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Zeolite-Mediated Cation Exchange Enhances the Stability of mRNA during Cell-Free Protein Synthesis

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Abstract The addition of zeolite particles enhances the stability of mRNA molecules in a cell-free protein synthesis system. When 20 μg/μL of zeolite (Y5.4) is added to a reaction mixture of cell-free protein synthesis, a substantial increase in protein synthesis is observed. The stabilizing effect of zeolite is most clearly observed in an *in vitro* translation reaction directed by purified mRNA, as opposed to a coupled transcription and translation reaction. Upon the addition of zeolite in the *in vitro* translation reaction, the life span of the mRNA molecules is substantially extended, leading to an 80% increase in protein synthesis. The effect of zeolite upon the mRNA stability appears be strongly related to the cation exchange (potassium to sodium) reaction. Our results demonstrate the possibility of modifying this biological process using heterogeneous, non-biological substances in a cell-free protein synthesis system.

Keywords: zeolite, ion-exchange, sodium, potassium, cell-free protein synthesis, translation

INTRODUCTION

Due to its outstanding flexibility, simplicity and throughput, cell-free protein synthesis is drawing attention as an alternative tool for the genome-wide translation of genetic information [1]. The open nature of this technique conveniently enables the potential analysis of multiple factors affecting the events of protein synthesis [2]. It also carries a potential to be used for studying the effectors of protein synthesis in a miniaturized format [3-5]. In addition, cell-free protein synthesis can also provide a useful tool for validating the results from transcriptome analysis [6,7]. Therefore, extensive research [8,9] has been undertaken in recent years in an attempt to modify and optimize the reaction conditions of batch cell-free protein synthesis systems for practical applications of this method.

Providing an optimal ionic environment is important for the efficient production of proteins in a cell-free protein synthesis system. Among the ions used in cell-free protein synthesis, monovalent cations are known to play important roles in various aspects of protein synthesis including the maintenance of the ribosome structure [7], binding of tRNA to ribosomes [8] and peptidyl transfer reactions [9-12]. Previously, it was reported that certain types of synthetic zeolite enhanced the efficiency of cell-free protein synthesis in a study using an *E. coli* extract [13]. It was subsequently found that this enhancement mainly resulted from an ion exchange reaction between the added zeolites and the reaction mixture. For example, protein synthesis was also enhanced when potassium ions in the reaction mixture were substituted with sodium ions, and the addition of zeolite did not further enhance the protein synthesis in the sodium-substituted reaction mixture [14].

In this study, we explored how monovalent cations affect the efficiency of a coupled cell-free protein synthesis reaction. From the comparative analyses of the effects of zeolite on transcription and translation, we discovered that the presence of zeolite had little effect on the efficiency of transcription. In contrast, the addition of zeolite remarkably enhanced protein synthesis in the *in vitro* translation reactions using purified mRNA. It appeared that the stimulation of the translational efficiency resulted from the extended life-span of the mRNA molecules. Similar enhancements in mRNA stability and protein synthesis were obtained when potassium ions in the reaction mixture were directly replaced with sodium ions, instead of relying on the mediation by the zeolite. The functional stability of mRNA is known to be one of the most critical factors affecting the efficiency of cell-free protein synthesis [15,16], and our results demonstrate that modifying the ionic environment during protein synthesis can regulate the ribonucleolytic activity of cell-free extracts.

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MATERIALS AND METHODS

Preparation of mRNA and Plasmids

Plasmid pK7CAT [17] was used as the template for the cell-free protein synthesis reactions in this work. This plasmid encodes the ORF of CAT under the control of the T7 promoter. For the experiments involving *in vitro* translation, mRNA was prepared according to the previously reported protocols [18] and was purified by sequential precipitation with 3 M lithium chloride and ethanol.

