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5S rRNA-assisted DnaK refolding

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ABSTRACT

Although accumulating evidence has revealed that most proteins can fold without the assistance of molecular chaperones, little attention has been paid to other types of chaperoning macromolecules. A variety of proteins interact with diverse RNA molecules *in vivo*, suggesting a potential role of RNAs for folding of their interacting proteins. Here we show that the *in vitro* refolding of a representative molecular chaperone, DnaK, an *Escherichia coli* homolog of Hsp70, could be assisted by its interacting 5S rRNA. The folding enhancement occurred in RNA concentration and its size dependent manner whereas neither the RNA with the reverse sequence of 5S rRNA nor the RNase pretreated 5S rRNA stimulated the folding *in vitro*. Based on our results, we propose that 5S rRNA could exert the chaperoning activity on DnaK during the folding process. The results suggest an interesting possibility that the folding of RNA-interacting proteins could be assisted by their cognate RNA ligands.

Introduction

The *in vitro* refolding experiments by Anfinsen demonstrated that the final native conformation of a protein is encoded by its amino acid sequence [1]. Nevertheless, proteins can encounter misfolding and aggregation during their folding processes [2]. Molecular chaperones have been known to assist protein folding inside the cells by preventing misfolding and aggregation through recognition and shielding of exposed hydrophobic regions of their substrates proteins [3,4]. From the concept of the 'assisted' folding *in vivo*, traditionally protein folding *in vivo* has been understood on the basis of the principles obtained from molecular chaperones. However, accumulating evidence suggests that the majority of proteins can fold without the assistance of the known molecular chaperones. For instance, neither DnaK nor the trigger factor is essential for folding of proteins or cell viability at normal growth temperatures such as 30 and 37 °C [5,6]. Moreover, both the proteome-wide analysis of the GroEL chaperonin and the genetic study of physical loss of GroEL have revealed that it assists the folding of only a minority of proteins inside the cells [7,8]. These results suggest that there might be other factors yet to be discovered that assist protein folding inside the cells.

In addition to the classical molecular chaperones including DnaK, trigger factor and GroEL, ribosome and its components including 50S subunit, 23S rRNA, and the domain V of 23S rRNA have been shown to exhibit protein chaperoning activity *in vitro* in a trans-acting mode [9–11]. Recently, we demonstrated that RNAs could exert chaperoning effect on the proteins carrying an RNA-binding domain (RBD) [12,13]; when RBD is fused to aggregation-prone proteins, the binding of cognate RNA to the RBD could enhance the solubility and proper folding of the passenger proteins. Mechanistically, highly negative charge and the steric hindrance offered by the bound RNA were suggested to contribute to the stabilization of aggregation-prone proteins against aggregation [12,13], in good accordance with the previous reports on the solubilizing mechanism of highly soluble macromolecules such as N-terminal domains of multi-domain proteins and molecular chaperones [14,15]. A variety of RNA molecules that interacts with proteins inside the cells imply that RNA-mediated protein folding might be ubiquitous.

Basically, in many RNA–protein complexes, RNA molecules could induce folding of their interacting proteins in the complex. The folding of many natively unfolded proteins could be dictated by the bound RNA ligands. For example, *Bacillus subtilis* ribonuclease P protein which is disordered in solution at low ionic strength becomes structured upon binding of anions [16]. A local helical structure of the antitermination protein N of bacteriophage λ is induced upon binding of its cognate RNA, *boxB* RNA [17]. Additionally, flexible loop of the ribosomal protein L11 becomes ordered upon binding to ribosomal 23S rRNA [18].

Although the RNA binding-coupled folding has been reported in several natively unfolded proteins, still the chaperoning role of RNAs in the folding of their cognate proteins that are stable without RNA ligand binding has not yet been investigated. DnaK, the

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Escherichia coli homolog of Hsp70 chaperone, is known to interact with 5S ribosomal RNA of 120 nucleotides in size as a specific ligand [19]. Using DnaK and 5S rRNA as a model system, here we investigated potential chaperoning effect of the 5S rRNA on DnaK and showed that 5S rRNA could assist the refolding of DnaK *in vitro*.

Materials and methods

Protein expression and purification. For purification of DnaK, hexa-histidine tag was inserted at its C-terminus. The *E. coli* strain of HMS174(DE3)pLysE (Novagen) was used as an expression host. The expressed DnaK proteins were purified on Ni-affinity chromatography using HiTrap Chelating HP column (Amersham Biosciences). After analyzing the eluted fractions by SDS–PAGE, the fractions containing DnaK were pooled and dialyzed against the refolding buffer (50 mM Tris–Cl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$). The concentration of purified protein was determined according to the BCA method (Pierce).

In vitro synthesis of RNAs. The genes for 5S rRNA, 23S rRNA and 16S rRNA were obtained by PCR amplification of *E. coli* genomic DNA, using the sequence specific oligonucleotides. To synthesize RNA *in vitro*, the DNA sequences encoding rRNAs were placed under the T7 promoter. The primers used in the PCR were designed to insert BglII and HindIII sites at the 5′ or 3′ ends of the DNA templates. The DNA templates were treated with BglII and HindIII, and RNAs were produced using the RiboMAX™ large scale RNA production systems-T7 (Promega). After the reaction, RNAs were precipitated by addition of 1 volume of citrate-saturated phenol (pH 4.7): chloroform: isoamyl alcohol (125:24:1). The supernatant of the mixture was collected after centrifugation at 12,000 rpm for 2 min, and added 1 volume of chloroform: isoamy alcohol (24:1). After vortexing and centrifugation, the supernatant of the mixture was transferred to a fresh tube, and added 0.1 volume of 3 M Sodium acetate (pH 5.2) and 1 volume of isopropanol. The pellet was obtained by centrifugation at 12,000 rpm for 15 min. Following washing of the pellet with 1 ml of 70% ethanol (Sigma), the pellet was dried under vacuum, suspended in DEPC-treated water (Sigma). The unincorporated rNTPs were removed by spin columns, Illustra™ MicroSpin G-25 (GE healthcare).

