Prolonging Cell-Free Protein Synthesis by Selective Reagent Additions

Article *in* Biotechnology Progress · June 2000

DOI: 10.1021/bp000031y · Source: PubMed

2 authors:

Dong-Myung Kim
Chungnam National University
146 PUBLICATIONS **2,749** CITATIONS
SEE PROFILE

James Swartz
Stanford University
124 PUBLICATIONS **5,349** CITATIONS
SEE PROFILE

Prolonging Cell-Free Protein Synthesis by Selective Reagent Additions

Dong-Myung Kim and James R. Swartz*

Department of Chemical Engineering, Stanford University, Stanford, California 94305

Factors causing the early cessation of protein synthesis have been studied in a cell-free system from *Escherichia coli*. We discovered that phosphoenol pyruvate (PEP), the secondary energy source for ATP regeneration, and several amino acids are rapidly degraded during the cell-free protein synthesis reaction. The degradation of such compounds takes place even in the absence of protein synthesis. This degradation severely reduces the capacity for protein synthesis. The lost potency was completely recovered when the reaction mixture was supplied with additional PEP and amino acids. Of the 20 amino acids, only arginine, cysteine, and tryptophan were required to restore system activity. Through repeated additions of PEP, arginine, cysteine,and tryptophan, the duration of protein synthesis was greatly extended. In this fed-batch reaction, after a 2 h incubation, the level of cell-free synthesized chloramphenicol acetyl transferase (CAT) reached 350 μg/mL, which is 3.5 times the yield of the batch reaction. Addition of fresh magnesium further extended the protein synthesis. As a result, through coordinated additions of PEP, arginine, cysteine, tryptophan, and magnesium, the final concentration of cell-free synthesized CAT increased more than 4-fold compared to a batch reaction. SDS−PAGE analysis of such a fed-batch reaction produced an obvious band of CAT upon Coomassie Blue staining.

Introduction

Cell-free protein synthesis systems provide rapid and convenient tools for expression of rDNA proteins (*1*−*4*). Previously considered only for laboratory-scale synthesis, this approach is now being considered as a promising alternative to conventional in vivo expression systems (*5*−*10*). As the miscellaneous metabolic reactions to maintain the viability and homeostasis of living cells are not required, ideally, all of the metabolic resources can be directed to the exclusive synthesis of proteins encoded on the template DNA (or RNA). Thus, this approach has the potential to become a more straightforward and efficient method for protein synthesis. Unlike in vivo gene expression, reaction conditions for the cell-free system can be directly manipulated to provide an environment optimized for expression of proteins in their native conformations. For instance, through such measures as control of redox potential of the reaction mixture and introduction of molecular chaperones or foldases, this system is expected to offer efficient synthesis of hard-to-fold or aggregation-prone proteins (*11*). Another promising aspect of the cell-free system is that it can be readily expanded to a multiplexed format for the rapid and simultaneous expression of various genetic materials (*12*). Along with its capability to incorporate unnatural amino acids into protein structure, this feature of cell-free protein synthesis offers great potential for the field of protein engineering (*13*−*16*).

However, all of the above potentials can be realized only when a cell-free system can offer sufficient productivity. Unfortunately, conventional cell-free systems have suffered from poor yields of protein synthesis, mainly as a result of their short reaction periods. In general, in an *Escherichia coli* batch cell-free system, protein synthesis lasts no more than 20 min. The reason for the early halt of protein synthesis has remained unanswered.

Recently, we found phosphatase activities of the cell extract rapidly degrade phosphoenol pyruvate (PEP) during the incubation periods. It was also found that the concentrations of certain amino acids, arginine, cysteine, and tryptophan, decrease significantly during the incubation.

Here we report that the repeated additions of these compounds allow a substantial extension of the reaction period with a coordinate increase in protein yield. In addition, the inhibitory effect of accumulating phosphate was relieved significantly by adding more magnesium. As a result, when the reaction was fed with a mixture of PEP and amino acids every 20 min and with magnesium acetate at 60 min, the final yield of CAT in the cell-free system reached 420 μg/mL after a 2 h reaction.

Materials and Methods

PEP and *E. coli* total tRNA mixture were purchased from Boerhinger-Mannheim. L-[U-^{3}H]leucine and [5,6-^{3}H]-UTP were from Amersham. All other reagents were obtained from Sigma. T7 RNA polymerase was prepared from the culture of *E. coli* strain BL21 (pAR1219) according to the slightly modified procedures of Davanloo et al. (*17*). Plasmid pK7CAT, which includes the bacterial CAT sequence between the T7 promoter and T7 terminator, was used as a template for protein synthesis.

