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Cell-free production of active *E. coli* thioredoxin reductase and glutathione reductase

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Abstract ***Escherichia coli*** **thioredoxin reductase (TR) and glutathione reductase (GR) are dimeric proteins that require a flavin adenine dinucleotide (FAD) cofactor for activity. A cell-free protein synthesis (CFPS) reaction supplemented with FAD was used to produce TR at 760 μg/ml with 89% of the protein being soluble. GR accumulated to 521 μg/ml in a cell-free reaction with 71% solubility. The TR produced was fully active with a specific activity of 1390 min^{-1}. The GR had a specific activity of 139 U/mg, which is significantly more active than reported for GR purified from cells. The specific activity for both TR and GR decreased without FAD supplementation. This research demonstrates that CFPS can be used to produce enzymes that are multimeric and require a cofactor.**

Key words: Cell-free protein synthesis; Thioredoxin reductase; Glutathione reductase; Flavin adenine dinucleotide; Specific activity

1. Introduction

Current methods for producing recombinant proteins including bacterial fermentation and mammalian cell culture will not be able to keep pace with the exponentially growing amount of genetic information. These methods require extensive time and labor for cloning, transformation, fermentation and downstream processing and are not easily adaptable to a multiplexed format. In contrast, cell-free protein synthesis (CFPS) has great potential as a high-throughput expression system.

Expression of many different proteins can be done in a multi-well plate by simply adding different polymerase chain reaction (PCR) products to each well containing a mixture of cell extract and other required reagents [1]. The lack of a physical barrier, the cell wall, makes the reaction environment accessible to manipulation in CFPS. The reaction conditions can also be varied over a wider range because living cells are not required. Often, biological activities of synthesized proteins can be determined directly in the CFPS reaction in the plate. If purified proteins are desired, CFPS shortens the time required for preparing purified proteins [2].

CFPS is now achieving protein synthesis yields on par with conventional technologies. The yield in a batch reaction has been increased to 700 μg/ml. Most work with CFPS has been done with a simple enzyme, chloramphenicol acetyl transferase (CAT), which is monomeric and contains no cofactors. In order for CFPS to reach its potential, it must be able to synthesize more complex proteins including ones with disulfide bonds, prosthetic groups or unnatural amino acids [3]. Recently, the expression of a catalytic Fab fragment, which includes disulfide bonds, has been reported [4]. There has yet to be a report of a multimeric enzyme that requires a cofactor for enzymatic activity being produced using CFPS.

Thioredoxin reductase (TR) and glutathione reductase (GR) are members of a family of flavoproteins that transfer electrons from a pyridine nucleotide to a specific disulfide-containing substrate. These enzymes are key components in two pathways that keep the cytoplasm of *Escherichia coli* reduced. In the thioredoxin system, thioredoxin reduces disulfide bonds in proteins in the cytoplasm, including recycling of the essential enzyme ribonucleotide reductase [5]. In this process thioredoxin becomes oxidized and TR uses the reducing equivalents provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) to regenerate the reduced thioredoxin [6].

GR is also a key enzyme in maintaining the reducing environment in the cell. GR uses reducing equivalents of NADPH to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH) [7]. This buffer assists in maintaining the sulfhydryl redox potential in the cytoplasm.

Structurally, both TR and GR are homodimers with one molecule of flavin adenine dinucleotide (FAD) and one pair of redox-active cysteines per monomer. The structure and enzymatic mechanism of TR have been studied in detail [6,8,9]. It has a FAD-containing domain and a NADPH-containing domain, and these domains exhibit a large conformational change during catalysis with the NADPH domain rotating by 67° [8].

In spite of the complexity of these enzymes in both structure and function, this research demonstrates that CFPS techniques can generate active TR and GR. To our knowledge, this is the first example of using CFPS to produce an active multimeric enzyme containing cofactors.

