Synthesis of chloramphenicol acetyltransferase in a coupled transcription–translation in vitro system lacking the chaperones DnaK and DnaJ

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Abstract **A trimeric enzyme chloramphenicol acetyltransferase (CAT_I) has been synthesized in the Zubay system genetically depleted from DnaK and DnaJ. Most of CAT formed in the system fail to assemble into an active trimer. Instead CAT is accumulated in either a GroEL-bound complex or as an inactive monomer. Addition of purified DnaK and DnaJ to the system prior to the start of protein synthesis leads to the increase of the specific activity of formed CAT. A portion of exogenous DnaK and DnaJ added to the system associate with nascent polypeptide chains in the ribosomes. DnaK also comigrates with 50S-ribosomal subunits.**

Key words: Protein folding; Chaperone; Chloramphenicol acetyltransferase; DnaK; DnaJ, in vitro protein synthesis

1. Introduction

Molecular chaperones are proteins that through a transient interaction with a variety of other proteins assist in their folding, assembly–disassembly or transportation to proper cellular locations (for review see [1]). Chaperones are classified into several families based on their molecular weights and similarity in structure. For the last few years chaperones of Hsp70 and Hsp60 families were the subjects of the most extensive research.

Members of the Hsp70 family are highly conserved ubiquitous proteins that can be found in all known species from *E. coli* to humans in various cellular compartments: cytoplasm, mitochondria, chloroplasts and endoplasmic reticulum. In *E. coli* Hsp70 family is represented by protein DnaK. In many well studied processes DnaK works together and interacts with its 'cohort' proteins: DnaJ and GrpE [2]. In cases of λ and P1 phage replication, DnaK and the cochaperones assist in disassembly of the quaternary protein structures [3–4]. In two other examples the action of DnaK, DnaJ and GrpE leads to reactivating of heat inactivated enzymes: firefly luciferase [5] and RNA-polymerase [6]. DnaK binds with various affinities to short peptides and protein fragments in extended conformations [7–10]. The exact mechanism of DnaK action is not clear and may not be the same for different cases.

It is widely accepted that molecular chaperones participate in folding of newly synthesized proteins. Less clear are the particular functions of different chaperones and their place in the folding pathway. Eukaryotic Hsp70 family chaperones: SSB1, SSB2 in *Saccharomyces cerevisiae* [11] and Hsp73 in mammalian cells [12] have been found in association with nascent polypeptides. Previously we reported the association of a fraction of cellular DnaK and DnaJ with nascent chains in translating ribosomes and posttranslational interaction of many full-size newly synthesized proteins with Hsp60 family chaperone GroEL in *E. coli* cells [13]. But in general, especially taking into account that dnaK null mutants are viable, the data in support of the role of bacterial DnaK in folding of newly synthesized proteins remain still scarce and mostly indirect.

In the present study we have constructed a coupled transcription–translation in vitro system completely lacking both DnaK and DnaJ and studied the effect of the added purified chaperones on the activity of de novo synthesized enzyme chloramphenicol acetyltransferase (CAT_I). CAT is an important widely used enzyme (for review see ref. [14]). It is active as a trimer and only anecdotal data about its de novo folding and assembly are available (for example see ref. [15]).

2. Materials and methods

2.1. Strains, plasmids and proteins

As a strain with a deletion of both *dnaK* and *dnaJ* for making the Zubay system we used a derivative of PK101 [16] selected by the ability to grow at LB-medium. We used MG1655 as a wild type strain [16]. As a source of CAT_I in the system we used a plasmid pACYC184 commonly used as a cloning vector. DnaK protein, anti-DnaK and anti-DnaJ antibodies were kindly provided by A. Gragerov. DnaJ was a generous gift of R.McMacken.

2.2. Synthesis of CAT in vitro

We used the coupled *E. coli* system developed by Zubay [17] with modifications by Pratt [18] with the exception that calcium acetate was not included into the system. For the kinetics experiments all components of the sample for each separate time-point were mixed in a separate tube in total volume of 30 μl at 0°C. Where appropriate DnaK and DnaJ were added up to concentrations of 70 μg/ml (near 3% of total protein) and 8 μg/ml, respectively. The reaction of in vitro protein synthesis was started by placing the tube into a water-incubator at 37°C and after appropriate time 8 μl were withdrawn for analysis in a 8–20% gradient SDS-PAGE and further quantitation of the storage phosphor image (Molecular Dynamics PhosphorImager) of [^{35}S]methionine labeled CAT; the remaining 22 μl were diluted in 100 μl of 0.25 M Tris-HCl pH 8.0 (such dilution completely blocked the protein synthesis and posttranslational assembly of CAT in the system); then CAT activity was determined as published [19].