S30 extract was prepared from *E. coli* K12 (strain A19) according to the procedures of Pratt (*18*). The standard reaction mixture consists of the following components:

* E-mail: swartz@chemeng.stanford.edu.

10.1021/bp000031y CCC: $19.00
Published on Web 05/16/2000

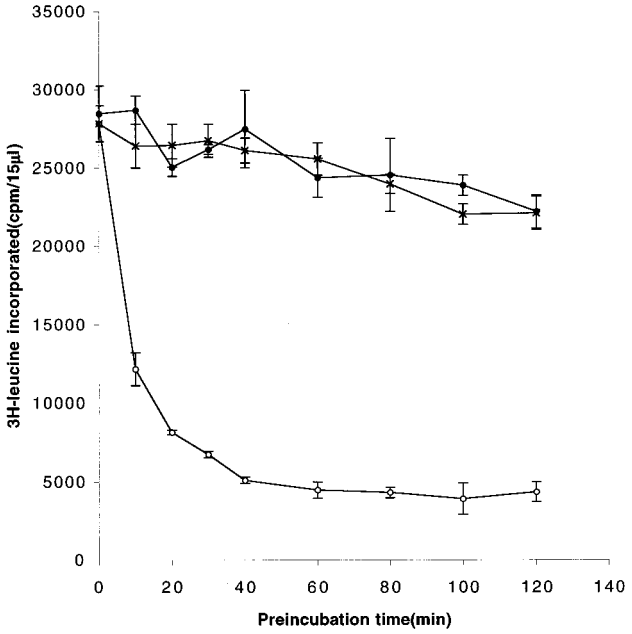

Figure 1. Preincubations of the different parts of reaction mixture. Aliquots (14 μL) of a standard reaction mixture were incubated in different tubes. At the same time, 3.6 μL aliquots of S30 extract and 10.4 μL aliquots of the balance of the reaction mixture were incubated. Protein synthesis was initiated by addition of the missing components along with DNA at the given time points. After an additional 30 min incubation, each tube was withdrawn and the TCA-insoluble radioactivity was measured: ○, preincubation of the entire reaction mixture; ●, preincubation of S30 extract; ×, preincubation of the reaction mixture without S30 extract.

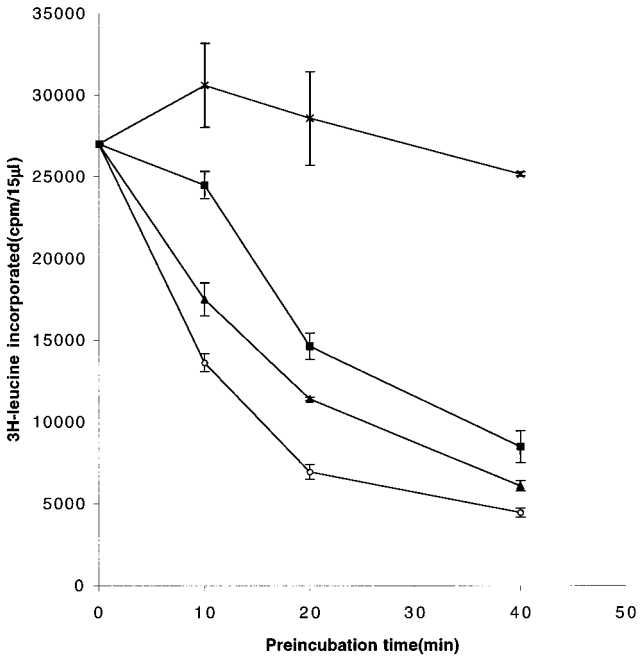

Figure 2. Preincubation of reaction mixture without PEP and/or amino acids. PEP, amino acids, or PEP/amino acids was removed from the preincubation mixture and added along with DNA at the end of the preincubation periods. Following an additional 30 min incubation, each tube was withdrawn and TCA-insoluble radioactivity was measured: ○, preincubation of the entire reaction mixture; ■, preincubation of the reaction mixture without PEP; ▲, preincubation of the reaction mixture without amino acids; ×, preincubation without PEP and amino acids.

