 300 aa in the bacterial ribosome)^{32,46} for building itself, leading to the continuous reproduction and evolution of the mirror-image ribosome.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or codes.

Preparations of flexizyme-charged tRNAs for aaRS-free translation

The 21 tRNAs were individually charged with cognate amino acids by the 46-nt dinitro-flexizyme or the 45-nt enhanced flexizyme ([Supplemental experimental procedures](#)). The charging reactions were quenched by 0.3 M NaOAc and were precipitated. The pellets were purified by either 70% ethanol wash or a Shimadzu Prominence HPLC system (Japan) ([Figures S5A and S5B](#)). Symmetry Shield RP18 columns (3.5 μm , 4.6 \times 150 mm and 3.5 μm , 4.6 \times 100 mm) (Waters, MA, US) were used for HPLC purification, with elution conditions described in the literature.⁴⁷ The fractions containing flexizyme-charged tRNAs were precipitated, dissolved in 10 mM NaOAc at pH 5.2, and the concentration was measured by the Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, US). The desired amounts of tRNAs were then mixed and precipitated again by ethanol. The pellets were air-dried and stored at -80°C until use.

Cell-free *in vitro* translation

The cell-free *in vitro* translation mix was prepared according to the previously reported method¹⁷ with the following modifications: recombinant IF1, IF2, IF3, EF-Ts, EF-Tu, EF-G, RF-2, RF-3, RRF, and methionyl-tRNA formyltransferase (MTF) proteins were expressed in the *E. coli* strain BL21 with an N-terminal TEV-cleavable His-tag, purified by Ni-NTA Superflow resin (Senhui Microsphere Tech., Suzhou, China), cleaved by the tobacco etch virus (TEV) protease (Sigma-Aldrich, MO, US), further purified by ion-exchange chromatography, and exchanged into a buffer containing 50 mM HEPES at pH 7.6, 100 mM potassium glutamate, 10 mM magnesium acetate, 7 mM β -mercaptoethanol, and 30% glycerol. Buffer components and small

molecule ingredients were prepared as described in the literature.⁴⁸ The aaRS-free *E. coli* ribosome was purchased from New England Biolabs (MA, US).

AaRS-free translation of protein enzymes

The 20-codon DNA templates (Table S4) for chicken lysozyme, *Gaussia* luciferase, and *E. coli* TrpRS were synthesized and assembled using Genewiz (Jiangsu, China) and cloned into the pUC-57 vector. The DNA plasmids were double digested and purified by 1% agarose gel prior to use. Upon retrieval from -80°C , the dried flexizyme-charged tRNA pellets were washed twice with 70% ethanol and dissolved in 10–20 μl of 1 mM NaOAc at pH 5.2. The dissolved tRNA mix was then added to the aaRS-free translation mix that had been pre-incubated at 37°C for 5 min, with the final DNA template concentration at ~ 10 ng/ μl . For the translation of lysozyme and luciferase, ~ 1 μM of flexizyme-charged tRNAs were used for each translated codon; for the translation of TrpRS, ~ 1 μM of flexizyme-charged FAM-labeled Phe-tRNA^{fMet}, ~ 0.4 μM of flexizyme-charged tRNAs for each Cys and Pro codon, and ~ 0.2 μM of flexizyme-charged tRNAs for each remaining codon were used (overall tRNA concentrations shown in Table S1). The control experiments without DNA template were performed using identical flexizyme-charged tRNA concentrations, whereas the control experiments with uncharged tRNAs used 30 μM (each) for tRNA^{Asn}, tRNA^{Glu}, tRNA^{Lys}, tRNA^{Ile}, and 5 μM (each) for the other tRNAs, as well as 100 μM (each) for the free amino acids. Translation reactions were incubated at 37°C for 2 h for lysozyme and luciferase and 4 h for TrpRS. For the analysis by 15% SDS-PAGE, a 10 μl aliquot was sampled from the translation reaction, mixed with 2 μl of 6 \times protein loading dye and heated at 98°C for 3 min for loading. The Alexa Fluor 488-labeled Benchmark fluorescent protein standard was purchased from Thermo Fisher Scientific (MA, US). The gels were scanned by Typhoon FLA 9500 (GE Healthcare, UK) operated under Cy2 mode.

