Substrate Replenishment Extends Protein Synthesis With an In Vitro Translation System Designed to Mimic the Cytoplasm

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Abstract: Cytoplasmic mimicry has recently led to the development of a novel method for cell-free protein synthesis called the "Cytomim" system. In vitro translation with this new system produced more than a 5-fold yield increase of chloramphenicol acetyl transferase (CAT) relative to a conventional method using pyruvate as an energy substrate. Factors responsible for activating enhanced protein yields, and causes leading to protein synthesis termination have been assessed in this new system. Enhanced yields were caused by the combination of three changes: growing the extract source cells on 2× YTPG media versus 2× YT, replacing polyethylene glycol with spermidine and putrescine, and reducing the magnesium concentration from conventional levels. Cessation of protein synthesis was primarily caused by depletion of cysteine, serine, CTP, and UTP. Substrate replenishment of consumed amino acids, CTP, and UTP extended the duration of protein synthesis to 24 h in fed-batch operation and produced 1.2 mg/mL of CAT. By also adding more T7 RNA polymerase and plasmid DNA, yields were further improved to 1.4 mg/mL of CAT. These results underscore the critical role that nucleotides play in the combined transcription–translation reaction and highlight the importance of understanding metabolic processes influencing substrate depletion. © 2004 Wiley Periodicals, Inc.

Keywords: cell-free protein synthesis; cytoplasmic mimicry; nucleotide; amino acid; combined transcription–translation; fed-batch reaction

INTRODUCTION

Cell-free protein synthesis is well suited to help satisfy the growing need for more efficient protein production methods. The well-controlled and flexible environment of the cell-free system offers several advantages over conventional in vivo technologies (Jermutus et al., 1998; Shimizu et al., 2001; Jewett et al., 2002). In brief, cell-free translation offers an attractive and convenient approach to produce properly folded proteins on a laboratory scale, incorporate unnatural or labeled amino acids, screen PCR fragment libraries in a high-throughput format, and express pharmaceutical proteins (Noren et al., 1989; Hanes and Pluckthun, 1997; Kigawa et al., 1999; Nakano et al., 2000; Norais et al., 2001; Kiga et al., 2002; Guignard et al., 2002; Sawasaki et al., 2002; Takahashi et al., 2002; Jewett and Swartz, 2004b).

Recently, we reported the development of the Cytomim system, a new method for empowering cellular catalysts to produce a desired protein in vitro (Jewett and Swartz, 2004a). This method addressed several limitations of conventional cell-free systems and has increased the utility of this technology by achieving greater expression of active protein products at a low cost. In this article, we have sought to understand two features of this new system. One was to identify the changes that enabled the greater than 5-fold increase in protein expression relative to conventional approaches using pyruvate as a secondary energy source. The second was to examine the reason for protein synthesis termination in the batch reaction.

To establish the requirements for activating the cell-free transcription and translation system, we investigated the effects of each individual change made in the development of the Cytomim system. Previously, we had only established that the new magnesium concentration, one of the seven changes, was essential (Jewett and Swartz, 2004a). Here, we have determined two additional changes that were necessary for the yield increase.

To identify the causes of reaction termination, we investigated substrate stabilities in the Cytomim system. Previous analyses reported by Kim and Swartz (2000a) illustrated that depletion of several substrates—including arginine, tryptophan, cysteine, and the secondary energy source, PEP—can lead to cessation of protein synthesis in cell-free systems. Guided by this knowledge and the original work of Spirin and co-workers (1988) suggesting that batch reactions are limited by substrate supply, we explored energy source, amino acid, and nucleotide stability during a Cytomim batch reaction.

We report that three primary changes activated cell-free protein synthesis in the Cytomim system. Furthermore, we determined that the secondary energy source (pyruvate),

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cysteine, serine, threonine, asparagine, glutamine, CTP, and UTP were all depleted during a batch reaction. Substrate depletion was overcome by repeated addition of limiting substrates over a 24-h fed-batch incubation. This approach extended protein biosynthesis and doubled the final yield of CAT relative to the control reaction without additions.

