Final Article Available at: https://doi.org/10.1021/acssynbio.9b00456

A bi-functional polyphosphate kinase driving NTP regeneration and reconstituted cell-free protein synthesis

Running title: Simultaneous regeneration of ATP and GTP by a polyphosphate kinase

Po-Hsiang Wang,[a‡] Kosuke Fujishima,[ab‡*] Samuel Berhanu,[a#] Yutetsu Kuruma,[ac#] Tony Z. Jia,[ad] Anna N. Khusnutdinova,[e] Alexander F. Yakunin[ef] and Shawn E. McGlynn[ad*]

[a] Earth-Life Science Institute, Tokyo Institute of Technology, Tokyo, Japan.

[b] Graduate School of Media and Governance, Keio University, Fujisawa, Japan

[c] JST, PRESTO, Saitama, Japan

[d] Blue Marble Space Institute of Science, Seattle, Washington, U.S.A

[e] Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada.

[f] Centre for Environmental Biotechnology, Bangor University, Bangor, Wales, United Kingdom.

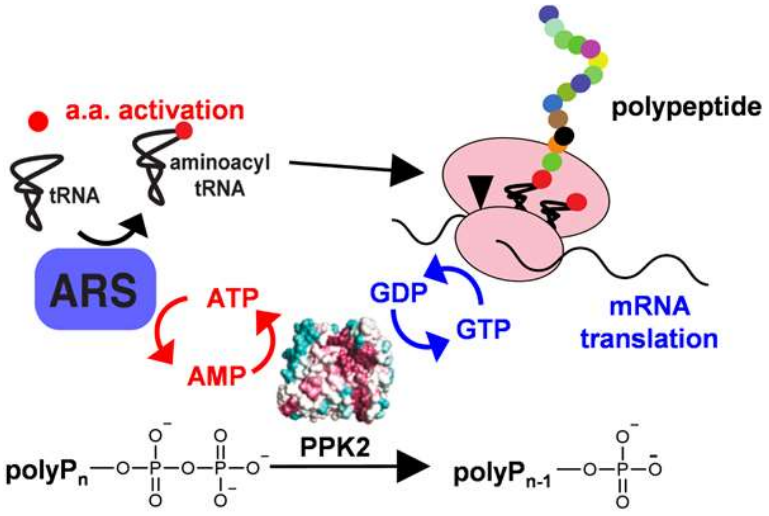
* To whom correspondence should be addressed: E-mail: fuji@elsi.jp; mcglynn@elsi.jp.

‡ P.-H.W. and K.F. contributed equally to this work.

Current affiliation: Institute for Extra-Cutting-Edge Science and Technology Avant-garde Research (X-star), JAMSTEC, Yokosuka, Japan

Abstract

Cell-free protein synthesis systems reconstituted from individually purified enzyme components (*i.e.*, the PURE system) allow the expression of toxic proteins, hetero-oligomeric protein subunits and proteins with non-canonical amino acids with high levels of homogeneity. In these systems, an artificial ATP/GTP regeneration system is required to drive protein synthesis, which is accomplished using three kinases and phosphocreatine in the PURE system. Here, we demonstrate the ability to replace these three kinases with a single bi-functional *Cytophaga hutchinsonii* polyphosphate kinase, which phosphorylates nucleotides in an exchange reaction from polyphosphate. This system results in a two-fold faster initial mRNA translation efficiency than that of the three-kinase system, along with a comparable final protein yield (~0.35 mg/mL). The single-kinase system is also compatible with expression of heat-sensitive firefly luciferase at 37°C. The single enzyme-based NTP regeneration approach described here could facilitate future applications of the PURE systems and their variants for high-throughput protein expression.



Keywords: family II polyphosphate kinase; reconstituted cell-free protein synthesis; NTP regeneration; functional protein expression.

Introduction

A major problem in contemporary biology is that a sizable fraction of proteins coded in sequenced genomes do not have functional annotation [1]. A high-throughput protein expression platform would facilitate functional characterization and annotation, potentially to the extent of total proteome synthesis [2]. Cell-free protein synthesis systems derived from cell lysates [3,4] or from reconstituted purified components (*i.e.*, the PURE system) have yielded fundamental biochemical insights [5–7] and also allow an opportunity for high-throughput expression of a multitude of proteins [8,9]. As opposed to *in vivo* protein expression in organisms like *E. coli*, cell-free systems allow protein synthesis in an environment free of destructive nucleases and proteases [7], along with the flexibility for adjusting conditions and integrating multiple biochemical reactions to cope with protein toxicity, complexity and co-factor requirements [5]. These features allow functional expression of membrane proteins, hetero-oligomeric protein subunits, proteins with non-canonical amino acids and toxic proteins with high levels of homogeneity [10,11]. In addition, the PURE system reconstituted from purified components allows protein expression using linear DNA, thereby bypassing multiple steps of plasmid cloning and organism culturing. These properties substantially shorten the procedure for protein production and prevent the occurrence of undesirable gene mutations. Furthermore, the PURE system has been applied to studying protein synthesis in non-traditional, *i.e.*, synthetic, settings such as in artificial cells [12–15].