In vitro refolding assay of DnaK. Purified DnaK was denatured in 4 M guanidine hydrochloride at 25 °C for 2 h. Under this condition, the protein was reported to lose its secondary and tertiary structures completely [20]. The denatured protein was refolded by 50-fold dilution of the denatured mixtures in the refolding buffer (50 mM Tris–Cl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$) in the presence or absence of RNAs. After incubation for 15 min at 37 °C, the activity of the refolded DnaK was analyzed by measuring ADPs accumulated by ATPase activity of the DnaK. For starting the ATPase reaction, ATP (Sigma) was added to the final ATP concentration of 200 μM. After the reaction at 37 °C for 30 min, the accumulated ADPs were measured by ADP hunter™ plus assay kit (Amersham) according to manufacturer's protocol.

Results

In vitro refolding of DnaK in a 5S rRNA dosage-dependent manner

Refolding studies were performed with the purified DnaK and *in vitro* synthesized 5S rRNA. Following denaturation by guanidine hydrochloride, the unfolded DnaK was diluted into final concentration of 2.45 μM in the buffer containing various concentrations of 5S rRNA. The ATPase activity of DnaK in the refolding sample, as monitored by the accumulation of ADP (see Materials and methods), was used for the calculation of the refolding yields of DnaK.

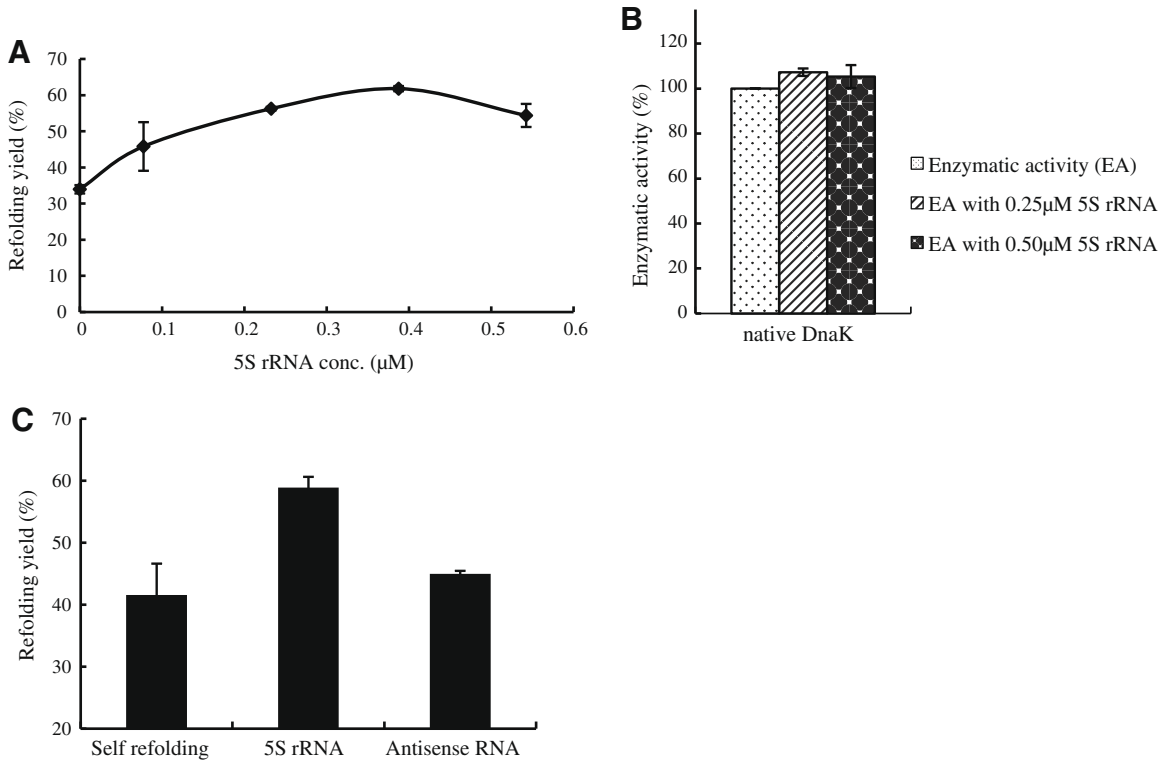

Fig. 1. 5S rRNA-assisted DnaK refolding *in vitro*. (A) The refolding yields of DnaK in a dosage-dependent manner of 5S rRNA. The purified DnaK was unfolded in 4 M guanidine hydrochloride, and refolded in various concentrations of 5S rRNA (0.077, 0.23, 0.4 and 0.54 μM). The refolding was monitored by measuring the accumulated ADPs. The refolding yields are shown as relative to the same amount of native DnaK. In all refolding experiments, the final concentration of DnaK was 2.45 μM. The assays were performed in duplicates, and the average values of refolding yields were shown. (B) The effect of 5S rRNA on the enzymatic activity of native DnaK. The mean values of the enzymatic activity were from three independent experiments, and error bars represent the standard deviations. (C) The 5S rRNA-specific chaperoning effect on the DnaK refolding. The refolding yields in the absence of RNA and in the presence of RNA (5S rRNA or antisense RNA of 5S rRNA at the final concentration of 0.4 μM) are compared.