Cell-Free Protein Synthesis System

S30 extract from *E. coli* strain A19 was prepared according to the procedures described by Pratt [19]. Unless otherwise specified, the standard reaction mixture used for the cell-free protein synthesis contained 57 mM Hepes/KOH at pH 8.2, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 0.64 mM cAMP, 210 mM potassium glutamate, 36 mM NH_4OAc, 16 mM $Mg(OAc)_2$, 0.17 mg/mL *E. coli* total tRNA mixture, 34 μg/mL folinic acid, 0.3 U/mL pyruvate kinase, 0.46 mM [^{14}C]leucine (0.27 GBq/mmol), 2% polyethylene glycol-6000, 28 mM phosphoenolpyruvate, 24% (v/v) S30 extract, and deionized water to make up the desired reaction volume. As the template for protein synthesis, 50 μg/mL of purified mRNA (*in vitro* translation) or 6.7 μg/mL of the plasmid (coupled transcription and translation) was used. For the coupled transcription/translation system, 33 μg/mL T7 RNA polymerase was added to the reaction mixture for the *in situ* generation of mRNA. In the experiments to examine the effect of zeolite, zeolite Y 5.6 was used as described previously [13]. All of the cell-free protein synthesis reactions were conducted at 37°C for 2 h. The amount of the cell-free synthesized protein generated was estimated from the radioactivity of TCA-insoluble products using a liquid scintillation counter [6].

Analysis of mRNA during Cell-Free Protein Synthesis

The electrophoretic analysis of mRNA was conducted as described by Ingle and Kushner [14] with minor modifications. At the indicated time points, 20 μL samples were withdrawn from the reaction mixture and analyzed on a 5% polyacrylamide/7 M urea gel using methylene blue staining.

RESULTS AND DISCUSSION

Zeolite Increases mRNA Levels during Protein Synthesis in a Coupled Transcription/Translation Reaction

It was previously reported that zeolites enhanced protein synthesis in a coupled transcription/translation reaction utilizing S30 extract derived from *E. coli*. Although the stimulation of protein synthesis was shown to be related to the zeolite-mediated exchange of sodium and potassium ions [14], it was not clear which step in the entire process of protein synthesis was affected by the addition of zeolite.

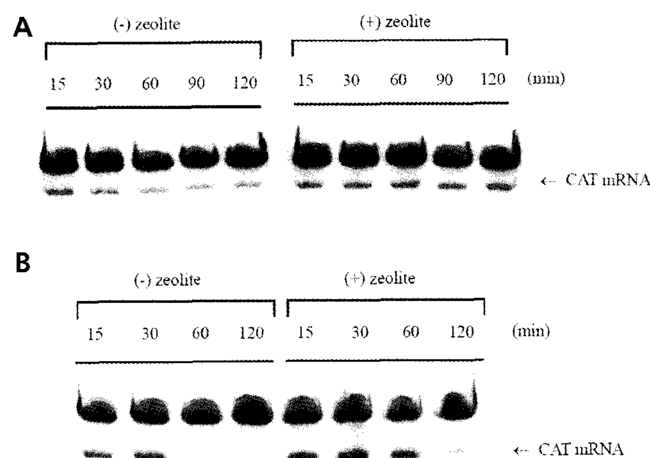

Fig. 1. Effect of zeolite on mRNA levels in S30 extract with (A) or without (B) transcription-coupled translation. Reaction mixtures containing the plasmid pK7CAT were incubated at 37°C in the absence or presence of zeolite. At the indicated time points, samples of 20 μL were removed from the reaction mixture. mRNA samples were isolated as described by Ingle and Kushner [20] with minor modifications. The samples isolated were analyzed by denaturing electrophoresis on a 5% polyacrylamide/7 M urea gel and the urea gels were stained with methylene blue.