In mRNA translation, formation of each peptide bond requires one ATP for tRNA aminoacylation by aminoacyl-tRNA synthetases (producing an AMP) and two GTPs for elongation and translocation of polypeptides (producing two GDPs) [16]. In contrast to cellular organisms that have intrinsic biochemical

pathways to regenerate nucleoside triphosphates (NTP), regeneration of ATP and GTP in cell-free systems requires an artificial NTP regeneration system (*e.g.*, [3,7]). In the PURE system, NTP regeneration is accomplished by three coupled reactions involving creatine kinase, myokinase and nucleoside diphosphate kinase, which transfer the phospho-moiety of phosphocreatine to ADP, AMP and GDP, respectively (**Figure 1A**) [7]. Harmonizing the activities of the three kinases, each with distinct kinetic properties, is challenging because myokinase and nucleoside diphosphate kinase each consume ATP produced by creatine kinase. Furthermore, the PURE system employs a high concentration of phosphocreatine to keep the system moving in the forward direction [8].

Family II polyphosphate kinase (PPK2) is a family of phosphotransferases capable of transferring the phospho-moiety of inorganic polyphosphate(polyP) to nucleoside mono- and diphosphates using metal cations (*e.g.*, Mg^{2+}) as the cofactor [17–21]. Class III PPK2s are capable of phosphorylating both nucleoside mono- or diphosphates, and have been applied to *in vitro* ATP regeneration [22]. PolyP has been used to enhance ATP regeneration in cell-lysate-based cell-free protein synthesis systems [23,24]; nevertheless, NTP regeneration in the cell-free system requires the amalgamation of multiple kinases and substrates and has not been applied to reconstituted systems comprised of purified components. Recently, a novel, highly active class III PPK2 from *Cytophaga hutchinsonii*, CHU0107 (ENA accession ABG57400), capable of phosphorylating AMP, ADP, GMP and GDP to the corresponding nucleoside diphosphates and triphosphates was structurally and biochemically characterized [17].

Results and Discussion

In this study, we investigated the possibility using polyP and the bi-functional polyP kinase CHU0107 for NTP regeneration in a reconstituted cell-free protein synthesis system. The K_m of CHU0107 for AMP (0.60 ± 0.10 mM) and GDP (2.75 ± 0.55 mM) are within an order of magnitude, suggesting a potential for simultaneous phosphorylation of AMP to ATP via ADP and GDP to GTP [17]. Moreover, the truncated variant of CHU0107 without the C-terminal tail (Leu285-Asp305; denoted as CHU0107t) exhibited a two-fold higher activity in phosphorylation of both AMP (45 U/mg) and GDP (10 U/mg) compared to the wild-type protein, rendering CHU0107t a possible alternative for NTP regeneration in the PURE system (**Figure 1B**).

We first determined the appropriate polyP concentration for NTP regeneration using customized PURE system (PURExpress®, GeneFrontier Corp.) reagents free of phosphocreatine, ATP, GTP and the three kinases. We observed polyP-dependent AMP phosphorylation to ATP via ADP [17], and ATP production was enhanced with the increase in polyP concentration from 10 mM to 50 mM (based on the molar content of phosphate monomer) (**Figure S1A**). Therefore, we used 50 mM polyP in the PURE system for subsequent experiments. Next, we applied CHU0107t for simultaneous polyP-dependent regeneration of ATP and GTP in the PURE system. The K_m of CHU0107t with AMP (22 ± 3 μM) or with GDP (0.29 ± 0.02 mM) measured under PURE system conditions were more than one order-of-magnitude lower than the reported values for the wild-type CHU0107 (AMP = 0.60 ± 0.10 mM; GDP = 2.75 ± 0.55 mM) (**Figure S1B**). The two-fold higher k_{cat} of CHU0107t with AMP (45/s) or GDP (7.7/s) compared to that of wild-type CHU0107 under the PURE system conditions is consistent with the previously reported data [17].

As a reporter for protein synthesis in the PPK2-based PURE system, we selected the superfolder green fluorescent protein (sfGFP; ~26.5 kDa) [25]. To directly compare the NTP regeneration efficiency between the three-kinase system and the PPK2-based system, we utilized mRNA as the template to synthesize proteins, avoiding depletion of the NTP pool by mRNA synthesis. Production of sfGFP was estimated using fluorescence emission at 518 nm in round bottom 96-well microplates (20 microliter reactions) in a real-time PCR system. We first performed sfGFP synthesis using 3 mM GTP and 0.2 mM ATP since the K_m of CHU0107t with AMP is ~15 times lower than that with GDP (the concentration for both ATP and GTP in the commercial PURE system is 3 mM). The PPK2-based PURE system showed time-dependent sfGFP production against the mRNA-free negative control (**Figure S2A**). Moreover, most of the sfGFP was produced within the first five hours of reaction (**Figure S2G)**. The sfGFP production is consistent with the visible green color of reaction mixtures after incubation and was also confirmed using SDS-PAGE (**Figure 2A**). In order to improve the protein yield, we tested multiple concentrations of Mg^{2+}, polyP and CHU0107t in the PPK2-based PURE system and determined the optimal concentrations to be 18 mM Mg^{2+}, 1 μM CHU0107t and 50 mM polyP (**Figures S2A–C**). sfGFP production was almost undetectable when CHU0107t was not added to the reaction mixtures (**Figures S2B**). In the range of 0.05–0.45 mM ATP and 0.9–2.7 mM GTP, concentration changes did not appear to have a significant effect on sfGFP production; therefore, 0.1 mM ATP and 1 mM GTP were used for subsequent experiments (**Figures S2D,E**). After optimization, the PPK2-based PURE system produced sfGFP with a final concentration corresponding to 168 ± 4 μg/mL (determined using purified recombinant sfGFP standard), which is two times lower than the sfGFP production in the PURExpress positive control (**Figure 2B,C**).

