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A Note on Eukaryotic Cell-Free Protein Synthesis

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Abstract

The use of expensive high-energy phosphate compounds and exogenous enzymes to power protein synthesis limits eukaryotic cell-free protein synthesis. In a Saccharomyces cerevisiae crude extract CFPS platform, we demonstrate the capacity to regenerate ATP by using glucose as a secondary energy substrate. In comparison to the CrP/CrK system, batch reactions with 16 mM glucose and 25 mM phosphate produced 3.64 0.35 g mL1 of active luciferase, resulting in a 16% increase in relative protein yield. Efficient eukaryotic CFPS platforms can be developed on the basis of our demonstration.

Keywords: Phosphate compounds; Exogenous enzymes; Eukaryotic cell; CrP/CrK system

Introduction

Cell-free protein synthesis is a new field that makes it possible to make proteins without breaking down the cells. Rough cell lysates, or separates, are utilized all things being equal. The development of CFPS has encountered significant difficulties in providing chemical energy for the formation of peptide bonds and amino acylation of TRNAs [1]. Historically, donors of phosphate bonds with high energy; acetyl phosphate, creatine phosphate, and phosphoenolpyruvate, among others, have been utilized. The addition of pyruvate kinase, creatine kinase, or acetate kinase, or the endogenous presence of these enzymes in the cell extract, is required for ATP regeneration in these instances. Sadly, it has been demonstrated that the rapid production of phosphate from these high-energy compounds inhibits CFPS. In addition, batch reactions that make use of these substrates with secondary energy typically only produce a brief burst of ATP. Additionally, the high cost of phosphorylated energy compounds restricts their use in industry. New, low-cost secondary energy regeneration systems are needed to overcome these limitations [2].

By substituting glucose for PEP in the lysate, the E. coli CFPS platform has been able to fuel highly active CFPS from non-phosphorylated energy substrates in the last ten years. Glycerol drives CFPS at a much lower cost and generates more ATP per secondary energy substrate molecule, both of which are primarily made possible by advancements made by Swartz and colleagues. For instance, glucose has a 2:1 molar ratio of ATP to its secondary energy metabolite, whereas CrP and PEP have ratios of 1:1. Starch, maltodextrin, and maltose are just a few of the slowly metabolized glucose polymers that have been used by numerous groups to fuel E. coli-based CFPS as an extension of the pioneering works mentioned above [3].

The majority of eukaryotic CFPS platforms have been restricted to the use of high-energy phosphate secondary energy substrates, whereas E. coli-based CFPS systems have been developed from non-phosphorylated energy substrates, making it possible to develop numerous novel applications in industrial biotechnology and rapid prototyping. This includes, for instance, a CFPS system based on yeast that makes use of creatine phosphate and creatine phosphokinase to drive protein synthesis [4]. In this study, we sought to determine whether crude yeast cell extracts could be used to test the feasibility of initiating glycolysis and thereby regenerating energy, cofactors, and the support system required to fuel highly active protein synthesis. Not only is it important for CFPS applications to be able to use glucose as a fuel, but it can also increase the impact of cell-free synthetic biology by joining the rapidly increasing number of reports that highlight the ability to co-activate multiple biochemical systems in an integrated cell-free platform. We show that it is to be sure conceivable to drive yeast CFPS responses with glucose, as well as other glycolytic intermediates and non-phosphorylated energy sources, and have arrived at amalgamation yields of 1.05 ± 0.12 μg mL−1 dynamic luciferase with 16 mM glucose [5]. We improved our glucose energy system by incorporating cyclic AMP and exogenous phosphate, achieving batch yields of 3.64 0.35 g mL-1 active luciferase after demonstrating the synthesis of luciferase from glucose as the sole secondary energy substrate. According to our knowledge, this is the first instance of using the native glycolytic pathway to power a eukaryotic CFPS reaction. This makes it possible to create low-cost eukaryotic CFPS platforms from a variety of host organisms for a wide range of uses.

Materials and Methods

The energy regeneration system was replaced with glycolytic intermediates, but the yeast extract preparation, CFPS reactions, and luciferase quantification were carried out as previously described. Since CFPS yields are known to be magnesium-dependent, the concentration of magnesium glutamate added to CFPS reactions was optimized for each extract [6]. Fructose-1, 6-bisphosphate, pyruvate, glucose-6-phosphate, 3-phosphoglyceric acid, phosphoenolpyruvate, fructose-1, and CFPS reactions containing 0–25 mM glucose in conjunction with the CrP/CrK energy regeneration system were also put through their paces. The reaction mixture contained phosphate and 0.15 mM cAMP when indicated. The conditions of the reaction are listed in Supplemental. Ethanol was subjected to HPLC analysis in the manner previously mentioned. The nucleotide analysis was carried out as previously described, with the exception of adjusting the gradient for buffer B to: 0 min, 0%; 10 min, 30%; 50 min, 80%; 55 min, 100%; End after sixty minutes.

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Results

We wanted to use non-phosphorylated energy substrates to start glycolysis and central metabolism, which would fuel yeast CFPS. Given that Eduard Buchner discovered in 1897 that yeast extract could convert sugar into ethanol and carbon dioxide, we anticipate that this metabolism will be active. In the beginning, we tested six distinct glycolytic intermediates for their capacity to fuel both transcription and translation in batch CFPS reactions containing 15 L and were conducted at 21 °C for four hours [7]. Fructose 1,6-bisphosphate, phosphoenolpyruvate, glucose, 3-phosphglyceric acid, pyruvate, and glucose 6-phosphate were the six intermediates at concentrations ranging from 0 to 30 mM. The CFPS reaction was programmed to synthesize luciferase as a model reporter protein, and the use of the cap-independent translation initiation leader sequence made it possible to combine transcription and translation. Surprisingly, our findings demonstrated that yeast CFPS reactions can be powered with FBP or PEP from glycolytic intermediates upstream of pyruvate, with concentrations of 1.04 0.45 and 1.62 0.10 mL-1, respectively [8]. Only pyruvate was unable to function as a secondary energy source among the six glycolytic intermediates. The failure of pyruvate to drive CFPS was supposed because of the absence of ATP recovering force of pyruvate alone in aging metabolic cycles.

