Rapid Expression and Purification of 100 nmol Quantities of Active Protein Using Cell-Free Protein Synthesis

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Two strategies for ATP regeneration during cell-free protein synthesis were applied to the large-scale production and single-column purification of active chloramphenicol acetyl transferase (CAT). Fed-batch reactions were performed on a 5−10 mL scale, approximately 2 orders of magnitude greater than the typical reaction volume. The pyruvate oxidase system produced 104 nmol of active CAT in a 5 mL reaction over the course of 5 h. The PANOx system produced 261 ± 42 nmol, about 7 mg, of active CAT in a 10 mL reaction over the course of 4 h. The reaction product was purified to apparent homogeneity with approximately 70% yield by a simple affinity chromatography adsorption and elution. To our knowledge, this is the largest amount of actively expressed protein to be reported in a simple, fed-batch cell-free protein synthesis reaction.

Introduction

Cell-free protein synthesis offers a method to exploit biological processes without intact cells. It is a convenient tool for the production of active recombinant DNA (rDNA) proteins and has several advantages over in vivo protein expression systems (*1*−*4*). The productivity of cell-free systems has increased more than 100 times over the past decade, and this technology is becoming more than just a laboratory research technique. The surge in productivity of cell-free systems is founded on a new understanding of energy source limitations present in cell-free reactions. However, three main challenges still remain for a broader acceptance of cell-free protein synthesis as a general method for the production of rDNA proteins. One of these is gaining a more fundamental understanding of the processes involved in obtaining properly folded proteins in vitro. Another is continuing to understand and remove the limitations that lead to short reaction times in batch reactions. The third is the demonstration that cell-free systems can be operated at increased production scales. This work addresses producing proteins in vitro on a milliliter, rather than microliter, scale in a batch system using two recently developed energy systems. The Yokoyama group has recently shown the utility of continuous exchange cell-free methods in preparing milligram quantities of protein for crystal structure determination (*5*−*7*). However, production of milligram quantities of protein using a batch approach has not been pursued.

One of the schemes to regenerate energy is the pyruvate oxidase system (*8*). Kim and Swartz reported that pyruvate could be utilized to regenerate ATP during a cell-free protein synthesis reaction. In this energy regeneration system, the pyruvate oxidase enzyme from either *Lactobacillus* or *Pediococcus* sp. is added to the cell-free reaction mixture to convert pyruvate into acetyl-phosphate. Molecular oxygen is required for this oxidation reaction. Acetyl-phosphate regenerates ATP via endogenous acetate kinase present in the cell extract prepared from *E. coli*. The most notable feature of this system is that phosphate released during protein synthesis is recycled into acetyl-phosphate (Figure 1A). This attribute allows for numerous additions of the energy source without inhibition of the protein synthesis reaction via phosphate accumulation. Previous attempts to scale-up the protein synthesis reaction using the pyruvate oxidase energy regeneration system were unsuccessful. It was reported that scaling up a batch reaction from 15 to 120 μL decreased the protein synthesis yield by more than 60% (*9*). Most likely, the simple batch configuration utilized for these reactions was limited by transfer of oxygen into the reaction solution. Since 33 mM pyruvate is initially added and the solubility of oxygen in our reaction mixture is about 0.25 mM, significant oxygen transfer is necessary for the production of acetyl phosphate from pyruvate. This limitation is expected to be worse at even larger scales because of the reduction in surface-to-volume ratio.

More recently, Kim and Swartz reported another novel method for energy regeneration, the PANOx system (*9*). This system combines the conventional PEP system with the addition of nicotinamide adenine dinucleotide (NAD) and coenzyme A (CoA) to utilize both PEP and pyruvate to regenerate ATP (Figure 1B). The cofactors are necessary to exploit the endogenous enzymes present in the *E. coli* extract for energy regeneration from pyruvate. The PANOx system also increases the amino acid concentration as compared to the traditional PEP system (*8*) and supplements the reaction mixture with sodium oxalate, which has been shown to enhance protein synthesis yields in batch reactions (*10*). Attempts to scale-up the PANOx system have not been previously carried out, although the challenges associated with the addition of an exogenous enzyme and oxygen supply are not present.

In this work, production of 100 nmol quantities of active chloramphenicol acetyl transferase (CAT) is reported with the use of both the pyruvate oxidase and the PANOx energy regeneration system. To our knowledge,

