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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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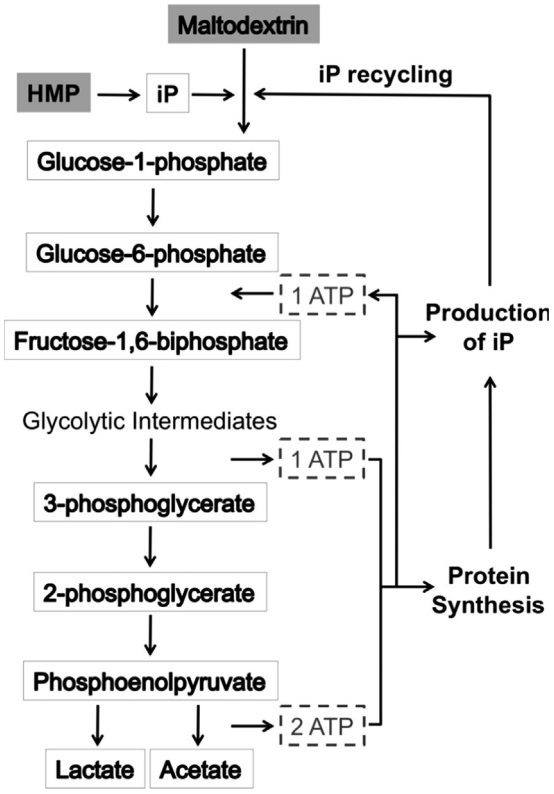

Fig. 1. Schematic of the metabolism based on maltodextrin and hexametaphosphate for ATP production and iP recycling. Chemicals shaded in gray are added to the cell-free reaction.

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

composition, was carried out for 7 h at 37 °C. Successively 30 μL from the mini-culture was added to 60 mL of 2xYT (chloramphenicol and phosphates) and grown for 8 h at 37 °C. 7 mL from the last culture was inoculated into 700 mL of the 2xYT with phosphates and incubated at 37 °C until OD600 = 1.5–1.6 (doubling time ≈ 0.5 h). Cells were harvested and washed with the S30A buffer (Caschera and Noireaux, 2013) and the lysate was prepared using a cell-press. After clarification at 30,000*g* the cytoplasmic extract was incubated 90min at 37 °C to remove endogenous DNA and messenger RNAs. The extract was dialyzed at 4 °C for 1 h against S30B buffer (14 mM Mg-Glutamate, 150 mM K-Glutamate buffered to pH 8.2 with Tris 2 M) using 10 KDa molecular mass cut-off cassettes. The total protein concentration in the cellular extract (≈ 28 mg/ml) was measured by Bradford.

The cytoplasmic crude extract was 33% in volume of the cell-free reaction with a final protein concentration of ≈ 9.2 mg/mL. The other 66% volume of the reaction was composed of water, 6 mM Mg-glutamate, 60 mM K-glutamate, 2% PEG-8000 (v/v), 3.5 mM of each of the 20 canonical amino acids, hexametaphosphate (at different concentrations), maltose or maltodextrin at 15 mM and 35 mM respectively, and the reaction buffer (RB). The RB was prepared with the following components: 50 mM Hepes pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, and 1 mM spermidine. Maltodextrin added in the reaction mixture was calculated by taking the same molecular mass of maltose (360.31 g/mol). All the chemicals listed were purchased from Sigma-Aldrich. Cell-free reactions were performed at 29 °C in 1.5 mL tubes (12 μl) and protein yields measured at room temperature in 384-well plate (10 μl). Kinetics measurements of cell-free expression (5 μL) were taken at 29 °C in polypropylene 96-well plate with V-bottom shape sealed with a plastic cap to avoid evaporation. The concentration of synthesized proteins (deGFP) was determined through fluorescence measurements (Ex 485 nm; Em 528 nm) using a multimode plate reader H1m (BioTeK). The calibration line, used to retrieve the protein concentration (Supplementary Fig. 1), was built using recombinant eGFP (reGFP) purchased from Cell Biolabs Inc.

2.4. SNARF-5F kinetic measurements of pH change

The fluorescent dye SNARF-5F (5-(and-6)-carboxylic acid, Invitrogen) is sensitive to pH changes. The emission ratio at 580/640 nm (excitation at 514 nm) was used to build a calibration curve (Supplementary Fig. 2), used to compute the pH during *in vitro* protein synthesis. The dye was dissolved at 10 mM in DMSO and stored at −20 °C. The dye was diluted to 1 mM in water before use and added into cell-free reactions to a final concentration of 0.1 mM, as previously described (Caschera and Noireaux, 2013). The inhibitor of the glycolic pathway: 2-deoxy-D-glucose, dissolved in water at the desired concentration, was purchased from Sigma-Aldrich. In order to avoid interference between fluorescence spectra of the dye and the reporter protein, a plasmid encoding for the non-fluorescence protein luciferase was used: pBEST-OR2-OR1-Pr-UTR1-Luc-T500 (Shin and Noireaux, 2010).