57 mM Hepes-KOH (pH 8.2); 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 1mM DTT; 0.64 mM cAMP; 200 mM potassium glutamate; 80 mM $NH_4(OAc)$; 15 mM $Mg(OAc)_2$; 34 μg/mL folinic acid; 6.7 μg/mL plasmid; 33 μg/mL T7 RNA polymerase; 500 μM each of 20 unlabeled amino acids and 0.21 μM [^{3}H]leucine (5.81 TBq/mmol); 2% PEG 8000; 24 mM PEP; and 0.24 volumes of S30 extract. Reactions of 15 μL were run for given time periods in a waterbath set at 37 °C. The amount of synthesized protein was estimated from the measured cold TCA-insoluble radioactivities as described earlier (*6*). Radioactivities of samples were measured in a liquid scintillation counter (Beckman LS3801). For conducting preincubation experiments, different combinations of reaction components were incubated in the absence of DNA, and protein synthesis was initiated by adding DNA, along with non-preincubated parts of the reaction mixture after the indicated periods of preincubation.

Quantitative analysis of inorganic phosphate was carried out according to the procedures of Saheki et al. (*19*) after minor modifications, in which 2 μL samples were taken and mixed with 15 μL of 20% SDS solution and 43 μL of water. After sequential addition of 670 μL of solution A (100 mM zinc acetate, 15 mM ammonium molybdate, pH 5.0) and 170 μL of solution B (10% ascorbic acid), each tube was incubated in a 30 °C gyratory incubator for 15min. Finally, the concentration of inorganic phosphate was estimated from the measured OD_{850} of the sample and standard curve.

Concentrations of amino acids were measured by an amino acid analyzer (system 6400, Beckman).

Most experiments were done in duplicate. Error bars indicate the two values, with the average value being plotted.

Results

As is typical for a cell-free system of batch configuration, our standard reaction showed a sudden stop of protein synthesis at around 20 min. To examine whether the halt of reaction is related to protein synthesis, we have conducted a series of preincubation experiments as follows. A preincubation mixture was prepared by mixing standard reaction components described in the Materials and Methods except that plasmid was omitted from the complete mixture. Next, 14 μL aliquots of the mixture were placed in eppendorf tubes, and preincubation was started by placing them in a water bath set at 37 °C. Following a given time period of preincubation, each tube received 1 μL (6.7 ng) of plasmid to initiate protein synthesis. After a 30 min incubation after the addition of plasmid, 100 μL of 0.1 N NaOH solution was added to terminate the reaction and to hydrolyze aminoacyl-tRNAs. TCA-insoluble radioactivity of each sample was measured as described. As shown in Figure 1, the amount of synthesized protein rapidly decreased depending on the length of preincubation. The synthesis yield in a tube that had undergone a 40 min preincubation was less than 20% that of a control reaction that had not been preincubated. Interestingly, when the preincubation was carried out with only S30 extract and the balance of the reaction mixture was added along with the DNA, the yield of protein synthesis was relatively stable over the preincubation periods examined. Similarly, preincubation of the chemical mixture (protein synthesis was initiated by addition of S30 extract and DNA) produced no significant reduction of synthesis yield. These results suggest that (i) both the S30 extract and the other chemical components of the reaction mixture are stable by themselves under the reaction conditions; (ii) the potency of the cell-free protein synthesis system begins

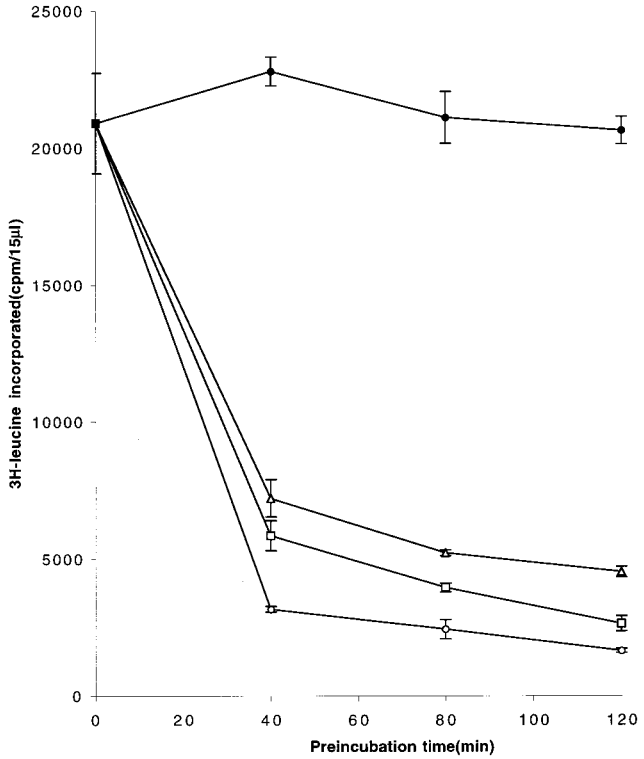

Figure 3. Effect of PEP/amino acids supplementation after preincubation of the reaction mixture. A preincubation mixture (reaction mixture minus DNA) was prepared and dispensed into different tubes in 15 μL aliquots**.** Following the indicated time periods of preincubation, additional PEP (□), amino acid (△), or the mixture of PEP and amino acids (●) was added together with DNA. The concentrations of the added PEP and amino acids were the same as their initial values (24 mM and 0.5 mM each, respectively); ○, control reaction without any supplementation.

