Demonstration of the role of the DnaK chaperone system in assembly of 30S ribosomal subunits using a purified in vitro system

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ABSTRACT

Recently, there has been controversy regarding the ability of the DnaK chaperone system to facilitate *Escherichia coli* 30S subunit assembly at otherwise nonpermissive conditions. Here, we present additional data indicating that purified DnaK chaperone assembled 30S subunits are functional. Additionally, explanations for reported differences are discussed.

Keywords: DnaK chaperone system; 16S rRNA; ribosome assembly

Ribosome assembly is a highly complicated process that, despite years of work and the continuing efforts of many groups, is still not very well understood (for overview, see Williamson 2003). One avenue of research has been directed toward identifying extrinsic factors that facilitate assembly (for examples, see Alix and Guerin 1993; Charollais et al. 2003). Recently, Alix and Nierhaus (2003) reported experiments that appear to contradict our previous results showing that the DnaK chaperone system facilitates 30S subunit assembly under otherwise nonpermissive conditions (Maki et al. 2002). Here, we take the opportunity to reexamine the experiments performed by Alix and Nierhaus (2003) and to offer alternative explanations for the reported differences.

To briefly summarize our findings (Maki et al. 2002), we first searched for cellular factors that facilitate 30S subunit assembly in vitro. Toward this end, we focused on a well-known stall in assembly that occurs at low temperature and results in the production of an assembly intermediate, the 21S, reconstitution intermediate (RI) particle (Held and Nomura 1973; Traub and Nomura 1968). RI contains a subset of the small subunit ribosomal proteins and requires heat activation to assemble a full complement of proteins into a functional 30S particle. We observed that treatment of the intermediate with S100 extract at the nonpermissive temperature resulted in the formation of a peak that cosediments with 30S subunits, suggesting that factors that facilitate assembly were present in the extract. A nonribosomal protein was found associated with the intermediate, and N-terminal sequencing in combination with Western blot analysis identified this protein as DnaK. The purified DnaK chaperone system (DnaK, its two cochaperones, DnaJ and GrpE, and ATP) was shown to be sufficient to convert the sedimentation of the 21S RI particle to something resembling a 30S peak at the nonpermissive temperature. The appropriate ribosomal proteins were associated with this 30S peak after sucrose gradient sedimentation and purification. The purified particle was shown to have tRNA binding activity significantly above that of the assembly intermediate, although not as robust as the tRNA binding activity of 30S subunits formed by heat activation. Additionally, we demonstrated that DnaK selectively interacts with a subset of small subunit ribosomal proteins, of which S4 showed the strongest binding. Lastly, it was observed that overexpression of S4 could partially rescue phenotypic defects observed for a temperature-sensitive allele of *dnaK* (*dnaK756*). From these results, we concluded that the DnaK chaperone system facilitates 30S subunit assembly.

In their paper, "DnaK-facilitated ribosome assembly in *Escherichia coli* revisited," Alix and Nierhaus (2003) used two main approaches to test our conclusions. In the first approach, they assessed the kinetics of in vitro 30S subunit

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reconstitution at various temperatures in the presence and absence of the DnaK chaperone system by monitoring poly(U)-directed polyphenylalanine synthesis. The same levels of polyphenylalanine synthesis in the presence and absence of purified chaperone components were observed at all tested temperatures. In the second approach, ribosomal components from an *E. coli dnaK* null strain (BB1553; grown at the permissive temperature) were compared to wild-type components. Ribosomal subunits, both 30S and 50S, reconstituted with components isolated from the *dnaK* knockout strain were shown to participate in polyphenylalanine synthesis. Also, ribosomal proteins isolated from the *dnaK* null strain grown under permissive conditions were shown to be similar to proteins isolated from a wild-type strain. Additionally, ribosomal subunits from both the *dnaK* knockout and wild-type strains were stable when incubated up to 50°C in a 1-mM $MgCl_2$, 200-mM NH_4Cl buffer. From these studies, Alix and Nierhaus (2003) conclude, "The DnaK chaperone family is not sufficient to facilitate reconstitution of 30S subunits…"