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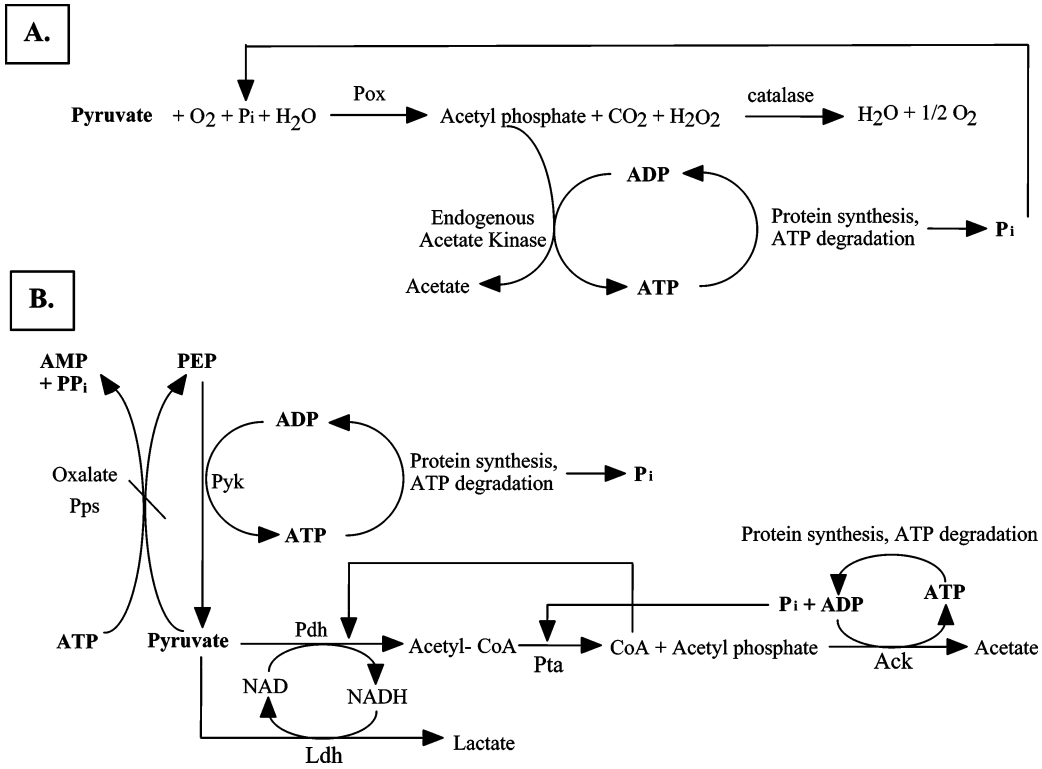

Figure 1. Proposed mechanisms for secondary energy regeneration systems. (A) Pyruvate oxidase system. Pyruvate oxidase catalyzes the formation of acetyl phosphate from pyruvate, requiring molecular oxygen. Endogenous acetate kinase, present in the cell extract, transfers the phosphate group from acetyl phosphate to regenerate ATP. The phosphate generated during protein synthesis is recycled. (B) PANOx system. The conventional PEP system is coupled with ATP generation from pyruvate. This requires the addition of coenzymeA and NAD. Sodium oxalate is used to inhibit the energy wasting reaction that converts pyruvate to PEP. Abbreviations: acetate kinase (Ack), adenosine triphosphate (ATP), lactate dehydrogenase (Ldh), nicotinamide adenine dinucleotide (NAD), phosphoenolpyruvate (PEP), phosphoenolpyruvate synthase (Pps), pyruvate dehydrogenase (Pdh), phosphotransacetylase (Pta), pyruvate kinase (Pyk), pyruvate oxidase (Pox).

this is the first account describing large scale cell-free protein production using a fed-batch format. Rapid, single-step affinity purification of CAT is also demonstrated. These results begin to suggest the possibility for large-scale production of protein pharmaceuticals using cell-free synthesis systems. In addition, these data suggest the feasibility of high-throughput batch protocols for generating sufficient quantities and purity of proteins for structure determination by NMR or X-ray cystallography.

Materials and Methods

Cell-Free Protein Synthesis. Coupled transcription-translation reactions were carried out in 1.5 mL eppendorf tubes or stirred glass beakers at 37 °C. The pyruvate oxidase reaction was performed in a 10 mL beaker, and the PANOx scale-up reactions were performed in a 25 mL beaker. Plasmid pK7CAT was used as a template for protein synthesis. pK7CAT encodes for chloramphenicol acetyl transferase (CAT), with the CAT gene cloned between the T7 promoter and the T7 terminator (*9*). The plasmid was purified using a QIAGEN Plasmid Maxi Kit (Valencia, CA). N-Terminal histidine-tagged T7 RNA polymerase was prepared from *E. coli* strain BL21 (using a modified form of the plasmid pAR1219 (*11*), containing the additional sequence for six N-terminal histidine residues) according to the procedures of Swartz et al. (*12*). S30 extract for the pyruvate oxidase system was prepared from *E. coli* strain A19, a derivative of K12, as described earlier (*2*). Extract for the PANOx system was prepared from *E. coli* strain A19 Δ*tonA* Δ*speA* Δ*tnaA met*$^+$. Extract preparation was carried out as above. However, cells were grown on a glucose and phosphate media, 2 x YTPG (*13*). 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, and removing the *tonA* gene encoding the ferrichrome-iron receptor to protect against phage infection. When the cell-extract from this mutant A19 strain was used in a 3-h PANOx batch reaction, comparable amounts of CAT were synthesized relative to reactions when the cell extract was prepared from the original A19 strain (data not shown). Extracts from cells grown on 2 x YTPG were shown to produce about 37% more protein and have reduced phosphatase activity relative to those from cells grown on 2 x YT (*13*). The standard reaction mixture contains the following components: 57 mM Hepes-KOH (pH7.5), 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP, 1 mM dithiothreitol (DTT), 200 mM potassium glutamate, 80 mM ammonium acetate, 12 mM magnesium acetate, 34 μg/mL folinic acid, 170.6 μg/mL *E. coli* tRNA mixture, 6.7 μg/mL plasmid, 33 μg/mL T7 RNA polymerase, 11 μM L-[U-^{14}C]-leucine, 2% poly-(ethylene glycol) 8000, 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 a Bradford assay using a commercially available assay reagent (Bio-Rad, Hercules, CA). Bovine serum albumin was used as the standard. In the pyruvate oxidase energy regeneration system, 33 mM sodium pyruvate, 0.5 mM each of 20 unlabeled amino acids, 6 U/mL pyruvate oxidase, 6.7 mM potassium phosphate, 3.3 mM thymidine