MATERIALS AND METHODS

Cell-Free Protein Synthesis

Combined transcription–translation reactions were carried out in 1.5-mL Eppendorf tubes at 37°C. Plasmid pK7CAT was used as a template for protein synthesis. pK7CAT contains the nucleotide sequence of the chloramphenicol acetyl transferase (CAT) gene, which has been cloned between the T7 promoter and the T7 terminator (Kim and Swartz, 2001). CAT has a molecular mass of 25,662 Daltons. Plasmids encoding for β-galactosidase and luciferase were also used. These constructs also contain the T7 promoter and terminator. Plasmids were purified using a QIAGEN Plasmid Maxi Kit (Valencia, CA). N-terminal histidine-tagged T7 RNA polymerase was prepared from *Escherichia coli* strain BL21 [using a modified form of the plasmid pAR1219 (Davanloo et al., 1984), containing the additional sequence for six N-terminal histidine residues]. Histidine-tagged T7 RNA polymerase was purified using a metal affinity column. Cell extract was prepared from *E. coli* strain A19 Δ*tonA*Δ*speA*Δ*tnaA*Δ*endA met*$^+$, a derivative of K12. Markerless deletions were achieved by following the method of Datsenko and Wanner (2000). These modifications include reverting a methionine auxotrophy back to a methionine prototrophy, removing the *speA* gene encoding arginine decarboxylase to stabilize arginine concentrations, removing the *tnaA* gene encoding tryptophanase to stabilize tryptophan concentrations, removing the *tonA* (*fhuA*) gene encoding a ferrichrome-iron receptor to protect against phage infection, and removing the *endA* gene encoding endonuclease I to stabilize the plasmid DNA (Michel-Reydellet et al., in press). Cells were grown in a 10-L fermenter to an OD (600nm) of 3.3, and cell extract was prepared as previously described (Jewett et al., 2002). When noted in the text, cells were grown on 2× YT media (Sambrook et al., 1989). Otherwise, cells were grown on a glucose and phosphate media, 2× YTPG (Kim and Choi, 2000). The 2× YTPG media contains the components of 2× YT media plus additional 22 mM KH_2PO_4, 40 mM K_2HPO_4, and 100 mM glucose. All cell-free reactions contained the following components: 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 34 μg/mL folinic acid, 170.6 μg/mL *E. coli* tRNA mixture, 13.3 μg/mL plasmid, 100 μg/mL T7 RNA polymerase, 5 μM l-[U-^{14}C] leucine, 2 mM each of 20 unlabeled amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.26 mM coenzyme A (CoA), 33 mM sodium pyruvate, and 0.24 volume of S30 extract. Solute concentrations for the conventional system control also included 57 mM HEPES-KOH (pH 7.5), 2% (w/v) polyethylene glycol 8000 (PEG), 175 mM potassium glutamate, 10 mM ammonium glutamate, 20 mM magnesium glutamate, and 2.7 mM sodium oxalate. Solutes added to the Cytomim system included 130 mM potassium glutamate, 10 mM ammonium glutamate, 8 mM magnesium glutamate, 1.5 mM spermidine, 1 mM putrescine, and 4 mM sodium oxalate. There was approximately an additional 3.3 mM magnesium, 14.4 mM potassium, 2.4 mM TRIS, and 23.5 mM acetate in each reaction originating from the cell extract. The final total *E. coli* protein concentration in each reaction was 9.6 ± 0.7 mg/mL, as determined by a Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Fed-batch experiments were performed at 33°C. Details are given in the figure legend. *E. coli* total tRNA mixture was purchased from Roche Molecular Biochemicals (Indianapolis, IN). l-[U-^{14}C]leucine was from Amersham Pharmacia Biotechnology (Uppsala, Sweden). All other reagents were obtained from Sigma (St. Louis, MO).

Product Determination

The amount of synthesized protein was determined by the incorporation of ^{14}C-leucine into TCA-precipitable radioactivity using a liquid scintillation counter (Beckman LS3801, Beckman Coulter, Fullerton, CA). Soluble and insoluble protein amounts were determined as previously described (Kim and Swartz, 2001).

Nucleotide and Organic Acid Concentration Measurement

High-performance liquid chromatography (HPLC) analysis was used to quantitate nucleotide and organic acid concentrations. For nucleotide analysis, 5% trichloroacetic acid (TCA) at 4°C was added to the cell extract reaction mixture in a 1:1 volumetric ratio. TCA-precipitated samples were centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant was collected and twenty-microliter samples were applied to a Vydac 302IC4.6 column (Vydac, Hesperia, CA) for analysis with an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA). Separation was carried out at a flow rate of 2 mL/min. The mobile phase started with 100% of a 10 mM phosphate buffer (1:1 molar ratio of NaH_2PO_4/Na_2HPO_4 adjusted to pH 2.6 with glacial acetic acid) and 0% of a 125 mM phosphate buffer solution (1:1 molar ratio of NaH_2PO_4/Na_2HPO_4 adjusted to pH 2.8 with glacial acetic acid). A linear gradient from 0% to 100% of the 125 mM phosphate buffer was applied for minutes 2 through 25, then maintained at 100% for 2 min, and then returned to 0% in a linear gradient for 3 min. Nucleotides were detected at 260 nm. Nucleotide concentrations were determined by comparison to a calibration obtained with nucleotide standards.

For pyruvate analysis, 5 mM sulfuric acid at 4°C was added to the cell extract reaction mixture in a 10:1 vol-

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umetric ratio. Acid-precipitated samples were centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant was collected and thirty-microliter samples were applied to a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA) for analysis on an Agilent 1100 series HPLC system. Separation was carried out at 55°C using a 5 mM sulfuric acid running buffer at a flow rate of 0.6 mL/min. Pyruvate was analyzed using duel UV and refractive index detection. Concentrations were determined by comparison to a standard calibration.

