GroEL and GroES Increase the Specific Enzymatic Activity of Newly-Synthesized Rhodanese If Present during in Vitro Transcription/Translation†

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ABSTRACT: Enzymatically active mammalian rhodanese, a mitochondrial matrix enzyme, which has been found to require assistants for efficient refolding in vitro, has been synthesized from a plasmid in a cell-free, fractionated, coupled transcription/translation system derived from *Escherichia coli*. The bacterial chaperonins, GroEL and GroES, along with the rhodanese substrate thiosulfate greatly enhance the specific enzymatic activity of the rhodanese polypeptide that is formed. Indirect evidence suggests that the effect of the GroEL/ES chaperonins is on ribosome-bound nascent peptides. The in vitro transcription/translation system produces sufficient amounts of rhodanese to provide a system for studying factors that control the initial steps in folding of nascent proteins.

Chaperonins and heat shock proteins have been implicated in various cellular functions [for reviews, see Georgopoulos (1992) and Gething and Sambrook (1992)]. These include aiding in the correct folding of denatured proteins as well as maintaining proteins in an unfolded form during cytosolic transport and membrane translocation. Recently, it was shown that the eukaryotic chaperone, hsp70, may interact with the ribosome-bound nascent polypeptide chain, thus, presumably preventing misfolding or aggregation of the formed product (Beckmann et al., 1990; Nelson et al., 1992).

We have shown previously that the *Escherichia coli* chaperonins GroES and GroEL act in concert to promote the correct folding of the mitochondrial sulfurtransferase enzyme rhodanese (Mendoza et at., 1991a). Experiments presented here suggest that these two chaperonins may prevent the misfolding of newly-synthesized rhodanese by interacting with the nascent polypeptide chain. We used a modified version (Kudlicki et al., 1992) of the cell-free transcription/translation system introduced by Zubay (Zubay, 1973). This system is well suited since it contains no endogenous activity interfering with the rhodanese assay. Furthermore, pretreatment of the S30 under the conditions used eliminates the endogenous DNA and mRNA in this fraction. In these studies, we also use a ribosomal fraction (RF)[1] which is purified from the S30 fraction (Kudlicki et al., 1992), in place of the S30. This RF is more efficient than S30, yet it contains less soluble proteins than the latter fraction. Additionally, nonlinearized rhodanese-encoding plasmid has been used in this system, resulting in less degradation of DNA and thus greater protein formation.

Rhodanese has emerged as an excellent model for studies involving protein folding with either chaperonins (Mendoza et al., 1991a; Martin et al., 1991; Bochkareva et al., 1992), detergents (Tandon & Horowitz, 1989), mixed micelles (Zardeneta & Horowitz, 1992a), or liposomes (Zardeneta & Horowitz, 1992b). Rhodanese is a monomeric 33-kDa sulfurtransferase enzyme which is found in the matrix of mammalian mitochondria. It contains four cysteine residues, yet it has no disulfide bonds (Ploegman et al., 1978). Its only posttranslational processing is the cleavage of its N-terminal methionine residue (Miller et al., 1991). Folding of rhodanese can be easily monitored by measuring enzymatic activity since its sulfurtransferase activity is lost in the first stage of denaturation (Tandon & Horowitz, 1989). Finally, this enzyme is well suited for these studies since conditions are known where unfolded rhodanese will not spontaneously fold into an active species without the assistance of ancillary factors (Mendoza et al., 1991b).

In the present work, we show that GroES and GroEL, along with the rhodanese substrate thiosulfate greatly enhance the activity of rhodanese synthesized in the coupled transcription/translation system.

MATERIALS AND METHODS

Materials

E. coli tRNA and nucleoside triphosphates were purchased from Boehringer Mannheim; [^{14}C]leucine was obtained from NEN–Du Pont; all other biochemicals (e.g., pyruvate kinase, amino acids, phosphoenol pyruvate, rifampicin, folinic acid) were purchased from Sigma; X-ray film (Hyperfilm) was from Amersham, as was the detection kit for peroxidase-linked second antibody. All other chemicals were of reagent grade.

E. coli K12 (strain A19) was a gift from Drs. Wittmann and Nierhaus (Berlin, Germany). A plasmid containing both chaperonins (pGroESL) was obtained from Dr. G. H. Lorimer (Du Pont Corp., Wilmington, DE).

GroEL antibodies were kindly provided by Dr. J. Gould-Kostka (FDA, Bethesda, MD).