2.5. Lactate, acetate, oxaloacetate and glucose assays

The lactate, acetate, oxaloacetate and glucose concentrations during cell-free protein synthesis were determined using enzymatic colorimetric assays purchased from BioVision, Inc. (USA) according to the manufacturer instruction. The experiment was performed with a multimode plate reader H1m (BioTeK) using 96-well plates.

2.6. ATP and iP assay

The ATP concentration during cell-free protein synthesis was determined using the kit ENLITEN ATP assay purchased from Promega Corporation. Aliquots of the cell-free reaction were collected at different time intervals and diluted accordingly before bioluminescence measurements. The experiment was performed with a multimode plate reader H1m (BioTeK) using 96-well plates.

The inorganic phosphate assay was performed as described before (Caschera and Noireaux, 2013). This is a spectrophotometric assay based on ascorbic acid reducing the complex formed between phosphate and ammonium molybdate, with the presence of Zinc acetate under mild pH condition (Saheki et al., 1985). Absorbance measurements at 850 nm were taken in disposable 1 mL plastic cuvette with the Genesys 10UV spectrophotometer (Thermofisher).

3. Results and discussion

Our goal was to design and characterize a novel, low-cost metabolic pathway to energize cell-free TX–TL reactions. The novel metabolic scheme (Fig. 1) is based on the initial phosphorylation of maltodextrin (Mx) with the inorganic phosphate (iP) released by the hexametaphosphate (HMP) hydrolysis to produce glucose-1-phosphate, which is processed in the glycolytic pathway to produce ATP. The yield of deGFP was measured quantitatively by fluorescence using a calibration line (Supplementary Fig. 1).

3.1. HMP hydrolysis and optimal condition for fueling in vitro protein synthesis

Hexametaphosphate undergoes hydrolysis in water producing orthophosphate (iP) and trimetaphosphate, which is then slowly hydrolyzed into orthophosphate. To depolymerize into ortophoshate, trimetaphosphate must be first converted into triphosphate. Such conversion is temperature dependent and rapid at 100 °C (Bell, 1947). First, HMP was prepared in water at room temperature and tested as phosphate donor in the cell-free expression system supplemented with 1.5 or 3.5 mM amino acids (Supplementary Fig. 2). An HMP concentration range, 0.1–1.5 mM, was tested and the resulting deGFP yield was measured. The solution of HMP, kept at room temperature, was tested once a day for four 4 days. The HMP solution was incubated at 100 °C for 5 min just before use and afterwards kept at RT (≈ 25 °C) before being tested again (Supplementary Figs. 3 and 4). The concentration of maltose and maltodextrin was chosen on the basis of our previous optimization (Caschera and Noireaux, 2013). We found that about 40 μM (≈ 1 mg/mL) of active deGFP protein is produced with 3.5 mM amino acids, 15 mM maltose and HMP ≈ 1 mM. Once the optimal conditions were determined, the HMP concentration range was performed with 1.5 mM or 3.5 mM amino acids and with maltose (Fig. 2A) or maltodextrin, (Fig. 2B).

The highest yield, ≈ 45 μM (1.14 mg/mL), was observed for 35 mM maltodextrin, 3.5 mM amino acids, and a concentration of HMP of 0.8–1.2 mM. With our previous system based on maltose or maltodextrin coupled to 3-PGA (Caschera and Noireaux, 2013), we were able to achieve ≈ 1.4 mg/mL of active protein using the same plasmid.

3.2. Kinetics of in vitro deGFP synthesis with HMP

Kinetics measurements of deGFP expression with different concentrations of HMP were performed with 1.5 mM (Supplementary Fig. 5) or 3.5 mM amino acids, and with 15 mM maltose (Fig. 3A) or 35 mM maltodextrin (Fig. 3B).