Biochemical characterization of aaRS-free translated protein enzymes

AaRS-free translation of chicken lysozyme, *Gaussia* luciferase, and *E. coli* TrpRS were performed with Met-tRNA^{fMet}. The translation mix for chicken lysozyme was diluted with an equal volume of a 2 \times folding buffer containing 0.1 M sodium phosphate and 0.1 M NaCl at pH 7.5, incubated for 24 h at room temperature, and assayed by the EnzChek Lysozyme Assay Kit (Thermo Fisher Scientific, MA, US). The translation mix for *Gaussia* luciferase was diluted with an equal volume of a 2 \times folding buffer containing 6 mM reduced and 4 mM oxidized glutathione at pH 7.3, shown previously to facilitate disulfide bond formation in recombinant *Gaussia* luciferase,²⁵ incubated for 16 h at room temperature, and assayed by the Pierce *Gaussia* Luciferase Glow Assay Kit (Thermo Fisher Scientific, MA, US) according to manufacturer's instructions. For the translation of *E. coli* TrpRS, Cy5-tRNA^{Trp} was prepared by enzymatic ligation of two synthetic oligos (Table S5), and purified by 10% denaturing PAGE as described in the literature.⁴⁹ A mixture of 2 μM Cy5-tRNA^{Trp}, 250 μM tryptophan, and 1 mM ATP was added to the reaction mix after completion of translation, incubated for 1 h at 37°C , quenched by 0.3 M NaOAc, and phenol-chloroform extracted. The charged samples were analyzed by 8% acid PAGE and scanned by Typhoon FLA 9500 operated under Cy5 mode. A sample of 2 μM of uncharged Cy5-tRNA^{Trp} and 2 μM of Cy5-tRNA^{Trp} charged by 100 nM of recombinant *E. coli* TrpRS were used as standards. All control experiments lacking DNA template, or with uncharged tRNAs and free amino acids, were assayed under identical conditions.

Mirror-image tRNA charging

Mirror-image tRNA charging was performed using the same aminoacylation procedure as described in Supplemental experimental procedures, except that L-tRNA and

L-flexizyme concentrations were scaled down to 2 and 10 μM , respectively. The mirror-image tRNAs were transcribed by the synthetic D-Dpo4-5m-Y12S polymerase, and the natural tRNAs were synthesized either by a recombinant Y12S mutant of Dpo4 (L-Dpo4-5m-Y12S) (tRNA^{Ala}, tRNA^{Gly} and tRNA^{Lys}) or by the T7 RNA polymerase (tRNA^{Phe}). The tRNA-charging yields were determined by ImageJ (<https://imagej.nih.gov/ij>) using the integrated peak area of charged tRNAs relative to the total tRNAs.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.chempr.2021.01.017>.

ACKNOWLEDGMENTS

We thank L. Han, M. Han, Y. Li, J. Liao, K. Wang, and B. Xiao for assistance with the experiments. This work was supported in part by funding from the National Natural Science Foundation of China (21925702, 21750005, and 81803072), the Tsinghua-Peking Center for Life Sciences (CLS), the Beijing Advanced Innovation Center for Structural Biology, and the Beijing Frontier Research Center for Biological Structure.

AUTHOR CONTRIBUTIONS

J.C. and M.C. performed the experiments. J.C. and T.F.Z. analyzed the results and wrote the manuscript. T.F.Z. designed and supervised the study.

DECLARATION OF INTERESTS

The authors have filed a provisional patent application related to this work.

Received: September 2, 2020

Revised: November 26, 2020

Accepted: January 20, 2021

Published: March 11, 2021

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