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Abbreviations: CAT, chloramphenicol acetyl transferase; CFPS, cell-free protein synthesis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide; GR, glutathione reductase; GSSG, glutathione oxidized; PCR, polymerase chain reaction; TR, thioredoxin reductase

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2. Materials and methods

2.1. Cloning

A 100 μl PCR consisted of 1 μg *E. coli* genomic DNA, 60 pmol forward and reverse primers, 5% dimethylsulfoxide, 0.8 μl of 25 mM deoxyribonucleoside triphosphate (dNTP), 1 μl Herculase HotStart DNA polymerase (Stratagene, La Jolla, CA, USA), and 10 μl of 10× Herculase reaction buffer. The reaction was carried through 25 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s. For TR the primers were trxBCATfrontend (5′-TTCGAACGAACATATGGAGAAAAAAATCACTGGCACGACCAAAC-3′) and trxBReverse (5′-GGGGTCGACTTTTGCGTCAGCTAAACC-3′). For GR they were gorCATfrontend (5′-TTCGAACGAAATTAATGGAGAAAAAAATCACTACTAAACACTATGATTACATCG-3′) and gorReverse (5′-GGGGTCGACACGCATTGTCACGAACTC-3′).

The PCR products were eluted in 50 μl water from a GFX PCR purification kit (Pharmacia). The TR insert was digested with *Nde*I and *Sal*I, while the GR insert was digested with *Ase*I and *Sal*I. Restriction endonucleases were purchased from New England Biolabs and suggested protocols were followed.

The vector pK7His contains a kanamycin resistance gene, a T7 promoter, and *Nde*I and *Sal*I cloning sites with a His6 tag, stop codon, and T7 terminator following the *Sal*I site. 5 μg of the plasmid was digested at 37°C overnight with 60 U *Nde*I, 60 U *Sal*I, 6 μl of 10 μg/ml bovine serum albumin (BSA), 6 μl *Sal*I unique buffer, and DI water to 60 μl. The digested vector was dephosphorylated with 0.27 U calf intestine alkaline phosphatase (Invitrogen) for 30 min at 37°C according to suggested protocols. Correctly digested vector was separated on a 1% agarose gel. The band was excised and purified using a GFX PCR purification kit.

The ligation reaction was performed with 0.2 pmol vector and a 3-fold excess of the insert. The 20 μl reaction contained pk7His vector, PCR insert, 1 U T4 DNA ligase (Invitrogen) and 1× ligase buffer in a 20 μl reaction. Ligation took place at 16°C overnight.

The ligation mixture was transformed into $CaCl_2$ competent *E. coli* TG1 cells and plated on Luria–Bertani medium with kanamycin (LB-Kan) [10]. Plasmid preparations (Qiagen, Miniprep) were performed for several clones. The success of the cloning was determined by endonuclease digestion and DNA sequencing. Plasmid preparations were performed with Qiagen Maxi-Prep kits.

This same procedure was used to clone the native forms of the TR and GR genes into a construct for CFPS. These constructs do not have the first five amino acids of CAT or the polyhistidine tag. The primers for TR were trxBForward (5′-TTCGAACGAACATATGGGCACGACCAAAC-3′) and trxBSTOP (5′-GGGGTCGACTTATTTTGCGTCAGCTAAACC-3′). The primers for GR were gorForward (5′-TTCGAACGAAATTAATGACTAAACACTATGATTACATCG-3′) and gorSTOP (5′-GGGGTCGACTTAACGCATTGTCACGAA-3′). The PCR products were cloned into the pK7 vector which lacks the His6 tag found in pK7His.