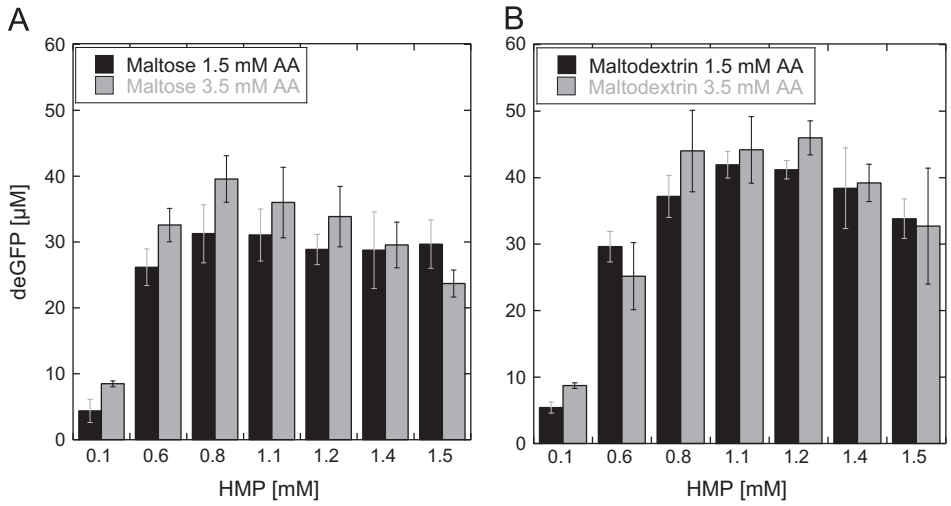

Fig. 2. deGFP yield achieved with (A) 15 mM maltose or (B) 35 mM maltodextrin and different concentrations of HMP. The concentration of amino acids was 1.5 mM or 3.5 mM with 6 nM of the plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500. Cell-free reactions (12 μL) were incubated at 29 °C for 18 h before measurements in a 384-well plate. Error bars represent the standard deviation of three experiments.

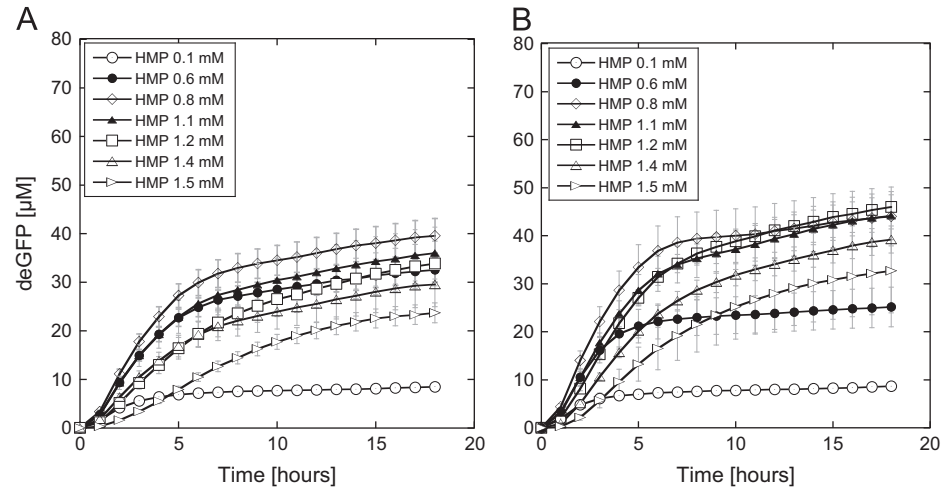

Fig. 3. Kinetics of *in vitro* deGFP synthesis with 15 mM maltose (A) or 35 mM maltodextrin (B) and different concentrations of HMP (3.5 mM amino acids, 6 nM plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500). Kinetics of cell-free reactions (5 μL) were incubated at 29 °C in sealed 96-well plate with conical bottom for 18 h during plate reader measurements. Error bars represent the standard deviation of three experiments.

Overall, protein synthesis is faster with maltodextrin as carbon source and decreases with higher concentration of HMP. With maltose, 0.8 mM HMP and 3.5 mM amino acids the synthesis last up to 8 h (Fig. 3A). With maltodextrin, HMP between 0.8–1.2 mM and 3.5 mM amino acids the synthesis is faster (5 h) reaching also higher protein yield. Overall, this metabolism is faster than the maltodextrin-3-PGA system showing higher protein yield after 9–10 h of incubation (Caschera and Noireaux, 2013). Most likely, the immediate availability of iP triggers the phosphorylation of maltodextrin (degradation) for subsequent activation of the glycolytic pathway.

3.3. *pH change during in vitro protein synthesis and accumulation of organic acids (lactate and acetate)*

During protein synthesis the pH decreases due to the production of organic acids: lactate and acetate. We previously reported a pH drop with the maltose-3-PGA system using the pH sensitive dye SNARF-5F (Caschera and Noireaux, 2013). We used SNARF-5F to monitor the pH profile during protein synthesis with maltose and maltodextrin in the presence of HMP as phosphate molecule donor (Fig. 4). The pH was retrieved using a calibration line and the fluorescence measurements (Supplementary Fig. 6).

