Prolonging Cell-Free Protein Synthesis with a Novel ATP Regeneration System

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Abstract: A new approach for the regeneration of adenosine triphosphate (ATP) during cell-free protein synthesis was developed to prolong the synthesis and also to avoid the accumulation of inorganic phosphate. This approach was demonstrated in a batch system derived from *Escherichia coli*. Contrary to the conventional methods in which exogenous energy sources contain high-energy phosphate bonds, the new system was designed to generate continuously the required high-energy phosphate bonds within the reaction mixture, thereby recycling the phosphate released during protein synthesis. If allowed to accumulate, phosphate inhibits protein synthesis, most likely by reducing the concentration of free magnesium ion. *Pediococcus* sp. pyruvate oxidase, when introduced in the reaction mixture along with thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD), catalyzed the generation of acetyl phosphate from pyruvate and inorganic phosphate. Acetyl kinase, already present with sufficient activity in *Escherichia coli* S30 extract, then catalyzed the regeneration of ATP. Oxygen is required for the generation of acetyl phosphate and the H_2O_2 produced as a byproduct is sufficiently degraded by endogenous catalase activity. Through the continuous supply of chemical energy, and also through the prevention of inorganic phosphate accumulation, the duration of protein synthesis is extended up to 2 h. Protein accumulation levels also increase. The synthesis of human lymphotoxin receives greater benefit than than that of chloramphenicol acetyl transferase, because the former is more sensitive to phosphate inhibition. Finally, through repeated addition of pyruvate and amino acids during the reaction period, protein synthesis continued for 6 h in the new system, resulting in a final yield of 0.7 mg/mL. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **66**: 180–188, 1999.

Keywords: in vitro protein synthesis; cell-free protein synthesis; chloramphenicol acetyl transferase (CAT); human lymphotoxin; adenosine triphosphate (ATP) regeneration; phosphoenol pyruvate (PEP); acetyl phosphate; pyruvate oxidase; pyruvate; inorganic phosphate

INTRODUCTION

For several decades, in vitro protein synthesis has served as an effective tool for lab-scale expression of cloned or synthesized genetic materials. In recent years, it appears that the in vitro protein synthesis system has been reconsidered as a possible alternative to conventional recombinant DNA technology (Jermutus et al., 1998; Rattan and Kristensen, 1990; Stiege and Erdmann, 1995). Because many aspects of cellular metabolism are no longer required, the in vitro protein synthesis system has potential advantages in the production of cytotoxic, unstable, or insoluble proteins. Also, through simultaneous and rapid expression of various proteins in a multiplexed configuration (e.g., 96-well plates), this technology can provide a valuable tool for development of important research and industrial proteins. In addition, various kinds of unnatural amino acids can be incorporated efficiently into proteins for specific purposes (Noren et al., 1989). However, despite all its promising aspects, the in vitro system has not been accepted as a practical alternative, due mainly to the short reaction period, which causes a poor yield of protein synthesis.

The development of a continuous-flow in vitro protein synthesis system by Spirin et al. (1988) proved that the reaction could be extended up to several hours. Since then, several studies have reproduced and improved this system (Endo et al., 1992; Kigawa et al., 1991, 1995).

Despite the extended reaction period of protein synthesis, however, the Spirin system is limited by such problems as high cost of operation, operational complexity, and significantly lowered rates of protein synthesis. Recently, Kim and Choi (1996a) reported that the merits of batch- and continuous-flow systems could be combined by adopting a "semicontinuous operation" using a simple dialysis membrane reactor. They were able to reproduce the extended reaction period of the continuous-flow system while maintaining the synthesis rate of a conventional batch system. The final yield reached 1.2 mg/mL. Most recently, Kigawa et al. (1999) reported a yield of 6 mg/mL in chloramphenicol acetyl transferase (CAT) synthesis by modifying this system.

On the other hand, significant improvements have also been made in the conventional batch system (Kawarasaki et al., 1995; Kim et al., 1996b; Kudlicki et al., 1992). Although the semicontinuous system maintains the initial rate of protein synthesis over extended periods, the conventional batch system still offers significant advantages; for instance, convenience of operation, easy scale-up, and excellent repro-

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ducibility. Also, the batch system can be readily conducted in multiplexed formats to express various genetic materials simultaneously. Patnaik and Swartz (1998) reported that the initial specific rate of protein synthesis could be enhanced to a level similar to that of in vivo expression through extensive optimization of reaction conditions. It is notable that they achieved such a high rate of protein synthesis using the conventional cell extract prepared without any condensation steps (Kim et al., 1996b; Nakano et al., 1996). Their result implies that further improvement of the batch system, especially in terms of the longevity of the protein synthesis reaction, would substantially increase the productivity for batch in vitro protein synthesis. However, the reason for the early halt of protein synthesis in the conventional batch system has remained unclear.

Kim and Choi (1996a) proposed that accumulation of low-molecular-weight byproducts could inhibit protein synthesis. They argued that the continuous removal of the inhibitory byproduct(s), as well as the continuous supply of substrates for protein synthesis, enabled the continuous or semicontinuous reaction system to support protein synthesis over long reaction periods. We believe that if we can understand and prevent the mechanism of such inhibition, an efficient batch system can be developed. During our investigation, we found that phosphoenol pyruvate (PEP), which is the most widely used energy source for regeneration of adeosine triphosphate (ATP) in an *Escherichia coli* in vitro protein synthesis system (see Fig. 1A), is very rapidly degraded during the incubation period. It was found that, after a 30-min incubation, more than 70% of the initial amount of PEP was degraded to pyruvate and inorganic phosphate by the action of phosphatase(s) present in the S30 extract. This result strongly suggests the possibility that the loss of ATP regenerating potential is one of the causes for the early halt of protein synthesis.

In this report, we demonstrate that the duration of protein synthesis can be greatly extended by introducing a novel scheme for ATP regeneration. Instead of exogenous addition, we developed a method for the in situ generation of the required high-energy phosphate bonds.