Based on the observation that sfGFP production in the PPK2-based PURE system became much slower after one hour of incubation (note that the fluorescence emission intensity of sfGFP is not linear at concentrations >10 μg/mL) (**Figures 2B,S2F**), we hypothesized that this might be due to either the loss-of-function of CHU0107t or the depletion of polyP over time. We also tested the potential influence of Mg^{2+} concentrations during incubation since a previous study reported synergistic effects of the phospho-moiety donor and Mg^{2+} when added to the PURE system [6]. Therefore, in another set of experiments we added either additional CHU0107t (1 μM), Mg^{2+} (3 mM) or polyP (10 mM) to the reaction mixtures after one hour of incubation (values are the final concentrations of the added component). After additional incubation for 70 min, polyP supplementation enhanced the sfGFP production by ~25% compared to the (+) control which had only buffer added (**Figure 2D**), while addition of CHU0107t showed only a minor effect. In contrast, additional Mg^{2+} hampered the production of sfGFP. Thus, our data suggest that the reduction in sfGFP production in the PPK2-based PURE system over time is affected by polyP depletion. We therefore analysed time-dependent polyP consumption in the reaction mixtures using a toluidine blue-stained TBE-urea gel (6%) [26]. We found that after two hours of incubation, the longer-chain fraction of the added polyP 25-mer mixture was mostly consumed, while the shorter-chain fraction remained (**Figure S3**), suggesting that CHU0107t has a preference to use long-chain polyP as substrate in the PPK2-based PURE system.

To investigate possible enhancement of protein synthesis by longer chain polyP, we replaced the original polyP 25-mer mixture with a polyP 100-mer mixture (Kerafast®) at the same concentration (50 mM based on the molar content of phosphate monomer) as the energy source in the PPK2-based PURE system. With longer polyP substrates, the system produced over 350 μg of sfGFP per mL (351 ± 3 μg/mL), roughly

a two-fold increase compared to the reaction mixtures using short chain polyP (**Figure 2C**). This modification resulted in a final sfGFP production comparable to yield of the PUREfrex positive control (~90%) (**Figure 2C**). Moreover, the PPK2-based PURE system with the polyP 100-mer exhibited a two-fold faster mRNA translation efficiency than the PUREfrex positive control in the first hour of incubation, along with a protein concentration of ~0.2 mg/mL after the first four (**Figures 2B,S2F**). The enhanced mRNA translation efficiency in the PPK2-based PURE system is consistent with the mRNA-stabilizing effect of long-chain polyP reported previously [24,27]. Nevertheless, sfGFP production in the PPK2-based PURE system with the polyP 100-mer became much slower after three hours of incubation, while sfGFP production in the PUREfrex positive control continued in the first five hours, resulting in the superior final yield (~0.4 mg/mL; **Figure 2C, S2G**).

The versatility of the PPK2-based regeneration system in protein synthesis applications was then tested using a heat-sensitive enzyme, *Photinus pyralis* firefly luciferase (~62 kDa), as a model protein. The *P. pyralis* firefly luciferase is unstable at temperatures greater than 30ºC [27], whereas the PURE system is generally operated at 37˚C. We detected firefly luciferase bioluminescence from both reaction mixtures of the PUREfrex positive control and the PPK2-based system incubated at 37°C for three hours (**Figure 3A**), respectively. Both systems exhibited a final concentration of active firefly luciferase corresponding to ~50 µg/mL of a commercial firefly luciferase standard (1×10^{11} U/mg, **Figure S4**), which is ~30% lower than the final concentration of active firefly luciferase in reaction mixtures incubated at 30°C (**Figure 3A**). Firefly luciferase production was also confirmed using SDS-PAGE (**Figure 3B**). Moreover, supplementing the native *E. coli* chaperones DnaKJ and GrpE to the PPK2-based PURE system doubled the active firefly

luciferase production at 37°C (**Figure 3A**). Altogether, our data suggest that the PPK2-based PURE system is compatible with functional expression of certain heat-sensitive proteins.