Amino Acid Analysis

Amino acids were analyzed using an AAA-DIRECT™ system from Dionex (Sunnyvale, CA). Five percent TCA at 4°C was added to the cell extract reaction mixture in a 1:1 volumetric ratio. TCA-precipitated samples were centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant was collected and diluted 250 times. Twenty-microliter samples were applied to an AminoPac™ (Dionex, Sunnyvale, CA) column for HPLC analysis, and gradient elution anion-exchange method was conducted according to the manufacturer's recommendations. Amino acids were detected using a gold working electrode with pulsed electrochemical detection (PED). Amino acid concentrations were determined by comparison with a calibration standard.

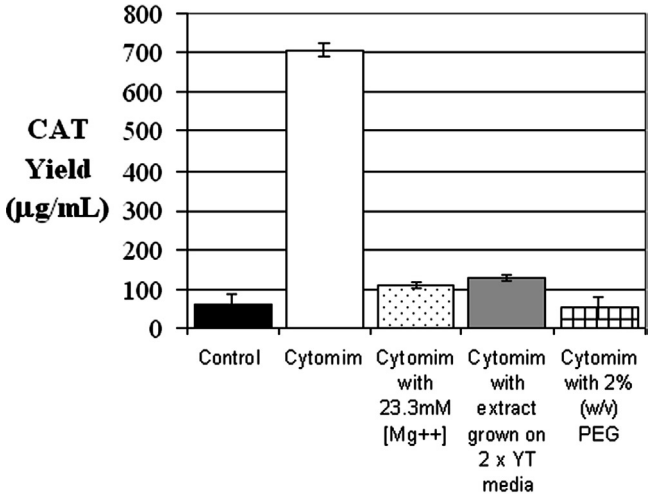

Figure 1. Changes required for protein expression using the Cytomim system. Batch reactions (15 μL) were carried out for 6 h (37°C). CAT expression was monitored by ^{14}C-leucine incorporation. Error bars represent the standard deviation for 3–8 separate reactions. From left to right: closed bar, control (the conventional cell-free system with pyruvate as an energy source); open bar, the Cytomim system (features include extract derived from source cells grown on 2× YTPG, 1.5 mM spermidine, 1 mM putrescine, and 11.3 mM magnesium); spotted bar, Cytomim system with 23.3 mM magnesium (magnesium concentration used in conventional control); shaded bar, Cytomim system with extract derived from cells grown on 2× YT (source cells used in conventional control); checkered bar, Cytomim system with 2% (w/v) PEG 8000 in place of 1.5 mM spermidine and 1 mM putrescine (conventional system uses 2% (w/v) PEG).

RESULTS

Requirements for Activating the Cytomim System

As previously reported (Jewett and Swartz, 2004a), the environmental conditions used in the Cytomim system resulted from an effort to better mimic the intracellular environment of an *E. coli* cell. The HEPES buffer and polyethylene glycol (PEG) were removed, spermidine and putrescine were added, acetate salts were replaced with glutamate salts, and ammonium and magnesium concentrations were significantly reduced. Moreover, the extract source cells were grown on 2× YTPG media rather than 2× YT. Because several changes were made, the specific modifications leading to the unexpectedly high productivity of the Cytomim system were unknown. We examined protein expression by restoring each individual compositional change back to the condition in the conventional system and then measuring the synthesis of chloramphenicol acetyl transferase (CAT).

Previously, we had shown that reducing the magnesium concentration from conventional levels was critical in activating the Cytomim system (Jewett and Swartz, 2004a). This is evident in Fig. 1, which shows batch-reaction CAT yields for a control reaction (the conventional system using pyruvate as an energy substrate), the Cytomim system, and the Cytomim system with the magnesium concentration present in the conventional method. This study allowed us to conclude that two additional changes were also necessary for the dramatic increase in protein expression. The next critical change for Cytomim activation was use of a different growth media than traditionally employed. The extract source cells used for the Cytomim system were grown on media containing glucose and phosphate following the protocol of Kim and Choi (2000). According to their report, extract prepared on 2× YTPG produced 37% more protein than extract grown on the conventional 2× YT. In the case of the Cytomim system, use of extract from cells grown on 2× YTPG medium resulted in about a 500% increase in protein production (Fig. 1). This clearly indicates that using source cells grown on 2× YTPG is vital for the Cytomim system. The remaining essential modification was removal of polyethylene glycol from the standard reaction mixture. Reactions with 2% (w/v) PEG produced only about 8% of the CAT yield as compared to the Cytomim system (Fig. 1). Changing from acetate to glutamate salts, removing the HEPES buffer, and reducing ammonium concentrations were not required to activate the new system (data not shown).