Pyruvic acid is a pivotal intermediate of the glycolytic pathway. Some organisms are able to condense pyruvate with inorganic phosphate to produce acetyl phosphate using pyruvate oxidase (EC 1.2.3.3) (Muller et al., 1993). Because acetyl phosphate can be used as an energy source for protein synthesis (Ryabova et al., 1995), the pyruvate oxidase reaction provides the basis for a new scheme to regenerate ATP that has been hydrolyzed in the process of protein synthesis (see Fig. 1B).

Perhaps the most striking advantage of this system is that the inorganic phosphate released in the process of protein synthesis is recycled into acetyl phosphate. Because inorganic phosphate is one of the possible inhibitory byproducts of protein synthesis, we expected that this scheme would prolong the period of protein synthesis by avoiding the accumulation of inorganic phosphate.

Here, we report that this strategy allowed the reaction period for protein synthesis to be extended up to 2 h. In the expression of chloramphenicol acetyl transferase (CAT), although the initial rate of protein synthesis in the new system was significantly lower than with the conventional system, remarkable improvement in the longevity of the reaction enabled a higher yield of protein synthesis as compared with a conventional system using PEP. To our knowledge, this is the first report describing a multihour bacterial protein synthesis reaction of batch configuration.

Furthermore, we show that the benefits of the new system are most striking in the expression of certain proteins particularly sensitive to the concentrations of inorganic phosphate and magnesium. In the case of human lymphotoxin

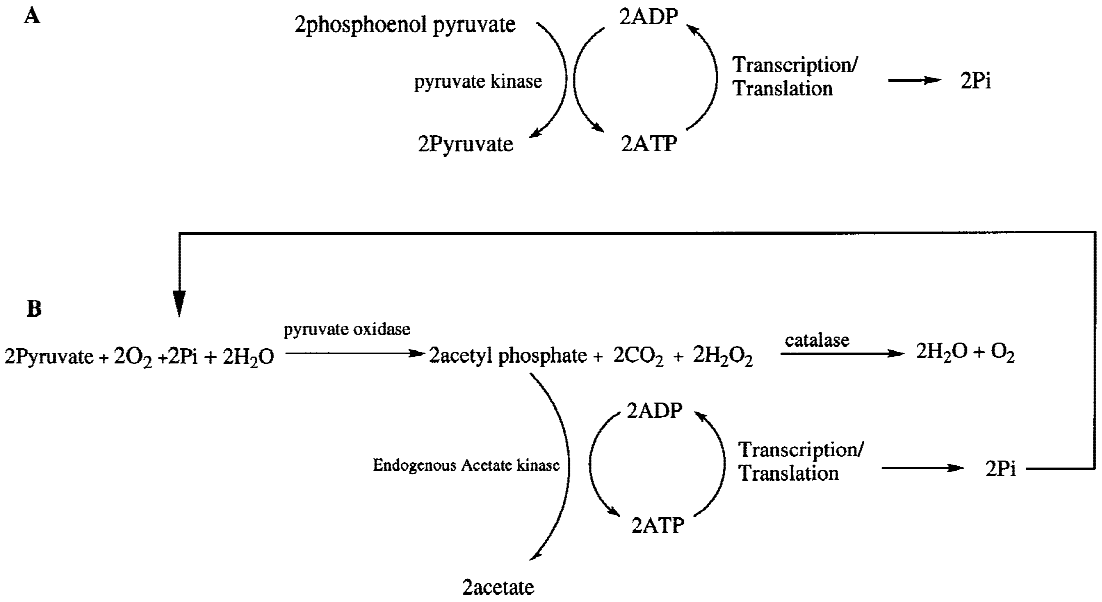

Figure 1. Regeneration of ATP in *in vitro* protein synthesis systems. (A), Conventional scheme for energy regeneration using phosphoenol pyruvate(PEP) and pyruvate kinase (PK). (B) A new scheme proposed in this report using pyruvate, pyruvate oxidase (Pox), and endogenous acetate kinase (AcK).

(hLT), which showed an expression level strongly dependent on the concentrations of inorganic phosphate and magnesium, the final yield after a 2-h incubation increased by 100% compared with the conventional reaction using PEP.

To our surprise, the longevity of protein synthesis in the new system was further extended up to 6 h when the concentrations of pyruvate and amino acids were maintained through repeated additions during the incubation periods.

What makes this new system more attractive is that the cost for pyruvate is almost negligible compared with the conventional energy sources such as phosphoenol pyruvate, creatine phosphate, and acetyl phosphate. Thus, with lower cost and greater yields, this new strategy for ATP regeneration can significantly improve the economics of cell-free protein synthesis.

MATERIALS AND METHODS

PEP and *E. coli* total tRNA mixture were purchased from Boerhinger-Mannheim (Indianapols, IN). L-[U–^{14}C]-leucine (11.7 GBq/mmol) , L-[U–^{3}H]-leucine (4.14 TBq/mmol), and [5,6-^{3}H]-UTP (0.37 TBq/mmol) were from Amersham Pharmacia Biotechnology (Uppsala, Sweden). All the other reagents were obtained from Sigma (St. Louis, MO). T7 RNA polymerase was prepared from the culture of *E. coli* strain BL21 (pAR1219) according to the procedures of Davanloo et al. (1984). Plasmid pK7CAT, which includes the bacterial CAT sequence between the T7 promoter and T7 terminator (Kigawa et al., 1995), was used as a template for CAT synthesis. For hLT synthesis, plasmid pK7LT was constructed by replacing the CAT sequence of pK7CAT with the human lymphotoxin sequence. S30 extract was prepared from *E. coli* K12 (strain A19) as described earlier (Kim et al., 1996b). The standard reaction mixture consists of the following components: 57 m*M* HEPES-KOH (pH 8.2), 1.2 m*M* ATP, 0.85 m*M* each of GTP, UTP, and CTP, 1 m*M* DTT, 0.64 m*M* cAMP, 200 m*M* potassium glutamate, 80 m*M* $NH_4(OAc)$, 15 m*M* $Mg(OAc)_2$, 34 mg/mL folinic acid, 6.7 μg/mL plasmid, 33 μg/mL T7 RNA polymerase, 500 μ*M* each of 20 unlabeled amino acids, and [^{14}C]-leucine (11.0 μ*M*) or [^{3}H]-leucine (1.2 μ*M*), 2% Polyethylenglycol (PEG) 8000, 32 m*M* PEP, and 0.24 volume of S30 extract. In reactions in which pyruvate was used as the energy regenerating compound, PEP was removed from the standard reaction mixture and 32 m*M* pyruvate, 6 U/mL pyruvate oxidase, 6.7 m*M* potassium phosphate, 3.3 m*M* TPP, and 0.3 m*M* FAD were added. Reactions were run for given time periods in 15- to 60-μL volumes in a waterbath set at 37°C. In the experiments to extend the protein synthesis reaction through supplementation, a mixture of pyruvate and 20 amino acids was added to the reaction every hour. The amount of synthesized protein was estimated from the measured TCA-insoluble radioactivities as described earlier (Kim et al., 1996a) using a liquid scintillation counter (Beckman LS3801). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, samples were loaded on a 16% SDS-PAGE gel (Novex) with standard molecular weight markers. Resulting gels were stained with Coomassie brilliant blue following the standard procedures. In cases where autoradiography was required, Kodak XoMat film was exposed to a dried gel overnight and developed in an automatic X-ray film developer.