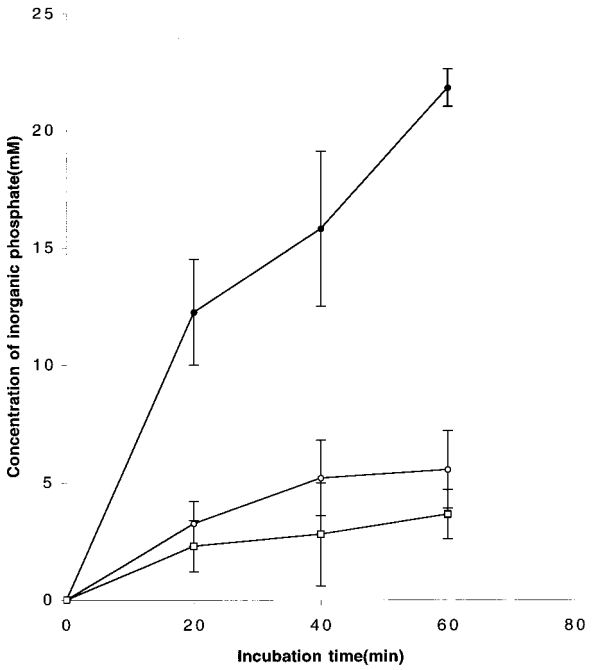

Figure 4. Accumulation of inorganic phosphate in the reaction mixture. A standard reaction mixture without DNA was prepared and incubated at reaction temperature. Next, 5 μL samples were taken at the given time points and assayed for inorganic phosphate concentration (●) as described in the Materials and Methods. As control reactions, the same reaction mixtures but without PEP (○) and S30 extract (□) were incubated and phosphate concentration was monitored.

to deteriorate when the S30 extract is mixed with the balance of the reaction mixture at the reaction temperature; and thus, (iii) certain reactions between S30 extract and reaction components cause the deactivation of the system. The dramatic loss of potency during the preincubation led us to hypothesize that these reactions are closely related to the early halt of protein synthesis in a typical cell-free system.

To search for the possible reactions that cause the deactivation, each individual reaction component was sequentially removed from the entire preincubation mixture and added along with the DNA after the preincubation periods. Two reaction components were found whose removal from the preincubation mixture relieved the loss of protein synthesis potency. As shown in Figure 2, when PEP was removed from the preincubation mixture and added along with DNA, the potency of protein synthesis was marginally recovered. Similarly, removal of the amino acid mixture increased the level of residual activity after preincubation. Surprisingly, when both PEP and the amino acid mixture were excluded from the preincubation mixture, preincubation periods up to 2 h did not decrease the subsequent synthesis yield.

Thus, it appears that deterioration of the PEP and the amino acids is catalyzed by the S30 extract of the cell-free system. We examined if the complete system that had lost its activity after preincubation (standard reaction mixture minus DNA) could be restored by the addition of more PEP and amino acids. As shown in Figure 3, similar to the above results of delayed additions, the level of protein synthesis was restored when a mixture of additional PEP and amino acids was given along with the DNA. This result strongly suggests that the inactivation during the preincubation period is due the degradation of PEP and amino acids.

It had been found in our laboratory that PEP is easily degraded in an *E. coli* cell-free system by phosphatase activities of the S30 extract (*20*). A typical time course of inorganic phosphate concentration is given in Figure 4. The rapid degradation of the secondary energy source results in the rapid depletion of ATP, which in turn, limits protein synthesis. However, as was shown above, addition of more secondary energy source alone was not sufficient to restore the protein synthesis. Both additional energy source and a new supply of amino acids were required.

Although it was calculated that the initial concentration of amino acids (0.5 mM of each amino acid) is excessive compared to the typical yield of protein synthesis in a batch system (less than 1 μM), it seems that the enzymes involved in amino acid metabolism retain sufficient activities under the present reaction conditions to significantly reduce the concentrations of certain amino acids.

