Mimicking the *Escherichia coli* Cytoplasmic Environment Activates Long-Lived and Efficient Cell-Free Protein Synthesis

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Abstract: Cell-free translation systems generally utilize high-energy phosphate compounds to regenerate the adenosine triphosphate (ATP) necessary to drive protein synthesis. This hampers the widespread use and practical implementation of this technology in a batch format due to expensive reagent costs; the accumulation of inhibitory byproducts, such as phosphate; and pH change. To address these problems, a cell-free protein synthesis system has been engineered that is capable of using pyruvate as an energy source to produce high yields of protein. The ''Cytomim'' system, synthesizes chloramphenicol acetyltransferase (CAT) for up to 6 h in a batch reaction to yield 700 μg/mL of protein. By more closely replicating the physiological conditions of the cytoplasm of *Escherichia coli*, the Cytomim system provides a stable energy supply for protein expression without phosphate accumulation, pH change, exogenous enzyme addition, or the need for expensive high-energy phosphate compounds. © 2004 Wiley Periodicals, Inc.

Keywords: cell-free protein synthesis; pyruvate; cytoplasmic mimicry; phosphate and pH homeostasis; polyamines; combined transcription – translation

INTRODUCTION

Cell-free protein synthesis exploits the catalytic machinery of the cell to produce active proteins in vitro (Jermutus et al., 1998; Jewett et al., 2002; Shimizu et al., 2001; Yokoyama et al., 2000). This technology is recognized as a succesful protein production method on a laboratory scale and boasts several advantages over in vivo expression platforms. One key feature of cell-free expression systems is that they lack a cell wall, which constitutes a barrier to system modification and control in vivo. An open system provides the opportunity to directly manipulate reaction conditions for the most favorable expression of properly folded proteins. Other advantages of in vitro systems include: altering tRNA levels to reflect the codon usage of the expressed gene (Jiang et al., 2001), achieving single-step purification and recovery of protein products (Alimov et al., 2000; Jewett and Swartz , in press; Lamla et al., 2002), and incorporating unnatural or isotope labeled amino acids into proteins (Kigawa et al., 2002; Kigawa et al., 1999; Noren et al., 1998).

Previous work has shown that maximum protein expression requires adequate substrate supply (particularly nucleoside triphosphates and amino acids), a homeostatic environment, catalyst stability, and the removal or avoidance of inhibitory byproducts (Kim and Swartz, 2000a, 2001). Not surprisingly, these optimal characteristics are similar to the in vivo state of a rapidly growing *Escherichia coli* cell. The inability of prokaryotic cell-free systems to replicate these features of the cytoplasmic environment has hampered the productivity of this technology. One commonly recognized limitation is providing the translational machinery with sufficient chemical energy, in the form of ATP, to fuel protein synthesis in a homeostatic manner. For example, the conventional PANOx energy regeneration system (Kim and Swartz, 2001) produces 700 μg/mL of chloramphenicol acetyl transferase (CAT) using phosphoenolpyruvate (PEP) as an energy substrate; however, the batch reaction accumulates phosphate. Accumulation of this byproduct can inhibit protein synthesis (Kim and Swartz, 1999). Phosphate formation can be circumvented by using pyruvate to fuel protein production (Kim and Swartz, 2001). Alternatively, one can provide a sink to remove phosphate from the reaction with a continuous exchange cell-free system (CECF) (see Jewett et al., 2002 for review). Unfortunately, both of these approaches are not ideal solutions to alleviate byproduct formation. Previous attempts utilizing pyruvate have only 120 μg/mL of CAT in a batch reaction (Kim and Swartz, 2001). CECF systems lack the simplicity and high-throughput capability of the batch system and use expensive reagents inefficiently.

To address reaction homeostasis, reagent cost, and cytoplasmic mimicry, we developed a new method for prokaryotic in vitro translation called the Cytomim system. We reasoned that providing a set of reaction conditions that more closely reflects the cytoplasmic composition of the

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host organism, *E. coli*, would be beneficial for energy regeneration, protein synthesis, and protein folding in vitro. Several changes were made to the physicochemical environment of the conventional cell-free system. Unnatural components, such as pH buffers and polyethylene glycol (PEG) were removed and the ionic composition of the reaction mixture was altered to more closely replicate the physiological environment of the cytoplasm. In addition, two naturally occuring *E. coli* polycations involved in modulating cellular function, spermidine and putrescine, were incorporated. Finally, rather than employ an expensive high-energy phosphate compound (i.e., PEP), which leads to inhibitory byproduct formation, pyruvate was used to regenerate ATP.