Energy delivery is a critical aspect of cell-free protein synthesis [3,24,28,29]. The single kinase-based system described herein simplifies (from three kinases system to single kinase) and potentially cheapens the cost of reagent preparations (by using polyP instead of phosphocreatine), while yielding protein amounts comparable to that of the three-kinase system. The described system also exhibits a high initial rate of mRNA translation suitable for high throughput protein expression. Further optimization of the reaction components and conditions may result in the betterment of commercial kits, leading to wider applications of protein synthesis from reconstituted systems for high-throughput protein expression. The single kinase-based NTP regeneration approach developed in this study might also be applied to drive other biochemical processes simultaneously requiring ATP and GTP under *in vitro* conditions, *e.g.*, gluconeogenesis [30]. Given that polyP is a ubiquitous and ancient energy source as well as an active metabolic regulator for all cellular organisms [26,27], incorporation of the PPK2-based NTP regeneration system into synthetic biomembrane vesicles could lead to artificial cell and proto-cell systems more akin to their natural counterparts. The use of long-chain polyP as substrate for NTP regeneration in the artificial cells is also cost-effective since long-chain polyP can be readily obtained from insoluble phosphate glass using an established simple two-step purification method [24].

Methods

(Methods for heterologous protein expression and purification; characterization of kinetic properties of recombinant CHU0107t; *in vitro* RNA transcription; electrophoretic analysis of toluidine blue-stained TBE-Urea gel are shown in **Supporting Information**)

Experimental Materials. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless specified otherwise. The customized PUREfrex reagents free of ATP, GTP, phosphocreatine, Mg^{2+} and NTP-regenerating kinases (creatine kinase, myokinase and nucleoside diphosphate kinase) as well as the linear DNA of codon-optimized *Photinus pyralis* luciferase (RCSB-PDB ID: 1LCI) were provided by GeneFrontier Corp. (Kashiwa, Chiba, Japan). The pETDuet-1 plasmid carrying the recombinant super folder green fluorescent protein (sfGFP) gene (RCSB-PDB ID: 2B3P) was purchased from GenScript (Nanjing, China). The DNA sequences for recombinant sfGFP and firefly luciferase are shown in the **Appendix**.

Cell-free protein synthesis using the polyP-based NTP regeneration system. GFP and firefly luciferase were synthesized using a customized PUREfrex system following the manufacturer's standard protocols (https://www.genefrontier.com/en/solutions/purefrex/lineup/purefrex-2-0/). Briefly, in an ice bath, solution I (reaction buffer mixtures free of NTPs, magnesium acetate and creatine phosphate; 10 μL), solution II (enzyme mixtures free of creatine kinase, myokinase and nucleoside diphosphate kinase; 1 μL) and solution III (ribosome; 2 μL) were mixed with the components to final concentrations of (i) recombinant CHU0107t (44–1600 nM), (ii) sfGFP mRNA: 0.7–1.4 μg; (iii) firefly luciferase mRNA: 3 μg, (iv) ATP (50−250 μM), (v) GTP (0.45−2.7 mM), (vi) magnesium acetate (12−27 mM) and (vii) sodium polyphosphate (50−80 mM)

to a final volume of 20 µL (the components of solution I, II and III are available at https://www.genefrontier.com/files/PF001_contents Oct2016.pdf). Cell-free protein synthesis was performed at 37°C and the reaction mixtures were kept on ice after incubation before further SDS-PAGE analysis or firefly luciferase activity assays. Note that the stock solutions for the sodium polyphosphate 25-mer mixture (0.5 M), the sodium polyphosphate 100-mer mixture (0.5 M; Kerafast®, Boston, USA) and magnesium acetate (240 mM) were pre-adjusted to pH 7.5 in HEPES-K^+ buffer (50 mM) to prevent a pH shift in the PURE system reaction mixture. The SDS-PAGE gel images for all the reaction components and products are available in the **Appendix**.

Real-time measurement of cell-free sfGFP synthesis. The time-dependent sfGFP production in the polyP-based PURE system was monitored based on its characteristic fluorescence emission (blue light at 518 nm) using a real-time PCR system (StepOnePlus, Applied Biosystems®, Foster City, CA, USA). The MicroAmp™ optical 96-well reaction plate (Applied Biosystems®) (round bottom with a well volume of 0.2 mL) containing reaction mixtures (20 µL in each well) were covered with an optically transparent film, and incubated at 37ºC for 2–5 h. The fluorescence emission of sfGFP was recorded every 10 min using the FAM™ filter (excitation: 494 nm; emission: 518 nm). The recombinant sfGFP standards and the PURE system reaction mixtures were always kept on ice before and after incubation in the real-time PCR system. The sfGFP production (~27 kDa) was examined by 12% SDS-PAGE (Novex®, Thermo Fisher Scientific, Waltham, MA, USA) and Coomassie Blue staining. Final sfGFP concentrations in the polyP-based PURE system reaction mixtures were determined using a standard curve generated by serial-diluted recombinant sfGFP standards, which has a linear range of 0–10 µg/mL (standard curve available in the **Appendix**).