triphosphate (TPP), and 0.3 mM flavin adenine dinucleotide (FAD) were also added. In the PANOx energy regeneration system, 33 mM phosphoenolpyruvate (PEP), 2 mM each of 20 unlabeled amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.26 mM CoenzymeA (CoA), and 2.7 mM sodium oxalate were added. Phosphoenolpyruvate (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 obtained from Sigma (St. Louis, MO). Oxygen concentrations were measured with a small oxygen probe (Microelectrodes, INC., Bedford, NH).

Product Determination. The amount of synthesized protein was determined by the incorporation of ^{14}C-leucine into TCA-precipitable radioactivity using a liquid scintillation counter. Total protein yield was measured by adding 100 μL of 0.1 N sodium hydroxide to 15 μL of the cell-free protein synthesis reaction mixture. The sodium hydroxide treated samples were incubated at 37 °C for 20 min. Fifty microliters of the treated sample was allowed to soak into each of two separate pieces of Whatman 3MM chromatography paper and dried under a heat lamp for 1 h. One piece of filter paper in each pair was placed into a beaker on ice and covered with 5% (v/v) trichloroacetic acid (TCA) at 4 °C to precipitate the proteins onto the filter paper. After 15 min, the solution was exchanged with fresh TCA. This incubation was performed a total of three times. Following the third precipitation, the filter papers were covered once with 100% ethanol for 10 min at room temperature, and then they were removed from the beaker and allowed to dry under a heat lamp. Radioactivity of both the TCA-precipitated (washed) and non-TCA-precipitated (unwashed) samples was measured using a liquid scintillation counter (Beckman LS3801). The fraction of incorporated leucine (washed/unwashed) was used to determine the amount of protein synthesized. To determine the soluble protein yield, 30 μL of the reaction mixture was centrifuged at 12 000*g* for 15 min at 4 °C. Ten microliters of the supernatant was combined with 100 μL of 0.1 N NaOH, and TCA precipitation was carried out as described above.

Protein Activity Assay. The enzymatic activity of synthesized CAT is determined by the spectrophotometric method described by Shaw (*14*). Samples from a protein synthesis reaction were centrifuged at 12 000*g* for 15 min at 4 °C. The collected supernatant was diluted 40 times with water at 4 °C. A 1 mL assay solution was prepared and prewarmed to 37 °C for 5 min, in a 1 mL cuvette. This solution contained 0.1 mM chloramphenicol, 0.1 mM acetyl-coenzyme A, 0.4 mg/mL 5,5′-dithiobis-2-nitrobenzoic acid, and 100 mM Tris-HCl (pH 7.8). Ten microliters of the diluted sample was added to 990 μL of the assay mixture, and the rate of absorbance increase was measured at 412 nm and 37 °C. This absorbance value is adjusted for a negative control (a cell-free reaction that has been incubated without a plasmid) and divided by the extinction coefficient of free 5-thio-2-nitrobenzoate (13.6 mM^{-1} cm^{-1}) to yield the units of CAT activity within the cuvette. One unit is defined as 1 μmol of chloramphenicol acetylated per minute.

ATP Concentration Measurement. Two methods were used to measure ATP concentration. A firefly luciferase assay was used for the small scale reactions and high performance liquid chromatography (HPLC) analysis was used for the large scale reactions. Sample preparation for both methods was the same. Five percent TCA at 4 °C was added to the cell extract reaction mixture in a 1:1 volumetric ratio. TCA precipitated samples were centrifuged at 12 000*g* for 15 min at 4 °C. The supernatant was collected. Samples for the firefly luciferase assay were diluted with luminescence buffer 500−1000 times. Luminescence buffer contains 20 mM TRIS acetate, 5 mM magnesium acetate, 1 mM DTT, and 0.1 mM EDTA (Sigma, St. Louis, MO). Diluted samples were added to an opaque microtiter plate well containing 0.2 μg/mL luciferase (Promega, Madison, WI), 500 μM luciferin (Molecular Probes, Eugene, OR), and 0.2% bovine serum albumin (Sigma, St. Louis, MO). The intensity of the luminescence was measured in a plate luminometer (ML 3000, Dynatech Laboratories, Chantilly, VA).