2.2. Cell-free production

Cell-free reactions were performed using the PANOx system [11]. The total volume was either 50 μl or 1 ml and contained: 16 mM magnesium acetate, 80 mM ammonium acetate, 230 mM potassium glutamate, 57.2 mM HEPES–KOH (pH 7.5), 1.2 mM adenosine triphosphate (ATP), 0.86 mM guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytosine triphosphate (CTP), 34 μg/ml folinic acid, 170 μg/ml tRNA, 2 mM of the 20 amino acids, 30 mM phosphoenol pyruvate, 0.33 mM nicotinamide adenine dinucleotide, 0.27 mM coenzyme A, 2.7 mM oxalic acid, 2% polyethylene glycol 8000, 4.2 μM ^{14}C-Leu, 70 μg/ml T7 RNA polymerase, 6.8 μg/ml plasmid, 1 mM dithiothreitol (DTT), and 0.24 volume S30 extract. FAD was added at various concentrations up to 20 μM. The reaction was incubated at 37°C for 4 h. 50 μl reactions were performed in an Eppendorf tube. 1 ml reactions were incubated as a thin film in a well of a six-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ, USA; Falcon 35-3046). The cell extract was produced from *E. coli* K12 (strain A19 Δspe ΔtnaA ΔtonA ΔendA met$^+$) as described earlier [12], except that the cells were grown on 2× yeast tryptophan peptone glucose (YTPG) media (Jewett and Swartz, submitted).

Some CFPS reactions contained the following cytoplasmic chaperones; DnaJ (Stressgen, San Diego, CA, USA; SPP640) 0.4 μM, DnaK (SPP630) 1.0 μM, GrpE (SPP650) 0.4 μM, and GroEL/ES (SPP625) 0.4 μM. The periplasmic chaperone Skp was produced in-house [13] and added at 0.3 mg/ml.

Soluble protein was isolated by centrifuging at 12 000 × *g* for 10 min and collecting the supernatant. The amount of total and soluble protein synthesized was measured by TCA-insoluble radioactivities as described by Kim et al. [14] using a liquid scintillation counter (Beckman LS3801).

2.3. Purification

TR and GR were purified using a 1 ml Ni-chelation column (Amersham Biosciences, HisTrap). The column was first washed with 15 ml of wash buffer (50 mM sodium phosphate buffer pH 7.0, 300 mM NaCl). It was then loaded with 1 ml of 0.1 M $NiSO_4$ and equilibrated with 15 ml of wash buffer. The soluble portions of the 1 ml cell-free reactions were combined for TR and GR. Either the TR or GR was then applied to the column. Impurity proteins were washed away with 10 ml of wash buffer containing 20 mM imidazole. The products were eluted with 10 ml of wash buffer supplemented with 300 mM imidazole. Approximately 300 μl fractions were collected.

The load, wash, and elution fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) gels (Invitrogen, NuPAGE 10% bis-tris). DTT and sodium dodecyl sulfate (SDS) were included as suggested by the manufacturer. Staining was with Coomassie blue. Radioactive leucine incorporation was used to quantify the concentration of purified protein.

2.4. Activity assays

The activity assay for TR contained the following components: 50 mM NaH_2PO_4 pH 7.6, 1.5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM glucose 6-phosphate, 200 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 300 μM NADPH, 3 μM *E. coli* thioredoxin (EMD Biosciences; Darmstadt, Germany), and 0.2 U glucose 6-phosphate dehydrogenase (Sigma) [9]. Approximately 500 ng of TR was added to 1 ml of the assay mixture and the increase in absorbance at 412 nm was measured for 90 s at 37°C. DTNB produces two molecules of nitrothiobenzoate when it is reduced. The extinction coefficient of nitrothiobenzoate (13 600 M^{-1} cm^{-1}) was used to calculate the rate of reaction.

The GR assay contains 100 mM TAPS buffer pH 8.1 (Sigma), 3.75 mM GSSG, and 150 μM NADPH [7]. Approximately 500 ng of GR was added to 1 ml of the assay mixture and the decrease in absorbance at 340 nm was measured for 120 s at 25°C. The extinction coefficient for NADPH was measured to be 5714 M^{-1} cm^{-1} and was used to calculate the reaction rate. A unit of GR is defined as the amount of enzyme required to reduce 1 μmol NADPH/min [15].