Quantitative analysis of inorganic phosphate was carried out according to the procedures of Saheki et al. (1985) after minor modifications. Two-microliter samples were taken and mixed with 15 μL of a 20% SDS solution and 43 μL water. After sequential addition of 670 μL solution A (100 m*M* zinc acetate, 15 m*M* ammonium molybdate, pH 5.0) and 170 μL solution B (10% ascorbic acid), each tube was incubated in a 30°C gyratory incubator for 15 min. Finally, the concentration of inorganic phosphate was estimated from the measured OD_{850} of sample and standard curve.

ATP concentration was measured in a continuous-flow luminometer (Lumitec, St. John's Associates, Beltsville, MD). Using a peristaltic pump, a luciferase solution (0.1 mg/μL luciferase and 125 μ*M* luciferin) and the diluted reaction sample were pumped separately into the reaction coil of the luminometer at 0.5 mL/min, where they were mixed to initiate the luminescence reaction. The intensity of luminescence was linear with respect to ATP concentration over the range of 0 to 6 μ*M*.

RESULTS

Nonproductive Degradation of PEP

The concentration of inorganic phosphate in the standard reaction mixture initially containing 32 m*M* PEP was measured throughout the reaction period of protein synthesis as described in the previous section. As shown in Figure 2, the concentration of inorganic phosphate in the reaction mixture increased linearly during the incubation period. The accumulation of inorganic phosphate took place at almost the same rate, even without addition of plasmid to the reaction mixture, demonstrating that protein synthesis does not contribute significantly to the generation of phosphate. (The background level of protein synthesis due to contaminating mRNA and chromosomal DNA was negligible in our system. The CPM value of a negative control reaction without plasmid DNA was less than 10% of a reaction programmed with pK7CAT [data not shown].)

The rate of phosphate accumulation was just as high even when no nucleotides were added, suggesting direct hydrolysis of the PEP.

Such a nonproductive degradation reduces the amount of PEP available for ATP regeneration and also increases the accumulation of phosphate. Without S30 extract in the incubation mixture, the accumulation of inorganic phosphate was negligible. These results suggest that PEP is a major target of phosphatases and that the uncoupled PEP hydrolysis can significantly reduce protein synthesis yields.

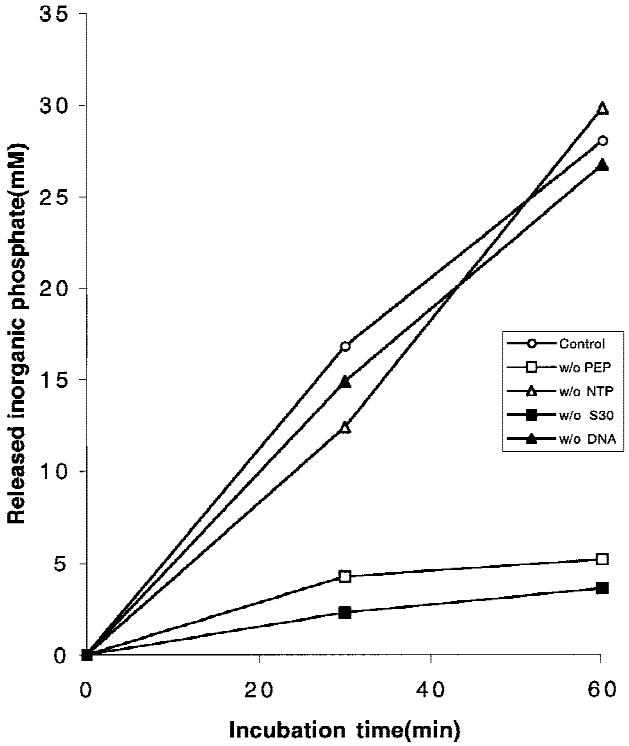

Figure 2. Accumulation of inorganic phosphate in the reaction mixture under various conditions. Standard reaction mixture (control) and reaction mixtures devoid of the indicated components were prepared and incubated. Two-microliter samples were taken at the given timepoints and the concentration of inorganic phosphate was measured as described in "Materials and Methods."

In Situ Generation of Acetylphosphate Using the Pyruvate Oxidase Reaction

The aforementioned results indicate that, for a 30-min protein synthesis reaction, as much as 60% of the energy source can be wasted. This will limit ATP regeneration and could explain, at least in part, the early halt of the in vitro protein synthesis reaction. Besides PEP, creatine phosphate and acetyl phosphate have been used successfully as secondary energy sources in various in vitro protein synthesis systems of eukaryotic and prokaryotic origins. Both of these substrates supported protein synthesis in our system as efficiently as PEP (data not shown). However, similar to PEP, they lose their high-energy phosphate bond during the incubation with S30 extract (data not shown). Thus, it appears that as long as we rely on the direct use of phosphate bond energy sources to regenerate ATP, the nonproductive depletion of the energy source and accumulation of inorganic phosphate is almost inevitable. To attain an extended reaction period in the batch system, a different approach for ATP regeneration was needed. We looked for a system that does not require a high concentration of compounds with high-energy phosphate bonds; that is, a system that can regenerate the high-energy phosphate donor in situ as well as ATP.