Methods

Rhodanese Plasmid Preparation. The rhodanese plasmid was prepared using a pET-11d (Novagene) vector in which

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[1] Abbreviations: RF, ribosomal fraction; DTT, dithiothreitol; Tris–acetate, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol acetate; Tris-HCl, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol chloride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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the coding sequence for rhodanese is under the control of the T7 promoter (Miller et al., 1991).

Chaperonin Purification. The chaperonins GroEL and GroES were expressed and purified from *E. coli* containing the plasmid pGroESL as previously described (Goloubinoff et al., 1989). Purified protein was dialyzed against 50 mM Tris-HCl (pH 7.5), 1 mM DTT, made 10% (v/v) in glycerol, and stored at −80 °C. Concentrations were calculated using extinction coefficients (280 nm) of 1190 M^{-1} cm^{-1} for GroES and 13 900 M^{-1} cm^{-1} for GroEL (Viitanen et al., 1990) and molecular masses of 10 kDa and 60 kDa, respectively.

Transcription/Translation. The system used to transcribe and translate the rhodanese plasmid [cf. Kudlicki et al. (1992)] contained, in a total volume of 30 μL, 55 mM Tris–acetate (pH 7.8), 2 mM DTT, 1 mM ATP, 0.8 mM each GTP, CTP, and UTP, 2% poly(ethylene glycol)-6000, 25 mM phosphoenol pyruvate, 0.4 mM cAMP, 36 mM NH_4OAc, 72 mM KOAc, 16 mM $Mg(OAc)_2$, 0.5 mM EDTA, 42 μM [^{14}C]leucine (40 Ci/mol) and 83 μM all other amino acids, 0.035 mg/mL folinic acid, 0.33 mg/mL *E. coli* tRNA, 0.17 mg/mL rifampicin, 0.01 mg/mL pyruvate kinase, 0.04 mg/mL T7 polymerase, 0.06 mg/mL rhodanese plasmid, and either 5 μL of the S30 fraction or 1 A_{260} unit of the ribosomal fraction (RF). Sodium thiosulfate or GroEL and GroES were added to the transcription/translation reaction mixture for some experiments as indicated. Note that the S30 fraction was used in all experiments, unless indicated otherwise. After a 30-min, 37 °C incubation, the total protein product formed was quantified by measuring the amount of radioactivity in a trichloroacetic acid precipitated fraction as described (Kudlicki et al., 1987). The amount of rhodanese synthesized (assuming all protein formed was rhodanese) was calculated on the basis of the radioactivity and the number of leucine residues per rhodanese (=24). Total active rhodanese synthesized was determined by assaying an aliquot of the reaction mixture as described below. Aliquots from the reaction mixture were analyzed by SDS–PAGE, followed by autoradiography as described earlier (Kudlicki et al., 1987).

The S30 fraction was prepared as previously described (Zubay, 1973), from *E. coli* K12 (A19), while the RF was purified from the S30 as described (Kudlicki et al., 1992).

Rhodanese Assay. Rhodanese which was used as a standard in assays and polyacrylamide gels was purified as previously described (Horowitz, 1978). The enzyme's activity was assayed with the substrate, thiosulfate, as described (Sorbo, 1953; Wang & Volini, 1973) and quantified by measuring the absorption at 460 nm of the complex between the product, thiocyanate, and ferric ions. Molar amounts of active rhodanese was determined from its molecular mass of 33 kDa and the definition established earlier (Jarabak & Westley, 1974), where 1 μg of enzyme gives 1.0 OD unit at 460 nm after a 1-min, 25 °C incubation in the assay described above.

Immunological Assay for GroEL. Fractions containing GroEL were subjected to SDS–PAGE. The proteins from the gel were electrophoretically transferred to a PVDF membrane (Westran, Schleicher & Schuell) by semidry blotting using Bio-Rad's TransBlot cell. The membrane was consecutively incubated with 4% milk powder, GroEL monoclonal antibodies, and anti-mouse antibodies to which peroxidase was covalently attached (Amersham). The membrane was developed using Amersham's ECL (enhanced chemiluminescence) reagents and procedure. The ELISA was carried out as previously detailed (Fullilove et al., 1984) except that the same GroEL-containing fractions were analyzed with the same antibodies indicated above. ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (from Zymed) together with H_2O_2 was the substrate in the ELISA.