Without maltose added to the cell-free reaction the pH is stable around 7.9 (Fig. 4A), suggesting that the metabolism for producing and regenerating ATP is not activated. Conversely, with maltose the metabolism is activated and characterized by a pH drop to 6.5 after 4 h of cell-free protein synthesis. Interestingly, the pH changes also in the presence of 2-deoxy-D-glucose, which is a glucose analog inhibiting the glycolytic pathway. This indicates that the glycolytic pathway is not inhibited. This suggests that under the conditions described (high concentration of iP+maltose) the metabolism is mainly based on glucose-1-phosphate. Indeed, accumulation of glucose during protein synthesis is very low $\leq$0.5 mM (Supplementary Fig. 7). Conversely, in a previous work we showed that maltose metabolism is inhibited with 2-deoxy-D-glucose added before starting the reaction (Caschera and Noireaux, 2013), thereby suggesting that in this case, the metabolism is first based on glucose and 3-PGA, and later on the recycling of iP accumulated during protein synthesis. The reaction without DNA suggests that maltose phosphorylation occurs mainly through the iP derived from HMP hydrolysis rather than the iP accumulated during protein synthesis.

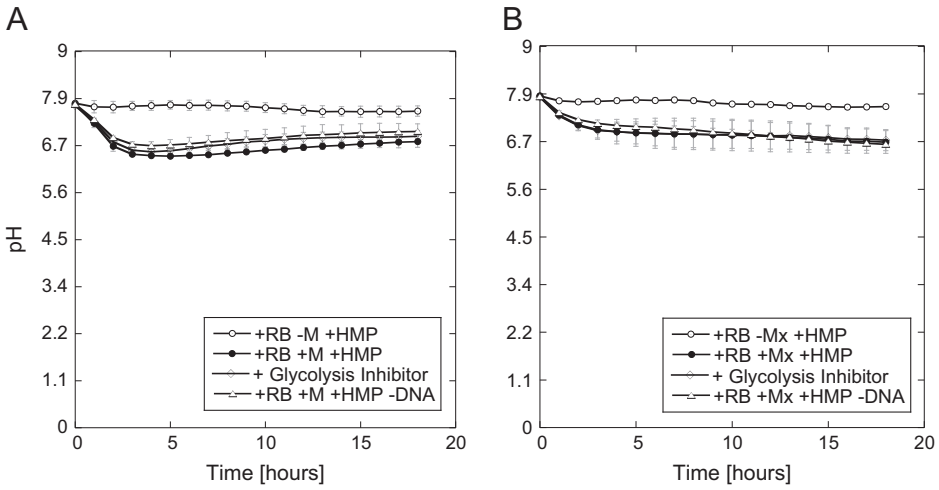

Fig. 4. Kinetics of pH change during cell-free protein synthesis. (A) 15 mM maltose (B) 35 mM maltodextrin. In both cases 3.5 mM amino acids, 0.8 mM HMP and 6 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-Luc-T500 were used in solution. The inhibitor 2-deoxy-D-glucose was used as a control (12 mM). Cell-free reactions (5 μL) were incubated at 29 °C in sealed 96-well plate with conical bottom for 18 h during plate reader measurements. Error bars represent the standard deviation of three experiments.

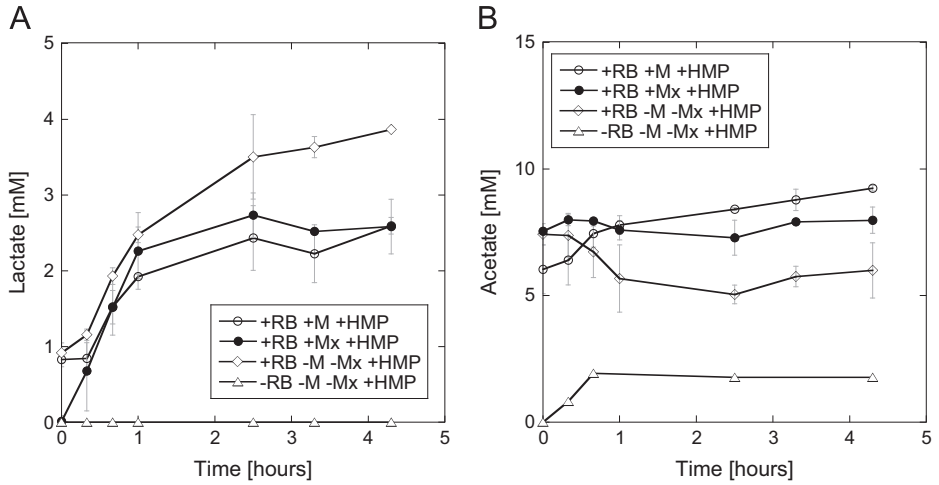

Fig. 5. Time course of lactate (A) and acetate (B) concentrations during cell-free protein synthesis. Cell-free reactions (12 μL) were incubated at 29 °C and snap frozen in liquid nitrogen before determination of the organic acid concentrations through enzymatic colorimetric assays. The reactions were prepared with 15 mM maltose, 35 mM maltodextrin, 3.5 mM amino acids, 1.1 mM HMP and 6 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-Luc-T500. Error bars represent the standard deviation of two experiments.