Twenty microliter samples were applied to a Vydac 302IC4.6 column (Hesperia, CA) for analysis with an Agilent 1100 series HPLC system (Palo Alto, CA). Separation was carried out at a flow rate of 2 mL/min. The mobile phase started with 100% of a 10 mM phosphate buffer (1:1 molar ratio of NaH_2PO_4/Na_2HPO_4 adjusted to pH 2.6 with glacial acetic acid) and 0% of a 125 mM phosphate buffer solution (1:1 molar ratio of NaH_2PO_4/Na_2HPO_4 adjusted to pH 2.8 with glacial acetic acid). A linear gradient of 0% to 100% of the 125 mM phosphate buffer was applied from 2 to 25 min, maintained at 100% for 2 min, and returned to 0% in a linear gradient for 3 min. Nucleotides were detected at 260 nm. For both assays, ATP concentrations were determined by comparison to a calibration obtained with ATP standards.

Protein Purification. CAT was isolated from a 10 mL PANOx fed-batch reaction using a chloramphenicol caproate affinity resin (Sigma). The cell-free reaction mixture was centrifuged at 12 000*g* for 20 min at 4 °C. The supernatant was collected and diluted 2-fold with a loading buffer, 50 mM Tris-HCl, pH 7.8, and filtered through a 0.2 μm PES filter. This sample was loaded onto the 3 mL column at 4 °C using a peristaltic pump at a flow rate of 0.5 mL/min with the aid of a column flow adapter. The column (10 cm long, 1 cm i.d.) had been packed with 3 mL of resin according to procedures provided by the manufacturer and equilibrated with 5 column volumes (15 mL) of the loading buffer. After the sample was loaded, 20 mL of a wash buffer, 50 mM Tris-HCl, pH 7.8, 300 mM sodium chloride, was used to remove nonspecifically bound protein. CAT was then eluted using 20 mL of a solution containing 50 mM Tris-HCl, pH 7.8, 300 mM sodium chloride, and a linear gradient of 0−8 mM chloramphenicol. The elution buffer was passed through the column at a flow rate of 1 mL/min. The eluent was collected in 1.5 mL fractions. The fractions containing CAT were determined by Coomassie blue staining of a 10% SDS−PAGE gel. Eleven fractions containing the purified enzyme were pooled and dialyzed for 2 h at 4 °C (MWCO 6-8000) (Spectrum, Rancho Dominguez, CA) against 2 L of a buffer containing 5 mM Tris-HCl, pH 7.8, 1 mM DTT. The buffer was exchanged, and the incubation was repeated once. The dialyzed sample was centrifuged at 25 000*g* for 20 min at 4 °C and then concentrated 6-fold for enhanced determination of purity by SDS−PAGE analysis using a Centriprep10 (Millipore, Bedford, MD).

Results

CAT Production Using the Pyruvate Oxidase System. The pyruvate oxidase energy regeneration

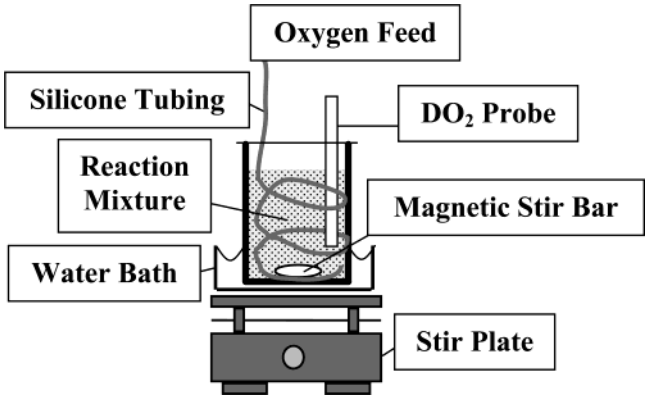

Figure 2. Reactor configuration used for the pyruvate oxidase system. A small piece of stainless steel wire was threaded through a 20 cm long piece of silicone tubing. Ten centimeters of the tubing was immersed in the cell-free reaction mixture by coiling inside the reactor. This tubing was pressurized with pure O_2 to deliver the oxygen necessary for the regeneration of ATP within the cell-free protein synthesis reaction. A water bath was used to maintain a 37 °C temperature. Note: the experimental setup for the PANOx system was similar except that it did not contain the oxygen feed, silicone tubing, or the dissolved oxygen probe.

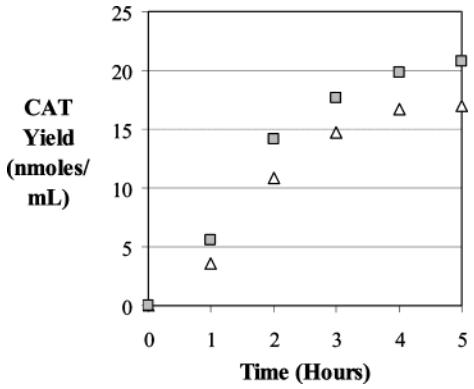

Figure 3. Time course of chloramphenicol acetyl transferase (CAT) accumulation in a 5 mL pyruvate oxidase reaction. Dissolved oxygen was supplied through the walls of a piece of silicone tubing; 0.5 mM of each amino acid was added every hour and 17 mM of sodium pyruvate was added approximately every 20 min after sudden rises in the DO_2 concentration. Triangles, total amount of CAT expressed as monitored by ^{14}C-leucine incorporation. Squares, active amount of CAT as determined by enzymatic assay (assuming a specific activity of 125U/mg (*14*)). The measured specific activity, based upon soluble CAT as determined by ^{14}C-leucine incorporation, was 153 ± 6 U/mg.

system was used to express CAT in a 5 mL fed-batch reaction. Oxygen was constantly supplied by diffusion. It was delivered through the walls of a 10 cm long piece of silicone tubing (3.2 mm o.d., 0.8 mm i.d.) immersed in the reactor (Figure 2).