Pyruvate oxidase (EC 1.2.3.3) plays an important role in the aerobic growth of lactobacteria by catalyzing the oxidative decarboxylation of pyruvate in several steps. Importantly, in the presence of the cofactors, TPP and FAD, this enzyme catalyzes the condensation of pyruvate and inorganic phosphate to generate acetyl-phosphate, which can serve as an energy source in our in vitro protein synthesis system. This reaction was tested to determine if acetyl phosphate could be continuously provided while avoiding the accumulation of inorganic phosphate. The inorganic phosphate produced either from protein synthesis or by degradation of acetyl phosphate would be recycled to generate another molecule of acetyl phosphate. The acetyl phosphate would, in turn, regenerate the required ATP. A simplified diagram for this strategy is given in Figure 1B. In addition, because both acetyl phosphate and ATP are continually generated and depleted, the peak concentration of both is likely to be kept low enough to discourage nonproductive hydrolysis.

Expression of CAT in the Pyruvate Oxidase System

To examine this scheme under the reaction conditions for in vitro protein synthesis, the required compounds were introduced into the reaction mixture at different concentrations and incubated at 37°C for 1 h. As shown in Figure 3, TCA precipitable radioactivity suggests that the new strategy for ATP regeneration does support CAT synthesis. The amount of synthesized CAT increased with increasing concentrations of exogenous pyruvate oxidase and leveled off at 6.6 U/mL pyruvate oxidase. Although relatively insensitive to the concentration of FAD, the yield of protein synthesis responded sharply to the concentrations of TPP and inorganic phosphate giving maximal protein synthesis at 3.3 m*M* and 6.6 m*M*, respectively. The yield of protein synthesis was almost negligible when pyruvate oxidase was removed from the reaction mixture.

After a 1-h incubation, the final amount of synthesized CAT reached about 120% of that for the PEP reaction. We then examined the time course of protein synthesis. Strikingly, as shown in Figure 4A, we found that the protein synthesis reaction continued up to 2 h in the pyruvate system, whereas the reaction using PEP slowed drastically after 20 min.

The time course of ATP concentration coincided nicely with the duration of protein synthesis. As shown in Figure 4B, the ATP level in the reaction mixture decreased rapidly in the absence of any secondary energy sources. Complete depletion of ATP was observed within 10 min. Although the addition of PEP could significantly increase ATP concentration, it was not sufficient to prevent a rapid decrease of ATP concentration. In contrast, although the average level was lower than in the PEP system, pyruvate and pyruvate oxidase maintained a stable ATP concentration for over 60 min.

As a result, despite the significantly reduced initial rate of protein synthesis (192 μg/mL · h, compared with 300 μg/