Adding CoA and NAD to the reaction mixture was reported necessary for the production of about 120 μg CAT/mL using pyruvate as a secondary energy source in the conventional system (Kim and Swartz, 2001). These cofactors are involved in the conversion of pyruvate to acetyl phosphate using endogenous pyruvate dehydrogenase (*pdh*), the proposed mechanism of energy regeneration from pyruvate. Expression without both cofactors had

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formerly produced 30 μg CAT/mL from 33 mM pyruvate. The Cytomim system is not as dependent on addition of these cofactors (Table I). The removal of CoA had no significant effect, and the removal of NAD reduces synthesis by only about 10–15%. The manner in which the cells were grown may have had an impact on these results.

Substrate Profiles for the Cytomim System

After establishing the requirements for activating the Cytomim system, we explored potential causes for the arrest of protein synthesis during a batch reaction. Historically, cell-free protein synthesis systems have been plagued by early termination of protein expression. For example, initial work in the field utilized PEP batch reactions that lasted no longer than 20 min. Nonproductive energy source consumption, energy exhaustion and amino acid depletion have been identified as primary reasons for reaction termination in the batch system (Kim and Choi, 2000; Kim and Swartz, 2000a). We investigated the stability of these substrates in the Cytomim system.

Previous work demonstrated that consumption of arginine, cysteine, and tryptophan is one factor associated with protein synthesis cessation in vitro (Kim and Swartz, 2000a). The present study utilized extract prepared from a genetically engineered host strain that displays significantly lower rates of arginine and tryptophan degradation (Michel-Reydellet et al., in press). Although these amino acids were stabilized, cysteine stability was still expected to be a problem. Additionally, the degradation of other amino acids may have contributed to synthesis termination in this more natural system.

Thus, concentrations of all twenty amino acids were investigated in the Cytomim system during the course of a standard batch reaction. Twelve of the amino acids maintained relatively constant values around 2 mM, their initial concentration. Some amino acids, however, changed significantly (Fig. 2A). Cysteine and serine were entirely depleted during the first hour of synthesis. Threonine was exhausted from the reaction by the fifth hour. Glutamine and asparagine decreased rapidly over the first hour and then stabilized. Alanine increased to about 4 mM over the first hour, most likely due to formation from pyruvate, and then decreased to approximately 1.5 mM. Glutamate, which has an initial concentration of about 160 mM, appeared to decrease as well, although this value was not precisely quantified due to method constraints. Finally, aspartic acid increased linearly to about 20 mM during the reaction (Fig. 2B). These data suggest that the protein synthesis reaction may be constrained by the depletion of cysteine, serine, and possibly threonine.

Rapid consumption of the secondary energy source that supplies ATP for protein synthesis has also been shown to