The dissolved oxygen concentration was monitored with a small probe and maintained at 5 ± 2% DO_2 relative to air saturation. Approximately every 20 min, the dissolved oxygen concentration would rapidly increase. This indicated a decrease in the oxygen consumption rate and signaled the exhaustion of pyruvate. Adding 17 mM sodium pyruvate returned the dissolved oxygen concentration to its original baseline. There were 16 additions of 17 mM pyruvate over the course of the reaction. In accordance with previous fed-batch data demonstrating increased yields relative to a batch reaction, 0.5 mM of each amino acid was also fed to the reaction every hour (*8*). After about 4 h, as the cumulative amount of sodium pyruvate added to the system increased, the dissolved oxygen concentration slowly increased and could no longer be sustained at 5% DO_2. Ten millimolar phosphate was added to the system at 4.5 h. Phosphate addition returned the baseline to 5% DO_2.

Figure 3 shows the CAT accumulation over time as determined by TCA precipitable radioactivity and the CAT enzymatic activity assay. The final yield of CAT after a 5-h incubation, given by the average of the total and active synthesized protein, was about 19 nmol/mL (480 ± 40 μg/mL). The soluble fraction of CAT was 67 ± 2% (data not shown) and the active amount of protein produced was 104 nmol. The active yield was determined by using the published value for CAT activity of 125 U/mg (*14*). When calculated on this basis, the amount of active protein produced was greater than the total amount of CAT synthesized as determined by radioactive counting. Possibly the total yield of protein synthesis was underestimated. The measured specific activity, based upon soluble CAT as determined by ^{14}C-leucine incorporation, was 153 ± 6 U/mg, which is greater than the published value.

CAT Production and Small Scale Development Using the PANOx Energy System. More recently, Kim and Swartz reported the development of the PANOx system (*9*). The promising yields, convenience, and lack of need for oxygen supply and exogenous enzyme addition relative to the pyruvate oxidase system prompted the shift to use the PANOx system for further scale-up. Prior to extending the use of this energy regeneration method to large scale, the system was reinvestigated. Protein synthesis using the PANOx conditions was previously shown by Kim and Swartz to produce approximately 14 nmol/mL CAT (350 μg/mL) over a 2 h incubation on a 120 μL scale. Smaller reactions (15 μL) were found to produce approximately 21 nmol/mL CAT (520 μg/mL) with synthesis extending for a longer period of time than at the 120 μL scale.

The amount of synthesized protein measured in this work was greater than previously reported values. Figure 4 shows the typical time course of synthesis for a PANOx batch reaction. The yield for a 15 μL reaction was 28 ± 2.3 nmol/mL (700 ± 57 μg/mL) after a 3 h reaction period. The 33% increase in yield from previously described values is believed to be caused by two factors. One is the fermentation growth media used in extract preparation. Using 2 x YTPG has been shown to increase yields in batch reactions by most likely reducing phosphatase activity (*13*). The second factor is a greater attention to the quality control of reagent preparation, particularly PEP. The simple batch experiment has been repeated hundreds of times with yields on the order of data presented here since the initial publication by Kim and Swartz (data not shown). Protein synthesis with a 120 μL reaction, an 8-fold increase in scale, resulted in 21.5 ± 2 nmol/mL (540 ± 50 μg/mL) of synthesized CAT (Figure 4). This was nearly 50% greater than the previously reported value. Both the 15 and the 120 microliter reactions produce about the same concentration yield of product through 1.5 h. After 1.5 h, the smaller scale reaction continues to produce protein while the production in the larger reaction stops. The reason for the limitation at larger scales is believed to be due in part to energy limitation, although other unknown reasons may exist.

Previously, fed-batch operation of the PANOx system had a dramatic effect on protein synthesis. Hourly additions of magnesium acetate, 20 amino acids, and

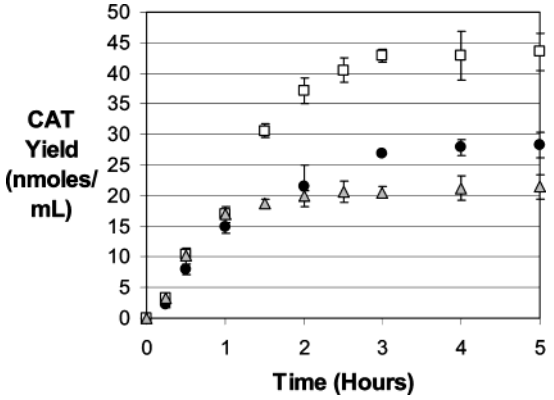

Figure 4. Time course of chloramphenicol acetyl transferase (CAT) accumulation in a small-scale PANOx reaction. The total amount of CAT is shown for a batch and a fed-batch reaction. CAT expression was determined by ^{14}C-leucine incorporation. Error bars indicate the high and low of 4−6 separate experiments. For the fed-batch reactions, 33 mM phosphoenolpyruvate, 6 mM magnesium acetate, 2 mM unlabeled amino acids, and 11 μM ^{14}C-leucine were added at 1, 2, and 3 h. Circles: batch 15 μL scale. Triangles: batch 120 μL scale. Squares: fed-batch 120 μL scale.