There are two main differences in the functional experiments performed by Alix and Nierhaus (2003) and those performed by us (Maki et al. 2002) that warrant discussion. The first involves the manner in which the chaperone-treated reconstituted 30S subunits are handled prior to being assayed for function. Alix and Nierhaus (2003) assayed crude 30S subunit reconstitution mixtures for polyphenylalanine synthesis capability. In marked contrast, we used reconstituted 30S particles that were sucrose-gradient purified and then washed and concentrated on molecular sieving filters (molecular weight cut-off of 100,000; Centricon 100s) in tRNA binding assays (Maki et al. 2002). It appears that assaying purified particles is important for monitoring function of different populations of reconstituted 30S subunits, as we, too, have difficulties detecting activity of crude reconstitution mixtures prepared in the presence of the DnaK chaperone system at low temperature. In the absence of purification, it is possible that DnaK remains bound to the 30S subunit, inhibiting function. Indeed, Western blot analysis reveals that DnaK is still bound to particles that were reconstituted at low temperature in the presence of the DnaK chaperone system and then isolated from a sucrose gradient (Fig. 1, lane 2). Conversely, when the same sucrose-gradient purified particles are concentrated on Centricon 100s and washed with reconstitution buffer (containing 330 mM KCl) prior to Western blot analysis, significantly less DnaK is found associated with the particles (Fig. 1, lane 3). To allow for semiquantitative results, equal amounts of particles were examined and serial dilution of samples was also performed. Additionally, the same results were obtained using various reconstitution conditions; however, at higher reconstitution temperatures, the amount of DnaK that remains bound appears to be somewhat diminished. These results suggest that the observed differences in functional capability of reconstituted 30S subunits could be the result of the presence of DnaK. Our use of purified 30S particles is also relevant to the question raised by Alix and Nierhaus (2003) of possible assembly during our tRNA binding assay. Given our purification procedure, only proteins that were stably associated with the 16S rRNA-containing particle were present in the tRNA binding assay. This precludes the binding of additional nonassociated proteins during the tRNA binding assay at 37°C. Thus, incubation at 37°C alone could not account for our results. Our previous results in conjunction with those presented here suggest that removal of DnaK from 30S subunits during their assembly is an important step in functional 30S subunit formation.

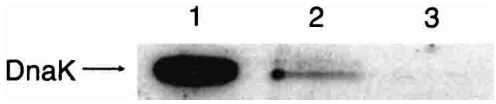

FIGURE 1. Western blot analysis of in vitro reconstituted 30S particles. Reconstituted 30S subunits were formed at 15°C under the following conditions: 16S rRNA:DnaK:DnaJ:GrpE 1:1:1:2. The resulting particles were applied to 10%–40% sucrose gradients (Culver and Noller 1999); the peak was collected and split into two equal fractions. One fraction was directly precipitated, whereas the second was concentrated and washed (with a buffer containing 330 mM KCl) on a Centricon 100 sieving filter prior to precipitation. Equal fractions were probed with monoclonal anti-DnaK antibody from StressGen Biotech. (Lane *1*) 50 ng of purified DnaK from StressGen Biotech. (Lane *2*) Sucrose-gradient purified reconstituted 30S particle. (Lane *3*) Sucrose-gradient purified and Centricon 100 treated reconstituted 30S particle.