The same experiments were performed with maltodextrin (Fig. 4B). As with maltose, without maltodextrin in the reaction mixture the pH is stable, around 7.5. When maltodextrin is added to the reaction the pH decreases to 7.3 after 2 h of incubation and remains stable around 7.3 for 12 h. However, when DNA is not added to the reaction, the pH drops to ≈ 7.3 within the first 2 h, and then to 6.8 over the observation time (18 h). This suggests that the production of the organic acids and the accumulation of iP are responsible for the decrease of pH. As in the case with maltose, when the inhibitor of the glycolysis is added to the reaction, no inhibition of the pathway is observed. This confirms that with maltodextrin the metabolism for ATP production is provided by glucose-1-phosphate (accumulation of large amount of glucose is not observed, Supplementary Fig. 7), the product of the enzymatic reaction catalyzed by maltodextrin phosphorylase (Chao et al., 1969). The change in pH is due to the accumulation of inorganic acids, primarily lactate and acetate, during *in vitro* metabolism (Fig. 5A and B).

Interestingly, without maltose or maltodextrin in solution lactate reaches a concentration up to 4 mM, as a result of the activity of the lactate dehydrogenase (Calhoun and Swartz, 2007). With maltose or maltodextrin the accumulation of lactate decreases, most likely due to larger concentrations of glycolytic intermediates, such as pyruvate. Indeed, greater concentrations of acetate are observed with maltose and maltodextrin in solution up to 8 and 7 mM respectively (Fig. 5B). The accumulation of acetate is a consequence of the ATP-regeneration system based on the endogenous enzymes pyruvate dehydrogenase, phosphotransacetylase and acetate kinase (Calhoun and Swartz, 2007). Moreover, organic acids accumulation (Fig. 5A and B) is the result of a metabolic activation given by the components present in the reaction buffer (RB).

The results described above show that with maltodextrin higher protein yields and synthesis rates are achieved compared to maltose (Figs. 2B and 3B). Most likely, this result is due to a better homeostatic condition, *e.g.* lower levels of organic acids produced and therefore stable pH. In addition, efficient production of glucose-1-phosphate, bypassing the production of glucose, gives one less ATP consumed.

3.4. *In vitro protein synthesis and oxidative phosphorylation*

The slight change in pH (Fig. 4A and B) and the modest accumulation of organic acids (Fig. 5A and B), may suggest that oxidative phosphorylation is an important process involved in the

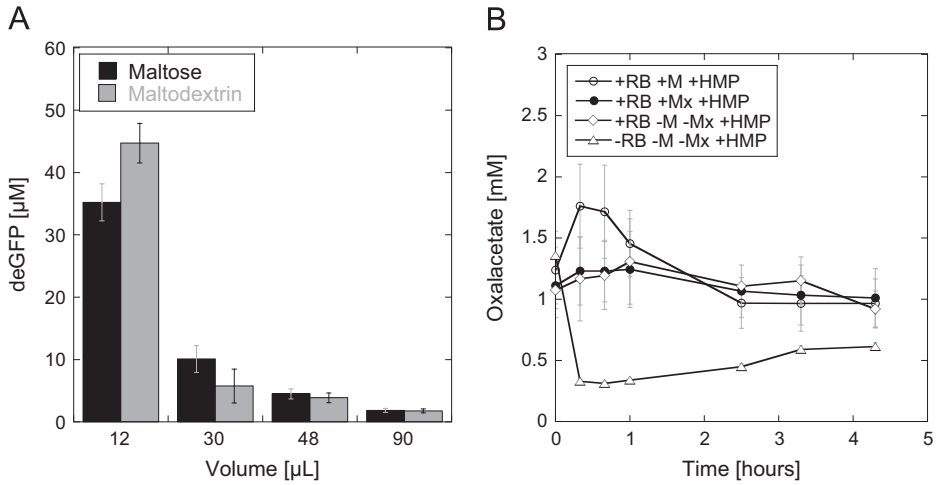

Fig. 6. (A) Cell-free synthesis of deGFP in batch-mode varying the reaction volume with 15 mM maltose or 35 mM maltodextrin, 3.5 mM amino acids, 1.1 mM HMP and 6 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 in solution. Cell-free reactions were incubated at 29 °C for 18 h before measurements in a 384-well plate. Error bars represent the standard deviation of three experiments. (B) Time course of oxaloacetate concentration during cell-free protein synthesis with 15 mM maltose or 35 mM maltodextrin, 3.5 mM amino acids, 1.1 mM HMP and 6 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-Luc-T500. Cell-free reactions (12 μL) were incubated at 29 °C and snap frozen in liquid nitrogen before determination of the organic acid concentrations through enzymatic colorimetric assays. Error bars represent the standard deviation of two experiments.

