Chaperonin releases the substrate protein in a form with tendency to aggregate and ability to rebind to chaperonin

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Abstract To know whether the protein released from chaperonin GroEL/ES is in a form committed to the native state or still an aggregatable non-native one, two experiments were carried out. Dilution of the [GroEL–substrate protein] binary complex prior to ATP addition significantly improved the yield of folding, suggesting that the released protein has a tendency to aggregate. When *N*-ethylmaleimide treated GroEL, which can form the binary complex but not release the bound protein, was added to the binary complex prior to ATP addition, productive folding was severely inhibited, indicating that the protein released from GroEL/ES can bind to *N*-ethylmaleimide treated chaperonin. These data favor the 'reservoir' or 'reversion' model, in which GroEL/ES acts as a buffer of folding intermediate or mediates reversion of a misfolded protein to a less folded primitive form, rather than the 'marsupium' model in which folding of the substrate protein proceeds in chaperonin.

Key words: Chaperonin; GroEL; Protein folding; Rhodanese

1. Introduction

The *Escherichia coli* heat shock protein GroEL and its homologs in other organisms belong to a chaperonin (or hsp60) family of molecular chaperones [1–4]. They are well conserved in amino acid sequences, ubiquitous, and indispensable for living cells [1,5,6]. GroEL is composed of two stacked heptamer rings of 60 kDa subunits [7–9], and its crystal structure has been determined recently by Braig et al. [10]. Isolated 60 kDa subunit has primitive chaperone activity [11,12]. GroEL captures labile folding intermediate to form binary complex and, with aid of ATP and the co-chaperonin (*E. coli* GroES), the intermediate is slowly released from GroEL/ES to continue productive folding [13–15].

There seems to be a general agreement that the major role of chaperonin to assist protein folding is prevention of aggregate formation of unfolded or partially folded proteins [16]. We discussed two possible models to explain how chaperonin can do so [17]. One model, which we call 'marsupium' (kangaroo's bag) model (Fig. 1a), assumes that the central cavity provides a closed microcompartment in which protein folding proceeds without risk of aggregation until the protein takes a structure which no longer have tendency to aggregate. According to this model, the released protein (I_2) can not rebind to chaperonin. In the marsupium model the role of chaperonin appears to be a folding catalyst. On the contrary, in the second model, which we call the 'reservoir' model, the role of chaperonin is simple, just to keep folding intermediate (I_1) concentration in the solution low by rapid binding and ATP-regulated slow release (Fig. 1b). Since aggregation is highly dependent on folding intermediate concentration, this can be enough to suppress aggregation (see computer simulations [17,18]). Recently, the third model ('reversion' model) was postulated by Todd et al. (Fig. 1c) [19]. It assumes that chaperonin binds misfolded form (I_2) of substrate protein, which tends to aggregate, reverts the form to a less folded one (I_1), and releases it to resume partitioning between native and misfolded forms. Key differences between the marsupium model and other two models exist in whether or not the form of protein released from chaperonin (or other form derived from the released form) has tendency to aggregate and whether or not it can rebind to chaperonin.

The explanation of chaperonin function based on the marsupium model has been widely accepted since it can explain functional meaning of a central cavity of chaperonin [20–22]. However, several results that favor the reversion model were published recently [19,23]. Here, starting the folding reaction from the binary complex between chaperonin and folding intermediate of rhodanese, we have observed significant improvement of folding yield by dilution of the binary complex concentration prior to the addition of MgATP, and rebinding of proteins released from the binary complex to another chaperonin. These results are readily explained by the reservoir and reversion models but not by the marsupium model.