The second difference between our experiments (Maki et al. 2002) and those of Alix and Nierhaus (2003) involves the chosen functional assay. In our previous studies, we used tRNA binding to monitor the function of reconstituted 30S subunits. We chose this assay because tRNA binding is an inherent function of the 30S subunit and thus is highly sensitive to defects in its assembly and structure. In contrast, Alix and Nierhaus (2003) monitored polyphenylalanine synthesis, an assay that is dependent on the presence of natural 50S subunits, which can often mask deficiencies in 30S subunit function. The polyphenylalanine synthesis experiments performed by Alix and Nierhaus (2003) involved the addition of an *E. coli* crude, high-speed supernatant fraction, S150 (Alix and Nierhaus 2003). We previously demonstrated that all of the DnaK chaperone components are present in an S100 extract (Maki et al. 2002) and subsequently have prepared S150 extract as described by Alix and Nierhaus (2003) (Nierhaus 1990). Western blot analysis of S150 extract reveals that DnaK, DnaJ, and GrpE are all present at levels comparable to those found in S100 extract (Fig. 2). Thus, although the authors went to some lengths to determine that the 30S subunits used to prepare the components for these experiments were devoid of DnaK, they appear to have overlooked another source of chaperone contamination. Hence, the question of whether the presence of the DnaK chaperone system alters the kinetics of the

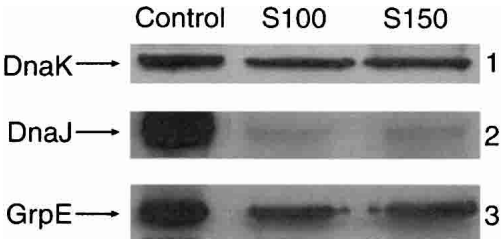

FIGURE 2. Western blot analysis of *Escherichia coli* extracts for the DnaK chaperone system. Control, 0.5 μg of purified protein (DnaK, DnaJ, or GrpE) purchased from StressGen Biotech. S100, 20 μg S100 extract; S150, 20 μg S150 extract. (Lane *1*) Membrane probed with monoclonal anti-DnaK antibody. (Lane *2*) Membrane probed with polyclonal anti-DnaJ antibody. (Lane *3*) Membrane probed with polyclonal anti-GrpE antibody. All antibodies were purchased from StressGen Biotech. S100 prepared as reported in (Maki et al. 2002). S150 prepared as reported by Nierhaus (1990).

30S subunit assembly at various temperatures remains unanswered.

We have now monitored function of sucrose-gradient purified and washed reconstituted 30S subunits, using a completely defined polyphenylalanine synthesis assay. This system utilizes purified translation factors and precharged tRNAs (Southworth et al. 2002), eliminating the requirement for S100 or S150 extract and thus circumventing the chaperone contamination problem. These experiments demonstrate that 30S subunits assembled at low temperature in the presence of the DnaK chaperone system are more active than the 21S intermediate, but less active than 30S subunits formed by heat activation (Fig. 3). Whereas the percent activity of the chaperone-treated 30S particle relative to heat-activated 30S subunits is similar to what we reported for tRNA binding, the activity of RI is greatly increased relative to our reported results. For these experiments, we performed the reconstitutions at 20°C, following the protocol of Alix and Nierhaus (2003), which might partially account for this increased activity; in our hands, at 20°C, a subpopulation of 21S particles are able to form 30S particles in the absence of any additional factors. These changes in the reconstitution conditions and in the functional assay performed, which is dependent on the activity of natural 50S subunits, result in an apparent lower level of stimulation by the DnaK chaperone system. However, in these experiments, what is actually decreased is the dynamic range of these measurements. Nonetheless, the results of our polyphenylalanine synthesis assays (Fig. 3) are consistent with our previous conclusions that the DnaK chaperone system facilitates assembly of functional 30S subunits under otherwise nonpermissive conditions.