3. Results and discussion

3.1. CFPS

The first step in producing active TR and GR is to clone the genes for these enzymes into a template suitable for cell-free expression. We have observed in our lab that adding the first five amino acids of CAT to the N-terminus of a protein often increases production of that protein in CFPS. We postulate that the 5′ end of the mRNA for many genes may participate with the 5′ untranslated region to form strong secondary structure, which inhibits translation initiation. The 5′ terminus of CAT typically provides reliable expression in our CFPS system. Therefore, the first five codons of CAT were cloned before the TR or GR genes. In the future, a better understanding of translation initiation in the CFPS system may eliminate the need to add the N-terminal amino acids of CAT. In fact, Section 3.5 will show that the amino acids of CAT proved to be unnecessary for TR and GR.

Also, six histidines were added to the C-terminus of the proteins as a purification tag. Examination of the crystal structures of the enzymes complexed with their substrates and cofactors shows that both the N-terminus and C-terminus of both proteins are exposed [8,16]. Therefore, one would not expect the addition of the histidine tag or the N-terminal amino acids of CAT to interfere with folding or activity.

A variety of experimental conditions were explored to maximize first the quantity of soluble TR and GR produced, and second the amount of active TR and GR produced. TR and GR were initially produced in 50 μl reactions. In the base case, 20 μM FAD was added to the reaction mixture, which was incubated at 37°C for 4 h. The dependence upon FAD concentration for the production of TR and GR was investigated at concentrations of 8, 2, and 0 μM FAD (Figs. 1 and 2). An arm of the investigation also examined production at 30°C instead of at 37°C. Production at a lower temperature might be expected to decrease the amount of total protein produced, but to increase the amount of soluble protein by encouraging the correct folding of a larger percentage of the protein [17]. All of these reactions were performed in triplicate on three different days ($n = 9$). Finally, the effect of the chaperones GroEL/ES, GrpE, DnaJ, DnaK, and Skp on protein folding was examined. Skp has been shown to aid in folding of periplasmic proteins [18], but also could be beneficial in folding cytoplasmic proteins like TR and GR as well. The data presented for chaperones represent only one trial ($n = 1$), but the results are consistent with those from similar reactions. The base case reaction was also scaled up to 1 ml total volume. The large scale reaction was performed in triplicate on one day ($n = 3$). The average total and soluble yields are presented in Figs. 1 and 2. The error bars are ± one standard deviation.

The data show that the production of TR and GR by CFPS is not largely affected by temperature, FAD concentration, or the presence of chaperones. The percent solubility remains unchanged as well. It is not surprising that the use of a lower temperature or the addition of chaperones did not increase solubility because a large percentage of the protein was soluble in the base case. Including all trials and conditions, TR accumulated to 760 ± 93 μg/ml total protein with 678 ± 83 μg/ml soluble protein. GR was produced at 521 ± 69 μg/ml with 371 ± 50 μg/ml being soluble. For GR, 722 ± 4 μg/ml of protein was produced in the 1 ml reaction. This concentration of protein is significantly higher (95% confidence interval) than that produced in the 50 μl reactions. We have observed an increase in protein production at the 1 ml scale for other proteins. The cause is unclear, but increased production is only observed when the reaction is carried out as a thin film in a six-well tissue culture plate.

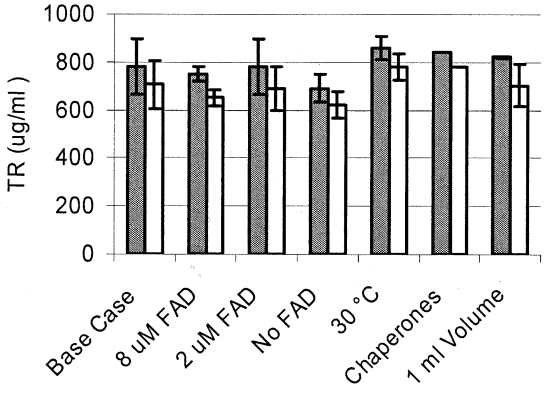

Fig. 1. Total (closed bars) and soluble (open bars) TR produced using CFPS. The base case is 20 μM FAD, 37°C incubation for 4 h, and 50 μl total volume. Deviations from the base case include incubation at 30°C, addition of chaperones, variation of the FAD concentration, and increasing the reaction volume to 1 ml. Data are averages over many trials with error bars of ± one standard deviation.