synthesis of ATP and therefore in the system energetic. Jewett et al. have demonstrated the presence of inverted membrane vesicles (IMVs), accessorized with the complex for the electron transport chain and the F_1 F_0 –ATPase, in cellular lysates. Such supramolecular structures are active in ATP synthesis and regeneration (Jewett et al., 2008). Interestingly, we can hypothesize that also in our system oxidative phosphorylation is an important process for efficient protein synthesis. Indeed, protein synthesis depends on oxygen availability (Fig. 6A). The cell-free reactions were prepared in batch-mode at different volumes to decrease the surface area/volume ratio and depth of diffusion. When the volume increases the protein yield decreases demonstrating that a proper level of oxygenation is essential for the system energetic. Moreover, oxidative phosphorylation is coupled to the tricarboxylic acid cycle (TCA cycle) for synthesis of NADH. The concentration of oxaloacetate, an intermediate of the cycle, during *in vitro* protein synthesis was measured (Fig. 6B).

Oxaloacetate is slightly accumulated during the first hour up to 1.6 mM with maltose in solution, whereas its concentration is kept constant at 1 mM either with maltodextrin and/or the components of the reaction buffer. Conversely, without polysugars and RB in solution the concentration of oxaloacetate drops below 0.5 mM, which indicates lower activity of the metabolic cycle. Additional demonstration that protein synthesis is oxygen dependent is given in Supplementary Fig. 8. Herein, an inhibitor (potassium cyanide, KCN) of cytochrome C oxidase, the enzyme of the electron transfer chain, is added in solution. In the experiment, deGFP yields with maltose and maltodextrin are compared with and without KCN in solution. We show that KCN inhibits the protein production, suggesting that oxidative phosphorylation is significant in our system.

3.5. Characterization of ATP regeneration and iP recycling with the maltodextrin-HMP metabolism

The efficiency of the novel ATP-regeneration system was determined by an ATP assay and an iP assay (Fig. 7A and B). The reaction buffer (RB) used to initiate cell-free expression is prepared with coenzyme, nucleoside triphosphate and an initial ATP concentration of 1.5 mM (see Section 2). The concentration of ATP was measured during protein synthesis with and without maltodextrin and RB (Fig. 7A). Without maltodextrin the concentration of ATP drops below 0.4 mM, and ATP is not regenerated without the reaction buffer components. Since the glycolytic pathway based on iP recycling is efficiently activated only with maltodextrin, it is observed that the level of ATP is kept constant at $\approx$ 1.2–1.5 mM, and therefore continuously available for energizing protein synthesis. However, a lower concentration of ATP is also regenerated only in the presence of HMP coupled to the components of the reaction buffer (Fig. 7A).

An efficient metabolism for cell-free expression should recycle iP because it inhibits the *in vitro* reaction by chelating magnesium ions (Kim and Swartz, 1999). In our previous study we showed that maltose is an efficient substrate for iP recycling (Caschera and Noireaux, 2013). Here, we also show that the new metabolism integrates iP recycling during protein synthesis (Fig. 7B). First, the phosphate donor HMP is added to the cell-free extract without the components of the reaction buffer. As a result, there is almost no iP (pyrophosphate) at the beginning of the reaction, but it is slowly accumulated overtime up to 7 mM (Fig. 7B). This suggests that HMP is hydrolyzed in triphosphate, after incubation at 100 °C (see Section 2). We hypothesize that the endogenous phosphatases carry out the dephosphorylation reaction producing iP. Conversely, when the components of the reaction buffer are added with HMP, the accumulation of iP is more evident, reaching a concentration of up to $\approx$ 15 mM (Fig. 7B). Likely this is due to the inorganic phosphate recycling for ATP regeneration. Instead, with maltodextrin, iP is recycled very efficiently to keep ATP at constant levels during protein synthesis.