2.3. Sucrose gradient fractionation of CAT-synthesizing mixture

The in vitro synthesis was performed in 150 μl for 30 min. Then 150 μl of a diluting buffer: 44 mM tris-acetate pH 8.2, 56 mM potassium acetate, 28 mM ammonium acetate and 12 mM magnesium acetate, containing or not (for mock-treatment) puromycin to the final concen-

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Abbreviations: CAT, chloramphenicol acetyltransferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence

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tration 5 mM were added (two-fold dilution of the system blocked the protein synthesis). The tubes were incubated for another 10 min at 37°C and placed on ice. Then the reaction mixtures were loaded on 14–40% sucrose gradients in the diluting buffer. The gradients were centrifuged at 40,000 rpm in SW 41 Beckmann rotor for 5 h at 4°C; 500 μl-fractions were collected, proteins were precipitated with trichloracetic acid, dissolved in SDS-PAGE sample buffer lacking Bromphenol Blue and glycerol, 260 nm absorption of each fraction was measured and then after the addition of Bromphenol Blue and glycerol the fractions were analyzed by 8–20% gradient SDS-PAGE and if appropriate by Western blotting against anti-DnaK or anti-DnaJ antibodies and visualized by using a conjugate of anti-rabbit antibodies with horse radish peroxidase and ECL substrate mixture (Amersham). [^{35}S]methionine labeled CAT was visualized by autoradiography or alternatively quantified by using Molecular Dynamics PhosphorImager.

3. Results and discussion

CAT_I was synthesized in the Zubay S30 system constructed from a strain bearing a deletion of *dnaK* and *dnaJ* genes. The system was characterized by a lower rate of protein synthesis and much lower CAT activity formed in comparison with a similar system derived from a wild type strain (not shown). As shown in Fig. 1, a long delay of about 20 min occurs between the appearance of full-size CAT chains and the appearance of CAT activity in the reaction mixture, so folding and assembly of active CAT trimers take a surprisingly long time in this system. Addition of the purified DnaK and DnaJ prior to the start of protein synthesis does not effect the rate of protein synthesis in the system but shortens the delay and leads to the increase of CAT activity at each separate time-point (Fig. 1). Actually, DnaK alone provides the main contribution into the stimulatory effect (not shown). Depending on the conditions of a particular in vitro synthesis reaction (for example the concentration of S30-extract) the stimulatory effect of DnaK may be stronger or weaker. The effect is the strongest if the specific CAT activity without DnaK is low but distinct from zero.

The stimulation of the specific CAT activity formed in the system by DnaK implies that some portion of the enzyme is synthesized inactive. To find out in what forms CAT protein

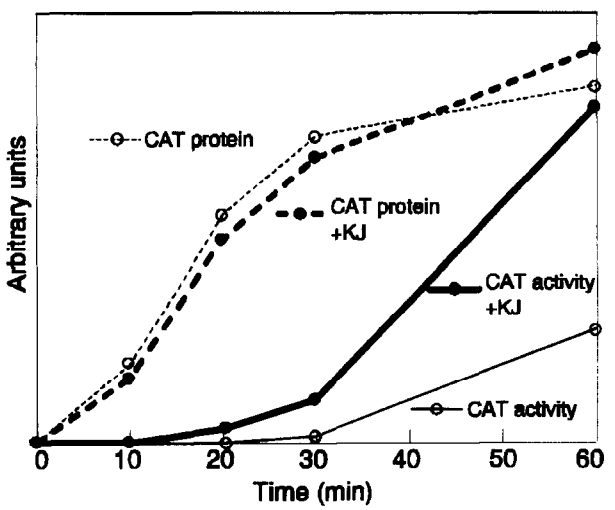

Fig. 1. Kinetics of formation of full-size CAT-protein (dashed line) and CAT activity (solid line) in the Zubay system in the presence (filled circles) and absence (open circles) of added purified DnaK and DnaJ. The Zubay system was constructed from the strain lacking *dnaK* and *dnaJ*.