As a next step, it was examined if the protein synthesis reaction can be extended through addition of more amino acids and energy source. During incubation of a standard reaction, PEP and amino acids were added to their initial concentrations every 20 min. Figure 5 shows that the repeated additions of PEP and amino acids did allow the extension of the reaction period to significantly increase the yield of CAT. Protein synthesis continued for 80 min, resulting in a 3.3-fold increase of its final yield compared to a control reaction.

To obtain a more detailed understanding of the amino acid degradation, the changes of amino acid concentrations were investigated during the course of a standard reaction. Interestingly, the concentrations of some amino

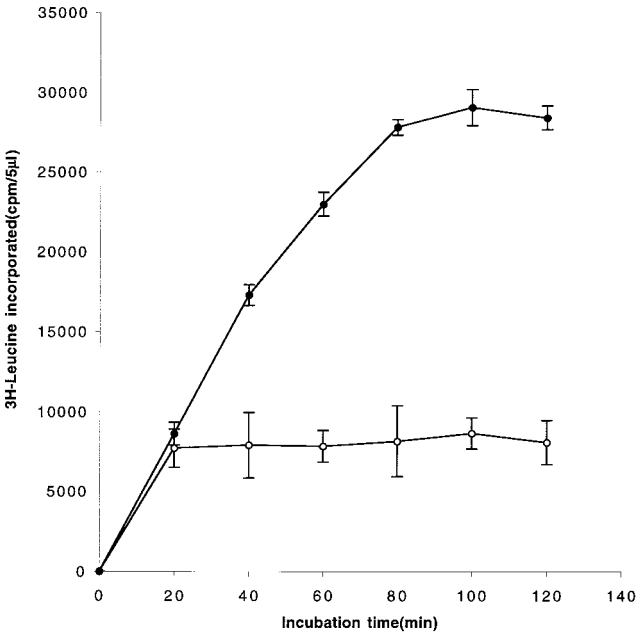

Figure 5. Supplementation of PEP and amino acids during protein synthesis. A standard reaction was carried out at a 60 μL volume. During the incubation period, the initial concentrations of PEP and amino acids were added to the reaction every 20 min. Prior to the additions, 5 μL samples were taken to measure the yield of synthesis. The same volumes of water were added to the control reaction: ○, standard reaction; ●, with additions.

acids increased dramatically, while others were depleted. In particular, alanine and aspartic acid and/or asparagine significantly increased in concentration. In contrast, arginine, cysteine, and tryptophan decreased to the point of depletion (Figure 6). No significant changes were observed in the concentration of the other 14 amino acids. A mixture of these three depleted amino acids was able to successfully replace the total amino acid mixture in extending protein synthesis.

Although the repeated additions of PEP and amino acids do extend protein synthesis, the longevity of the reaction is not as long as expected from the data of the preincubation experiments, which show the maintenance of activity for at least 2 h.

We think that such a discrepancy can be explained by the inhibitory effect of accumulating inorganic phosphate following the addition of PEP. Since a significant portion of added PEP is degraded by the cell extract, repeated addition of PEP continuously increases the level of inorganic phosphate in the reaction mixture. Most likely, accumulating inorganic phosphate limits protein synthesis by reducing the concentration of free magnesium. The postulated inhibitory effect of inorganic phosphate was confirmed by an experiment, with results shown in Figure 7. When the protein synthesis reaction was carried out with different initial concentrations of inorganic phosphate, the yield of protein synthesis began to decrease at around 40 mM of inorganic phosphate. This result agrees well with the results obtained after repeated PEP addition. In the reaction receiving PEP every 20 min, the results shown in Figure 4 suggest that the rate of synthesis begins to decrease at between 40 and 60 min when the level of inorganic phosphate released from PEP approaches 40 mM.

We attempted to reverse the inhibition by the addition of additional magnesium ion. As shown in Figure 8, additional magnesium did further extend the protein synthesis. As a result, the combination of magnesium supplementation and the periodic additions of PEP, arginine, cysteine, and tryptophan produced a yield of 420 μg/mL of CAT synthesis after a 2 h incubation. Synthesized CAT was visualized as a significant band by Coomassie Blue staining after SDS−PAGE analysis.