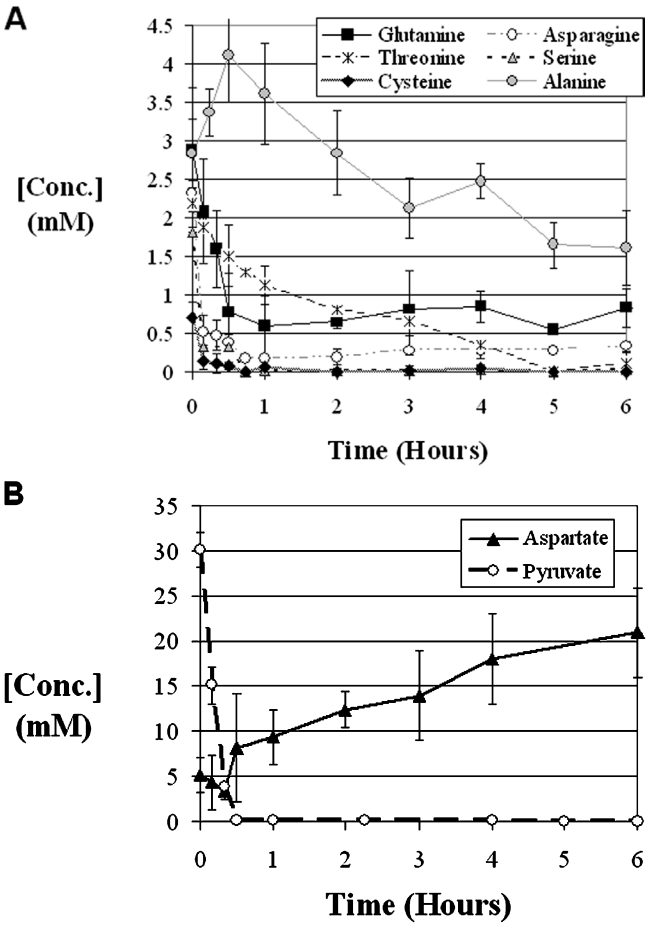

Figure 2. Substrate profiles in the cell-free protein synthesis system. Batch reactions (15 μL) synthesizing CAT were carried out for 6 h (37°C) using the Cytomim system, and samples were taken to measure amino acid or pyruvate concentrations. Error bars represent the standard deviation for 6–8 separate experiments. (**A**) Amino acid analysis was performed using a Dionex HPLC system. All 20 amino acids were monitored over the course of the reaction and those that demonstrated the most dramatic concentration change are shown (with the exception of glutamate, which was not quantified due to method constraints): closed squares, glutamine; open circles, asparagine; asterisks, threonine; closed triangles, serine; closed diamonds, cysteine; closed circles, alanine. (**B**) Aspartate analysis was performed using a Dionex HPLC system. Pyruvate analysis was carried out using a Bio-Rad Aminex HPX-87H ion-exchange column: closed triangles, aspartate; open circles, pyruvate.

Table I. Effects of synthesis with and without NAD and CoA in the Cytomim system.[a]

Expressed Protein	0.33 mM NAD	0.26 mM CoA	Relative protein production
CAT, β-gal, luciferase	x	x	1.00 ± 0.03
CAT		x	0.91 ± 0.05
CAT	x		1.02 ± 0.10
CAT			0.86 ± 0.03
β-gal			0.89 ± 0.05
Luciferase			0.76 ± 0.10

[a]Fifteen microliter reactions were performed for 6 hours (37°C), expressing CAT, β-galactosidase (β-gal), or bacterial luciferase. Error bars represent the standard deviation for 4 separate experiments. An "x" indicates the presence of that component. Protein production is given relative to a control with NAD and CoA. The control reactions produced 727 μg CAT/mL incubation, 622 μg β-gal/mL, and 704 μg luciferase/mL.

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be a limiting factor in cell-free translation (Kim and Choi, 2000; Kim and Swartz, 2000a). The concentration of pyruvate, the secondary energy source of the Cytomim system, was examined over the course of a 6-h batch reaction. This central metabolite was depleted from the reaction after 30 min (Fig. 2B). The depletion was independent of protein synthesis (data not shown). The brief presence of pyruvate relative to the duration of protein synthesis is very curious and indicates that there is most likely another energy source for ATP regeneration. Furthermore, it implies that pyruvate may not be necessary for protein synthesis in the Cytomim system.

The potential depletion of ATP, GTP, UTP, and CTP may also restrict productivity in cell-free systems. These nucleotides were investigated in a Cytomim reaction (Fig. 3A). Both GTP and ATP were significantly reduced over the first 30 min and then remained at constant levels. Strikingly, UTP and CTP were entirely depleted after the first hour. This important discovery is the first evidence of selective nucleotide triphosphate depletion. Total concentrations of the nucleotide phosphates also diminished over the first hour of the reaction (Fig. 3B). Understanding the critical role and metabolic fate of these compounds may suggest methods that help to increase the duration of synthesis in the batch reaction.