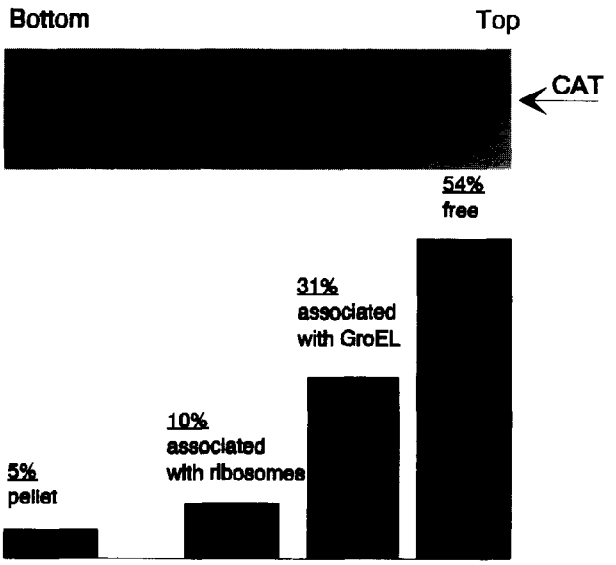

Fig. 2. Newly synthesized CAT moves in sucrose gradient as four different forms: pellet, associated with ribosomes, associated with GroEL and free. The CAT-synthesizing Zubay system constructed from the strain lacking *dnaK* and *dnaJ* was subjected to sucrose gradient centrifugation. An autoradiogram at the top shows SDS-PAGE of the sucrose gradient fractions. The fractions are arranged sequentially from the bottom to the top of the sucrose gradient. An arrow shows full-size CAT-protein. The positions of the GroEL and ribosomal peaks (not shown) are located exactly at the same place where the corresponding GroEL-associated and ribosome-associated peaks of CAT are. The diagram at the bottom illustrates the percentage composition of different forms of CAT in the reaction mixture. Note that the pellet fraction (the first left lane at the autoradiogram) was loaded on the gel 4-fold the regular amount and looks exaggerated.

is present in the system when it fails to reach the active state, the reaction mixture after the protein synthesis was analyzed by sucrose gradient centrifugation. As shown in Fig. 2 the aggregated portion comprises only about 5% of CAT protein, near 10% move in ribosomal fractions, about 31% comigrates with GroEL and near 54% stays at the top of the gradient. The further Superose12 size-column fractionation of the top fractions (not shown) revealed that the overwhelming majority of CAT protein from these fractions is represented by the inactive 26 kDa monomer. So in this particular system the major amount of CAT protein fails to assemble into an active trimer and instead accumulates in several inactive forms remaining mostly soluble. Actually the presence of CAT-protein that fails to assemble into an active trimer is not an absolutely unique feature of the system lacking DnaK and DnaJ. The same inactive forms of the enzyme but in different relative amounts can be found in the system generated from the wild type strain.

To provide a stimulatory effect it is necessary for DnaK to be present in the system from the beginning of protein synthesis. The addition of the chaperone after the termination of protein synthesis by tetracycline that was followed by the incubation up to 2 h has no effect on the activity of formed CAT (not shown). The action of DnaK at early time-points of synthesis and folding of CAT is consistent with the concept of cotranslational action of DnaK on nascent chains.

In an attempt to find out whether in our in vitro system exogenous DnaK and DnaJ associate with nascent polypeptide chains we fractionated the CAT synthesizing mixture contain-