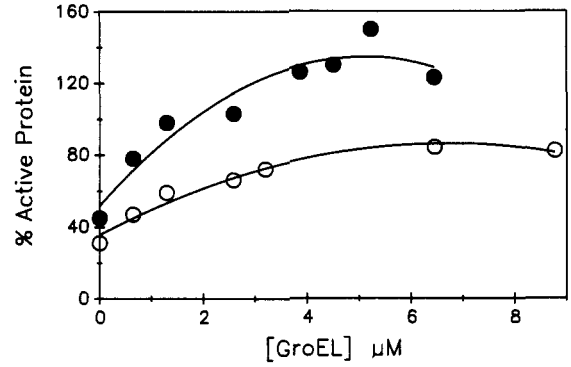

FIGURE 1: Percent of active rhodanese synthesized in the in vitro translation/transcription assay in the presence (filled circles) or absence (open circles) of 15 mM thiosulfate, at various concentrations of chaperonins. The abscissa represents the concentration of GroEL in the reaction; in each case the amount of GroES was 90% of that of GroEL. The ordinate represents the percent activity calculated as detailed under Methods.

Table I: Effect of Chaperonins and/or Thiosulfate on Protein Synthesis and Enzymatic Activity

additions	rhodanese synthesized (pmol)	enzyme activity [A_{460} units/(min·pmol); ×10^{-4}]
none	12.1	109
GroES	12.2	105
GroEL	12.0	103
GroES,GroEL	12.2	245
thiosulfate	5.8	162
GroES, GroEL, thiosulfate	4.9	358

RESULTS

The Activity of Newly Synthesized Rhodanese Is Modulated by GroEL and GroES. Rhodanese was synthesized in the presence and absence of thiosulfate from the rhodanese plasmid using the transcription/translation system detailed under Methods. Total protein synthesized and the amount of rhodanese activity were measured as described. The lower curve of Figure 1 shows that the percent of enzymatically active rhodanese synthesized in the absence of thiosulfate correlates with increasing chaperonin concentrations up to a GroEL concentration of approximately 6.45 μM, where the percent of active enzyme produced reached 84% and then leveled off. In these experiments we used a molar ratio of 1:0.9 GroEL to GroES; this ratio was found empirically to give the highest percentage of active enzyme under the conditions used. GroEL plus GroES had no effect on total protein synthesized in the system (Table I).

Both GroEL and GroES are required for enhancement of active enzyme production. Either protein alone is not sufficient to increase basal levels of the percentage of rhodanese synthesized as an active enzyme.

In a separate experiment, we showed that if protein synthesis was carried out in the absence of added chaperonins, and then the chaperonins were added immediately after the standard incubation time of 30 min at 37 °C, there was no effect on the specific activity of the synthesized rhodanese even after an additional incubation period. This result suggests that the nascent rhodanese peptide may misfold during synthesis or as it is released from the ribosome and that this enzymatically inactive enzyme cannot be reactivated by GroEL/ES. We have shown previously that the GroEL/ES system does not

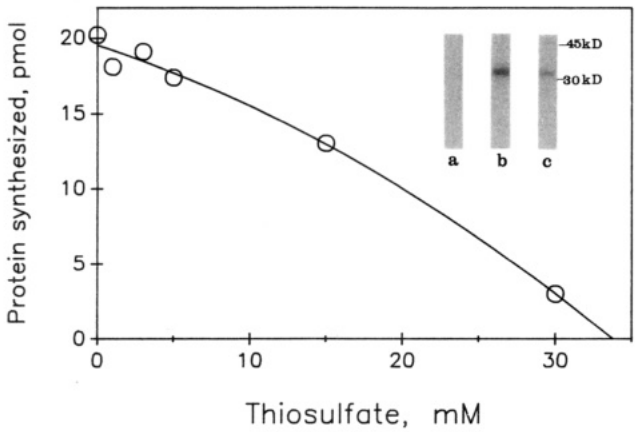

FIGURE 2: Transcription/Translation assay carried out as detailed under Methods, in the absence of chaperonins, using the rhodanese plasmid with the indicated amounts of sodium thiosulfate. Total protein synthesis was determined by the amount of [^{14}C]leucine incorporated. Insert: Reaction mixtures after coupled transcription/translation were analyzed by SDS–PAGE followed by autoradiography. The autoradiograms are shown. Tracks: (a) incubation mixture without plasmid; (b and c) with plasmid; (c) in the presence of 15 mM thiosulfate. The labels 30 kD and 45 kD indicate the positions of marker proteins, carbonic anhydrase and ovalbumin, respectively.

have an effect on inactive rhodanese that was misfolded into an inactive form during renaturation. (Mendoza et al., 1991c).