A final point of discussion is whether DnaK plays a role in ribosome biogenesis in vivo. Toward this end, Alix and Nierhaus (2003) examined ribosomes and ribosomal components formed in a *dnaK* null strain. They observed that there is virtually no difference in thermostability between ribosomes isolated from *dnaK* knockout or wild-type strains when the knockout is grown *under the permissive conditions* (Alix and Nierhaus 2003). This is not surprising, as Alix and colleagues have previously reported that "DnaK acts during ribosome assembly itself, and not by stabilizing mature ribosomes or protecting them from thermal injury (Alix and Guerin 1993)" (El Hage et al. 2001). Thus, although the thermostability of ribosomes isolated under permissive conditions from the Δ*dnaK* strain might be of interest, its bearing on our findings is questionable. Addressing our in vivo results, Alix and Nierhaus (2003) suggested that the partial suppression by S4 (at the nonpermissive temperature) of the altered polysome profiles in *dnaK756* (a temperature-sensitive allele of *dnaK*) is the result of its role as a translational repressor. This is a possibility; however, overexpression of S8, another translational repressor, did not yield the level of suppression observed with S4 (Maki et al. 2002). This suggests that changes in control of ribosome component production could not solely account for these results. Therefore, although the role of the DnaK chaperone system in ribosome biogenesis in vivo is still not well understood, it remains clear that a role for these chaperones in 30S subunit assembly can be demonstrated using a purified in vitro system.

The assembly of ribosomes is a highly complicated process. Given the importance of ribosome assembly to cell viability, there are likely many factors that are involved in

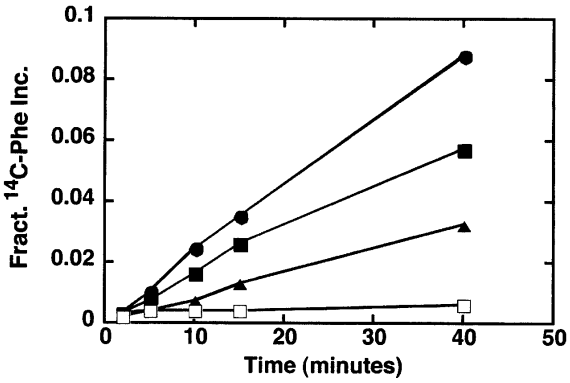

FIGURE 3. Polyphenylalanine synthesis by purified in vitro reconstituted *Escherichia coli* 30S particles. Reconstitutions were performed under the conditions of Alix and Nierhaus (2003). Polyphenylalanine generated by purified 30S particles reconstituted under normal, high-temperature (42°C) conditions (solid circles) compared to natural 50S subunits alone (open squares), or particles reconstituted at low temperatures (20°, 21S) (solid diamonds), or as for the 21S reconstitution but in the presence of the DnaK chaperone system (solid squares). Chaperone conditions were similar to those of Maki et al. (2002) (16S rRNA:DnaK:DnaJ:GrpE 1:1:1:2 with 1mM ATP). The polyphenylalanine synthesis was carried out essentially as described (Southworth et al. 2002), by incubating 30S particles and 50S subunits (0.3 μM) with poly-uridine (0.35 mg/mL), ^{14}C Phe-tRNA^{Phe}, His-tagged EF-G (0.3 μM), His-tagged EF-Tu (2 μM), GTP (1.4 mM), phosphoenolpyruvate (3.5 mM), and pyruvate kinase (14 μg/mL). Reactions were carried out in 80 mM K^+-HEPES pH 7.6, 13 mM $MgCl_2$, and 100 mM KCl.

this process. To date, our approach of searching for extrinsic 30S subunit assembly factors has focused on a small step in this assembly process and thus far led us to the DnaK chaperone system. As previously suggested (Maki et al. 2002), because ribosome biogenesis is of paramount importance and because *dnaK* is not essential (Paek and Walker 1987), it is likely that other factors involved in ribosome biogenesis have yet to be identified. This is consistent with conclusions of Alix and colleagues (El Hage et al. 2001): "DnaK is necessary [for ribosome biogenesis] above 42°C, but not below that temperature, *provided* that other HSPs are constitutively expressed…" Therefore, it follows that additional factors also facilitate ribosome assembly. We agree with Alix and Nierhaus (2003) that more sophisticated approaches for studying assembly are warranted and hope that advances can be made in this area. However, until such time, continued efforts using available approaches and minimal, purified systems will hopefully allow identification of an ensemble of ribosome biogenesis factors.

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