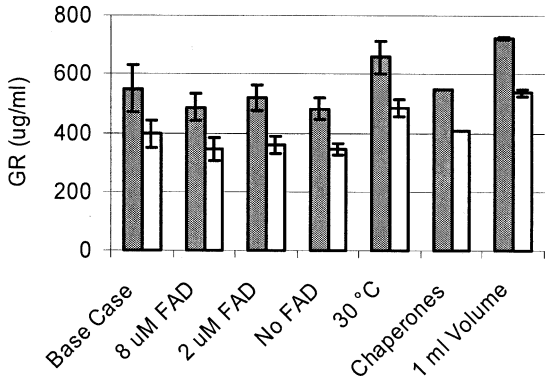

Fig. 2. Total (closed bars) and soluble (open bars) GR produced using CFPS. The base case is 20 μM FAD, 37°C incubation for 4 h, and 50 μl total volume. Deviations from the base case include incubation at 30°C, addition of chaperones, variation of the FAD concentration, and increasing the reaction volume to 1 ml. Data are averages over many trials with error bars of ± one standard deviation.

3.2. Activity

It has been shown that an appreciable amount of soluble TR and GR can be produced using CFPS. The specific activities of TR and GR were measured to determine if the proteins folded correctly, inserted the FAD cofactor, and became active. In the TR activity assay, TR reduces *E. coli* thioredoxin, which in turn reduces the molecule DTNB [9]. The course of this coupled reaction can be followed by observing the color change at 412 nm due to DTNB reduction to nitrothiobenzoate. The activity can be measured in these CFPS reactions without purifying the protein. The activity of a CFPS reaction with no TR plasmid added was subtracted as background. The measured TR activity was more than 50 times greater than background. The specific activity was measured for all samples of TR as shown in Fig. 3.

The TR specific activities for the base case, 30°C, chaperones, and 8 μM FAD are all the same. The average specific activity for these trials is 1390 ± 160 min^{-1} (measured at 37°C). The specific activity of the enzyme at 1 ml scale was 1251 ± 101 min^{-1}. This value is not significantly different than the specific activity at 50 μl. Prongay et al. measured a specific activity of 1320 min^{-1} using TR purified from *E. coli* using the same activity assay [9]. This suggests that the *E. coli* enzyme produced in the cell-free system is fully active. Williams measured the specific activity of TR to be 520 min^{-1} at 4°C and 2000 min^{-1} at 25°C using the same assay mixture as Prongay [19]. Assuming an Arrhenius relationship between specific activity and temperature, Williams predicts the specific activity to be 4230 min^{-1} at 37°C. The enzyme produced using CFPS is consistent with the findings of Prongay but not of Williams.

The TR specific activity results show no dependence of specific activity on reaction temperature or the addition of chaperones. However, there is a dramatic dependence on the FAD concentration. The enzyme synthesized in the presence of 8 μM FAD has normal activity, but when the FAD concentration is lowered to 2 or 0 μM, the specific activity decreases sharply. One would expect that TR molecules deprived

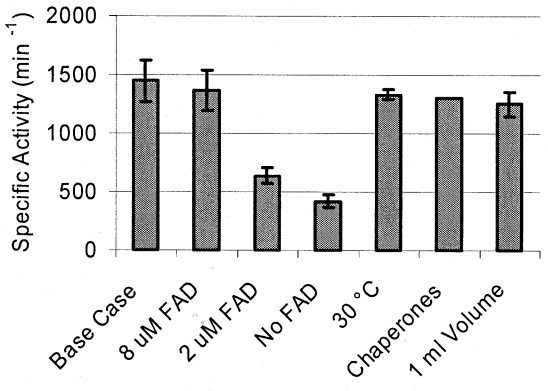

Fig. 3. Specific activity of TR produced using CFPS. The base case is 20 μM FAD, 37°C incubation for 4 h, and 50 μl total volume. Deviations from the base case include incubation at 30°C, addition of chaperones, variation of the FAD concentration, and increasing the reaction volume to 1 ml. Data are averages over many trials with error bars of ± one standard deviation.

of a FAD cofactor would be inactive because FAD is required to accept and donate electrons during the catalytic cycle [8]. The specific activity does not reach 0 min^{-1} when no FAD is added; most likely because a small amount of FAD is available from the *E. coli* extract.