Thiosulfate Increases Specific Activity of in Vitro Synthesized Rhodanese. Studies in the refolding of denatured rhodanese have shown that the substrate, sodium thiosulfate, is an essential component needed for the reactivation of this enzyme (Tandon & Horowitz, 1989). This appears to be related to the ability of this compound or other reductants to form a sulfane sulfur at cysteine 247 at the active site of the enzyme (Ploegman et al., 1978), a necessary structure for enzymatic activity (Westley, 1973). However, under certain experimental conditions, rhodanese may fold into an inactive form which has the spectral characteristics of a correctly folded species; addition of thiosulfate results in the recovery of enzymatic activity (Horowitz & Criscimagna, 1990). These results suggest that thiosulfate is not required for the initial folding steps, but perhaps only to produce the sulfane sulfur moiety in Cys-247, and any small final conformational changes which may result from the presence of the persulfide group at this amino acid. Therefore, we attempted to synthesize rhodanese from its plasmid under the conditions described above but in the presence of varying amounts of thiosulfate. Figure 2 shows that increasing amounts of thiosulfate inhibit total protein synthesis. About 50 mM thiosulfate gives maximal reactivation for the in vitro refolding of rhodanese; however, even 30 mM thiosulfate reduced total protein synthesis by 85%. We found that optimal enzymatic specific activity was obtained when 15 mM thiosulfate was included during the transcription/translation with or without chaperonins added (data not shown). The upper curve in Figure 1 shows that the percent of active enzyme synthesized reaches a maximum at approximately 4 μM GroEL (and therefore 3.6 μM GroES) in the presence of 15 mM sodium thiosulfate. Interestingly, thiosulfate alone increases the synthesized enzyme's specific activity significantly over basal levels. The results presented in Figure 1 indicate over 100% active enzyme which may be regarded as a misleading statement. The calculations are based on a definition given by Jarabak and Westley (1974) that 1 μg of rhodanese has an enzymatic activity of 1.0 A_{460} unit after incubation for 1 min at 25 °C. We kept this convenient definition even though our incubations were done at 37 °C.

The production of active rhodanese in the transcription/translation system using the rhodanese plasmid gives a linear increase over time in the amount of active enzyme synthesized for at least 13 min in the absence of chaperonins and in the presence of thiosulfate and at least 10 min in the absence of thiosulfate (data not shown). We have recently shown that this same system gives linear increases in protein synthesis over a 30-min period (Kudlicki et al., 1992).

Other Factors Which Affect Rhodanese Synthesis and Activity in the in Vitro Transcription/Translation System. Besides the amount of chaperonins and thiosulfate which, as shown above, greatly influence the synthesis and activity of newly synthesized rhodanese, there are other factors in the reaction mixture which have been optimized for the efficient production of active enzyme. One of these is the amount of S30 fraction used. Low amounts of this component gave low protein synthesis, while excess S30 (relative to the amount indicated under Methods) results in the production of higher amounts of protein, but correspondingly lower specific activities of the enzyme (data not shown). Rhodanese is produced at a much higher rate under the latter conditions. The amount or recycling rate of the chaperonins may become the limiting factor for correct folding of the nascent polypeptides assuming the proposed recycling of chaperonins occurs as proposed [see review by Georgopoulos (1992)]. Varying the amount of rhodanese plasmid in the system gave results similar to those described for variation of S30 fraction. The explanation may be similar to that proposed above.

In addition, synthesis was carried out using the ribosomal fraction (RF) rather than the S30. The RF was isolated from the S30 preparation by centrifugation thereby eliminating approximately 80% of the protein of the S30 fraction. This ribosome preparation has proven to be more effective than S30 in the expression of coding sequences from different plasmids (Kudlicki et al., 1992). This also occurs for the synthesis of rhodanese with coupled transcription/translation from the rhodanese plasmid. In the standard assay (see Methods) about 19.7 pmol of rhodanese was synthesized with S30, whereas 25.5 pmol of rhodanese protein was formed using the RF. However, very similar results were obtained with the RF as described above for the S30 fraction: suboptimal amounts of RF resulted in less rhodanese protein synthesized but higher specific activity of the enzyme produced. Nearly identical effects of thiosulfate and/or GroEL and GroES were observed as presented above for the S30 fraction (data not shown).