The results from this novel system are promising. Expression of CAT with this new method produced more than a fivefold yield increase relative to the PANOx system using sodium pyruvate as a secondary energy source. Improved economics, greater yields, phosphate homeostasis, and pH stability make the Cytomim system an attractive approach for in vitro translation.

MATERIALS AND METHODS

Cell-Free Protein Synthesis

Coupled 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 encodes for CAT, the CAT structural gene having been cloned between the T7 promoter and the T7 terminator (Kim and Swartz, 2001). CAT has a molecular weight of 25,662 Da. The plasmid was purified using a Plasmid Maxi Kit (Qiagen, Valencia, CA). N-terminal histidine-tagged T7 RNA polymerase was prepared from *E. 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), according to Swartz et al. (in press). S30 extract was prepared from *E. coli* strain A19 Δ*tonA* Δ*speA* Δ*tnaA* Δ*endA* *met*$^+$, a derivative of K12, as previously described (Jewett et al., 2002). However, cells were grown on a glucose and phosphate media, 2 × YTPG (Kim and Choi, 2000). It is important to note that the last step in cell extract preparation is repeated dialysis to bring the final ionic composition in the extract to 10 m*M* Tris-(hydroxymethyl)aminomethane (Tris; pH 8.2, with glacial acetic acid), 60 m*M* potassium acetate, and 14 m*M* magnesium acetate. Changes from the original A19 strain were carried out by P1-phage transduction and 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 trytophanase 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. The standard reaction mixture contains the following components: 1.2 m*M* adenosine triphosphate (ATP); 0.85 m*M* each of GTP, UTP, and CTP; 34 μg/mL folinic acid; 170.6 μg/mL of *E. coli* tRNA mixture; 13.3 μg/mL plasmid; 100 μg/mL T7 RNA polymerase; 5 μ*M* L-[U-^{14}C]-leucine; 2 m*M* each of 20 unlabeled amino acids; 0.33 m*M* nicotinamide adenine dinucleotide (NAD); 0.26 m*M* coenzyme-A (CoA); and 0.24 volume of S30 extract. The final total *E. coli* protein concentration in the reaction was 9.9 ± 0.7 mg/mL, as determined by Bradford assay using a commercially available assay reagent (Bio-Rad, Hercules, CA). Bovine serum albumin was used as a standard. In the PANOx energy regeneration system, 57 m*M* HEPES–KOH (pH 7.5), 2% (w/v) polyethylene-glycol 8000 (PEG), 200 m*M* potassium glutamate, 80 m*M* ammonium acetate, 16 m*M* magnesium acetate, 33 m*M* PEP, and 2.7 m*M* sodium oxalate were added. In the PANOx-SP system, 175 m*M* potassium glutamate, 10 m*M* ammonium glutamate, 20 m*M* magnesium glutamate, 33 m*M* PEP, 1.5 m*M* spermidine, 1 m*M* putrescine, and 2.7 m*M* sodium oxalate were added. Solutes added to the Cytomim system included 130 m*M* potassium glutamate, 10 m*M* ammonium glutamate, 8 m*M* magnesium glutamate, 33 m*M* sodium pyruvate, 1.5 m*M* spermidine, 1 m*M* putrescine, and 4 m*M* sodium oxalate. There was approximately an additional 3.3 m*M* magnesium, 14.4 m*M* potassium, 2.4 m*M* Tris, and 23.5 m*M* acetate in each reaction originating from the cell extract. See Table I for a summary of these three systems. Some experiments deviated from the conditions just described (see text). PEP and *E. coli* total tRNA mixture were purchased from Roche Molecular Biochemicals (Indianapolis, IN). L-[U-^{14}C]-leucine was from Amersham Pharmacia Biotechnology (Uppsala, Sweden). All other reagents were

Table I. Final concentrations of small molecules in the cell-free reaction for PANOx, PANOx-SP, Cytomim systems (concentrations listed account for small molecules contributed by the extract).

	PANOX system	PANOX-SP system	Cytomim system
Magnesium acetate (m*M*)[a]	19.3	3.3	3.3
Magnesium glutamate (m*M*)	0	20	8
Ammonium acetate (m*M*)	80	0	0
Ammonium glutamate (m*M*)	0	10	10
Potassium acetate (m*M*)[a]	14.4	14.4	14.4
Potassium glutamate (m*M*)	200	175	130
Phosphoenolpyruvate (m*M*)[b]	33	33	0
Sodium pyruvate (m*M*)	0	0	33
HEPES–KOH (pH 7.5) (m*M*)	57	0	0
Polyethylene-glycol (% [w/v])	2	0	0
Spermidine (m*M*)	0	1.5	1.5
Putrescine (m*M*)	0	1	1
Sodium oxalate (m*M*)	2.7	2.7	4
Tris (pH 8.2) (m*M*)[a]	2.4	2.4	2.4

[a]Includes carryover from cell extract.