Firefly luciferase activity assays. Firefly luciferase activity assays were performed following the established protocol published previously with some modifications [27]. Briefly, fresh *Photinus pyralis* luciferase standards (62 kDa; 10^{11} U/mg) were diluted to a final concentration of (10, 5, 2.5 and 1.25) x 10^8 U/mg using a Hepes-K^+ buffer (pH 7.5; 50 mM) containing NaCl (150 mM), 5% glycerol, dithiothreitol (DTT) (1 mM) and EDTA (1 mM). In a black 96-well polystyrene plate (Costar®, Corning, Corning, NY, USA), *Photinus pyralis* luciferase standards or the PURE system reaction mixtures (2 μL) were pipetted into the luciferase activity assay buffer (98 or 198 μL; 50-fold and 100-fold dilution) containing Hepes-K^+ (pH 7.5; 50 mM), 5% glycerol, sodium coenzyme A (0.1 mM), ATP (2 mM), $MgCl_2$ (5 mM), bovine serum albumin (0.1 mg/mL), D-sodium luciferin (50 μM), sodium EDTA (0.1 mM) and DTT (1 mM). The luciferase standards, reaction mixtures and activity assay buffers were always kept on ice before the bioluminescence measurement. Bioluminescence of luciferase was measured in a multimode plate reader (EnSpire®, PerkinElmer, Waltham, MA, USA) at 562 nm. The black 96-well plate was incubated at 25°C for 10 min with a bioluminescence scan every 20 s. The luciferase production in the PURE system was estimated by comparing the bioluminescence (the average values from 2–7 min) to that of the commercial luciferase standards (10^{11} U/mg).

Author contributions P.-H.W. and K.F. designed this research; A.K., A.F.Y. and P.-H.W purified the recombinant CHU0107t and recombinant sfGFP; P.-H.W. characterized kinetic properties of CHU0107t; P.-H.W. and K.F. developed polyP-based NTP regeneration system for cell-free protein synthesis; P.-H.W. and T.Z.J. performed the electrophoretic analysis of polyP; P.-H.W. performed the firefly luciferase activity

assays; P.-H.W., K.F., Y.K., S.B. and S.E.M participated in experimental design; all authors participated in the data analysis; P.-H.W. and S.E.M. wrote the paper with assistance from all authors. The authors declare no conflict of interest.

Acknowledgement This work is supported by JSPS KAKENHI Grant Numbers 10751084 to S.E.M, 16H06156 to Y.K. and JP18K14354 to T.Z.J. K.F. is supported by FirstLogic-Astrobiology donation program. A.K. and A.F.Y. are supported by the NSERC Industrial Biocatalysis Strategic Network grant IBN and Genome Canada (GAPP). We also gratefully acknowledge support of customized PURExpress reagents and codon-optimized linear DNA of *P. pyralis* firefly luciferase from Dr. Takashi Kanamori at GeneFrontier Corp. (Japan).

Abbreviations sfGFP, super-folder green fluorescent protein; NTP, nucleoside triphosphate; polyP, polyphosphate; PPK2, family II polyphosphate kinase; PURE system, reconstituted cell-free protein synthesis system.

References

(1) Hanson, A. D.; Pribat, A.; Waller, J. C.; de Crécy-Lagard, V. "Unknown" Proteins and "Orphan" Enzymes: The Missing Half of the Engineering Parts List--and How to Find It. *Biochem J* **2009**, *425* (1), 1–11.

(2) Sawasaki, T.; Ogasawara, T.; Morishita, R.; Endo, Y. A Cell-Free Protein Synthesis System for High-Throughput Proteomics. *Proc Natl Acad Sci USA* **2002**, *99* (23), 14652.

(3) Jewett, M. C.; Swartz, J. R. Mimicking the *Escherichia Coli* Cytoplasmic Environment Activates Long-Lived and Efficient Cell-Free Protein Synthesis. *Biotechnol Bioeng* **2004**, *86* (1), 19–26.

(4) Schwarz, D.; Junge, F.; Durst, F.; Frölich, N.; Schneider, B.; Reckel, S.; Sobhanifar, S.; Dötsch, V.; Bernhard, F. Preparative Scale Expression of Membrane Proteins in *Escherichia Coli*-Based Continuous Exchange Cell-Free Systems. *Nat Protoc* **2007**, *2*, 2945.

(5) Katzen, F.; Chang, G.; Kudlicki, W. The Past, Present and Future of Cell-Free Protein Synthesis.

Trends Biotechnol **2005**, *23* (3), 150–156.

(6) Li, J.; Zhang, C.; Huang, P.; Kuru, E.; Forster-Benson, E. T. C.; Li, T.; Church, G. M. Dissecting Limiting Factors of the Protein Synthesis Using Recombinant Elements (PURE) System. *Trans* **2017**, *5* (1), e1327006.

(7) Shimizu, Y.; Inoue, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Ishikawa, K.; Ueda, T. Cell-Free Translation Reconstituted with Purified Components. *Nat Biotechnol* **2001**, *19* (August), 751–755.