Discussion

The use of the conventional cell-free protein synthesis system has been limited by low yields, which mainly result from the short duration of reaction. Earlier reports have shown that the reaction period of a cell-free protein synthesis can be greatly extended by employing a continuous flow (*5*, *6*) or diffusional exchange of reaction buffer (*7*, *10*). However, the conventional batch system still carries many advantages over continuous or semi-

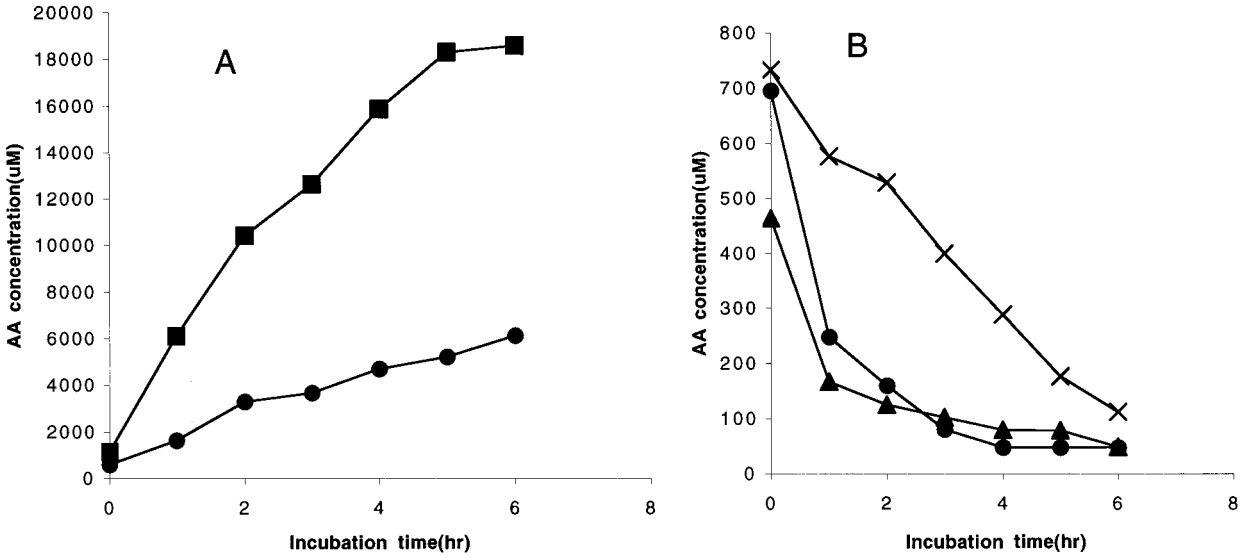

Figure 6. Analysis of amino acid concentrations during the incubation. During the incubation of a 900 μL standard reaction, 100 μL samples were withdrawn every hour to analyze the concentration of amino acids. Next, 100 μL of water and 200 μL of TCA (10%) solution were added to each sample to precipitate proteins. After centrifugation, 350 μL of supernatant was withdrawn and analyzed. Panel A: ■, alanine; ●, aspartic acid/asparagine. Panel B: ●, arginine; ▲, cysteine; ×, tryptophan. The other amino acid concentrations remained unchanged during the incubation.

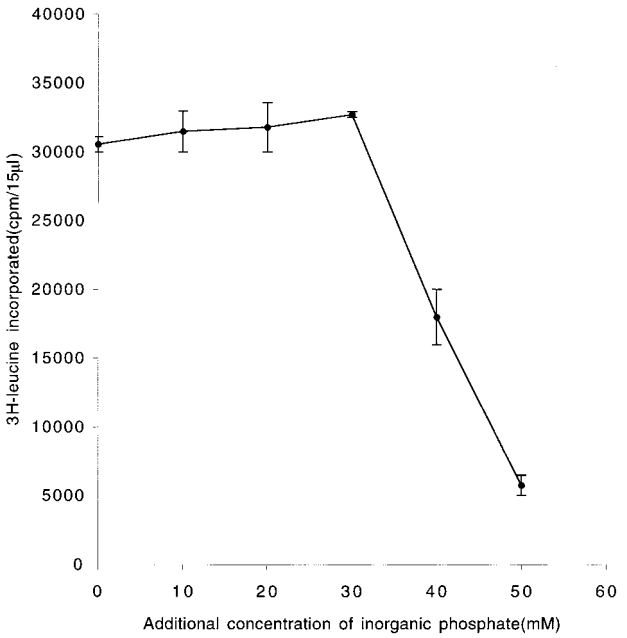

Figure 7. Effect of inorganic phosphate additions on protein synthesis. Standard cell-free protein synthesis reactions (15μL) were carried out with the addition of increasing concentrations of exogenous inorganic phosphate. After a 30 min incubation, the TCA-insoluble radioactivity of each tube was measured as described.