2. Materials and methods

2.1. Proteins

Bovine mitochondrial rhodanese (thiosulfate sulfurtransferase, 33 kDa monomer enzyme) type II and bovine serum albumin were purchased from Sigma. GroEL and GroES were purified as described previously from lysates of *E. coli* cells bearing the multicopy plasmid pACYC 184 carrying *groES-groEL* genes which was a kind gift from Dr. K. Ito [11]. Purified GroEL and GroES were stored as 65% ammonium sulfate precipitation at 4°C. Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [24]. Modification of the cysteine residues of GroEL with *N*-ethylmaleimide (NEM) was performed according to Mendoza [25]. Briefly, GroEL (16 μM as monomer) was incubated with 5 mM NEM in 25 mM Tris-HCl (pH 8) for 1 h at room temperature. The reaction with NEM was stopped by adding 2-mercaptoethanol. This modified GroEL was used after removal of excess NEM and 2-mercaptoethanol.

2.2. GroE-assisted rhodanese folding

Rhodanese was denatured in 25 mM Tris-HCl buffer, pH7.5, 5 mM dithiothreitol, 6 M guanidine HCl at room temperature for at least 30 min. The folding experiment consisted of four steps. The first step was formation of binary complex between GroEL and non-native state of rhodanese: denatured rhodanese was diluted (1.5 μM, final concentration) into the dilution buffer preincubated at 37°C containing 25 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 50 mM $Na_2S_2O_3$, 40 mM KCl, GroEL (1.45 μM as tetradecamer) and GroES (1.7 μM as heptamer).

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Abbreviations: NEM, *N*-ethylmaleimide; rhodanese, thiosulfate sulfurtransferase.

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The second step was addition of several components or dilution by adding the buffer: details for individual experiments are given in the legends to figures. The third step was to permit folding: MgATP was supplemented to the solution. The fourth step was assay of recovered rhodanese activity: at the indicated time after the MgATP addition, aliquot was withdrawn and rhodanese activity was determined according to Sörbo [26].

3. Results

3.1. Effect of concentration of binary complex on the folding yield

According to the reservoir model, when the folding assay is started from the binary complex ([cpn-I_1] in Fig. 1b) by the addition of MgATP, the yield of folding should be improved as the concentration of the binary complex is diluted because the concentration of folding intermediate (I_1 in Fig. 1b) depends on that of the binary complex and aggregation is greatly suppressed at low concentration of I_1. The same is true for the reversion model (Fig. 1c). To test the effect of concentration of the binary complex on the folding yield, we chose a monomeric enzyme, bovine rhodanese, as a substrate protein to avoid complication of subunit assembly step which is also concentration dependent [27,28]. Under the conditions adopted here (a final concentration of rhodanese concentration, 1.5 μM, at 37°C), denatured rhodanese cannot fold spontaneously from denatured state and forms aggregate [12,27,28]. A [GroEL-rhodanese] binary complex was formed by diluting the denatured rhodanese into the buffer containing GroEL, at a rhodanese: GroEL tetradecamer molar ratio of 1.2:1. Small excess of denatured rhodanese not captured by GroEL should aggregate under the condition. Once non-native rhodanese is captured by GroEL, the binary complex is stable [29] and supplement of MgATP triggers the release of bound rhodanese, resuming the folding of rhodanese (Fig. 2). When protein concentration of the binary complex was four-fold diluted prior to the addition of MgATP, the yield of folding was remarkably improved, from 44% to 90%. Since the fate of the released proteins is either productive folding or aggregate formation [28,30], the increase of productive folding should be a consequence of suppression of aggregate formation caused from lowering the concentration of the released proteins. Thus, improvement of the folding yield by prior dilution of the binary complex is reasonably explained by the reservoir model in which proteins released from the binary complex still have tendency to aggregate.