[b]PANOx system with pyruvate as an energy source had the same conditions as used in the PANOx system, except that 33 m*M* pyruvate was used instead of 33 m*M* PEP.

obtained from Sigma Co. (St. Louis, MO). pH was measured using a micro-pH probe (Model 9803BN, Orion, Beverly, MA).

Product Determination

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

Protein Activity Assay

The enzymatic activity of synthesized CAT was determined by the spectrophotometric method of Shaw (1975), as previously described by Kim and Swartz (2001).

ATP Concentration Measurement

A firefly luciferase assay was used to determine ATP concentration as previously described (Kim and Swartz, 2001).

Inorganic Phosphate Determination

Quantitative analysis of inorganic phosphate was performed according to the protocol of Saheki et al. (1985), after some modification (Kim and Swartz, 2000a).

RESULTS

Investigating Composition Used for Cell-Free Protein Synthesis

Unnatural Components

Previously, we reported that pyruvate could be used as a secondary energy source in a protein synthesis reaction (Kim and Swartz, 1999, 2001). This inexpensive energy substrate does not lead to phosphate accumulation and, in this respect, better mimics the homeostasis found in a rapidly growing cell. Unfortunately, protein expression yields of CAT using pyruvate in the PANOx system physicochemical environment are <20% of those produced when PEP is the energy substrate. These low production yields offset any benefits provided by using pyruvate.

Our objective was to alter elements of the in vitro system to better mimic the cell's cytoplasm in the hope of increasing protein production yields from pyruvate. We began by exploring the conventional solute composition of the PANOx system.

The first step in formulating more ''cell-like'' conditions was removing the unnatural components present in the system. The HEPES buffer was selected as the first target. Cell-free systems traditionally employ the use of buffers to stabilize pH. However, the pH tends to drop and is unstable when using secondary energy sources, such as PEP (Yao et al., 1999).

Protein biosynthesis was studied with and without a pH buffer. After a 3-h protein synthesis reaction with PEP as energy source, the different states not only produced equivalent yields of CAT (approximately 680 μg/mL) but also had identical pH profiles (Fig. 1). It is not surprising that the pH data were so similar for both cases considering that the pH of the reaction, which started at about 6.7, was almost 1 pH unit below the pK_a value of the HEPES buffer, 7.55. The initial pH of the reaction was altered and the pH was found to have a significant impact on protein yields. The optimum initial pH for CAT production is approximately 6.8 (data not shown). These results indicate that the pH of the solution is a key parameter to consider for the synthesis of proteins in vitro.

PEG is another artificial component traditionally used in cell-free systems. PEG helps to maintain stable message levels (data not shown) and may mimic macromolecular crowding affects within the cell-free reaction mixture (Record et al., 1998b). This might be beneficial because such macromolecular interactions are almost certainly lost after a 20-fold dilution (protein concentration in the cell-free system is 20-fold more dilute than the *E. coli* cytoplasm). Although providing some advantage, this polymer may negatively affect properties of the extract that are desirable for re-creating the in vivo environment. PEG was the second unnatural target to be removed from the conventional PANOx system.

Removal of PEG alone decreased synthesis yields (data not shown) and necessitated a means to better stabilize messenger RNA within the system. Naturally occurring polyamines are used in the cytoplasm of *E. coli* to modify

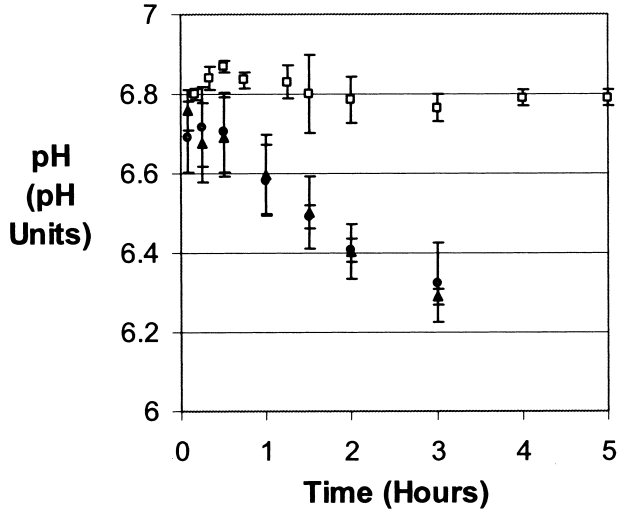

Figure 1. pH profile over the course of a cell-free reaction. pH was measured using a micro-pH electrode in 30-μL reactions. Error bars represent the standard deviation between three separate reactions. (●) Conventional PANOx reaction; (▲) PANOx reaction without HEPES buffer; (□) Cytomim reaction.