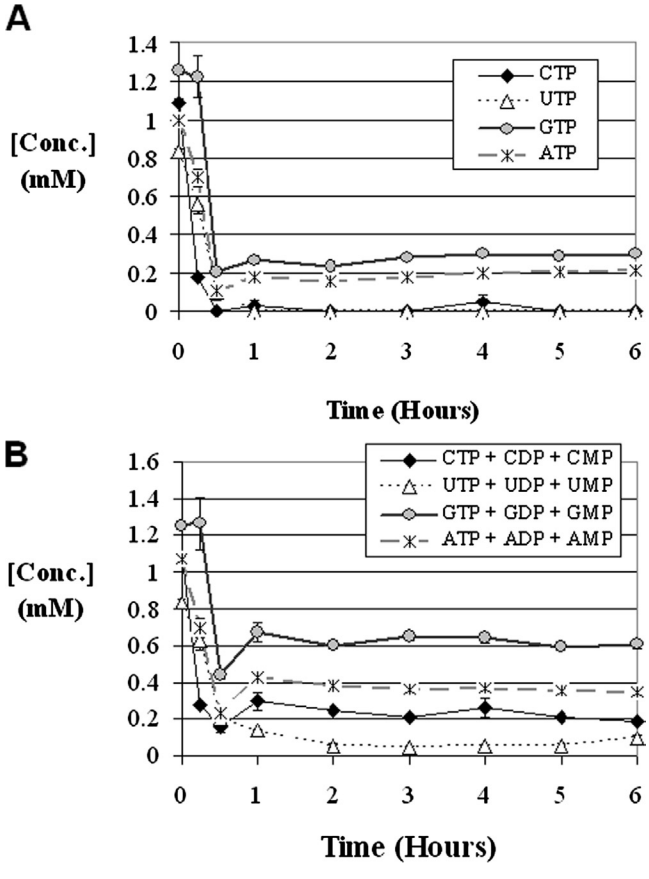

Figure 3. Nucleotide depletion in the Cytomim system. Batch reactions (15 μL) synthesizing CAT were carried out for 6 h (37°C), and samples were taken for nucleotide determination. Nucleotide concentration profiles were measured using HPLC analysis. Error bars represent the standard deviation for 3 separate reactions. (**A**) Triphosphate concentrations: closed diamond, CTP; open triangle, UTP; closed circle, GTP; asterisks, ATP. (**B**) Total nucleotide concentration: closed diamond, CTP + CDP + CMP; open triangle, UTP + UDP + UMP; closed circle, GTP + GDP + GMP; asterisks, ATP + ADP + AMP.

Extending the Duration of Protein Synthesis With Substrate Replenishment

Fed-batch experiments were performed to determine if the duration of protein synthesis could be extended by replenishing depleted substrates over the course of a 24-h reaction (Fig. 4). Consistent with the observation that

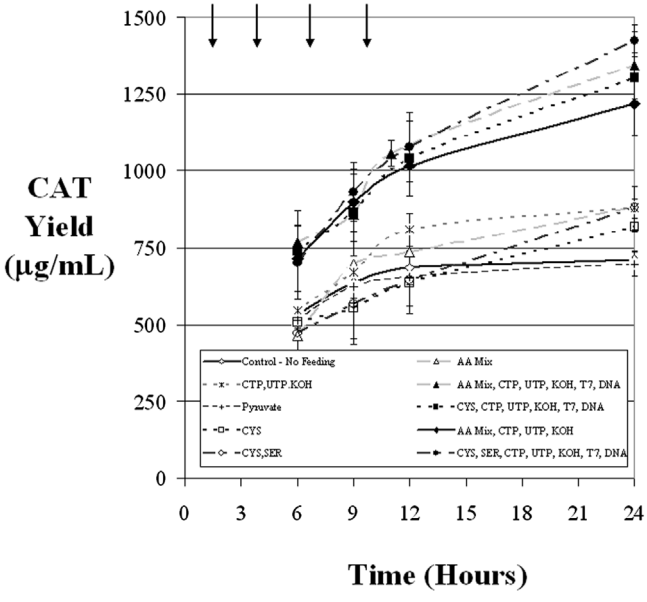

Figure 4. Fed-batch experiments with the Cytomim system. Reactions were carried out for 24 h at 33°C. Fifteen-microliter cell-free reactions were prepared in different tubes for every time point. At each time point, one tube was sacrificed in order determine the amount of expressed protein. CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the standard deviation from 3 to 8 separate experiments. The indicated substrates were added at 1.5, 3.5, 6.5, and 9.5 h (denoted with arrows on the figure). Substrates were added in the following concentrations: 33 mM pyruvate, 0.5 mM CTP, 0.5 mM UTP, 1.8 mM potassium hydroxide, 0.6 mM asparagine, 0.6 mM glutamine, 0.3 mM threonine, 2.4 mM cysteine, 1.2 mM serine, 12 mM potassium glutamate, 0.05 mg/mL T7 RNA polymerase, and 0.007 mg/mL pK7CAT plasmid. Water was added to the control reaction to ensure that there were no volume change effects. The amino acid mixture contained asparagine, glutamine, threonine, cysteine, serine, and glutamate. Potassium hydroxide was added simultaneously with UTP and CTP in order to avoid pH change. Legend: open diamonds, control, no additions; plus signs (+), pyruvate added; open triangles, amino acid mixture added; open squares, cysteine added; open circles, cysteine and serine added; asterisks, CTP, UTP, and potassium hydroxide added; closed diamonds, amino acid mixture, CTP, UTP, and potassium hydroxide added; closed triangles, amino acid mixture, CTP, UTP, potassium hydroxide, T7 RNAP, and pK7CAT added; closed squares: cysteine, glutamate, CTP, UTP, potassium hydroxide, T7 RNAP, and pK7CAT added; closed circles: cysteine, serine, glutamate, CTP, UTP, potassium hydroxide, T7 RNAP, and pK7CAT added.

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ATP concentration was maintained at a level sufficient for protein synthesis (~200 μM) during the six hour batch reaction, repeated additions of the secondary energy source, 33 mM pyruvate, had no impact on synthesis yields. On the other hand, replenishing nucleotides and amino acids allowed for the extension of the reaction period relative to a control without substrate feeding. Repeated additions of UTP and CTP or of an amino acid mixture containing threonine, asparagine, glutamine, cysteine, serine, and glutamate increased CAT expression. These results suggest that these substrates likely limit the batch reaction.