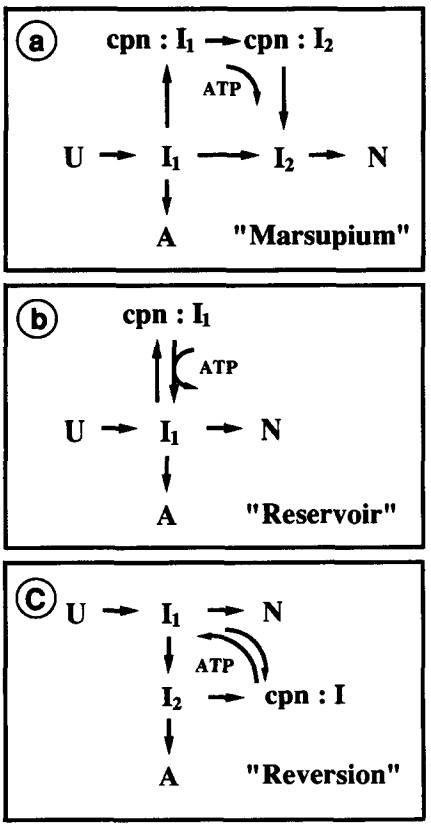

Fig. 1. Three possible models of chaperonin function. (a) Marsupium model. (b) Reservoir model. (c) Reversion model. 'U', 'I', 'N' and 'A' represent a completely unfolded protein, a folding intermediate, a protein with native structure, and aggregated proteins, respectively. 'cpn' indicates functional chaperonin and 'cpn:I' is a complex between chaperonin and a folding intermediate. Note that a key difference between marsupium model and the other two models exists in whether or not the form of protein released from chaperonin (I_2 of marsupium, I_1 of reservoir model) and the form derived from the released form (I_2 of reversion model) has tendency to aggregate. Steps connected with a unidirectional arrow indicate irreversible steps.

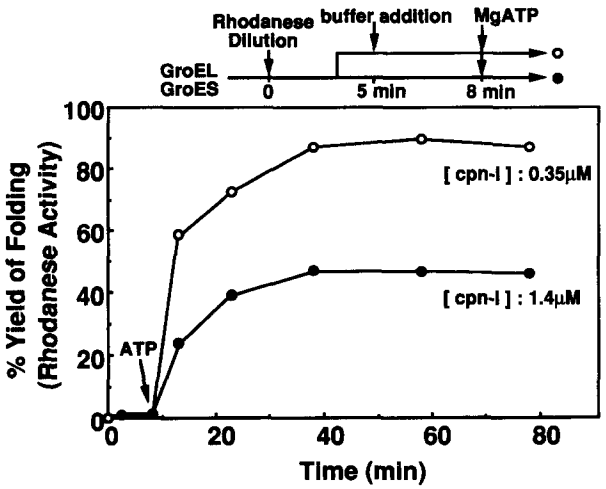

Fig. 2. Effect of concentration of the [GroEL–rhodanese] complex on the folding yield of rhodanese. Denatured rhodanese was diluted 25-fold to 1.5 μM at 37°C by injecting into the buffer containing 1.45 μM GroEL (as a 14-mer) and 1.7 μM GroES (as a 7-mer). After incubation for 5 min, a half of the solution was further diluted 4-fold by adding the chaperonin-depleted buffer and incubation was continued for 3 min. Then, MgATP (1 mM, final concentration) was added to the mixture. At indicated times, an aliquot was withdrawn and the recovered rhodanese activity was determined. Activities are shown as fractions of the calculated 100% activities for each concentration of native rhodanese.

3.2. Rebinding of released protein to NEM-treated GroEL

To test whether proteins released from the binary complex are capable of rebinding to another chaperonin molecule, we used a NEM-treated GroEL. It has been reported that GroEL treated with NEM is able to bind non-native rhodanese but can not release them even in the presence of both ATP and GroES [25]. In fact, NEM-treated GroEL could not mediate folding of rhodanese from the denatured state (Fig. 3, third column)