Table II. Optimization studies in both the PANOx-SP (with PEP) and Cytomim (with pyruvate) systems based on relative total CAT expression showing the trend for each component studied.

Ionic solute	Optimization profile (m*M*/fraction of maximum yield)					
PANOx-SP system						
Magnesium glutamate[a]	8/0.54	12/0.76	16/0.90	20/1.00	24/0.73	28/0.39
Potassium glutamate	100/0.63	125/0.79	150/0.93	175/1.00	200/0.94	225/0.92
Ammonium glutamate	0/0.90	10/0.95	20/0.98	50/0.99	80/1.00	100/0.91
Spermidine	0.75/0.85	1/0.92	1.5/1.00	2/0.94	5/0.54	
Putrescine	0/0.81	1/1.00	3/0.93	5/0.81	7/0.71	
Cytomim system						
Magnesium glutamate[a]	4/0.62	6/0.89	8/1.00	10/0.93	12/0.67	16/0.41
Potassium glutamate	0/0.20	25/0.52	50/0.69	100/0.88	130/1.00	200/0.95
Ammonium glutamate	0/0.86	10/0.92	50/0.94	75/1.00	100/0.93	150/0.84
Spermidine	0/0.53	0.5/0.90	1/0.97	1.5/1.00	5/0.48	10/0.15
Putrescine	0/0.95	1/1.00	3/0.80	7/0.58	11/0.43	15/0.23
Oxalate	0/0.70	1/0.83	2.7/0.89	4/1.00	7.5/0.96	10/0.74

Values given as '10/0.95' indicate that a 10 m*M* concentration of that ionic solute was added to the reaction and that 95% of the maximum expression of CAT was achieved at that particular concentration. Only one solute was varied at a time. All experiments were run using the conditions from Table I except for the component of interest. Fifteen-microliter PANOx-SP and Cytomim reactions were incubated at 37°C for 3 or 6 h, respectively. Listed concentrations do not account for the magnesium, acetate, and potassium contributed by the extract (see text). Optimal yields were approximately 700 μg/mL for the PANOx-SP system and 715 μg/mL for the Cytomim system.

[a]An additional 3.3 m*M* magnesium was carried over from the cell extract.

the function of and stabilize DNA, RNA, tRNA, and several other compounds (Igarashi and Kashiwagi, 2000; Tabor and Tabor, 1984). In addition, polyamines may be beneficial for protein synthesis. Jelenc and Kurland (1979) reported that the use of spermidine and putrescine in an in vitro translation system improved the fidelity of translation.

A series of optimization experiments were carried out to investigate the effects of substituting spermidine and putrescine for PEG in the cell-free system. Using the PANOx system without HEPES buffer, ranges of spermidine and then putrescine concentrations were examined with the objective of maximizing protein yield. The ''optimal'' concentrations of spermidine and putrescine for CAT expression were 1.5 m*M* and 1 m*M*, respectively (Table II). Cell-free transcription and translation with this modified PANOx system, lacking two artificial components (HEPES buffer and PEG), maintained the same protein expression yield relative to the previous approach with the conventional environment (Fig. 2, columns 1 and 2). The remaining ionic makeup was also investigated to determine whether changing it would have a positive effect on protein expression.

Ionic Solutes

In vivo, magnesium, potassium, ammonium, spermidine, and putrescine are used to balance the charge present from nucleic acid phosphate groups and other anionic species. Preservation of this ionic composition is essential for many protein–nucleic acid interactions and the proper function of biological processes of the cell, including protein synthesis (Jelenc and Kurland, 1979; Kurland, 1982; Record et al., 1998a, 1998b). In vitro, ionic solutes are added to the cell extract in an effort to reconstruct the natural environment present in living cells. One challenge with this approach is that, to mimic the approximate cation concentrations of the living cell, anionic species, generally glutamate and acetate, must be introduced at nonphysio-

