

Optimization of Programmed Suppression in a Cell-Free Protein Synthesis System with Unnatural Amino Acid *S*-(2-Nitrobenzyl)cysteine

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Abstract Unnatural amino acid *S*-(2-nitrobenzyl)cysteine was incorporated into human erythropoietin by using a programmed suppression of nonsense codon in a cell-free protein synthesis system. Several controlling factors affecting the operational efficiency of the suppression were investigated and optimized. The amount of suppressor tRNA and the concentration of Mg²⁺ were crucial not only for the efficiency but also for the control of the exact suppression. In addition, some general optimization factors are reported in order to improve the efficiency in an unnatural amino acid mutagenesis.

Key words: Cell-free protein synthesis, unnatural amino acid mutagenesis, erythropoietin, suppressor tRNA, salt concentration optimization

Heterologous gene expression in recombinant *Escherichia coli* is a common method to produce useful proteins. However, protein produced by this recombinant DNA system possesses numerous problems such as insoluble inclusion body formation or intracellular proteolytic degradation of the recombinant protein. Moreover, some proteins cannot be synthesized in recombinant cells because of their toxicities. A cell-free protein synthesis system is a promising alternative that can solve these problems, and recent progress in productivity enhancement has improved commercial feasibility for some proteins [11, 12, 16]. Besides the production of naturally occurring proteins, a cell-free protein synthesis system can be used for the production of proteins that contain unnatural amino acids. Since the pioneering work of Chapeville *et al.* [2], demonstrating that a tRNA bearing a noncognate amino acid could be employed for the elaboration of a modified

protein, increasing research activity has been devoted to the preparation of proteins containing synthetic amino acids at specific, predetermined positions. Several strategies have been developed and the incorporation of unnatural amino acid into a predetermined site of a protein is no longer a state-of-the-art skill. Generally, the strategies involve the use of misacylated tRNA to suppress nonsense codons in the mRNA [1, 4, 7, 13, 15,] or to read four bases as a codon (frame-shift) at a specific predetermined site [8] of the mRNA. By this method, also called “unnatural amino acid mutagenesis,” the actual mechanisms of peptide formation and translation initiation were investigated, and parameters that control the read-through of nonsense codons were characterized. In addition, several unnatural “caged” amino acids were successfully incorporated into a protein by unnatural amino acid mutagenesis. These caged substances, inactive precursor molecules that can be rapidly activated by photolysis, were used to confirm the active site for several proteins [3, 6, 14]. Previously, we reported that caged unnatural amino acid *S*-(2-nitrobenzyl)cysteine could be incorporated into a predetermined site of human erythropoietin [9]. However, there is no general rule as of yet to improve the incorporation efficiency of unnatural amino acid into a protein. The proposed optimization procedure in the present study may thus be used as a general guideline to improve the efficiency in an unnatural amino acid mutagenesis.

MATERIALS AND METHODS

Synthesis of Misacylated Suppressor tRNA

Suppressor tRNA charged with *S*-(2-nitrobenzyl)cysteine was synthesized as previously reported [9]. Briefly, N-(4-pentenoyl), *S*-(2-nitrobenzyl)cysteine cyanomethyl ester was synthesized, and then aminoacylated to dinucleotide

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pdCpA (as tetrabutyl ammonium salt form, Cruachem). Full-length suppressor tRNA was prepared by ligating aminoacyl pdCpA with tRNA(-CA) in which 3'-terminal CA is absent. Deprotecting 4-pentenoyl protecting group at α -amine of amino acid by iodine was carried out as described [9]. tRNA(-CA) was prepared from plasmid pSup-ala [9] by run-off transcription using T7 RNA polymerase.