PEP, substrates shown to be consumed or limiting during the protein synthesis reaction, more than doubled the CAT yield at the 120 μL scale (*9*). A fed-batch reaction at the 120 μL scale was therefore carried out before attempting the scale-up reaction. After 1, 2, and 3 h, the reaction was supplemented with 33 mM phosphoenolpyruvate, 2 mM of each unlabeled amino acid, 11 μM ^{14}C-leucine, and 6 mM magnesium acetate. Over 1 mg/mL (43 ± 3 nmol/mL) of CAT was made during the course of a 5 h reaction, more than doubling the amount of protein produced without the periodic addition of consumed substrates (Figure 4). The data over the first part of the reaction, which is dominated by energy regeneration from PEP, was similar for batch and fed-batch methods as well as at both volumetric scales. However, the rate of protein synthesis continued at its initial pace in the fed reactions beyond the first hour.

Larger Scale CAT Production and Purification using the PANOx System. The scale-up reaction using the PANOx energy regeneration system was simpler than the pyruvate oxidase system since there was no need to supply oxygen. Initially CAT was expressed in a 10 mL batch reaction. The final yield of CAT was 16.5 ± 0.6 nmol/mL. Seventy-five percent of the final synthesized protein was soluble, and 108 ± 4 nmol of active CAT was made (Figure 5). This was determined using the published value for CAT activity (*14*).

While the batch reaction produced greater than 100 nmol of active protein, it did not continue to synthesize protein for longer than 1 h at this scale. The 15 μL and the 120 μL scale reactions continued to synthesize protein for up to 3 and 1.5 h, respectively. Interestingly, the initial rate of protein production was nearly identical, about 16 nmol/mL·h, over the first hour for the 15 μL, 120 μL, and 10 mL scales. Upon closer investigation, it was determined that the ATP concentration had been entirely depleted after the first hour in the 10 mL batch reaction (Figure 6). Guided by the knowledge that ATP concentrations became limiting as the scale increased and supported by the encouraging data from the 120 μL scale, the fed-batch approach was adopted at the 10 mL scale to extend synthesis and increase yields.

The same feeding scheme exploited at the 120 μL reaction to supply fresh substrates was used at the 10 mL scale. This increased the available ATP for the reaction (Figure 6) and produced greater amounts of CAT than the batch method. The final yield of CAT after a 4 h incubation for three separate reactions was 31 ± 1.5 nmol/mL (780 ± 33 μg/mL) (Figure 5). All of the protein expressed over the first 1.5 h was soluble and active. The soluble amount of CAT by the end of the reaction was 76 ± 3%, and 261 ± 42 nmol of active CAT was expressed. The measured specific activity, based upon soluble CAT as determined by ^{14}C-leucine incorporation, was 123 ± 10 U/mg.

The 10 mL fed-batch PANOx reaction produced about 7 mg of active CAT. This product was isolated using single-step affinity chromatography. A chloramphenicol caproate resin achieved purification of CAT to a single visible band on a SDS−PAGE gel with 70% recovery

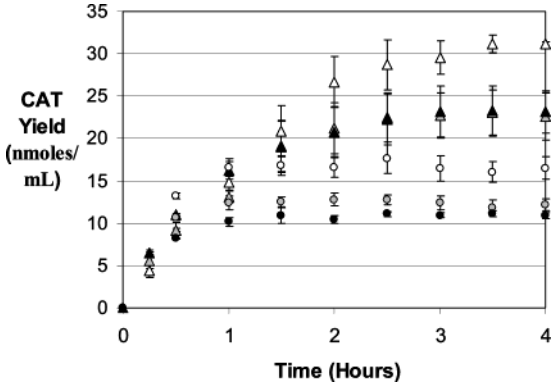

Figure 5. Time course of chloramphenicol acetyl transferase (CAT) accumulation in a 10 mL PANOx reaction. The total, soluble and active amounts of CAT are shown for a batch and a fed batch reaction. Error bars indicate the high and low of 2 separate batch experiments and 3 separate fed-batch experiments. For the fed-batch experiments: 33 mM phosphoenolpyruvate, 6 mM magnesium acetate, 2 mM unlabeled amino acids, and 11 μM ^{14}C-leucine were added at 1, 2, and 3 h. CAT expression was monitored by ^{14}C-leucine incorporation and enzymatic assay. The fed-batch reactions produced approximately 9 mg of total protein and 7 mg of active CAT. Note: by the end of the fed batch reactions, the volume had increased to approximately 11 mL as a result of reagent additions. Circles: batch. Triangles: fed-batch; open, total amount of CAT expressed as monitored by ^{14}C-leucine incorporation; grey, soluble amount of CAT expressed as monitored by ^{14}C-leucine incorporation; closed, active amount of CAT as determined by enzymatic assay (assuming a specific activity of 125 U/mg (*14*)).