Upon further investigation, it was determined that feeding both cysteine and serine or only cysteine alone enhanced synthesis yields as much or nearly as much, respectively, as adding all depleted amino acids (Fig. 4). Adding serine alone did not significantly improve protein synthesis (data not shown). In order for protein synthesis to be enhanced when only cysteine was added, there must be an intrinsic source of serine. One explanation is that sufficient serine is being synthesized to satisfy protein synthesis requirements. Another hypothesis is that proteins are being hydrolyzed to provide serine. However, this seems less probable since a general increase in other amino acids is not observed. It is not surprising that feeding glutamine and asparagine did not affect protein synthesis yields since these amino acids are not entirely depleted over the course of the reaction. The lack of expression increase upon threonine addition indicates that as with serine, sufficient threonine for protein expression must be scavenged or produced by enzymes within the extract.

When combined, repeated additions of amino acids, UTP, and CTP significantly enhanced protein synthesis over a 24-h reaction period to greater than 1.2 mg CAT/mL (Fig. 4). Yields could be further enhanced to approximately 1.4 mg/mL by addition of T7 RNAP and pK7CAT plasmid. T7 RNAP was previously shown to be degraded in the *E. coli*-based transcription and translation system by SDS-PAGE gel electrophoresis following metal chelation purification (N. Michel-Reydellet and J.R. Swartz, unpublished results).

DISCUSSION AND CONCLUSIONS

This work has characterized the requirements for activating high-level protein production in the Cytomim system, revealed several causes for the termination of protein synthesis, and demonstrated a doubling of CAT yields with a fed-batch protocol. These results underscore the importance of measuring metabolite concentrations and understanding active metabolic pathways in the cell-free reaction. Furthermore, they indicate that, in spite of our progress, we are not yet able to replicate essential features of the cytoplasmic environment. In vivo, rapidly growing cells continually regenerate energy and provide substrates necessary for internal regulation, rapid growth, and maintenance. We want to mimic this environment, one that is free of substrate limitations and inhibitory byproduct accumulation, in vitro. To do this, we still need to learn to maintain stable substrate concentrations. This work clearly demonstrates that substrate replenishment can be used as a strategy to overcome the degradation of building blocks used for mRNA and proteins. Another method to improve the productivity of cell-free systems is altering the cell extract by engineering a new source strain. Deletion of genes encoding nonessential enzymes that cause substrate degradation in vitro can be a powerful technique. This approach alleviates the need to replenish consumed resources and can increase batch production yields. For example, inactivating the *speA* and *tnaA* genes to avoid arginine decarboxylase and tryptophanase expression has stabilized arginine and tryptophan concentrations in the cell-free system (Michel-Reydellet et al., accepted).

Fed-batch experiments demonstrated that cysteine and serine were the primary amino acids responsible for the termination of protein synthesis in the Cytomim system (Fig. 4). Inactivation of the genes that encode the enzymes catabolizing cysteine and serine is being explored as a strategy to stabilize these amino acids. Several principal targets may lead to the reduction of cysteine. Cysteine desulfhydrase was one likely candidate. The glucose-containing media used in extract preparation enhances the rate of synthesis of this enzyme in vivo (Guarneros and Ortega, 1970). Unfortunately, deletion of the cysteine desulfhydrase gene was not effective in stabilizing cysteine in the cell-free system (Michel-Reydellet et al., in press). Other targets, such as glutamate cysteine ligate (Kim and Choi, 2000) and the family of cysteine desulfurase enzymes, are currently under investigation.

Serine exhaustion was almost as rapid as cysteine degradation. Serine deaminase activities convert serine into pyruvate in one step. The role of the two deaminase enzymes, the products of *sdaA* and *sdaB* genes (Su et al., 1989; Shao and Newman, 1993), is believed to be nonessential for growth in *E. coli* and these enzymes may likely responsible for the depletion of serine in the cell-free reaction. Mutant strains were constructed to stabilize serine concentrations within the in vitro translation system by deleting the *sdaA* and *sdaB* genes (Michel-Reydellet et al., in press). The rate of serine disappearance was reduced, although serine was still eventually depleted in reactions using extract from this new strain.

Depletion of glutamine, asparagine, and threonine has not previously been observed in the *E. coli*-based translation system. This effect is potentially due to nitrogen source catabolism. The Cytomim system has reduced the ammonium concentration by 70 mM relative to previously described methods (Kim and Swartz, 2001). It is plausible that the lower ammonium concentration causes the instability of these amino acids because glutamine, asparagine, and threonine can all be used as sources of nitrogen for the cell (Reitzer, 1996). While these three amino acids are diminished in the system, feeding them did not increase protein expression yields. This is most likely because they

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remain present at high enough concentrations to be above the threshold necessary for synthesis. At this time, we are not seeking genetic measures to stabilize these amino acids.