Cell-Free Translation and Suppression Reaction

Pure NTPs and UTP were obtained from Seikagaku Chemicals, Japan. Phospho(*enol*)pyruvate and an *E. coli* tRNA mixture were purchased from Boehringer-Mannheim. Poly(ethylene glycol) with an average molecular weight of 6000 was derived from Wako Pure Chemicals and used without further purification. Folinic acid, potassium glutamate, pyruvate kinase, and amino acids were purchased from Sigma. L-[U- 14 C]leucine (11.9 GBq/mmol) was obtained from ICN. Other reagents used in the cell-free protein synthesis were purchased from Wako Pure Chemicals. T7 RNA polymerase was prepared as described by Davanloo *et al.* [5]. S30 extract was prepared as described previously [10]. The standard 15 μ l incubation mixture contained: 57 mM HEPES/KOH (pH 8.2), 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 80 mM ammonium acetate, 0.17 mg/ml of *E. coli* total tRNA mixture (from strain MRE 600), 34 mg/ml of 1-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 6.7 μ g/ml of circular plasmid, 33 μ g/ml of T7 RNA polymerase, 0.3 U/ml of pyruvate kinase, 0.5 mM of each of the unlabeled amino acids, 28 mM phospho(*enol*)pyruvate, and 4 μ l of S30 extract. Reaction mixture was incubated at 37°C for 60 min, and the synthesized protein was analyzed by cold trichloroacetic acid precipitation method or Western blot analysis. Generally, samples of 15 μ l were taken for the assay of [14 C]leucine incorporation and 3 μ l for SDS-PAGE/Western blot analysis.

Western Blot

Samples separated by SDS-PAGE and SDS-urea-PAGE were transferred to nitrocellulose membrane (Amersham). Wild-type erythropoietin and the mutein were detected through sequential reaction with polyclonal anti-human erythropoietin (Sigma, 1/2000 in TBST), horseradish peroxidase conjugated anti-rabbit IgG (Amersham, 1/2000 in TBST), and enhanced chemical luminescence.

RESULTS AND DISCUSSION

General considerations in the optimization of a cell-free translation system may be investigated in the programmed suppression system as far as translation efficiency is concerned. However, a programmed suppression system

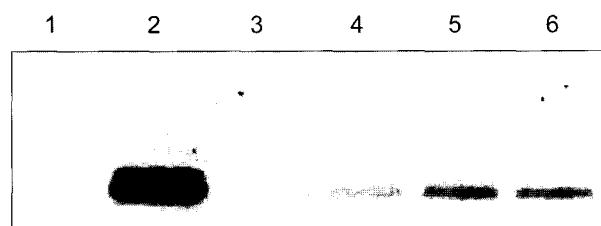


Fig. 1. The effect of the amount of suppressor tRNA on the programmed suppression.

The reaction was primed with: lane 1, no DNA; lane 2, pK7-EPO; lane 3, pN38am (TAATAA) and no suppressor; lanes 4-6, pN38am (TAATAA) and 0.1, 0.2, and 0.3 μ g/ μ l of suppressor, respectively.

utilizes suppressor tRNA which is not used in a normal translation system. The optimal amount of the suppressor tRNA was determined to assure an efficient programmed suppression. Figure 1 shows that the programmed suppression was induced by the action of the suppressor tRNA supplemented (lane 3 vs. 4-6), and that the synthesis of the suppressed product reached a plateau over the suppressor concentration of approximately 0.2 μ g/ μ l. Since the suppression might be limited by the amount of the suppressor, when optimization of other components of the cell-free translation system was carried out, an excess amount of the suppressor tRNA, *i.e.* 0.3 μ g/ μ l of suppressor, was used for further experiments.