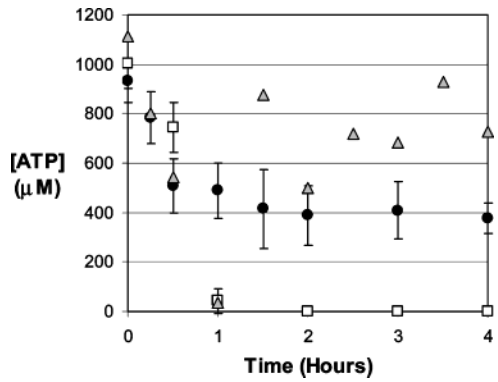

Figure 6. Time course of adenosine triphosphate concentration during large and small scale PANOx reactions producing CAT. ATP concentration was monitored with a firefly luciferase assay (small scale reactions) or by HPLC (large scale reactions). The small scale reactions were 15 μL and the large scale reactions were 10 mL in volume. Circles: batch small scale (3 experiments). Squares: batch large scale (2 experiments). Triangles: fed-batch large scale (1 experiment).

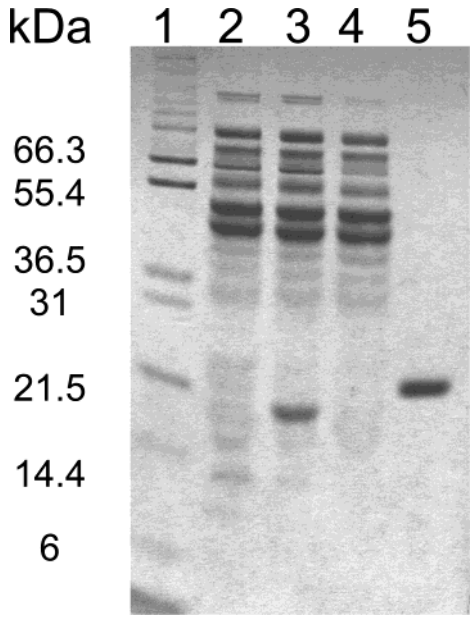

Figure 7. SDS−PAGE gel for samples from the affinity purification of CAT using a chloramphenicol caproate resin. Lane 1: Mark 12 Molecular Weight Marker (Invitrogen, Carlsbad, CA). Lane 2: reaction mixture from control reaction without plasmid addition. Lane 3: 10 mL PANOx fed-batch reaction sample after 4 h of CAT expression. Lane 4: Wash fractions before the elution with CAT extraction buffer. Lane 5: elution fractions of purified CAT after concentration to 1 mg/mL. The shift upward of the purified CAT band is believed to result from the protein no longer being in the high ionic strength cell-free reaction environment. The samples were run on a 10% SDS−PAGE gel and stained with Coomassie blue. There was a 70% recovery of CAT.

(Figure 7). The purified band of CAT was shifted upward relative to the band of CAT present in the cell-free reaction mixture. This is believed to be due to the different salt environment present in the reaction mixture. The specific activity, based upon total protein concentration in the soluble fraction as determined with a Bradford assay, increased from 6.9 U/mg total protein to 155 U/mg total protein after the purification.

Discussion and Conclusions

We have shown that two energy regeneration systems can be used in 5−10 mL batch reactions to produce 100 nmol quantities of active chloramphenicol acetyl transferase. To date, scale-up of cell-free systems has utilized continuous or semi-continuous formats with a reaction volume of about 1 mL (*5*, *15*, *16*). These formats provide a constant supply of substrates, such as the secondary energy source and amino acids, and a potential reservoir to remove inhibitory byproducts from the protein synthesis reaction, such as phosphate. While significantly increasing the duration of protein synthesis, these approaches are less desirable than batch systems because they can be inefficient in their use of expensive reagents and are more cumbersome for high throughput production of proteins. This study is the first to demonstrate the feasibility of protein production on a large scale using a batch format.

Delivering oxygen by diffusion and feeding degraded substrates to the pyruvate oxidase cell-free reaction overcame the limitation discovered in earlier scale-up attempts to produce 100 nmol quantities of CAT. The reason for the halt in synthesis in the pyruvate oxidase reaction remains unanswered. One explanation may be that the amount of acetate in the system became inhibitory. Batch experiments have shown that protein synthesis yield decreases in the presence of increased acetate concentrations (data not shown). As a result of pyruvate being converted into acetate, concentrations of acetate would have increased to approximately 400 mM over the course of the reaction. Another hypothesis is that increased sodium concentrations inhibited the coupled transcription-translation reaction. Sodium levels increased to 305 mM as a result of sodium pyruvate addition. Sodium is known to reduce transcriptional efficiency, and high levels of sodium may have led to a decrease in competent message production, limiting the system at the level of transcription.

