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A cost-effective polyphosphate-based metabolism fuels an all *E. coli* cell-free expression system

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ABSTRACT

A new cost-effective metabolism providing an ATP-regeneration system for cell-free protein synthesis is presented. Hexametaphosphate, a polyphosphate molecule, is used as phosphate donor together with maltodextrin, a polysaccharide used as carbon source to stimulate glycolysis. Remarkably, addition of enzymes is not required for this metabolism, which is carried out by endogenous catalysts present in the *Escherichia coli* crude extract. This new ATP regeneration system allows efficient recycling of inorganic phosphate, a strong inhibitor of protein synthesis. We show that up to 1.34–1.65 mg/mL of active reporter protein is synthesized in batch-mode reaction after 5 h of incubation. Unlike typical hybrid *in vitro* protein synthesis systems based on bacteriophage transcription, expression is carried out through *E. coli* promoters using only the endogenous transcription–translation molecular machineries provided by the extract. We demonstrate that traditional expensive energy regeneration systems, such as creatine phosphate, phosphoenolpyruvate or phosphoglycerate, can be replaced by a cost-effective metabolic scheme suitable for cell-free protein synthesis applications. Our work also shows that cell-free systems are useful platforms for metabolic engineering.

1. Introduction

DNA-dependent *in vitro* protein synthesis is becoming a popular technology for cell-free biology (Swartz, 2012). Linear or circular DNA programs are now executed in cell-free transcription–translation (TX–TL) mixtures for biomedical applications (He and Taussig, 2001), nanotechnology (Daube and Bar-Ziv, 2013), synthetic biology (Chappell et al., 2013; Iyer and Doktycz, 2014; Lentini et al., 2013; Niederholtmeyer et al., 2013; Shin and Noireaux, 2012; Sokolova et al., 2013; Sun et al., 2013) and metabolic engineering (Bujara et al., 2010; Ye et al., 2012). A direct consequence is the high demand for novel, efficient and cost-effective systems.

Cell-free protein synthesis platforms are open systems that provide direct access to complex biochemical networks, and controlled variations of the system's parameters. This type of approach allows quantitative characterization of biological networks and prototyping of metabolic pathways for production of valuable compounds (Goerke et al., 2008; Hodgman and Jewett, 2012; Krutsakorn et al., 2013; Liu et al., 2014).

Cell-free TX–TL requires adequate regeneration of ATP to work efficiently (Grandi, 2007; Kim and Kim, 2009). In conventional systems, ATP regeneration is achieved by adding external enzymes, co-factors and expensive phosphate donor molecules to cell-free reactions. However, many efforts have been made to reduce the cost of cell-free reactions. In particular, several metabolic schemes have been exploited to achieve novel cost-effective cell-free expression systems (Calhoun and Swartz, 2005; Jewett and Swartz, 2004b; Kim and Swartz, 2001; Kim et al., 2007a) for industrial applications (Swartz, 2006), thereby, for large-scale reactors and high-throughput experimentation (Spirin, 2004; Zawada et al., 2011). Nucleosides triphosphate and phosphate energy donors are the most expensive components in cell-free expression systems (Swartz, 2012). Cytoplasmic enzymes, present in the cellular extract, are exploited to regenerate nucleosides triphosphate, such as ATP and GTP (Swartz, 2012). Another significant reduction of the reaction cost is obtained by exploiting the central metabolism using polysaccharides, glucose, pyruvate, (Calhoun and Swartz, 2005; Jewett and Swartz, 2004b; Kim et al., 2011; Wang and Zhang, 2009), and oxidative phosphorylation with glutamate (Jewett et al., 2008). These novel systems bypass the

Abbreviations: TX–TL, transcription–translation; ATP, adenosine triphosphate; iP, inorganic phosphate; eGFP, enhanced green fluorescent protein; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; NAD, nicotinamide; CoA, coenzyme A; cAMP, adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol; M, maltose; Mx, maltodextrin; HMP, hexametaphosphate; poly(P), polyphosphate; RB, reaction buffer

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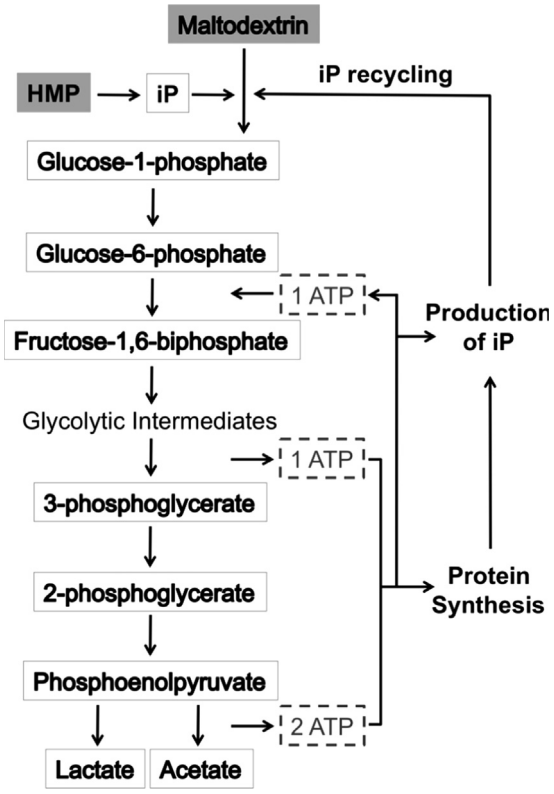

Fig. 1. Schematic of the metabolism based on maltodextrin and hexametaphosphate for ATP production and iP recycling. Chemicals shaded in gray are added to the cell-free reaction.

substrate level-phosphorylation based on expensive phosphate donors energy sources and externally added kinases (Kim and Swartz, 2001; Kim et al., 2006; Sitaraman et al., 2004).