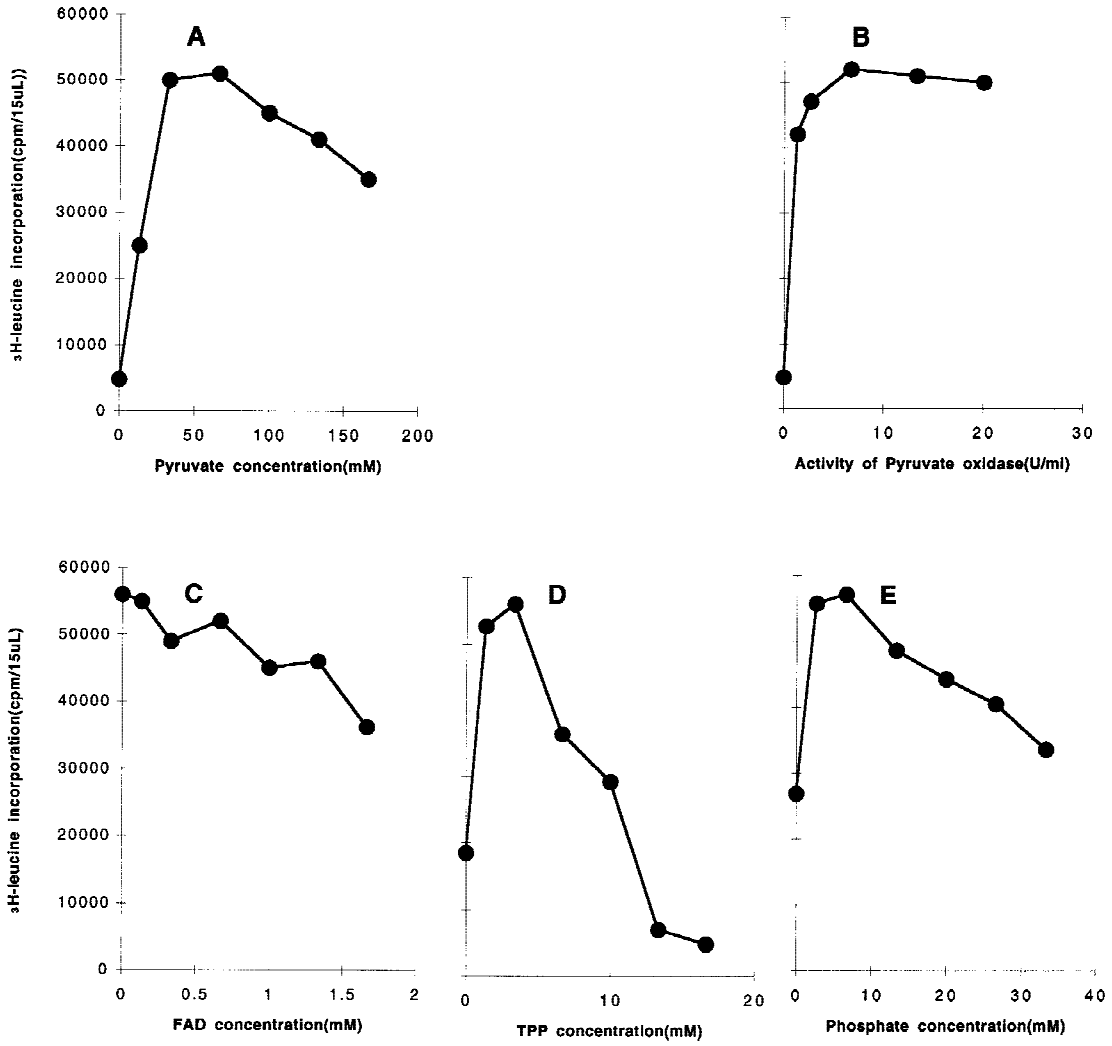

Figure 3. Use of pyruvate as energy source for protein synthesis. Standard reaction mixtures containing different concentrations of pyruvate, pyruvate oxidase, FAD, TPP, and inorganic phosphate were prepared and incubated for 1 h. The final amount of [^{3}H]-leucine incorporation was measured as described in "Materials and Methods." (A) 6.7 U/mL pyruvate oxidase, 0.3 m*M* FAD, 3.3 m*M* TPP, 6.7 m*M* inorganic phosphate. (B) 32 m*M* pyruvate, 0.3 m*M* FAD, 3.3 m*M* TPP, 6.7 m*M* inorganic phosphate. (C) 32 m*M* pyruvate, 6.7 U/mL pyruvate oxidase, 3.3 m*M* TPP, 6.7 m*M* inorganic phosphate. (D) 32 m*M* pyruvate, 6.7 U/mL pyruvate oxidase, 0.3 m*M* FAD, 6.7 m*M* inorganic phosphate. (E) 32 m*M* pyruvate, 6.7 U/mL pyruvate oxidase, 3.3 m*M* TPP, 0.3 m*M* FAD.

mL · h with the PEP-driven system), our new system was able to produce protein at a volumetric yield higher than the conventional system using PEP.

The generation of acetyl phosphate through this reaction cycle produces such byproducts as carbon dioxide, acetate, and hydrogen peroxide. It was observed that the generation of these byproducts did not significantly change the pH of the reaction buffer (data not shown). Moreover, hydrogen peroxide, possibly the most serious byproduct, does not seem to poison the system, as suggested by the prolonged, constant rate of protein synthesis. Presumably, our reaction system contains enough endogenous catalase activity to avoid hydrogen peroxide toxicity. Addition of exogenous catalase to the reaction mixture did not affect either the rate or duration of protein synthesis. An autoradiograph of samples taken from the PEP- and pyruvate-driven reactions showed that CAT was nearly the only protein synthesized during the reactions (data not shown).

Expression of Human Lymphotoxin in the New System

To examine further the potential of the new system, it was used for the expression of human lymphotoxin. Unexpectedly, the advantage of the new system appeared more striking with this protein. After a 2-h incubation, the expression level of lymphotoxin was twice that for the PEP system (Fig. 5A). After examining the effect of varying concentrations of magnesium, it was found that the expression of this protein is more sensitive to changes in magnesium concentration than is CAT (Fig. 5B). In addition, when different concentrations of exogenous inorganic phosphate were

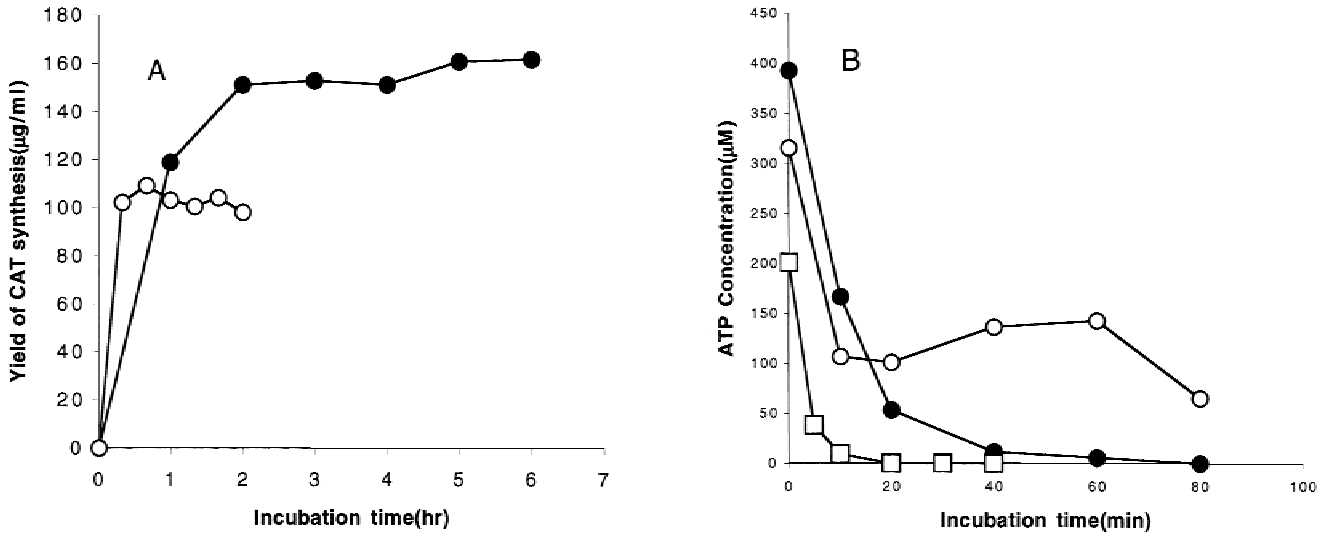

Figure 4. Time course of CAT synthesis and ATP concentration in the conventional system (○) and in our new system (●). Reaction mixtures (120 μL) of each system were prepared and incubated in a 37°C waterbath. (A) Ten-microliter samples were withdrawn every 30 min and the [^{14}C]-leucine-labeled radioactivity of protein was measured as described in "Materials and Methods." (B) Five microliters of reaction mixture was taken at the given timepoints, diluted with 495 μL of assay buffer, and ATP concentration was measured as described. (□) Time course of ATP concentration without any energy source.

added to the reaction mixture, lymphotoxin expression was more vulnerable to excess inorganic phosphate (Fig. 5C).