The 10 mL PANOx batch reaction, while expressing 100 nmol of active CAT, produced about 60% of the final concentration of total protein as compared to the 15 μL scale. This decrease in relative yield seems to be linked to a loss of ATP regenerating potential at large scale. This intriguing problem is not understood. It appears that the energy formation stemming from pyruvate is not efficiently harnessed in the 10 mL reaction. This is evident in Figure 6, which shows that ATP concentrations in a 10 mL reaction are entirely depleted after the first hour, whereas the ATP concentrations in the 15 μL reaction are maintained at about 400 μM over the course of the reaction. The proposed method of energy regeneration that is captured at small scale uses anaerobic reaction pathways surrounding pyruvate. It seems unlikely that these would be inactivated at large scale. However, HPLC analysis has shown that equimolar amounts of acetate and lactate, as depicted in Figure 1B, are not formed at the 15 μL scale. In fact the majority of pyruvate is converted to acetate (data not shown). Therefore, the system may rely on other reactions for the oxidation of NADH that are based on the need for oxygen. Scaling up reduces the surface area-to-volume ratio and may consequently decrease volumetric oxygen transfer into the reaction mixture. This may reduce the amount of energy regeneration potential of the PANOx system at large scale.

The 10 mL fed-batch PANOx reactions surpassed our initial goal of 100 nmol of active protein by more than 2.5-fold. Unfortunately, fed-batch PANOx reactions, both at small and large scale, terminate at around 2.5 h. This is most likely due to phosphate accumulation or magnesium deficit. High concentrations of phosphate can sequester magnesium from the cell-free reaction and are known to inhibit combined transcription-translation reactions at concentrations greater than 30 mM (*8*, *17*). Repeated additions of PEP would have led to 132 mM of phosphate accumulation by the end of the reaction. It is therefore not surprising that the reaction terminated after two feedings of 33 mM PEP. Poisoning the reaction with phosphate is the largest barrier to the fed-batch reaction scheme when using energy systems that exploit high energy phosphate compounds to regenerate ATP. Currently, a more suitable solution for maintaining homeostasis within the batch reaction is being sought.

We can estimate the ATP utilization efficiencies for each of the large scale reactions by assuming that 5 molecules of ATP are necessary for the addition of 1 amino acid to the growing polypeptide (*9*). For the pyruvate oxidase system, we estimate that 1 mM pyruvate produces 1 mM ATP. If all of the ATP produced from 33 mM pyruvate would be utilized for protein production, 750 μg/mL of protein would be expressed. The ATP

utilization efficiency, including all additions of pyruvate, was 7%. The ATP efficiency decreased with time and was 10% through the first 2 h. For the PANOx system, we estimate that there is 1.5 mM ATP produced per 1 mM PEP (*9*). Therefore, 33 mM PEP would produce approximately 1.1 mg/mL of synthesized protein if all of the energy went to protein production. The calculated ATP utilization efficiency of the 15 μL reactions is 60%; however, this efficiency was diminished at larger volumes. The efficiency of the 10 mL fed-batch reaction was approximately 18%. The final two additions of PEP did not provide significant benefit for protein synthesis.

One of the most notable results for in vitro protein expression was reported by Kigawa et al. (*5*). They described the production of 6 mg/mL of CAT in an 18 h reaction using a semicontinuous system and concentrated cell extract. The experimental setup contained 300 μL of the cell extract reaction mixture in dialysis tubing surrounded by an external solution of reagents of 3 mL that was exchanged once. If the volumetric productivity of the system is based on the total amount of reagents used rather than the cell extract reaction mixture, the final yield of CAT is reduced to about 300 μg/mL (1.8 mg CAT/6.3 mL). The fed-batch scale-up results from both energy regeneration systems presented here produced greater volumetric yields at a faster rate than the Kigawa et al. result.

It is interesting that the proportion of active to total protein produced decreases during the course of the reaction. For example, while the total amount of CAT produced in the 10 mL fed-batch reaction continues to increase at the same rate after 1 h, the amount of active and soluble protein appears to slow (Figure 5). Understanding the mechanism behind this phenomenon may be an important step in making cell-free systems even more productive.

The single-step affinity purification of CAT to a single intense band on a SDS−PAGE gel is a significant achievement. This underscores the potential advantages associated with purification of proteins from cell-free systems (*18*). Not only was the production and purification scheme carried out in a single day, but the product was isolated without the need for any other polishing steps. It should be emphasized that not all proteins have an available affinity partner for purification. In these cases high-throughput batch production and purification can be carried out in a number of more general ways. For example, Jiang et al. synthesized and isolated a single chain antibody fragment with a histidine tag (*19*). Alternatively, the PURE system, developed by Ueda and colleagues, offers a purification approach without tagging the protein product (*20*). Furthermore, purification may not be necessary for some applications. NMR structure analysis has been performed on ^{15}N-labeled proteins produced in a cell-free reaction without purification (*21*). Finally, it should be noted that protein production and purification can be combined in a continuous removal system (*22*).

The larger scale results yielding about 7 mg of active product are very promising. They indicate that cell-free protein synthesis may have a genuine potential for recombinant DNA pharmaceutical protein production and high-throughput expression of protein libraries. As this technology develops, it may only be a matter of time before production volumes surpass even the milliliter scale. The utility of cell-free systems will only continue to improve as we learn how to more efficiently harness the available metabolic resources for protein production and understand how to provide the optimal environment for folding to produce high yields of active complex proteins.

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