We then carried out time-course CFPS reactions using the three intermediates with the highest yields in order to gain a deeper comprehension of the dynamics of the system. This demonstrated that the choice of the glycolytic intermediate had no effect on the duration of the reaction but did affect the rate of protein synthesis; Negative control reactions with pyruvate or no secondary energy substrate produced little to no luciferase, and protein synthesis had stopped in all cases after 4 hours [9]. According to previous research, fermentation of the carbon from the glycolytic intermediates is anticipated to result in the production of ethanol. As a result, to ensure that glycolysis was operating for each carbon source, we measured the production of ethanol. When glucose, FBP, and PEP are used to power protein synthesis, we discovered, as expected, that ethanol is produced. In the presence of pyruvate, ethanol is also made, but no protein is made because there isn't enough ATP, as previously mentioned.

Determined to increment protein union yields, we next tried a double framework, in which glucose is utilized in blend with CrP/CrK. Beforehand, such a framework was shown by Kim to boost E. coli CFPS platform yields. Suddenly, we tracked down that the expansion of glucose to the CrP/CrK framework seriously hinders CFPS, with 10 mM glucose expansion bringing about an 89% decrease in protein union [10]. We reasoned that this could be a toxicity effect from the accumulation of ethanol or a pH drop, as was seen previously in E. coli CFPS platforms powered by glucose. In any case, we noticed no shift in pH during the direction of the response, and showed that ethanol isn't poisonous in that frame of mind at groupings of up to 25 mM, which far surpassed the normal ethanol created. By and large, non-useful energy utilization has been distinguished as one of the essential purposes behind early end of CFPS. As a result, we tracked the ATP pool over time with quantitative HPLC analysis. According to nucleotide analysis, the rapid ATP consumption that occurs when glucose is added to the reaction causes a decrease in the yields of protein synthesis. For instance, when 25 mM glucose is present, the reaction lasts for 15 minutes before all of the ATP has been used up, limiting the production of protein.

We reverted to the glucose-only system because the dual energy regeneration system could not be activated. An initial optimization revealed that the ideal substrate concentration is 16 mM glucose. Following that, we attempted to increase CFPS through a series of additional optimization experiments. We investigated the effects of the reaction temperature, the concentrations of magnesium glutamate, potassium glutamate, spermidine, and additives like cyclic AMP. Regardless of a thorough pursuit, we just saw that expansion of cAMP expanded yields, recommending that our unique circumstances for yeast CFPS caught a most extreme [11]. Our yields increased by 1.5 times when 0.15 mM cAMP was added, reaching approximately 1 g mL 1. Utilizing glucose and cAMP results in an intriguing trajectory for the kinetics of protein synthesis. Particularly, protein synthesis takes longer when glucose is used as an energy source, which we put down to the availability of ATP. In the first 30 minutes of the reaction, ATP is consumed quickly, but more than half is regenerated after 90 minutes.

We then looked into the use of carbon polymers that are slowly metabolized to slow the initial consumption of ATP since CFPS can be powered by glycolysis. With 1.4% starch, we demonstrated that soluble starch can fuel CFPS, albeit at much lower yields than the glucose system. With only 0.2 mM remaining after 30 minutes of the reaction, the use of starch had no effect on the initial consumption of ATP. When compared to glucose alone, our findings suggest that ATP regeneration restricts the utilization of starch [12]. When using starch, for example, the regeneration of ATP is less efficient than when using 16 mM glucose, which results in a lower protein yield. The fact that adding-glucosidase and amyloglucosidase enzymes did not increase the yields of protein synthesis suggests that our crude lysates have sufficient activity to metabolize starch.

Even though we demonstrated the principle using starch as an energy substrate, the glucose energy regeneration system maintained higher yields. As a result, we went back to the glucose system to look for parameters that might make more luciferase produced. Calhoun and Swartz previously demonstrated that phosphate limitation can occur during energy regeneration when non-phosphorylated energy substrates are utilized. When compared to their glucose-driven E. coli CFPS system alone, they discovered that the addition of 10 mM inorganic phosphate resulted in a threefold increase in CFPS yields [13]. We tested the addition of potassium phosphate, an inorganic phosphate of 0–50 mM, to our glucose-driven yeast CFPS system as a follow-up to this discovery. CFPS yields increased nearly 3.5 times when 25 mM inorganic phosphate was added, reaching 3.64 0.35 g mL 1.

Conclusion

We have created a new glucose and phosphate-based energy regeneration system for yeast CFPS. This clever methodology eliminates the requirement for a costly phosphorylated auxiliary energy source and maintains a strategic distance from inhibitory phosphate gathering. This is the first time a CFPS system based on eukaryotic cells has been powered by the natural metabolism of a non-phosphorylated energy substrate. Our novel glucose/phosphate system has increased the relative protein yield by 16%, despite our yields not exceeding those of yeast extract or the CrP/CrK system. A cost-effective eukaryotic CFPS platform for high throughput protein expression, synthetic biology, and proteomic and structural genomic applications holds promise if this platform is further optimized through host strain engineering, as was done in systems based on E. coli. In the years to come, we anticipate that yeast CFPS, along with other CFPS technologies, will emerge as a major player.

Conflict of Interest

None

Acknowledgement

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