The production of aspartate can be detrimental if it directs resources away from protein synthesis. Phosphoenolpyruvate synthetase (Pps) directs pyruvate toward PEP, which can lead to the formation of aspartic acid. This reaction wastes energy that is available for protein synthesis because conversion of ATP to AMP is necessary to drive the reaction. Addition of oxalic acid, a known inhibitor of Pps, was reported to enhance synthesis yields and reduce the formation of aspartate to less than 3 mM in the cell-free system (Kim and Swartz, 2000b). In the current system, which contains oxalate, aspartic acid concentrations increase to about 20 mM. Some of this may be due to formation via PEP; however, it is likely that oxaloacetate, the direct precursor to aspartate, is being formed by other metabolic pathways. Understanding these active pathways may help us to redirect more system resources towards the production of our protein product.

Discovering that UTP and CTP depletion leads to the cessation of protein synthesis is a significant advance. Phosphatase activity has been a primary concern for stabilizing the energy source for translation, ATP, but never before has the depletion of CTP and UTP been recognized. The function of phosphatases, many of which are periplasmic proteins, is to dephosphorylate a broad array of structurally diverse compounds. The presence of phosphatases in the extract is probably due to the unnatural decompartmentalization of the periplasmic and cytoplasmic spaces after cell breakage. Removing the activity of the deleterious enzymes responsible for CTP and UTP degradation is expected to improve the productivity of the system due to the lack of nonproductive nucleotide degradation.

We have identified three main deletion targets for UTP and CTP stabilization: 5′-nucleotidase (*ushA*) (Zimmermann, 1992), alkaline phophatase (*phoA*) (Coleman, 1992), and a nonspecific acid phophatase (*aphA*) (Rossolini et al., 1998). In particular, 5′-nucleotidase, which hydrolyzes nucleotide mono-, di-, and triphosphates, is a concern. Although not by genetic methods, previous work in reducing the effect of phosphatase activity on nucleotide hydrolysis has shown promise. Kang et al. (2000) removed the periplasmic proteases by first producing spheroplasts. This reduced the ATP hydrolysis activity by 30% and increased the duration of protein synthesis in batch mode. The effect of this approach on UTP and CTP stability was not explored. An approach to reduce alkaline and hexose phosphatase activity, initially carried out by Kim and Choi (2000), repressed the expression of these enzymes during cell growth by using high concentrations of glucose and phosphate in the growth medium (Malamy and Horecker, 1964; Dvorak et al., 1967). This also resulted in a reduction of ATP phosphatase activity by 30%. GTP, UTP, and CTP phosphatase activities were present in the extract, but the authors did not address or measure nucleotide degradation in the context of the cell-free expression system. Genetic measures are currently being used to inactivate the genes that encode for UshA, PhoA, and AphA.

Replenishing the system with T7 RNA polymerase does not appear to be as important for increased protein expression as feeding amino acids or nucleotides. Nevertheless, removing the deleterious activity in the crude cell extract degrading T7RNAP may enhance protein expression. T7 RNAP degradation is potentially due to the presence of the periplasmic protease OmpT, which is known to degrade T7 RNAP (Grodberg and Dunn, 1988). The whole-cell assay utilized by Grodberg and Dunn to analyze T7RNAP degradation is clearly different from the cell-free environment. However, OmpT is expected to be present in the crude extract used in the cell-free system and can thus cleave T7RNAP in vitro. Protease removal in cell extracts has been reported by Jiang et al. (2002). This genetic approach reduced the protein degradation of the final protein product, but was not used to stabilize T7 RNAP. Approaches are now being investigated to remove OmpT activity.

In addition to identifying factors limiting protein biosynthesis, we also determined three protocol changes that were necessary in combination to enable the unexpected protein yield increase from pyruvate in the Cytomim system. We do not yet fully understand why each change was necessary. The first protocol change, decreasing the magnesium concentration, was likely needed because the affinity of pyruvate for magnesium is much lower than that of PEP. The second, growth on 2× YTPG media, was probably essential to activate specific metabolic pathways that positively alter the catalytic composition of the cell extract. The third, removal of PEG, is perhaps the most interesting. Although it severely inhibits the Cytomim system, PEG does not inhibit conventional systems relying on PEP for energy regeneration. This suggests that energy regeneration in the new system relies on a pathway or enzyme in the extract that interacts with PEG that is not necessary in traditional systems. We anticipate that gaining a more fundamental understanding of how energy is supplied within the system will help better explain the rationale for each requirement.

This work has explored factors responsible for activating and terminating cell-free protein synthesis. We identified three modifications from conventional cell-free systems necessary to activate the Cytomim system. Additionally, we demonstrated that instability of cysteine, serine, UTP, CTP, and T7 RNA polymerase instability is a limitation for in vitro transcription-translation systems. This new knowledge will help direct future measures aimed at increasing the productivity and duration of cell-free reactions. For the moment, the tremendous flexibility offered by the cell-free approach allows for these limitations to be overcome with fed-batch methods to produce efficient, long-lived, and economical protein synthesis.

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