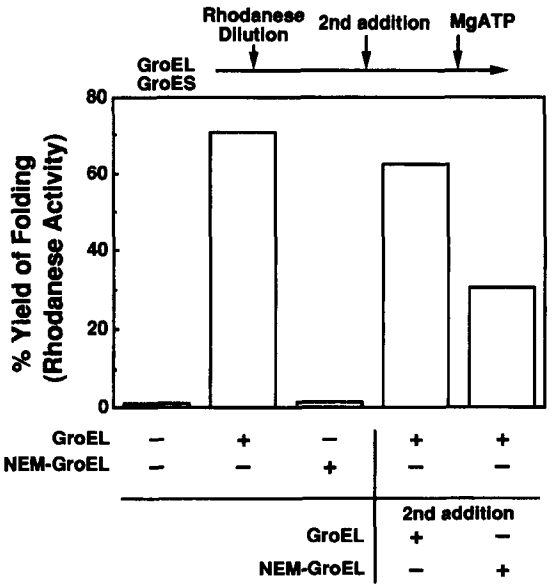

Fig. 3. Inhibition of GroE-dependent folding by NEM-treated GroEL. Denatured rhodanese was diluted 25-fold to 1.5 μM at 37°C by injecting into the buffer containing 0 μM (column 1), 1.45 μM (column 2, 4, 5) GroEL or 1.45 μM NEM-treated GroEL (column 3). All buffers also contained 1.7 μM GroES. The mixtures were incubated for 5 min and 10 μl of the dilution buffer containing following components was added to 25 μl of each of mixtures; buffer only (columns 1–3); GroEL (1.73 μM, final concentration, column 4); NEM-treated GroEL (1.73 μM, final concentration, column 5). After further incubation for 5 min, MgATP (2 mM, final concentration) was added to the mixture. At 60 min after the addition of ATP, an aliquot of the mixture was withdrawn and the recovered rhodanese activity was measured. An activity of the same amount of native rhodanese was taken as 100%.

under the condition where intact GroEL could (Fig. 3, second column). When the 2 molar excess of the NEM-treated GroEL (compared to native GroEL in the binary complex) was added into the solution containing preformed [GroEL-rhodanese] binary complex, folding of rhodanese was inhibited significantly (Fig. 3, fifth column). This means that the non-native rhodanese released from the binary complex is still recognizable by the NEM-treated GroEL. On the other hand, when the intact GroEL was added instead of NEM-treated GroEL to the binary complex, the inhibitory effect on the folding was very small (Fig. 3, forth column). This result is reasonable since the released non-native rhodanese can rebind to added intact GroEL, of course, but it can release again in the presence of MgATP.

4. Discussion

Our previous experiment using mainly the monomeric form of chaperonin showed that, when folding reaction is started from a binary complex between chaperonin and the substrate protein by addition of ATP, productive folding is significantly inhibited by prior addition of free chaperonin or the chaperonin 60 monomer [12]. We took this result, together with the fact that excess amount of chaperonin is inhibitory for protein folding [31–34], as an implication of the reservoir-like function of chaperonin [17]. The two characteristics of the protein released from the binary complex, tendency of aggregation and capability of rebinding to chaperonin, are easily explained according to the reservoir model and reversion model but are difficult, if not impossible, to explain by the marsupium model.

Recently, by using several mutants of GroEL that are capable of binding non-native proteins but not releasing them even in the presence of both ATP and GroES, it has been demonstrated that GroEL-mediated protein folding proceeds by multiple rounds of binding- release of non-native forms [23,35]. Similar conclusion was drawn from experiments using native GroEL [19,23]. Furthermore it has been reported that a eukaryotic cytoplasmic chaperonin, TCP-1, releases the target protein in a non-native form [36]. All these results are consistent with the reservoir and reversion models and discrimination between the two models should be the next problem to solve [19].

It should be appropriate to note a contradictory report on folding of the glutamine synthetase (GS) [37]. In the report, the [GS-GroEL] binary complex was concentrated before addition of MgATP but the yield of productive folding was not changed. The difference from our result may be explained by the assumption that the conditions for folding are different in the two proteins; 'permissive' condition for GS, while 'non-permissive' condition for rhodanese [38]. Under the permissive condition, where spontaneous folding could occur with aggregate formation being suppressed, the effect of concentration of released proteins is less obvious than that under non-permissive condition. Another possibility to explain the difference may be a difference in the subunit composition; rhodanese is a monomer protein, whereas GS is a dodecamer protein. As protein concentration increases, the yield of subunit assembly is increased which, in turn, can compensate the decrease of folding yield.

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