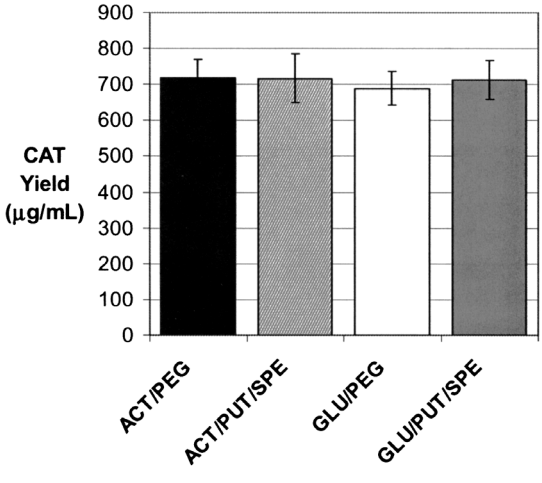

Figure 2. Expression of CAT using the PANOx system with four different ionic environments. Reactions (15 μL) were carried out for 6 h and CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the standard deviation from three individual experiments. All reactions were performed using the PANOx system without HEPES buffer. Reactions with glutamate salts contained 20 m*M* magnesium glutamate, 10 m*M* ammonium glutamate, and 175 m*M* potassium glutamate. Reactions with acetate salts contained 16 m*M* magnesium acetate, 80 m*M* ammonium acetate, and 200 m*M* potassium glutamate. Concentrations did not include the ionic effect from the cell extract. Reactions also investigated the effects of substituting the more natural spermidine and putrescine for 2% polyethylene-glycol (PEG). GLU, glutamate salts; ACT, acetate salts; PEG, 2% (w/v) PEG; PUT, 1.0 m*M* putrescine; SPE, 1.5 m*M* spermidine. Filled bar: acetate salts with PEG; hatched bar: acetate salts with putrescine and spermidine; open bar: glutamate salts with PEG; shaded bar: glutamate salts with putrescine and spermidine.

logical concentrations. For example, current protocols for cell-free systems use potassium glutamate concentrations of about 200 m*M*. Although the potassium concentration is in the physiologically relevant range, glutamate concentrations are never as great as potassium concentrations inside a living cell (Dinnibier et al., 1988; Record et al., 1998b). There is no apparent solution to this dilemma. However, Record et al. (1998b) suggested the use of glutamate as the preferred anionic species. Glutamate is the principal anion produced as a result of osmotic stress in *E. coli*, and it has been shown that protein–nucleic acid interactions are more favorable in glutamate than in acetate salt solutions (Record et al., 1998b).

Accordingly, salt concentrations added to the reaction were modified to better re-create the cytoplasmic condition. The effects of all solutes were explored utilizing the PANOx system with PEG replaced by 1.5 m*M* spermidine and 1 m*M* putrescine and without HEPES buffer. Acetate, which may be detrimental for protein synthesis (Aristidou et al., 1994), was entirely replaced with glutamate (except for that added with the cell extract). Substituting magnesium glutamate for magnesium acetate or ammonium glutamate for ammonium acetate had no significant effect on CAT yields (data not shown). Protein expression yields were nearly insensitive to ammonium glutamate concentrations between 0 and 100 m*M* (Table II). The ammonium concentration was therefore reduced to its cytoplasmic value of about 10 m*M* (Lubin and Ennis, 1964). Using the optimum magnesium and ammonium glutamate levels, potassium glutamate concentrations were also examined (Table II). The most protein was produced using 175 m*M* potassium glutamate.

Protein expression was studied utilizing this new ionic environment with minimal unnatural components, polyamines, and reoptimized ammonium, magnesium, and potassium glutamate concentrations. The glutamate salt environment produced 713 ± 54 μg/mL CAT. This is comparable to the amount of expressed protein using the traditional acetate salt environment with putrescine and spermidine (Fig. 2). If the glutamate salts were used with PEG rather than spermidine and putrescine, the yields were again similar (Fig. 2). The newly designed ''PANOx-SP'' system, which uses glutamate salts, PEP, spermidine, and putrescine (Table I), maintained equivalent protein expression yields relative to the original PANOx system.

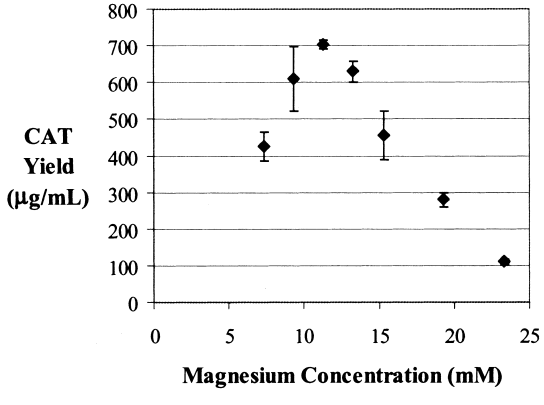

Figure 3. Magnesium dependence of the Cytomim system. Fifteen-microliter reactions were incubated for 6 h with increasing amounts of magnesium. The amount shown includes the magnesium from the extract. For example, 11.3 m*M* is due to 3.3 m*M* carryover from the extract and 8 m*M* directly added to the reaction. CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the standard deviation from three to eight separate experiments.