We assume that the more striking advantage of the new system in hLT expression is due to the higher sensitivity of hLT synthesis to the concentrations of magnesium and inorganic phosphate. As shown earlier, PEP is rapidly degraded and inorganic phosphate rapidly accumulates with the PEP system. Its concentration in the pyruvate system should remain stable.

Addition of Pyruvate and Amino Acids During Incubation

Although the new method of energy supply was able to support protein synthesis over significantly extended time periods, the length of the reaction was still much shorter than that of continuous or semicontinuous systems. From Figure 4, it seems that the ATP concentration limits protein synthesis in both the PEP and pyruvate systems. Assuming that the decrease of ATP concentration is a result of energy source exhaustion, periodic pyruvate additions were examined. As shown in Figure 6A, with hourly pyruvate additions, the ATP concentration was maintained stable for at least 4 h and a significant amount of ATP was detected, even after a 6-h incubation. On the other hand, in spite of the steady supply of ATP, the period of protein synthesis increased only by about 1 h. Thus, it seems that ATP depletion was not the only factor limiting protein synthesis.

Surprisingly, we found that protein synthesis continued for several hours when pyruvate was added to the reaction mixture along with a mixture of 20 amino acids. Upon repeated addition of amino acids as well as pyruvate, the

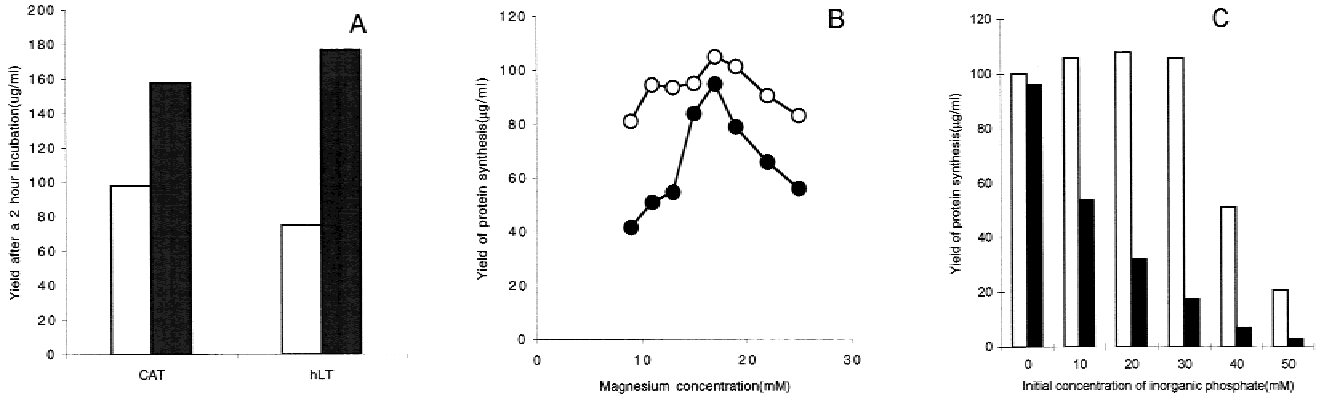

Figure 5. Expression of proteins using PEP or pyruvate as energy sources. (A) Plasmid pK7CAT and pK7LT were expressed for 2 h to produce CAT and hLT in a standard PEP (open bars) system or in our new system using pyruvate (filled bars). Final yields were measured by a standard TCA-precipitation method. (B) CAT (○) and hLT (●) were produced in the PEP system with varying concentrations of $Mg(OAc)_2$. (C) To the standard reaction mixture of PEP system, different concentrations of exogenous inorganic phosphate were added and incubated for 1 h. Open bars: CAT; filled bars: hLT.