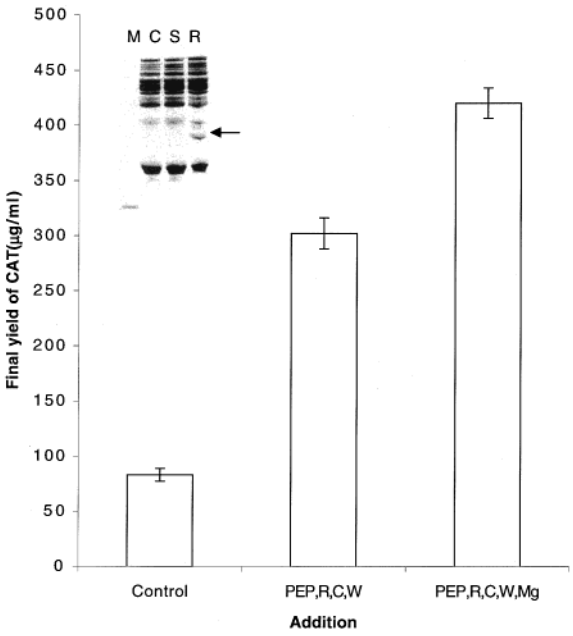

Figure 8. Addition of magnesium during a fed-batch reaction. During the incubation of a 15 μL standard reaction mixture, 0.24 μL of 1 M PEP and 0.15 μL each of 50 mM arginine, cysteine, and tryptophan were added every 20 min. The control reaction was supplied with the same volumes of water every 20 min. To examine the effect of additional magnesium, in addition to the repeated additions of PEP and amino acids, 0.25 μL of 900 mM magnesium acetate was added at 60 min. Inset, SDS−PAGE analysis of reactions. Lanes: M, molecular weight size markers; C, reaction without plasmid; S, standard reaction; R, standard reaction with the additions of PEP, arginine, cysteine, tryptophan, and magnesium. Arrow indicates the band of synthesized CAT.

continuous systems, such as simple reactor configuration, convenient manipulation of reaction conditions, and more efficient use of expensive reagents. As it can be easily expanded to a multiplexed format, it can be usefully employed for such purposes as expression of PCR fragment libraries and for adjusting crucial parameters to encourage simultaneous protein synthesis and folding.

On the basis of our present results, we propose that the extended protein synthesis in the continuous-flow or semicontinuous system is at least partly attributed to the maintenance of the concentrations of the secondary energy source and amino acids that are subject to degradation by cellular enzymes of the S30 extract. In this paper, we show that repeated additions of those substrates in a fed-batch mode can significantly extend the protein synthesis reaction. Also, the inhibitory effect of phosphate accumulation during the repeated addition of PEP can be relieved to some extent with additional magnesium ion. However, as was shown, the inhibitory effect of accumulating inorganic phosphate cannot be completely reversed by the supplementation of magnesium. The amount of CAT increased only by 118 μg/mL with the addition of magnesium, which is approximately 40% of the yield from the first 60 min. Thus, in addition to sequestering free magnesium, the magnesium phosphate or free phosphate may directly inhibit protein synthesis. Accordingly, to further extend the reaction period of protein synthesis through the continuous supply of secondary energy source, a new strategy that does not accumulate inorganic phosphate is required. We already have succeeded in developing such an ATP regeneration system by recycling inorganic phosphate to generate more secondary energy source during protein synthesis, and this new system was shown to provide a significantly extended reaction period (*20*).

In addition to the degradation of PEP and certain amino acids, the increase of alanine and aspartic acid/asparagine is also problematic, since they are generated most likely from PEP, which lowers the availability of PEP for ATP regeneration.

Probably the most straightforward method to improve the efficiency of substrate utilization would be to delete the degrading enzymes from the cell extract. The S30 extract can be prepared from an *E. coli* strain that has been genetically modified to knock out the enzymes responsible for the metabolism of PEP and amino acids. We expect the resulting cell-free protein synthesis system to have an improved efficiency due to the lack of nonproductive depletion of energy source and amino acids. We are now in the process of identifying and removing these harmful activities.

Acknowledgment

The authors thank Denise Woo and Rosemary Fernandez for their help with amino acid analysis. We also gratefully acknowledge support from the Charles Lee Powell Foundation and Genentech, Inc.

References and Notes

(1) Goodman, R. H.; Jacobs, J. W.; Habner, J. F. Cell-free translation of messenger RNA coding for a precursor of human calcitonin. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 932−938.