In this study, during the time-course analysis of the cell-free protein synthesis reactions conducted in the presence of zeolite, we found that the addition of zeolite significantly enhanced the stability of mRNA. In a control reaction involving coupled transcription/translation without the addition of zeolite, the amount of the full-length transcripts showed a gradual decrease over the incubation period, following an initial increase during the first 15 min. Upon the addition of zeolite (20 μg/μL), however, the amount of the full-length transcripts remained stable for substantially longer periods (Fig. 1A). The zeolite-mediated extension of mRNA life-span was also observed in a reaction mixture devoid of amino acids, indicating that the effect of zeolite was not dependent upon the translational events (Fig. 1B). It should be pointed out that the rate of mRNA disappearance was substantially slower in reaction mixtures containing amino acids, in both the presence and absence of zeolite, an effect most likely mediated through the protection of mRNA by the loaded ribosomes.

Zeolite-Mediated Retardation of the mRNA Degradation

In a coupled transcription/translation reaction, the amount of the full-length transcripts is determined by the relative rates of the transcription reaction and the degra-

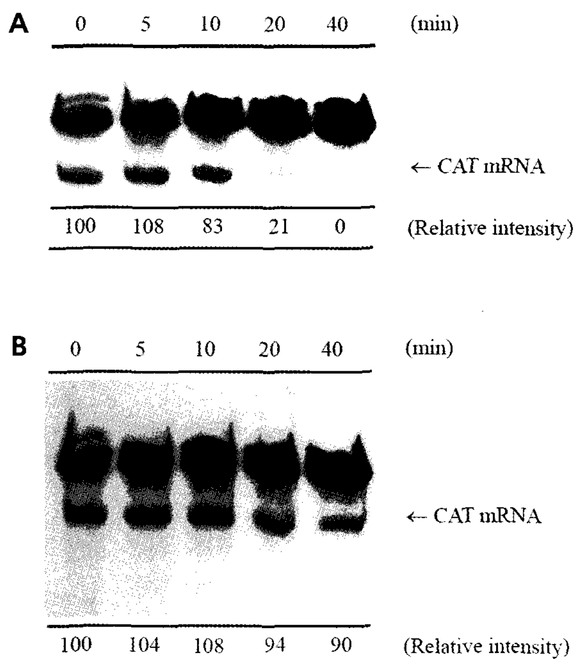

Fig. 2. Effect of zeolite on the stability of mRNA during *in vitro* translation. Reaction mixtures containing CAT mRNA instead of DNA were incubated at 37°C in the absence of zeolite (A) or in the presence of zeolite (B). At the indicated time points after incubation, samples of 20 μL were removed from the incubations. mRNA samples were isolated as described by Ingle and Kushner [20] with minor modifications. The samples isolated were analyzed by denaturing electrophoresis on a 5% polyacrylamide/7 M urea gel and the urea gels were stained with methylene blue. mRNA kinetics were analyzed on the denaturing gels. mRNA bands stained with methylene blue were quantified with a gel densitometer (Biorad GS-690). The amounts of mRNA were normalized to the amount at the start of incubation.

dation of the transcribed mRNA. In order to determine whether the increase in the mRNA level upon the addition of zeolite was due to an increase in the rate of transcription or to a decrease in the rate of mRNA degradation, the mRNA level was monitored in an *in vitro* translation reaction in which the protein synthesis was directed by the *in vitro* transcribed mRNA of CAT. Without the addition of zeolite, the full-length mRNA in the reaction mixture was almost completely degraded within 40 min (Fig. 2A). In contrast, the life-span of the mRNA was remarkably enhanced when zeolite was added prior to the incubation of the reaction mixture (Fig. 2B). The stabilizing effect of zeolite reached a plateau at 20 μg/μL, and 90% of the initial amount of intact mRNA was detected at the correct size after 40 min of incubation. Meanwhile, it was found in a separate experiment that the presence of zeolite did not affect the efficiency of the *in vitro* transcription reaction (data not shown). Taken together, these findings indicate that the increase in the mRNA level caused by the addition of zeolite resulted from the retardation of mRNA degradation.