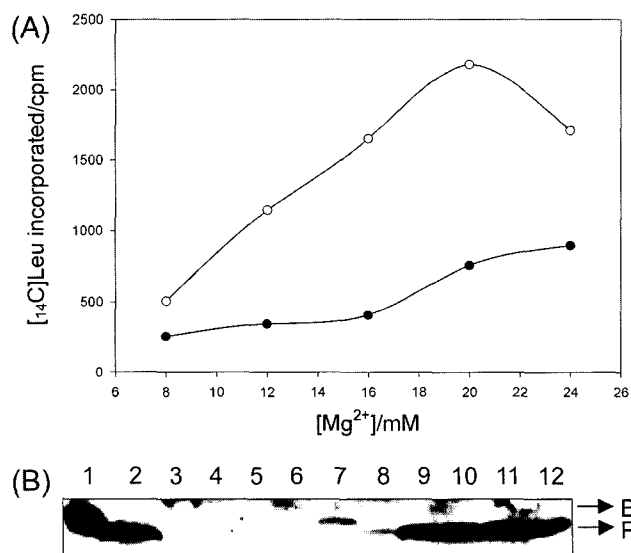


Fig. 2. The effect of [Mg^{2+}] on the background suppression and programmed suppression.

(A): Suppression reaction was primed with (\circ) or without (\bullet) suppressor tRNA. The amount of [14 C]leucine which was incorporated into the polypeptide was counted by liquid scintillation counting (LSC). (B): Each sample shown in (A) was assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blot. Lane 1, wild-type EPO; lane 2, mutein of EPO; lanes 3-7, data points marked as \circ in Fig. 2(A); lanes 8-12, data points marked as \bullet in Fig. 2(A). B, background suppression product; P, programmed suppression product.

For the next step, the optimal concentration of Mg^{2+} was investigated, since in a cell-free protein synthesis system, the concentration of Mg^{2+} is well known as a key factor for the efficient protein synthesis. The concentration of Mg^{2+} in a reaction mixture may influence not only the suppression yield but also the extent of background suppression (misreading by the malfunction of ribosome, which is the adaptation of noncognate tRNA as a decoder). Figure 2 exhibits the effects of different Mg^{2+} concentrations on EPO mutein synthesis. The amount of TCA-insoluble material (count per minute, cpm) showed the highest level at the Mg^{2+} concentration of 20 mM (Fig. 2A), which is much higher than the concentration of Mg^{2+} required in the synthesis of wild-type EPO (the concentration was 12 mM in our experiments, data not shown). However, misreading at a 20 mM or higher concentration of Mg^{2+} became dominant over the correct translation stop at the predetermined site (Fig. 2B). Therefore, an operational concentration of Mg^{2+} for the suppression was adjusted to 16 mM in order to avoid product heterogeneity. It is also important to set the concentration of Mg^{2+} differently with every change of S30, suppressor tRNA, and DNA batches. Monovalent cation, K^+ in this case, showed no influence on the misreading, but affected the translation efficiency (Fig. 3). The optimal concentration of K^+ in the suppression reaction was exactly identical to that required in the translation of wild-type EPO (data not shown).

Reaction kinetics was studied in the programmed suppression system. Translation reaction reached a plateau

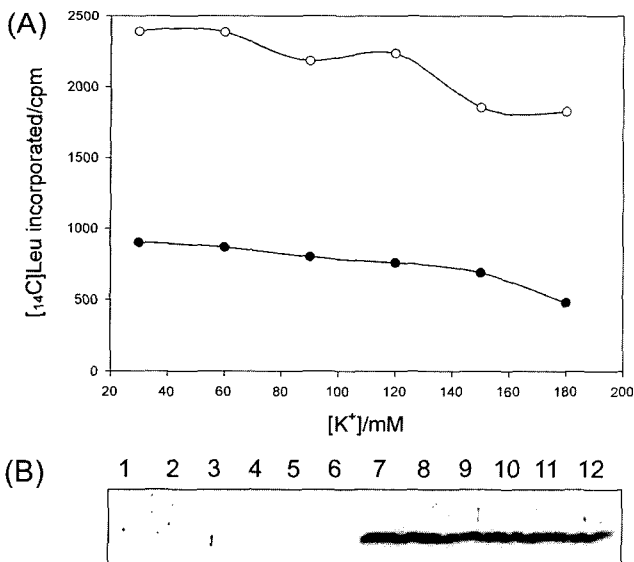


Fig. 3. The effect of $[K^+]$ on the efficiency of programmed suppression.