Providing a More Homeostatic Environment

One major limitation of cell-free systems is maintaining homeostasis within the reaction. It has already been shown that the pH of the reaction is not stable when using PEP as the energy substrate (Fig.1). In addition, accumulation of phosphate by consumption and degradation of PEP presents another deviation from homeostasis within the conventional PANOx system. Although it appeared that better mimicking the in vivo environment did not improve the PEP-based system, we evaluated the use of pyruvate as an energy source with this more natural environment. The magnesium concentration was reoptimized because of the higher affinity of PEP for magnesium relative to pyruvate and also because no significant phosphate accumulation was expected.

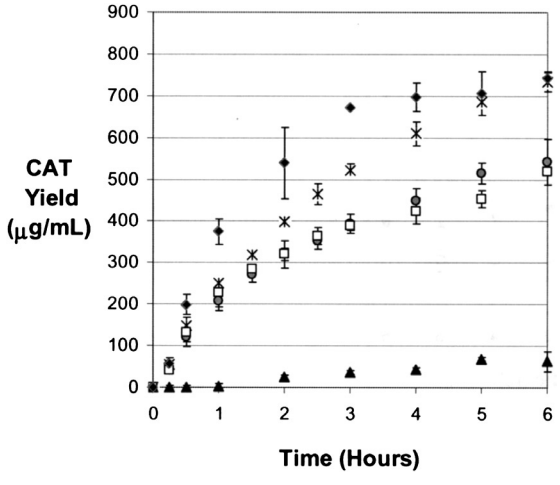

Figure 4. Expression of CAT comparing the PANOx-SP, Cytomim, and PANOx (using pyruvate as an energy substrate) systems. Reactions were carried out for 6 h and CAT expression was determined from ^{14}C-leucine incorporation and enzymatic activity assays. Fifteen-microliter reaction mixtures were prepared in a different tube for each timepoint. At each timepoint, one tube was sacrificed to determine the amount of expressed protein. Error bars represent the standard deviation from three separate experiments for the PANOx-SP and the PANOx with pyruvate systems and six individual experiments for the Cytomim system. (♦) Total yield from the PANOx-SP system using PEP as an energy source monitored by ^{14}C-leucine incorporation; (∗) total yield of CAT expressed as monitored by ^{14}C-leucine incorporation in the Cytomim system; (●) soluble yield of CAT expressed as monitored by ^{14}C-leucine incorporation in the Cytomim system; (□) active yield of CAT as determined by enzymatic assay in the Cytomim system; (▲) PANOx system using pyruvate as an energy source, total yield of CAT expressed as monitored by ^{14}C-leucine incorporation. Typical soluble and active yields of CAT for the PANOx and PANOx-SP systems using PEP as an energy source are approximately 65% to 70% of the total yield.

Remarkably, this new approach produced unexpectedly high CAT yields in a batch reaction (Fig. 3). Producing 700 μg/mL of CAT, the optimal magnesium concentration, 11.3 m*M*, is less than half that in the PANOx-SP system. This new method is called the Cytomim system. Spermidine, putrescine, oxalate, potassium glutamate, and ammonium glutamate optimizations of the Cytomim system were carried out with 11.3 m*M* magnesium (Table II). The most favorable conditions (Table I) were used to generate all data reported for the Cytomim system.

Figure 4 shows CAT accumulation over time as determined by TCA-precipitable radioactivity and enzymatic activity assays with the Cytomim system. The final yield of CAT after a 6-h incubation was 732 ± 23 μg/mL. This duration of synthesis is the longest ever reported for cell-free systems in batch mode. The soluble fraction of CAT was 76 ± 5% and all of the soluble protein was active. The measured specific activity, based on soluble CAT as determined by ^{14}C-leucine incorporation, was 124 ± 15 units/mg, which compared well with the published value of 125 units/mg (Shaw, 1975).

CAT expression using the Cytomim system with pyruvate was more than fivefold greater than the PANOx system using pyruvate as the energy source (Fig. 4). The yields were approximately the same as with the PANOx-SP system, which used PEP as an energy substrate (Fig. 4). The ATP concentration profiles for both systems were similar, although values for the Cytomim system were somewhat lower, consistent with the slower rate of protein expression (Fig. 5). ATP concentrations in both reactions remained >200 μ*M* over the course of each reaction. This suggests that an energy limitation is most likely not the direct cause of synthesis termination.