3.6. High yield protein synthesis as a function of plasmid concentration

The last step in the new HMP-maltodextrin metabolism characterization consisted of a plasmid concentration range to determine the highest protein achievable (Fig. 8). Two different reporters were expressed to show the versatility of our system. Further optimization of the genetic constructions may be needed to increase the yield of new interesting genes (Supplementary Fig. S9). The plasmid used in this work, pBEST-OR2-OR1-Pr-UTR1-deGFP-T500,

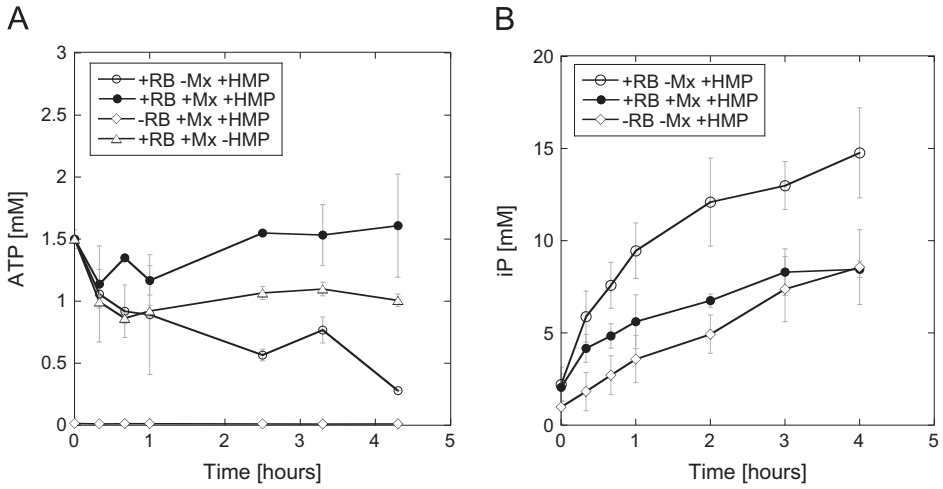

Fig. 7. Variation of (A) ATP concentration and (B) iP concentration during protein synthesis with 35 mM maltodextrin, 3.5 mM amino acids and 1.1 mM HMP (plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500, 6 nM). Cell-free reactions (12 μL) were incubated at 29 °C and snap frozen in liquid nitrogen before determination of ATP and iP concentrations. Error bars represent the standard deviation of two experiments.

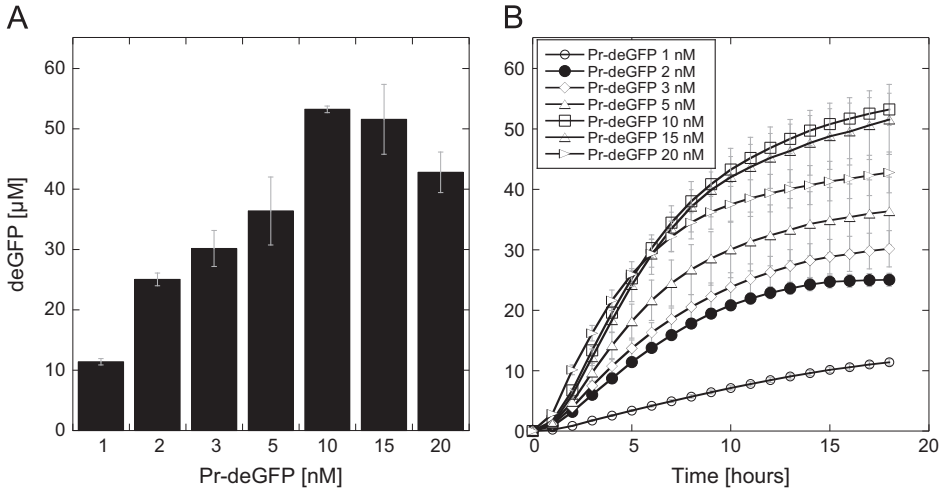

Fig. 8. Histograms of deGFP synthesis yield with different concentrations of pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 (A). Cell-free reactions (12 μL) were incubated at 29 °C for 18 h before measurements in a 384-well plate. Kinetics of cell-free reactions (5 μL) incubated at 29 °C in sealed 96-well plate with conical bottom for 18 h (B). In both cases 35 mM maltodextrin, 3.5 mM amino acids, 1.1 mM HMP and 6 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-Luc-T500 were used in solution. Error bars represent the standard deviation of three experiments.

Table 1
Cost analysis of different energy sources used to energize cell-free expression systems. The different cell-free expression systems are ranked by efficiency defined as: yield/$ (reagents cost from Sigma-Aldrich on-line catalog in June 2014).