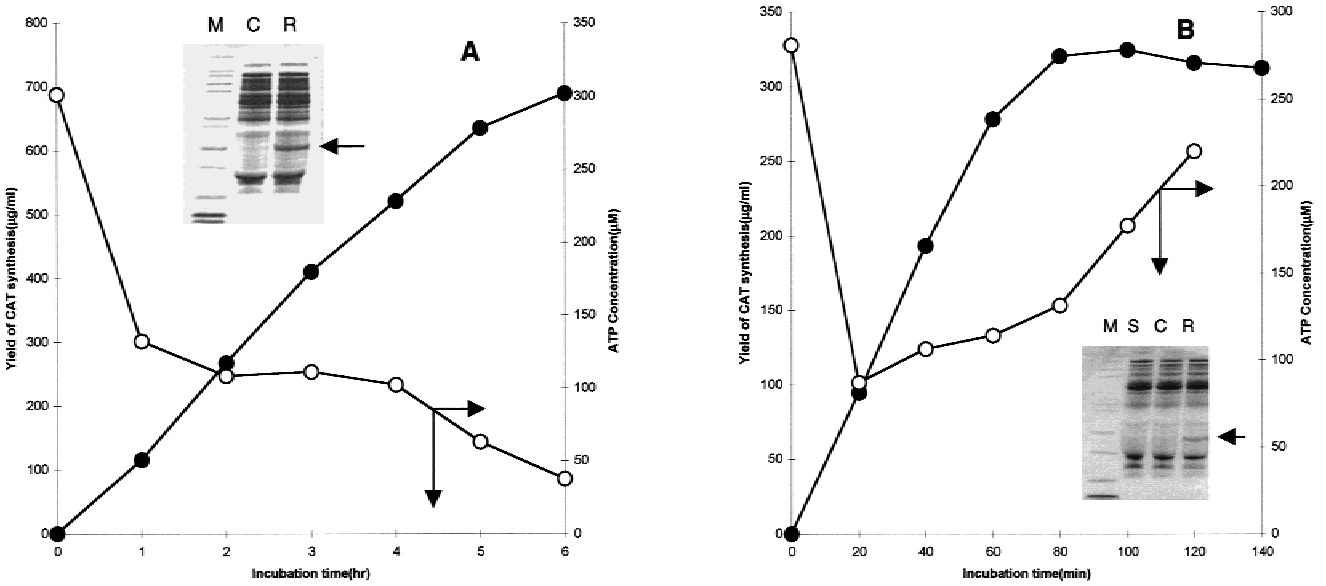

Figure 6. Expression of CAT with repeated addition of energy source and amino acids. Fifteen-microliter reaction mixtures for the pyruvate system (A) and the PEP system (B) were prepared in different tubes and placed in a waterbath at 37°C. (A) Each tube for the pyruvate system received 0.25 μL of 2 *M* pyruvate and 0.25 μL of amino acid mixture every hour. To monitor the time course of protein synthesis, one reaction tube was removed at each timepoint and TCA-insoluble radioactivity was measured. Five-microliter samples were withdrawn to measure ATP concentration. (B) Every 20 min, each tube received 0.5 μL of 1 *M* PEP and 0.25 μL of amino acid mixture. Protein synthesis and ATP concentration were measured as described. Insets to (A) and (B): SDS-PAGE of samples withdrawn at the end of each reaction. Lane M, standard molecular weight markers; lane C, standard reaction without any supplementation of amino acids and energy source; lane R, reaction with periodic supplementation of amino acids and pyruvate (A) or PEP (B).

amount of synthesized protein increased for over 6 h and the resulting yield reached 0.7 mg/mL (Fig. 6A). Such a product yield was sufficient to give an intense band on a SDS-PAGE gel stained with Coomassie Brilliant Blue dye. The stimulatory effect of amino acid addition is a rather unexpected result, because a calculation based on the protein synthesis yield of 150 μg/mL suggests that approximately only 65 μ*M* of each amino acid is required for protein synthesis, whereas the initial concentration of each amino acid in the reaction mixture is 500 μ*M*. This result thus indicates that certain amino acids are also degraded during the reaction period.

The ATP concentration could also be maintained in the conventional PEP system when the reaction was supplemented with PEP and amino acids. However, when the mixture of PEP and amino acids was added every 20 min, no significant increase in product yield was observed after 1 h (Fig. 6B).

As previously shown in Figure 5C, a high concentration of inorganic phosphate inhibits protein synthesis dramatically. Considering the rate of nonproductive phosphate generation from PEP (Fig. 2), after three additions of PEP, the inorganic phosphate in the reaction mixture will be at least 50 m*M*, which significantly inhibits protein synthesis. Thus, for the PEP system, phosphate accumulation ultimately limits its product synthesis.

Interestingly, the ATP concentration in the PEP system showed an obvious increase upon the repeated addition of PEP. Our working hypothesis is that, although the regeneration of ATP continues with the periodic supply of PEP, the cessation of protein synthesis, and possibly inhibition of other enzymes, reduces the rate of ATP usage in the PEP system, which then allows the concentration to increase. The increase is relatively small relative to the PEP addition because both PEP and ATP are being hydrolyzed by other reactions. In contrast, ATP concentration remained low in the pyruvate system after the halt of protein synthesis even with the supplementation of pyruvate. Because our experimental results show that the pyruvate oxidase remains active for as long as 10 h (data not shown), we must consider the possibility that the acetate kinase derived from the S30 extract is losing its enzymatic activity during the incubation period.

DISCUSSION

The findings described here demonstrate that the coupling of the in situ generation of an energy source (in this study acetyl phosphate) with the ATP regeneration reaction provides a steady supply of ATP for the protein synthesis reaction. This, in turn, allows the initial rate of protein synthesis to be maintained over greatly extended reaction periods. Our results suggest that the early halt of protein synthesis experienced by conventional systems can be largely attributed to the degradation of secondary energy sources as well as the degradation of ATP itself.

Conventional batch in vitro protein synthesis systems require relatively high initial concentrations of PEP (20 to 30

m*M*) for maximal yields of protein synthesis. Such a high concentration will increase the rate and extent of nonproductive degradation as it allows phosphatases with low affinities for PEP to bind and degrade the energy source. In contrast, the concentration of acetyl phosphate in the new system will not greatly exceed that of exogenously added inorganic phosphate (6.7 m*M*), although there may be marginal contributions of phosphate released from nucleotide triphosphates (1.2 m*M* ATP and 0.8 m*M* each of GTP, UTP, and CTP). More than likely, the acetyl phosphate concentration will be much lower because its generation appears to be rate limiting and the reported K_m value of acetate kinase for acetyl phosphate is 0.2 m*M* (Kessler and Knappe, 1996). Thus, a steady supply of acetyl phosphate, maintained at a rate-limiting concentration, in our system is expected to improve the efficiency of high-energy phosphate bond utilization.

In addition to the depletion of ATP regeneration potential, degradation of PEP leads to a stoichiometric accumulation of inorganic phosphate, which can act as an inhibitor on both the transcription and translation reactions for protein synthesis through sequestration of essential magnesium ion from the reaction mixture. However, the possibility cannot be excluded that the inhibitory effect of phosphate is due to a direct inhibition by magnesium phosphate rather than to the deprivation of magnesium. Because the stability constant of the magnesium complexes are about the same for PEP and inorganic phosphate (Dawson et al., 1986), the reaction of the first PEP addition should not change magnesium availability significantly. However, the inhibitory effect becomes most obvious when PEP is added repeatedly and excessive inorganic phosphate accumulates. In this case, both magnesium deprivation and magnesium phosphate inhibition are possible.

By employing a method that recycles the inorganic phosphate ion, we were able to avert this problem. Because the new strategy for ATP regeneration does not include any mechanism of phosphate accumulation, the secondary energy source can be added repeatedly to prevent the depletion of ATP. In addition, as was shown with the expression of human lymphotoxin, our new system would be most advantageous for proteins with an expression that is most sensitive to inorganic phosphate. This variable sensitivity to phosphate and magnesium concentration may also help to explain the need to determine the optimal magnesium concentration for each new protein. It is expected that the use of pyruvate as an energy source will allow more consistent performance from protein to protein.