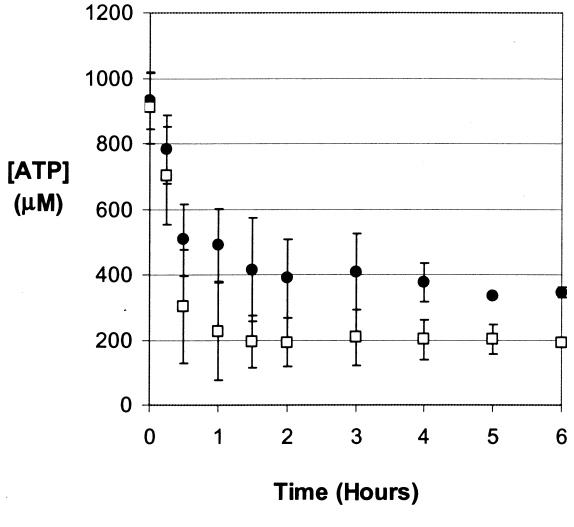

Figure 5. ATP concentration profile for PANOx-SP and Cytomim reactions. Reactions were carried out for 6 h and ATP concentration was determined with a firefly luciferase assay. Fifteen-microliter reaction mixtures were prepared in a different tube for each timepoint. At each timepoint, one tube was sacrificed to determine the ATP concentration. Error bars represent the standard deviation from three separate experiments. (●) PANOx-SP system; (□) Cytomim system.

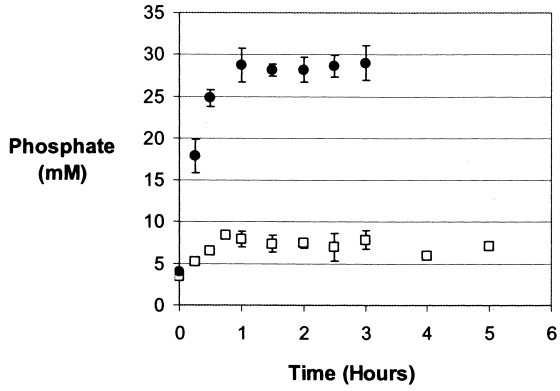

Figure 6. Phosphate profile over the course of a cell-free reaction. Phosphate accumulation was measured using a colorometric assay as described by Saheki et al. (1985). Error bars represent the standard deviation of three separate reactions. Fifteen-microliter reaction mixtures were prepared in a different tube for each timepoint. At each timepoint, one tube was sacrificed to determine the inorganic phosphate concentration. (●) PANOx-SP reaction; (□) Cytomim reaction.

Perhaps as important as producing significant protein yields over an extended batch reaction, the Cytomim system better imitates the intracellular environment. Not only is the ionic composition of the reaction more ''cell-like'' than traditional approaches, but the reaction maintains constant pH and does not accumulate inhibitory amounts of phosphate. Figure 6 compares the phosphate increase with time during a PANOx-SP reaction fueled by 33 m*M* PEP and a Cytomim reaction with 33 m*M* pyruvate. Approximately 30 m*M* of phosphate accumulated with the PANOx-SP system. Only 4 m*M* of phosphate accumulated over the course of the Cytomim reaction. Phosphate has been shown to inhibit the combined transcription and translation reaction at concentrations of >30 m*M* (Kim and Swartz, 1999). Phosphate levels with the Cytomim system were well below this value. The impact of reaction pH on protein synthesis yield was also examined with the Cytomim method. CAT expression was maximal at pH 6.8 (data not shown). Strikingly, when pyruvate was used as an energy source, the pH of the reaction was stable (Fig. 1).

DISCUSSION

This work has shown that re-engineering an in vitro system to better emulate the cytoplasm provides substantial advantages for protein synthesis when using pyruvate as an energy substrate as compared with a previously described system. None of the individual changes proposed are specifically new. However, carrying out the overall objective of better mimicking the intracellular environment of *E. coli* by reinvestigating the entire in vitro environment has not been explored previously in such depth.

Results with the redesigned PANOx-SP energy regeneration system were identical to those with the previous

PANOx method. The system produced similar yields, had similar problems with pH stability and phosphate accumulation, and made approximately the same amount of active protein (data not shown). Yields may have increased if the energy generation stemming from pyruvate had been better captured in the more natural environment, because pyruvate is the direct product of the PEP-to-ADP phosphotransferase reaction. However, the data do not indicate that this is the case. Perhaps the lack of homeostasis and the higher magnesium concentration in both the PANOx and PANOx-SP systems create a barrier to achieving better generation of energy from pyruvate.

The pH study with the PANOx system offers some valuable insights into protein production in vitro. The lack of pH stability when using PEP may contribute to the termination of protein synthesis in a batch system. By the end of the reaction, the pH decreased to below 6.5. Cells with an internal pH this low would most likely be impaired and this characteristic probably negatively affects the cell-free system as well. Controlling the pH of the reaction with base addition or a buffer with a pK_a near 6.8, such as Bis-Tris, is one approach for exploring the effects of pH change using PEP as an energy source.