Endogenous Chaperonins Are Present in S30 and RF Fractions. Basal levels of active rhodanese were produced in the transcription/translation system in the absence of added chaperonins with the S30 fraction and the RF, respectively. We attempted to determine if this basal activity was due to spontaneous correct folding of the nascent polypeptide or perhaps due to the presence of small amounts of endogenous chaperonins in the fractions used. The S30 and RF fractions as well as a sample of purified GroEL were analyzed by western blotting followed by immunodetection of the chaperonin using monoclonal anti-GroEL antibodies. The results showed that the amount of endogenous chaperonin in either fraction (S30 or RF) was approximately $^1/_8$ the amount of exogenously added GroEL (Figure 3). These ratios were confirmed by ELISA (data not shown). The results allow the conclusion that the amount of chaperonins may be crucial for the production of active rhodanese and suggest that the endogenous

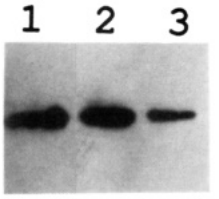

FIGURE 3: Detection of endogenous GroEL in the S30 and RF using monoclonal antibodies. Samples from either the S30, RF, or purified GroEL were analyzed by SDS–PAGE, followed by immunoblotting. The amount of the chaperonin was subjectively quantified from the blot. Lanes 1–3 represent 5 μL of S30, 1.5 A_{260} units of RF, and 0.4 μg of GroEL, respectively. These quantities represent 100%, 150%, and 6.6%, respectively, of the amounts present in the optimized transcription/translation assay.

levels of these factors may not be adequate to facilitate maximum correct folding of the nascent polypeptide under the standard conditions used in the transcription/translation system.

DISCUSSION

In this study we have shown that the chaperonins, GroEL and GroES, greatly influence the activity of rhodanese synthesized from a nonlinearized rhodanese plasmid in an in vitro transcription/translation system. Though we have not proven that these chaperonins bind the nascent polypeptide while it is bound to the ribosome, there is indirect evidence which suggests that this probably occurs. First, we have shown that GroEL binds to unfolded rhodanese but will not bind to the improperly folded inactive enzyme (Mendoza et al., 1991c). Thus, the large increase in enzymatic activity seen upon addition of chaperonins during refolding of denatured rhodanese must be due to the binding of an unfolded protein by GroEL and subsequent reactivation by GroES and ATP. If the synthesized protein is released from the ribosome prior to the addition of chaperonins, as for the experiments described in the text, then presumably the chaperonins would have no effect because they do not bind the misfolded protein. Experiments carried out in this study show, indeed, that chaperonins do not activate rhodanese protein when they are added after the standard incubation at which time most of the nascent peptides have been completed and released from the ribosomes. It has been reported that the eukaryotic chaperone hsp70 binds to the nascent, ribosome-bound polypeptide chain (Beckmann et al., 1990; Nelson et al., 1992). However, there is yet no evidence that its bacterial analogue, DnaK, a 63-kDa protein which has 48% sequence identity to hsp70 (Bardwell & Craig, 1984), binds to nascent polypeptides.

Experiments in which refolding of rhodanese was studied under the influence of DnaK in conjunction with DnaJ, GrpE, and ATP followed by transfer to GroEL led Langer et al. (1992) to the hypothesis that a similar sequence might occur on the ribosomes during protein synthesis. There are no experimental results yet to prove or disprove this hypothesis. Basal levels of active rhodanese were produced in the coupled transcription/translation system in which either the S30 or RF was used. Amounts of endogenous GroEL appeared to be nearly the same in both fractions (Figure 3). The effect of thiosulfate on the RF was similar to that obtained with S30. Strong inhibition of protein synthesis occurred with thiosulfate concentrations only about 30% as high as those found to optimally reactivate rhodanese during in vitro refolding. The reason for the strong inhibition by thiosulfate is not known: it may be on the transcription and/or translation level. We observed it with other plasmids tested in the coupled transcription/translation system, i.e., synthesis of bacterial dihydrofolate reductase or chloramphenicol–acetyl transferase (data not shown). The maximal percentage of active rhodanese synthesized in the presence of thiosulfate was about 10% lower in the RF compared to the S30 fraction. This may be due to the fact that the cruder S30 contains other chaperones or nonchaperone factors which affect the folding of the in vitro synthesized rhodanese.

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