In a separate set of reactions, TR had only 43% activity relative to the base case (20 μM) when FAD was left out of the reaction. To test for the possibility of post-translational FAD incorporation, an aliquot of the soluble fraction of the CFPS reaction conducted without additional FAD was harvested. This sample was incubated with 20 μM FAD for 4 h at 37°C. The specific activity increased to 55% of maximal activity (data not shown). These results suggest that, although a small fraction of the product can be post-translationally activated, cotranslational FAD incorporation is much more effective for producing an active reductase.

In the GR activity assay, GR directly reduces GSSG using reducing equivalents from NADPH [7]. The oxidation of NADPH can be monitored by the change in absorbance at 340 nm. As seen in Fig. 4, the specific activity of GR shows approximately the same trends as TR. The GR specific activity is not increased, and may even be decreased slightly when

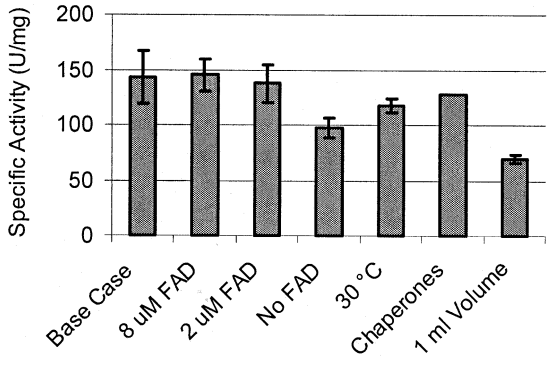

Fig. 4. Specific activity of GR produced using CFPS. The base case is 20 μM FAD, 37°C incubation for 4 h, and 50 μl total volume. Deviations from the base case include incubation at 30°C, addition of chaperones, variation of the FAD concentration, and increasing the reaction volume to 1 ml. Data are averages over many trials with error bars of ± one standard deviation.

produced at 30°C or in the presence of chaperones. The specific activity is also constant for 20, 8, and 2 μM FAD. It doesn't decrease until FAD is left out of the reaction and, in that case, it decreases less dramatically than for TR. Perhaps this is because approximately one half of the moles of protein are produced for GR relative to TR. Alternatively, the folding pathway for GR may produce a higher affinity for FAD than does the pathway for TR folding.

If the specific activity for all trials for the base case, 8 and 2 μM FAD, 30°C, and chaperones are averaged we find that the specific activity for GR is 139 ± 19 U/mg. Vanoni et al. used the same activity assay to measure the specific activity for GR purified from *E. coli*. They reported only 30.9 U/mg [7]. The reason for the discrepancy is unclear. We show below that the presence of the N-terminal CAT residues or the C-terminal histidines on the GR produced in CFPS does not increase the specific activity of the enzyme. A more likely hypothesis is that FAD may have been stripped from the protein during the four chromatography steps used by Vanoni to purify the native protein. This would cause them to observe less than maximal specific activity.

The specific activity of the GR produced in the 1 ml reactions was 70 ± 4 U/mg. This is less active than the protein produced at the 50 μl scale, but still significantly more active than reported by Vanoni et al. Further research will be needed to explain the differences in specific activity between GR produced in 50 μl CFPS reactions and that produced in vivo or in 1 ml CFPS reactions.

3.3. Purification

The three 1 ml reactions were combined for TR and for GR and each pool was purified using Ni-chelation chromatography. The purification resulted in a single major band on a SDS–PAGE gel (Fig. 5) for each protein. For TR the band appears at 36 kDa, which is the exact size of the TR monomer. The dimer does not appear because of the denaturing conditions of the gel. Fractions 5, 6, and 7 (lanes 4, 5, and 6 in Fig. 5) contained purified TR and were combined to give 1 ml of purified enzyme with a concentration of 1.4 mg/ml. The purified GR fractions also ran at the expected size. Fractions 5 and 6 (lanes 9 and 10 in Fig. 5) of the GR purification were combined to give 800 μl of enzyme at 1.2 mg/ml.