Finally, this system enables us to control easily the rate of protein synthesis by adjusting pyruvate oxidase activity in the reaction mixture. This will provide a useful method to study various aspects of protein synthesis, including protein folding as a function of the rate of protein synthesis.

Presently, we are carrying out experiments to understand why our system has a significantly lower initial rate of protein synthesis as compared with the conventional system using PEP. The lower level of ATP concentration during the initial phase of incubation may explain the poor rate of protein synthesis in the new system.

Our assumption at this point is that the rate of acetyl phosphate generation limits the regeneration rate of ATP. Because oxygen is a required reagent, the dissolved oxygen supply may limit the generation of acetyl phosphate. Because an equimolar amount of oxygen is required to oxidize pyruvate molecules while the solubility of oxygen is very low (approx. 0.25 m*M*), it is quite probable that the reaction step in which the acetyl-TPP-E-FAD_{red} is oxidized to acetyl-TPP-E-FAD_{ox} poses a rate limitation in acetyl phosphate production. Thus, we expect that the rate of protein synthesis can be improved by taking measures to increase the rate of oxygen transfer into the reaction mixture. If so, the rate of oxygen transfer to the reaction may be used as a control variable for manipulating the rate of protein synthesis.

We also believe that the system is limited by severe "wastage" of ATP by competing reactions. It is notable that our measured ATP concentrations are at least an order of magnitude lower than in vivo values. We are now working to identify the key reactions that hydrolyze ATP in addition to protein synthesis.

Our system also potentially provides economic benefits. As is well known, the high cost of in vitro protein synthesis systems has been one of the obstacles preventing its use as a commercial method for protein production. Among the reaction components, the high-energy phosphate bond compound contributes the largest fraction of the cost. A calculation based on the reagent prices shows that PEP accounts for more than 70% of the total reagent cost. Probably, the cheapest source of commercially available ATP regenerating compound would be acetyl phosphate. However, because the cost for pyruvate ($0.042/mmol) is almost negligible compared with acetyl phosphate ($3.2/mmol), not to mention PEP ($16.4/mmol), our system will greatly reduce the cost of reagents for the cell-free production of proteins. Although the commercial pyruvate oxidase used in this study is quite expensive ($0.8/unit), making the total cost still high, we expect that the cost for this enzyme can be eliminated by expressing it in the *E. coli* strain used for the preparation of S30 extract.

Through various efforts to enhance the initial rate of protein synthesis of our present system while maintaining the remarkably extended reaction period, we eventually aim to develop a cell-free protein synthesis system of batch configuration that enables economical, multiplexed production of families of bioactive proteins. In addition, such approaches can contribute to the development of new approaches for the economical, large-scale production of commercial proteins.

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References

Davanloo P, Rosenberg AH, Dunn JJ, Studier FW. 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc Natl Acad Sci USA 81:2035–2039.

Dawson RMC, Elliot DC, Elliot WH, Jones KM. 1986. Data for biochemical research. New York: Oxford University Press. p 399–415.

Endo Y, Otsuzuki S, Ito K, Miura K. 1992. Production of an enzymatic active protein using a continuous flow cell-free translation system. J Biotechnol 25:221–230.

Jermutus L, Ryabova LA, Pluckthun A. 1998. Recent advances in producing and selecting functional proteins by using cell-free translation. Curr Opin Biotechnol 9:534–548.

Kawarasaki Y, Kawai T, Nakano H, Yamane T. 1995. A long-lived batch reaction system of cell-free protein synthesis. Anal Biochem 226: 320–324.

Kessler D, Knappe J. 1996. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaecter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella* cellular and molecular biology. Washington, DC: American Society of Microbiology. p 199–205.

Kigawa T, Muto Y, Yokoyama S. 1995. Cell-free synthesis and acid-selective stable isotope labeling of proteins for NMR analysis. J Biomol Nucl Magn Res 6:129–134.

Kigawa T, Yabuki T, Yoshida Y, Tsutsui M, Ito Y, Shibata T, Yokoyama S. 1999. Cell-free production and stable-isotope labeling of milligram quantities of proteins. FEBS Lett 442:15–19.

Kigawa T, Yokoyama S. 1991. A continuous cell-free protein synthesis system for coupled transcription and translation. J Biochem 110: 166–168.

Kim D-M, Choi C-Y. 1996a. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. Biotechnol Prog 12:645–649.

Kim D-M, Kigawa T, Choi C-Y, Yokoyama S. 1996b. A highly efficient cell-free protein synthesis system from *Escherichia coli*. Eur J Biochem 239:881–886.

Kudlicki W, Kramer G, Hardesty B. 1992. High efficiency cell-free synthesis of proteins: refinement of the coupled transcription/translation system. Anal Biochem 206:389–393.

Muller YA, Schulz GE. 1993. Structure of the thiamine- and flavin-dependent enzyme pyruvate oxidase. Science 259:965–967.

Nakano H, Tanaka T, Kawarasaki Y, Yamane T. 1996. Highly productive cell-free protein synthesis system using condensed wheat-germ extract. J Biotechnol 46:275–282.

Noren CJ, Anthony-Cahill SJ, Griffith MC, Schultz PG. 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. Science 244:182–188.

Patnaik R, Swartz JR. 1998. *E. coli* based in vitro transcription/translation: in vitro specific rates and high yields in a batch system. BioTechniques 24:862–868.

Rattan SI, Kristensen O. 1990. Continuous gene expression in vitro: the Spirin system. Trends Biotechnol 8:275–276.

Ryabova LA, Vinokurov LM, Shekhovtsova EA, Alakhov YB, Spirin AS. 1995. Acetyl phosphate as an energy source for bacterial cell-free translation systems. Anal Biochem 226:184–186.

Saheki S, Takeda A, Shimazu T. 1985. Assay of inorganic phosphate in the mild pH range, suitable for measurement of glycogen phosphorylase activity. Anal Biochem 148:277–281.

Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB. 1988. A continuous in vitro translation system capable of producing polypeptides in high yield. Science 242:1162–1164.

Stiege W, Erdmann VA. 1995. The potentials of the *in vitro* protein biosynthesis system. J Biotechnol 41:81–90.