Although the new environment for synthesis did not have a dramatic effect on protein production when using PEP, cytoplasmic mimicry did produce surprisingly high protein yields from pyruvate. Using the Cytomim system, protein synthesis can be extended for up to 6 hours with stable maintenance of ATP in a batch operation. One consequence of increased reaction duration is that protein yields are as high as any batch system utilized to date, with substantial increases compared to previous work using pyruvate (Kim and Swartz, 2001). In addition to the significant yields, this new technology offers an economical advantage over conventional cell-free systems. The high-energy phosphate compounds used conventionally to regenerate ATP generally represent the highest costs associated with cell-free expression. This new system can eliminate this cost almost entirely. The current commercial cost of pyruvate is a mere 0.5% of the cost for PEP. Equivalent protein yields to the PANOx system, at a fraction of the cost, make the Cytomim system a very attractive method for cell-free protein synthesis.

Another advantage of the Cytomim system is that it maintains better homeostasis than conventionally used cell-free systems. First, this new system does not accumulate phosphate, which is known to inhibit protein synthesis. Operating the Cytomim system in fed-batch mode would allow numerous additions of pyruvate to regenerate ATP without inhibition by phosphate. This is not possible with conventional secondary energy sources such as PEP, which eventually poison the system (Jewett and Swartz, in press). Stability of pH is another attractive aspect of this new environment for synthesis. Returning the system to more ''cell-like'' behavior of consistent pH is believed to be beneficial for protein production, metabolic processes, and protein folding. Although the Cytomim system is more homeostatic, small molecules, such as acetate, still accumulate. In our future work, the challenge of metabolite accumulation will need to be addressed.

A central problem in cell-free expression is elucidating the most favorable environment for proper folding of complex proteins. Capturing a more physiological environment may provide better folding. We were initially discouraged that the amount of active CAT produced in the PANOx, PANOx-SP, and Cytomim systems was about the same, approximately 70% to 75%. However, it is possible that folding this specific protein in vitro, which requires no disulfide bonds, is not a good representative model. Tissue plasminogen activator (tPA) is a complex mammalian protein. The active domain of this protein contains nine disulfide bonds. Production of active v-tPA has been shown to increase in the new environment (Yin and Swartz, in press). Further studies with several different proteins are necessary to determine whether this is a general trend.

Mimicking the intracellular environment of living cells has produced significant improvements in cell-free expression; however, there some factors that remain to be addressed. First, oxalic acid is an unnatural component used in the cell-free reaction. It improves protein synthesis yields and is known to inhibit the phosphoenolpyruvate synthetase (*pps*) reaction, which converts pyruvate into phosphoenolpyruvate, wasting energy available for protein synthesis (Kim and Swartz, 2000b). Genetically modifying the host organism to delete the *pps* gene may alleviate the need to add this unnatural component. Second, the cell extract procedure should be modified to put the cell extract into a more natural ionic environment using glutamate salts without a pH buffer. This would eliminate the carryover of acetate and unnatural components into the reaction mixture.

Estimating ATP utilization efficiency for the Cytomim system leads to intriguing questions for energy regeneration in vitro. We can estimate the demand of ATP required for protein production by conservatively assuming that five molecules of ATP are necessary for the addition of one amino acid to the growing polypeptide (Kim and Swartz, 2001). For energy generation from pyruvate, we estimate that 1 m*M* pyruvate produces 0.5 m*M* ATP. This assumes that half of the pyruvate is converted to acetate, generating ATP, and the other half must go to lactate to regenerate NAD (Kim and Swartz, 2001). If all ATP produced from 33 m*M* pyruvate were to be utilized for protein production, 375 μg/mL of CAT would be expressed. Therefore, the actual yield for the Cytomim system would be 190% of the expected theoretical maximum. This abnormally high yield suggests the potential for new energy regeneration pathways and requires further investigation.

In summary, the Cytomim system is an attractive method for in vitro protein synthesis. It produces more than fivefold the amount of CAT compared with a previous system utilizing sodium pyruvate as an energy substrate. In addition, it overcomes two limitations of current cell-free systems by avoiding phosphate accumulation and maintaining a stable pH. Clearly, several questions remain to be addressed. In

particular, the reason for termination of protein synthesis must be determined. Because ATP does not appear to be limiting in the Cytomim reaction, other substrates, such as amino acids, messenger RNA, and the other nucleoside triphosphates, will need to be examined. However, even with the methods described herein, the ability to obtain significant protein yields with improved homeostasis from an inexpensive energy source is very promising.

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