(A): Suppression reaction was primed with (○) or without (●) suppressor tRNA. The amount of $[^{14}C]$ leucine incorporated into the polypeptide was counted by LSC. (B): Each sample shown in (A) was assayed by SDS-PAGE/Western blot. Lanes 1–6, data points marked as ○ in Fig. 3(A); lanes 7–12, data points marked as ● in Fig. 3(A).

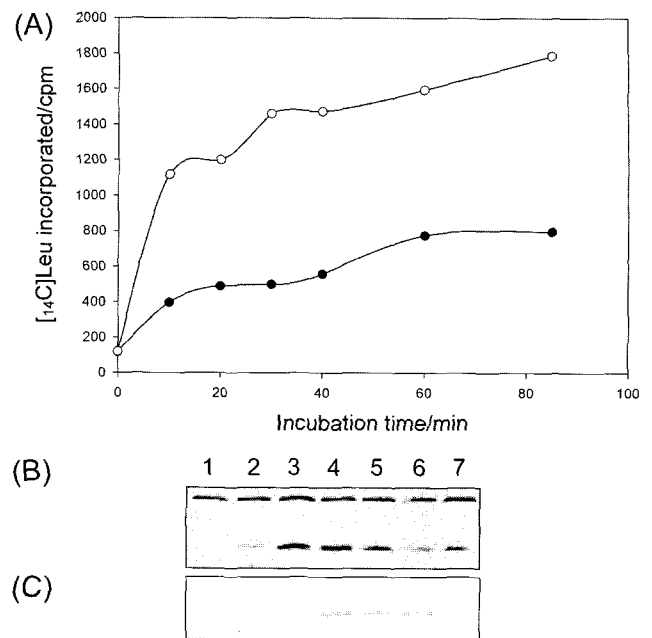


Fig. 4. Kinetics of programmed suppression.

(A): Suppression reaction was primed with (○) or without (●) suppressor tRNA. The amount of $[^{14}C]$ leucine incorporated into the polypeptide was counted by LSC. (B): Each sample marked as ○ in Fig. 4(A) was assayed by SDS-PAGE/Western blot. Upper bands correspond to nonspecific binding of S30 proteins to antibody used. (C): Each sample marked as ○ in Fig. 4(A) was assayed by SDS-PAGE/autoradiography. Dried gel was used to expose X-ray film for 7 days.

within 30–40 min in a typical cell-free protein synthesis system, because of the following possible reasons: substrate limitation, breakdown of mRNA, and circumstances not yet revealed. The reaction rate in the programmed suppression was greatly reduced in about 30 min, similar to the conventional cell-free protein synthesis system (Fig. 4A). Autoradiogram of the reaction mixture showed that programmed suppression stopped at 30 min (Fig. 4B). However, the reaction in the programmed suppression continued at a slow rate up to 80 min, resulting in a steady increase of the amount of TCA-insoluble materials. Therefore, the steady increase of the amount of TCA-insoluble material may be attributed to the fact that N-terminal peptide of the erythropoietin (resulting from the translation stop at the 38th codon) could be synthesized persistently. On the contrary, immunoblot analysis showed that the suppressed product underwent structural deformation as the incubation time increased, therefore, it was not recognized by the antibody used. For the production of a completely folded protein, the incubation period should be properly selected, even though the incubation time itself was not directly related to the production of the protein of interest.

In this report, several important operation parameters were investigated such as salt concentration, especially Mg^{2+} , incubation time, and suppressor tRNA concentration. The concentration of suppressor tRNA should be optimized to generate a maximal amount of programmed suppression product. Kinetic study revealed that the programmed suppression reaction could continue for longer than 60 min, when an appropriate supplementation of suppressor tRNA was made to the reaction. Based on these findings, a semi-batch type supplementation of suppressor tRNA for the programmed suppression reaction is strongly recommended.

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