Energy source	Reference	Cost per g	Yield of active protein (mg/mL)	(Yield/$)
Maltodextrin + KH_2PO_4	(Kim et al., 2011)	$0.40 + $0.22 = $0.62	1.7	2.74
Glutamate + KH_2PO_4	(Jewett et al., 2008)	$0.14 + $0.22 = $0.36	0.88	2.44
Glucose + KH_2PO_4	(Calhoun and Swartz, 2005)	$0.20 + $0.22 = $0.44	0.55	1.25
Maltodextrin + HMP	This work	$0.40 + $1.68 = $2.08	1.65	0.79
Pyruvate	(Jewett and Swartz, 2004b)	$5.18	1.4	0.27
Fructose-1,6-biphosphate	(Kim et al., 2007a)	$63.20	1.3	0.02
Glucose-6-phosphate	(Calhoun and Swartz, 2005)	$58.20	0.7	0.013
Glucose + creatine phosphate (CP)	(Kim et al., 2007b)	$0.20 + $156 = $156.2	1.5	0.0096
Creatine phosphate	(Kim et al., 2006)	$156	1.2	0.0077
Maltose + 3-phosphoglyceric acid (3PGA)	(Caschera and Noireaux, 2013)	$0.3 + $277 = $277.3	1.9	0.0068
3-phosphoglyceric acid (3-PGA)	(Shin and Noireaux, 2010)	$277	0.75	0.0027
Phosphoenolpyruvate (PEP)	(Kim and Swartz, 2001)	$323.50	0.75	0.0023

has an *E. coli* promoter specific to the housekeeping sigma factor 70 and a strong 5′ untranslated region. The plasmid was previously designed to exploit the endogenous *E. coli* RNA polymerase rather than the traditional T7 bacteriophage RNA polymerase (Shin and Noireaux, 2010). The concentration ranges in Fig. 8A and B show that protein productivity increases as a function of the plasmid concentration up to 10 nM.

The protein production decreases above 10 nM plasmid. An optimal concentration of 10 nM plasmid that delivers ≈ 53 μM active deGFP, *i.e.* 1.34 mg/mL, is determined. In addition, a range of

the molecular crowding agent PEG-8000 between 1.5% and 2% (v/v) shows that the protein yield can be further increased to ≈65 μM (1.65 mg/mL) of active protein in batch mode (Supplementary Fig. 10). Our system with 10 nM plasmid is comparable to the most efficient cell-free TX–TL systems currently available.

4. Conclusion

In this work we have shown that an efficient metabolic scheme, based on the recycling of inorganic phosphate and the phosphorylation of sugars, can be exploited for *in vitro* protein synthesis. In contrast to systems that require addition of enzymes (Kameda et al., 2001; Wang and Zhang, 2009), this new ATP-regeneration system uses only the enzymes found in the *E. coli* extract for phosphorylation of maltose or maltodextrin (Boos and Shuman, 1998). However, the existence of a maltose phosphorylase in *E. coli* has not yet been demonstrated. Interestingly, the expression of a heterologous maltose phosphorylase allows increasing conservation of ATP in *Saccharomyces cerevisiae* grown with maltose (de Kok et al., 2011). In our system, ATP is continuously regenerated from a polyphosphate molecule (HMP), a novel phosphate molecule donor much cheaper than conventional systems. This result provides also a demonstration of the presence of active polyphosphates in the cellular lysate. The cost of the main energy sources used in recent cell-free protein synthesis systems is listed in Table 1.

Maltodextrin, glutamate and glucose coupled to inorganic phosphate are the best energy sources for cost-effective *in vitro* protein synthesis. Cell-free expression systems fueled by non-phosphorylated glycolytic intermediates (maltodextrin, glutamate, glucose and pyruvate) are on average about 200 times more efficient than cell-free expression energized by expensive high-energy phosphate donor molecules (fructose-1,6-biphosphate, CP, 3-PGA and PEP). Moreover, the efficiency of the system presented herein, could be further improved by replacing expensive nucleoside triphosphates (NTPs) with nucleoside monophosphate (NMPs) and exploit the endogenous enzymes to regenerate NTPs (Calhoun and Swartz, 2005; Jewett et al., 2008)

The novel ATP-regeneration system presented in this work is suitable for semi- and continuous systems for *in vitro* protein synthesis (Spirin and Swartz, 2008), industrial applications (Swartz, 2006), high-throughput experiments (Caschera et al., 2011) and large-scale reaction using cell-free or enzyme technology (Butler, 1977). Our study also demonstrates that cell-free transcription–translation systems are valuable platforms for understanding and developing novel metabolic pathways (Zhang et al., 2007; Zhu et al., 2013).

Author contributions

F.C. performed the experiments; F.C. and V.N. analyzed the data and wrote the manuscript. The authors declare no competing financial interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.10.007.

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