3.4. Kinetics of purified GR and TR

The kinetic parameters of the purified enzymes were mea-

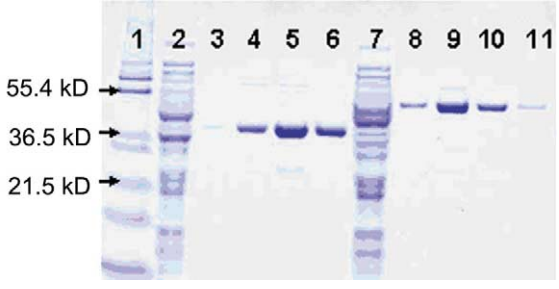

Fig. 5. Purification of TR and GR. Lane 1, MarkXII ladder (Invitrogen); lane 2, combined 1 ml TR CFPS reactions; lane 3, TR fraction 4; lane 4, TR fraction 5; lane 5, TR fraction 6; lane 6, TR fraction 7; lane 7, combined GR reactions; lane 8, GR fraction 4; lane 9, GR fraction 5; lane 10, GR fraction 6; lane 11, GR fraction 7.

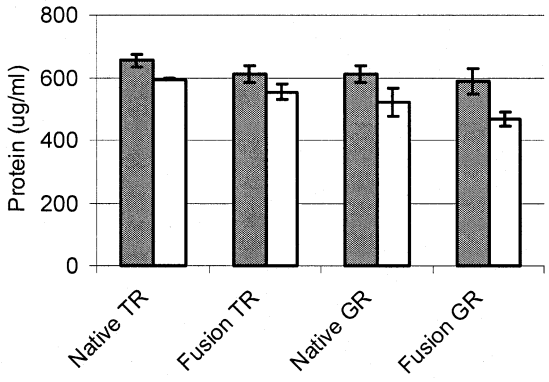

Fig. 6. Total (closed bars) and soluble (open bars) protein produced using CFPS. The base case CFPS reaction was used; 20 μM FAD, 37°C incubation for 4 h, and 50 μl total volume. Proteins include both the native TR and GR proteins as well as TR and GR fused with the first five N-terminal amino acids of CAT and a C-terminal His6 purification tag. Error bars represent ±one standard deviation for three trials.

sured to determine if the activity was changed during purification. The same activity assays were used for purified TR and GR as for unpurified TR and GR. The specific activity for purified TR remained unchanged at 1363 min^{-1}. The specific activity of GR purified from the 1 ml reactions was 69 U/mg, which is the same value obtained before purification. Thus, the specific activity did not change during the purification step.

3.5. CFPS of native proteins

TR and GR were recloned into the pK7 vector without the N-terminal amino acids of CAT or the C-terminal polyhistidine tag. The native proteins were produced to investigate the effects of the additional amino acids on the enzyme's productivity, solubility, and activity when produced in a cell-free system.

Fig. 6 shows that the productivity and solubility of TR and GR were unchanged between the native construct and the fusion protein in a side-by-side experiment ($n = 3$). The specific activities of both enzymes were also not changed for the native construct relative to the fusion protein (data not shown).

This experiment indicates that the N-terminal amino acids of CAT did not affect the performance of CFPS for TR or GR. Thus, the addition of the CAT front end may not be necessary to achieve adequate protein production in a CFPS reaction for all proteins.

3.6. Conclusions

This work demonstrates that complex enzymes such as TR and GR can be produced in CFPS. They are made in sufficient quantity during the simple batch reaction to appear as a major band on a PAGE gel before purification. Despite their complexity in structure and mechanism, both enzymes were shown to be fully active when produced in the cell-free system. This work extends the utility of CFPS to proteins that are multimeric and contain cofactors.

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