There are several types of phosphate donors, each exploiting a specific metabolic pathway, initiated by a precise enzyme catalyzing the initial phosphorylation reaction. Until now, the most favored ATP-regenerating systems have been acetyl phosphate and acetate kinase (Kondo et al., 1984; Ryabova et al., 1995), phosphoenol pyruvate (PEP) and pyruvate kinase (Crans et al., 1987), creatine phosphate and creatine kinase (Shih and Whitesides, 1977), 3-phosphoglycerate (Sitaraman et al., 2004) (3-PGA) and fructose-1,6-biphosphate (Kim et al., 2007a). However, these phosphate donor molecules are expensive, which may limit the potential of the cell-free expression technology for large scale preparation and industrial processes (Swartz, 2006).

In this work, an alternative cost-effective ATP production and regeneration system for cell-free metabolism is presented. We demonstrate that hexametaphosphate (HMP), a polyphosphate molecule $(NaPO_3)_{17}$, is efficiently used to fuel protein synthesis when coupled to a carbon source such as maltose or maltodextrin. In addition to reduce the cost of the ATP-regenerating system, we develop new knowledge of *in vitro* metabolic pathways and provide an alternative system to a rapidly growing research area (Carlson et al., 2012; Zhu et al., 2013).

Hexametaphosphate (Graham's salt) is a mixture of inorganic phosphate (iP) polymers with the hexamer $(NaPO_3)_6$ as base unit (Katchman and Van Wazer, 1954). Inorganic poly(P) is a versatile molecule with several functions (Achbergerova and Nahalka, 2011; Kornberg, 1995). It has been described as a possible precursor in prebiotic evolution (Kornberg, 1995), it is used in industry under the name of Calgon (calcium gone) and in the emergent field of synthetic biology (Achbergerova and Nahalka, 2011). Microbial cells commonly store poly(P) in the form of granules, which are degraded when chemical energy is required (Achbergerova and Nahalka, 2011). The enzymes involved in the biosynthesis and the utilization of poly(P) as energy source were first characterized by Kornberg and collaborators (Kornberg et al., 1956; Kornberg, 1957). This discovery elucidated important biochemical aspects of bacteria metabolism and also suggested novel applications in enzyme technology, where ATP-regenerating systems are required (Butler, 1977). An ATP regeneration system based on poly(P) for cell-free TX–TL has been already described (Kameda et al., 2001). Although interesting in principle, this system was not practical because two enzymes had to be added to the reactions. Polyphosphate-AMP phosphotransferase and polyphosphate kinase were first overexpressed in a bacteria strain and then purified. Herein, we present an ATP regeneration system that only exploits endogenous enzymes from the *Escherichia coli* extract to process hexametaphosphate as phosphate substrate, thus creating a novel low cost metabolism for fueling protein synthesis.

We recently showed that high-yield cell-free protein synthesis is achieved by coupling maltose or maltodextrin to 3-PGA (Caschera and Noireaux, 2013). In this work, we couple maltose or maltodextrin to HMP, to directly exploit the high-energy phosphoanhydride bond given in the poly(P) molecule (Fig. 1).

Maltodextrin is degraded through a phosphorylation reaction where HMP is the phosphate molecule donor. As a result of the phosphorylation step, glucose-1-phosphate is produced and subsequently processed in the glycolytic pathway to produce ATP. The presence of coenzymes, such as NAD and CoA, improves ATP regeneration and iP recycling, while lactate and acetate are produced as wastes (Calhoun and Swartz, 2005; Jewett and Swartz, 2004a). This novel metabolism works efficiently as it continuously recycle iP and keeps ATP at steady concentration for protein synthesis. Using this new low cost metabolism, we achieve a protein yield of 1.34–1.65 mg/mL (active reporter protein) in batch mode reaction. Kinetics of pH changes, organic acids accumulation, ATP and iP concentrations are measured during *in vitro* protein synthesis.

2. Materials and methods

2.1. Plasmid preparation: amplification, extraction and quantification

All the plasmids used in the experiments were amplified in *E. coli* KL740 cells and extracted using standard plasmid kits. Quantification was performed with the NanoDrop2000 (Thermofisher). Protein synthesis was carried out using the plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500, which was previously described as well as the reporter protein deGFP, 39.4 μM is 1 mg/mL. (Shin and Noireaux, 2012).

2.2. Hexametaphosphate preparation

Hexametaphophate (HMP) solid was dissolved in water and kept at 100 °C for 5 min before dilutions at desired concentration for cell-free reaction. HMP was purchased from Sigma-Aldrich with molecular weight average $(NaPO_3)_{17}$.

2.3. Cell-free reaction in batch mode (end points and kinetics measurements)

The *E. coli* crude extract was prepared as described before using a cell press to scale-up extract production (Caschera and Noireaux, 2013). The preparation of cell-free reactions was described before (Caschera and Noireaux, 2013). BL21 Rosetta2 cells were grown overnight at 37 °C on 2xYT agar plate with chloramphenicol and phosphates (40 mM phosphate dibasic and 22 mM phosphate monobasic solutions). A 5 mL mini-culture, same medium