(2) Jacobs, J. W.; Potts, J. T., Jr; Bell, N. H.; Habner, J. F. Calcitonin precursor identified by cell-free translation of mRNA. *J. Biol. Chem.* **1979**, *254*, 10600−10603.

(3) Amara, S. G.; Rosenfield, M. G.; Birnbaum, R. S.; Roos, B. A. Identification of the putative cell-free translation product of rat calcitonin mRNA. *J. Biol. Chem.* **1980,** *255*, 2645−2648.

(4) Mattingly, J. R., Jr.; Iriarte, A.; Martinez-Carrion, M. Structural features which control folding of homologous proteins in cell-free translation systems. The effect of a mitochondrial targeting presequence on aspartate aminotransferase. *J. Biol. Chem.* **1993**, *268*, 26320−26327.

(5) Spirin, A. S.; Baranov, V. I.; Ryabova, L. A.; Ovodov, S. Y.; Alakov, Y. B. ; A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **1988**, *242*, 1162−1164.
(6) Kim, D.-M.; Kigawa, T.; Choi, C.-Y.; Yokoyama, S. A highly efficient cell-free protein synthesis system from *Escherichia coli*. *Eur. J. Biochem*. **1996**, *239*, 881−886.
(7) Kim, D.-M.; Choi, C.-Y. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnol. Prog*. **1996**, *12*, 645−649.
(8) Patnaik, R; Swartz, J. R. *E. coli* based cell-free transcription/ translation: in vivo specific synthesis rates and high yields in a batch system. *BioTechniques* **1998**, *24*, 862−868.
(9) Nakano, H.; Tanaka, T.; Kawarasaki, Y.; Yamane, T. Highly productive cell-free protein synthesis system using condensed wheat germ extract. *J. Biotechnol*. **1996,** *46*, 275−282.
(10) Nakano, H.; Shinbata, T.; Okumura, R.; Sekiguchi, S.; Fujishiro, M.; Yamane, T. Efficient coupled transcription/ translation from PCR template by a hollow-fiber membrane bioreactor. *Biotechnol. Bioeng*. **1999**, *64(2)*, 194−199.
(11) Ryabova, L. A.; Desplancq, D.; Spirin, A. S.; Pluckthun, A. Functional antibody production using cell-free translation: Effects of protein disulfide isomerase and chaperones. *Nature Biotechnol*. **1997**, *15*, 79−84.
(12) Burks, E. A.; Chen, G.; Georgiou, G.; Iverson, B. Cell-free scanning saturation mutagenesis of an antibody binding pocket. *Proc. Natl. Acad. Sci*. **1997,** *94*, 414−417.
(13) Noren, C. J.; Anthony-Cahills, S. J.; Griffith, M. C.; Schultz, P. G. A general method of site-specific incorporation of unnatural amino acids into proteins. *Science* **1989**, *244*, 182−188.
(14) Kohno, T.; Kohda, D.; Haruki, M.; Yokoyama, S.; Miyazawa, T. Nonprotein amino acid furanomycin, unlike isoleucine in chemical structure, is charged to isoleucine tRNA by isoleucyl-tRNA synthetase and incorporated into protein. *J. Biol. Chem*. **1990**, *265*, 6931−6935.
(15) Chung, H. H.; Benson, D. R.; Schultz, P. G. Probing the structure and mechanism of Ras protein with an expanded genetic code. *Science* **1993**, *259*, 806−808.
(16) Ma, C.; Kuldlicki, W.; Odom, O. W.; Kramer, G.; Hardesty, B. Cell-free protein engineering using synthetic tRNA(Ala) with different anticodons. *Biochemistry* **1993**, *32*, 7939−7945.
(17) Davanloo, P.; Rosenberg, A. H.; Dunn, J. J.; Studier, F. W. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2035−2039.
(18) Pratt, J. M. Coupled transcription-translation in prokaryotic cell-free systems. In *Transcription and translation: a practical approach*; Hames, B. D., Higgins, S. J., Eds.; IRL Press: New York, 1984; pp 179−209.
(19) Saheki, S.; Takeda, A.; Shimazu, T. Assay of inorganic phosphate in the mild pH range, suitable for measurement of glycogen phosphorylase activity. *Anal. Biochem*. **1985**, *148*, 277−281.
(20) Kim, D.-M.; Swartz, J. R. Prolonging cell-free protein synthesis using a novel ATP regeneration system. *Biotechnol. Bioeng*. **1999**, *66(3)*, 180−188.

Accepted for publication March 28, 2000.

BP000031Y