In order to examine whether or not this enhanced stability of the mRNA resulted from the ion-exchange reaction mediated by zeolite, the *in vitro* translation reaction was conducted in a reaction mixture in which potassium glutamate was replaced by the same concentration of sodium glutamate. As shown in Fig. 3A, replacement of potassium with sodium enhanced the stability of mRNA to a similar extent to that observed in the case where zeolite was added. Both of the addition of zeolite and the replacement of cation species led to an approximately 70% increase of protein synthesis (Fig. 3B). Moreover, the addition of zeolite to the sodium-substituted reaction mixture did not further enhance the stability of mRNA or the protein synthesis

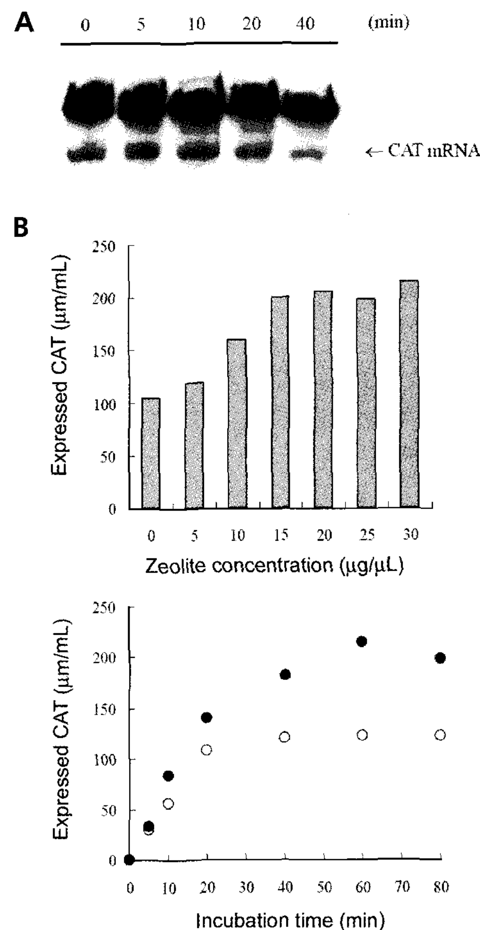

Fig. 3. Effect of sodium substitution on mRNA stability and the efficiency of translation. Potassium ions in the reaction mixture were replaced with sodium ions. The mixtures containing CAT mRNA were incubated at 37°C in the absence of zeolite. At the indicated time points after incubation, samples of 20 μL were removed from the incubations and the amount of residual mRNA was analyzed by denaturing electrophoresis on a 5% polyacrylamide/7 M urea gel (A). An increasing concentration of the zeolite Y 5.6 was added to the reaction mixture during *in vitro* translation and the final amount of the expressed CAT was measured (B, top). Time-courses of protein synthesis in the absence (open circles) or presence (closed circles) of the zeolite were estimated from radioactivity of the TCA-insoluble samples as described by Kim *et al.* [6] (B, bottom).

(data not shown), indicating that the effects of the sodium ion and zeolite coincide.

Cations neutralize the negative charges carried by the phosphate groups of nucleic acids, modulate the ionic atmosphere, and influence the conformation of nucleic acids by means of electrostatic forces. Therefore, it is plausible to suggest that cation-nucleic acid interactions would affect specific interactions between nucleic acids and proteins. We speculate that the sodium-bound mRNA assumes a secondary structure that was resistant to the nucleolytic activity of certain nucleases. It should be noted here that our results are not consistent with the previously reported work of Jung *et al.* [21] which showed that ion exchange does not enhance protein synthesis. We assume that the differing results stem from the fact that the two groups used different sources of translational machinery (S30 extract). While our system employs the S30 extract from *E. coli*, their experiments were based on wheat germ extract. Although a detailed mechanism is not clear at this moment, it appears that cell-free extracts respond to ionic conditions with wide latitude of variation depending on their sources.

In conclusion, we found that the stability of mRNA can be remarkably enhanced by the addition of zeolite particles to the reaction mixture, which in turn leads to an increase in protein synthesis. Our results also demonstrate the advantage of the cell-free synthesis system, in that it allows the efficiency of biological processes to be modified and improved through the introduction of heterogeneous, non-biological substances.

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