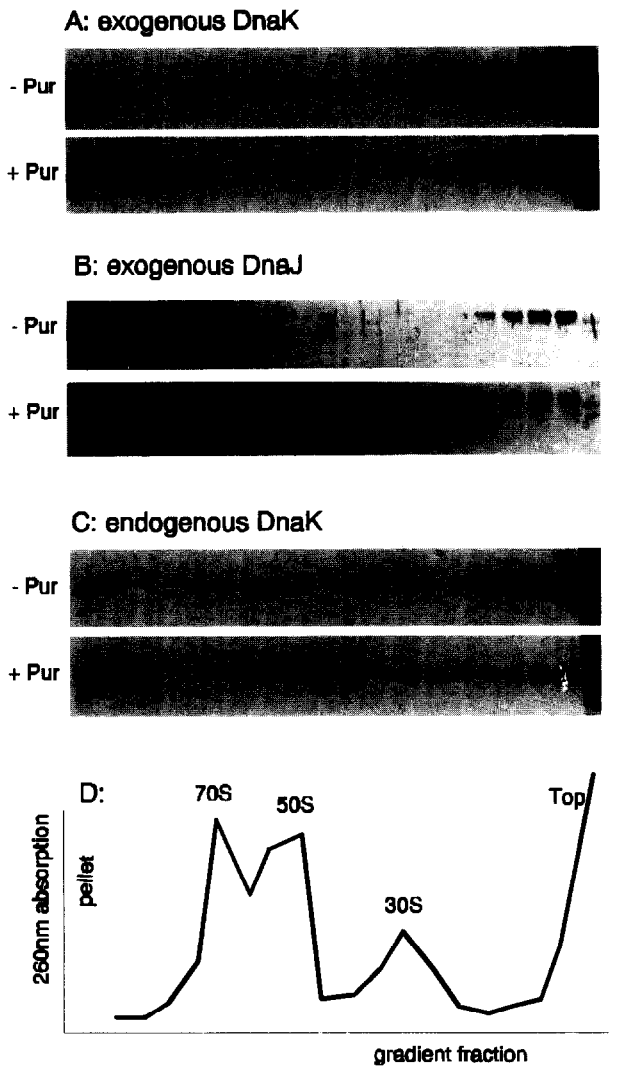

Fig. 3. Association of DnaK and DnaJ with ribosomal subunits. CAT-synthesizing Zubay system was subjected to sucrose gradient centrifugation then SDS-PAGE of the fractions and immunoblotting against anti-DnaK or anti-DnaJ antibodies. The presence or absence of puromycin treatment of the system before the centrifugation is designated as +Pur or −Pur, respectively. Pellet fraction is shown at the first left lane at each immunoblot. (A) Anti-DnaK immunoblots. The Zubay system was constructed from the strain lacking *dnaK* and *dnaJ*. Purified DnaK and DnaJ were added prior to the start of protein synthesis. (B) Anti-DnaJ immunoblots. The Zubay system was constructed from the strain lacking *dnaK* and *dnaJ*. Purified DnaK and DnaJ were added prior to the start of protein synthesis. (C) Anti-DnaK immunoblots. The Zubay system was constructed from the wild type strain. No chaperones were added. (D) Typical UV-260 nm absorbtion profile corresponding to the above sucrose gradients. The positions of 70S-ribosomes, 50S- and 30S-ribosomal subunits are designated.

ing exogenous DnaK and DnaJ in sucrose gradient and checked a possible comigration of the chaperones with ribosomes. As shown in Fig. 3a,b a part of both exogenous DnaK and DnaJ may be found in the area of the gradient where ribosomes migrate, and the major amount of the ribosome-associated chaperones are observed in the heavy part of the 70S-ribosomal peak. Almost all DnaK and DnaJ disappeared from the 70S-area after treatment of the system with puromycin suggesting that this fraction of the chaperones is bound to ribosomes via nascent polypeptide chains (Fig. 3a,b). Interestingly, when we fractionated the same way a system derived from the wild type strain containing endogenous DnaK and DnaJ we found that endogenous DnaK is distributed inside a wider area that includes the locations of both 70S-ribosomes and 50S-ribosomal subunits (Fig. 3c). DnaK from the 70S-area can be released by puromycin, indicating its binding to nascent chains, while 50S-associated DnaK remains bound to large ribosomal subunits after the puromycin treatment (Fig. 3c), implying binding independent of nascent chains. The physiological importance of the interaction of DnaK with large ribosomal subunits is not clear, but may reflect the role of DnaK in the assembly of ribosome or DnaK involvement in the ribosomal subunits association–dissociation cycle.

In general our results do not show that DnaK is indispensable for the formation of active CAT. Such statement would contradict the genetics data and our own observation that in appropriate experimental conditions a big amount of CAT activity can still be formed during in vitro synthesis even without DnaK and DnaJ. But our data suggest that DnaK can assist the formation of the active CAT-trimer in conditions where its effective de novo folding and assembly is prevented.

The exact mechanism of DnaK action in case of CAT is not clear. Earlier it was reported that the simultaneous addition of two purified chaperones DnaK and GroEL into the biochemically created chaperone-deficient Zubay system stimulates the formation of the active mitochondrial rhodanese by means of more efficient translation termination [20]. In case of CAT we did not observe any effect of DnaK on the amount of full-size CAT-chains associated with ribosomes (not shown).

Because the main side-product of in vitro CAT synthesis appeared to be inactive monomer it may be proposed that DnaK could interact with CAT protein at the early stage of its folding and either help to maintain the CAT monomer in the state competent for further trimerisation or help to convert it into such state.

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