(8) Kazuta, Y.; Matsuura, T.; Ichihashi, N.; Yomo, T. Synthesis of Milligram Quantities of Proteins Using a Reconstituted *in vitro* Protein Synthesis System. *J Biosci Bioeng* **2014**, *118* (5), 554—557.

(9) Niwa, T.; Ying, B.-W.; Saito, K.; Jin, W.; Takada, S.; Ueda, T.; Taguchi, H. Bimodal Protein Solubility Distribution Revealed by an Aggregation Analysis of the Entire Ensemble of *Escherichia Coli* Proteins. *Proc Natl Acad Sci USA* **2009**, *106* (11), 4201–4206.

(10) Singh-Blom, A.; Hughes, R. A.; Ellington, A. D. An Amino Acid Depleted Cell-Free Protein Synthesis System for the Incorporation of Non-Canonical Amino Acid Analogs into Proteins. *J Biotechnol* **2014**, *178*, 12–22.

(11) Kuruma, Y.; Ueda, T. The PURE System for the Cell-Free Synthesis of Membrane Proteins. *Nat Protoc* **2015**, *10*, 1328.

(12) Berhanu, S.; Ueda, T.; Kuruma, Y. Artificial Photosynthetic Cell Producing Energy for Protein Synthesis. *Nat Comm* **2019**, *10* (1), 1325.

(13) Nourian, Z.; Roelofsen, W.; Danelon, C. Triggered Gene Expression in Fed-Vesicle Microreactors with a Multifunctional Membrane. *Angew Chem Int Ed Engl* **2012**, *51* (13), 3114–3118.

(14) Furusato, T.; Horie, F.; Matsubayashi, H. T.; Amikura, K.; Kuruma, Y.; Ueda, T. *De novo* Synthesis of Basal Bacterial Cell Division Proteins FtsZ, FtsA, and ZipA Inside Giant Vesicles. *ACS Synth Biol* **2018**, *7* (4), 953–961.

(15) Forster, A. C.; Church, G. M. Towards Synthesis of a Minimal Cell. *Mol Syst Biol* **2006**, *2* (1), 45.

(16) Zavialov, A. V.; Ehrenberg, M. Peptidyl-TRNA Regulates the GTPase Activity of Translation Factors. *Cell* **2003**, *114* (1), 113—122.

(17) Berlicki, Ł.; Joachimiak, A.; Flick, R.; Burda, M.; Mucha, A.; Ruszkowski, M.; Batyrova, K.; Khusnutdinova, A. N.; Nocek, B. P.; Yakunin, A. F.; et al. Structural Insights into Substrate Selectivity and Activity of Bacterial Polyphosphate Kinases. *ACS Cat* **2018**, *8* (11), 10746–10760.

(18) Ishige, K.; Zhang, H.; Kornberg, A. Polyphosphate Kinase (PPK2), a Potent, Polyphosphate-Driven Generator of GTP. *Proc Natl Acad Sci USA* **2002**, *99* (26), 16684–16688.

(19) Parnell, A. E.; Mordhorst, S.; Kemper, F.; Giurrandino, M.; Prince, J. P.; Schwarzer, N. J.; Hofer, A.; Wohlwend, D.; Jessen, H. J.; Gerhardt, S.; et al. Substrate Recognition and Mechanism Revealed by Ligand-Bound Polyphosphate Kinase 2 Structures. *Proc Natl Acad Sci USA* **2018**, *115* (13), 3350–3355.

(20) Ogawa, M.; Uyeda, A.; Harada, K.; Sato, Y.; Kato, Y.; Watanabe, H.; Honda, K.; Matsuura, T. Class III Polyphosphate Kinase 2 Enzymes Catalyze the Pyrophosphorylation of Adenosine-5'-Monophosphate. *ChemBioChem*.

(21) Mordhorst, S.; Siegrist, J.; Müller, M.; Richter, M.; Andexer, J. N. Catalytic Akylation Using a Cyclic S-Adenosylmethionine Regeneration System. *Angew Chem Int Ed Engl* **2017**, *56* (14), 4037–4041.

(22) Kameda, A.; Shiba, T.; Kawazoe, Y.; Satoh, Y.; Ihara, Y. A Novel ATP Regeneration System Using Polyphosphate-AMP Phosphotransferase and Polyphosphate Kinase. *J Biosci Bioeng* **2001**, *91* (6), 557–563.

(23) Caschera, F.; Noireaux, V. A Cost-Effective Polyphosphate-Based Metabolism Fuels an All *E. coli* Cell-Free Expression System. *Meta Eng* **2015**, *27*, 29–37.

(24) Itoh, H.; Kawazoe, Y.; Shiba, T. Enhancement of Protein Synthesis by an Inorganic Polyphosphate in an *E. coli* Cell-Free System. *J Microbiol Meth* **2006**, *64* (2), 241—249.

(25) Pédelacq, J.-D.; Cabantous, S.; Tran, T.; Terwilliger, T. C.; Waldo, G. S. Engineering and Characterization of a Superfolder Green Fluorescent Protein. *Nat Biotechnol* **2006**, *24* (1), 79–88.

(26) Achbergerová, L.; Nahálka, J. Polyphosphate - an Ancient Energy Source and Active Metabolic Regulator. *Microb Cell Fact* **2011**, *10*, 1–14.

(27) Gray, M. J.; Wholey, W.; Wagner, N. O.; Cremers, C. M.; Mueller-schickert, A.; Hock, N. T.; Krieger, A. G.; Smith, E. M.; Bender, R. A.; Bardwell, J. C. A.; et al. Polyphosphate Is a Primordial Chaperone. *Mol Cell* **2014**, *53* (5), 689–699.

(28) Kim, H.-C.; Kim, D.-M. Methods for Energizing Cell-Free Protein Synthesis. *J Biosci Bioeng* **2009**, *108* (1), 1–4.

(29) Kim, D.-M.; Swartz, J. R. Prolonging Cell-Free Protein Synthesis with a Novel ATP Regeneration System. *Biotechnol Bioeng* **1999**, *66* (3), 180–188.

(30) Krebs Hans Adolf. The Croonian Lecture, 1963 Gluconeogenesis. *Proc R Soc Lond B Biol Sci* **1964**, *159* (977), 545–564.

Figure legends

Figure 1. NTP regeneration systems required for cell-free protein synthesis. (**A**) The conventional NTP regeneration system in the PURE system functions with phosphocreatine and three coupled kinases: CK, creatine kinase; MK, myokinase; NDK, nucleoside diphosphate kinase. (**B**) The single polyphosphate kinase-based NTP regeneration system for cell-free protein synthesis developed in this study. ARS, aminoacyl-tRNA synthetase; PPK2, polyP kinase family II.

Figure 2. Small-scale superfolder green fluorescent protein (sfGFP) synthesis using the PPK2-based PURE system in a 96-well microplate. (**A**) sfGFP production in reaction mixtures of PPK2-based PURE system after five hours of incubation, evidenced by the visible green color in the reaction mixtures (fluorescence emmision of sfGFP in a blue light gel illuminator) and SDS-PAGE analysis (~26.5 kDa)

(untrimmed images of 96-well plate and SDS-PAGE gel available in **Supporting Information**). mRNA (-), mRNA-free control; mRNA (+), positive control. The arrow indicates the position of sfGFP. (**B**) sfGFP production in the reaction mixtures with and without sfGFP mRNA, along with polyP in different lengths (polyP 25-mer or polyP 100-mer) as the energy source. The bars represent the range and the symbols represent the mean from duplicate trials. Note that the sfGFP fluorescence intensity is not linear above 10 µg/mL. (**C**) Final sfGFP concentrations (after 5 hours of incubation) were measured by dilution to the linear range. The * represents undetectable fluorescence. Time-dependent sfGFP production for five hours based on fluorescence intensity is shown in **Figure S2G**. The bars represent the range and the symbols represent the mean from duplicate trials. (**D**) Final sfGFP concentrations in reaction mixtures that received an extra addition of polyP, CHU0107t (PPK2), Mg^{2+} or only buffer (positive control) after one hour of incubation. All component stocks were dissoved in the same PURE system buffer and were added to reaction mixtures that were incubated for 60 min, and the reaction mixtures were further incubated for 70 min. Assays were performed in triplicate and the bars represent the standard error and the symbols represent the mean.

Figure 3. Functional expression of *P. pyralis* firefly luciferase (Luc) in the polyphosphate kinase (PPK2)-based PURE system. (**A**) Average bioluminescence activity (562 nm) in the supernatants of the PPK2-based PURE system reaction mixtures incubated for three hours with and without of firefly luciferase mRNA (mRNA(+) and mRNA (–)). Some reaction mixtures were supplemented with native *E. coli* chaperones (DnaK). Luc production in the three-kinase based PURExpress system was used as a positive control. Reaction mixtures were incubated at either 30°C or 37°C. The * represents undetectable bioluminescence activity. (**B**) SDS-PAGE analysis of functional Luc production (~62 kDa) in the supernatants of the PPK2-based PURE system after three hours of incubation (untrimmed SDS-PAGE gel image is available in **Supporting Information**). The arrow indicates the position of Luc. Assays were performed in triplicate and the bars represent the standard error and the symbols represent the mean.

Figures

Figure 1

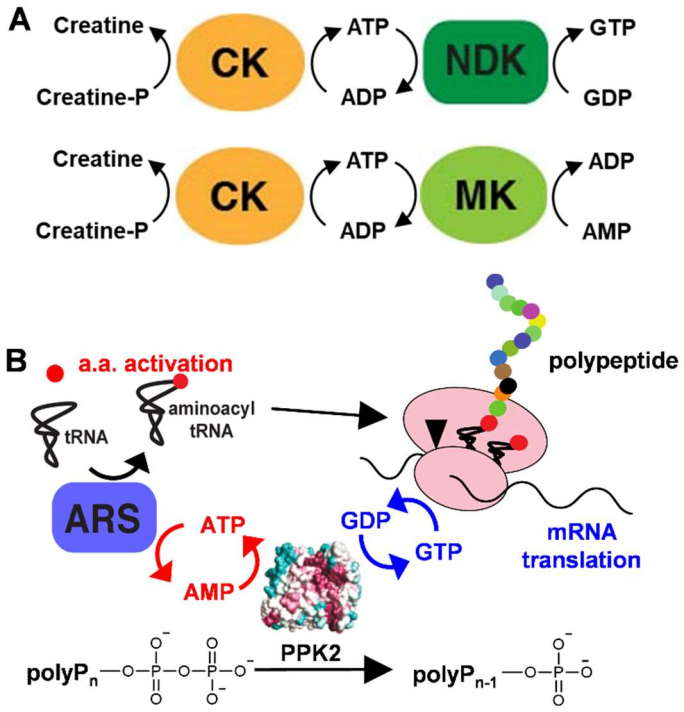
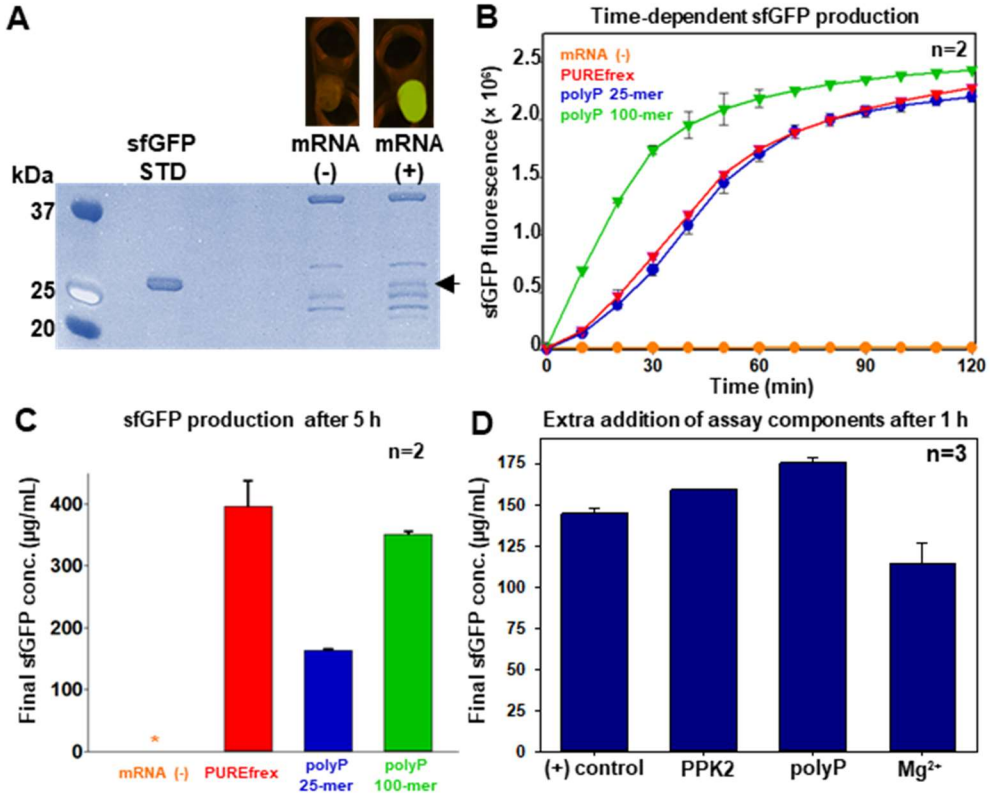
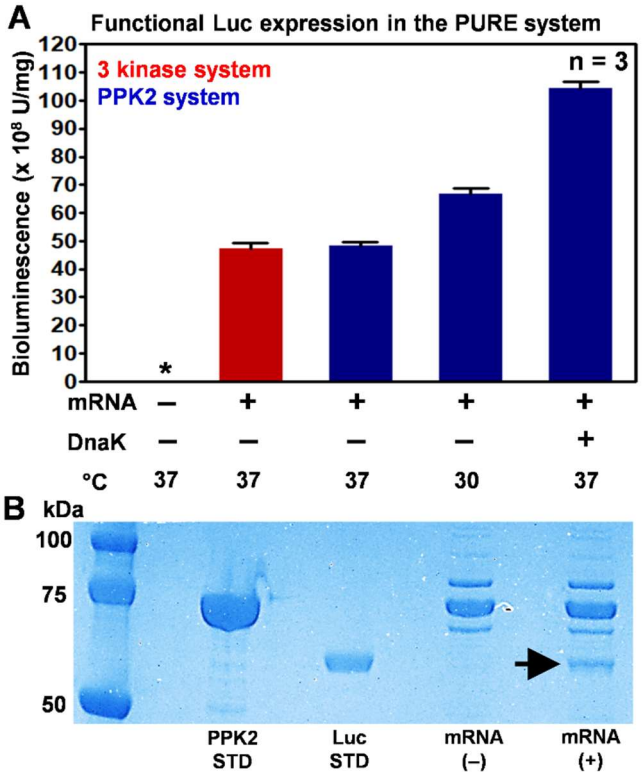


Figure 2



346 **Figure 3**



347