Symmetric GroEL-GroES complexes can contain substrate simultaneously in both GroEL rings

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Abstract Incubation of rhodanese with hche aperonins GroEL and GroES (1:2 $GroEL_{14}$:$GroES_7$ molar ratio) under functional and steady state conditions for ATP leads to the formation of a high proportion of rhodanese-bound symmetric complexes ($GroEL_{14}(GroES_7)_2$), as revealed by native electrophoresis. Aliquots of such samples were observed under the electron microscope, and the symmetric particles were classified using neuronal networks and multivariate statistical analysis. Three different populations of symmetric particles were obtained which contained substrate in none, one or both GroEL cavities, respectively. The presence of substrate in the symmetric complexes under functional conditions supports their role as active intermediates in the protein folding cycle. These results also suggest that symmetric GroEL-GroES complexes can use both rings simultaneously for folding, probably increasing the efficiency of the reaction.

Key words: Chaperonin; Symmetric GroEL-GroES complex; Protein folding; Electron microscopy; Image processing

1. Introduction

Chaperones are a family of proteins involved in the folding and assembly of newly generated proteins [1,2]. The best known members of this family are the so-called chaperonins, multimeric complexes of a protein with a molecular weight of approximately 60 kDa, which are present in both prokaryotic and eukaryotic organisms and which share an extensive homology [3]. The most studied member of this group is GroEL from *Escherichia coli*. GroEL is a 14-mer of approximately 800 kDa which requires for its folding activity the presence of ATP, Mg^{2+}, K^+ and the cochaperonin GroES, a heptameric ring of 70 kDa. The structures of both GroEL and GroES have been solved at atomic resolution [4,5]. The GroEL oligomer has a toroidal shape and is composed of two heptameric rings forming a cavity where unfolded polypeptides can bind [6,7]. As the same GroEL residues are involved in GroES and substrate binding [8], GroES may act in protein folding by directly displacing bound polypeptide and releasing it into the cavity. Recent results support the notion of substrate folding within the enclosed cage formed when GroES binds to one of the GroEL rings [9–11]. GroEL and GroES (from now on, the oligomers are referred to) form two different types of complex in the presence of ATP: an asymmetric GroEL-GroES complex where one GroES oligomer is bound to one of the ends of the GroEL oligomer [12] and a symmetric complex where two GroES oligomers are simultaneously capping both ends of the GroEL double ring [13–15]. Although the functional significance of the symmetric complexes is still a matter of debate, biochemical and biophysical evidence is mounting that symmetric complexes form part of the protein folding cycle [16–19]. Several models including the symmetric complexes have been proposed regarding the stoichiometry of the interaction between the substrate and the symmetric complexes in the functional cycle. Whereas some authors suggest that GroEL-GroES symmetric complexes probably bind substrate in one of the GroEL rings at any given time [19], others propose double occupancy with substrate of both GroEL rings of the symmetric complex [20,21].

In this work, GroEL-GroES symmetric complexes have been obtained in the presence of substrate and under steady state conditions for ATP [16], and aliquots of these samples have been subjected to electron microscopy, image processing and classification techniques to locate the substrate inside the symmetric GroEL-GroES complexes. Similar approaches have been used previously to map the substrate inside the cavity of the GroEL oligomer [12], the actin inside the cavity of the eukaryotic chaperonin CCT [22] and the DNA bound within the central hole of the T7 DNA helicase [23].

2. Materials and methods

2.1. Sample preparation

E. coli GroEL and GroES were purified from a pAR3 plasmid harboring strain [24] that overexpresses both GroEL and GroES, as described previously [13]. GroEL (0.35 μM) and GroES (1:2 oligomer molar ratio) were incubated in 50 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP, in the presence of urea-denatured rhodanese (5 : 1 rhodanese : GroEL oligomer molar ratio). As an ATP regenerating system, the solution also contained 20 mM glucose, 2 mM phosphoenolpyruvate and 20 μg/ml pyruvate kinase [16]. Rhodanese was labeled with iodine-125 ($Na^{125}I$ carrier-free; Amersham, Little Chalfont, UK) by the chloramine-T method [25]. When crosslinked, samples were incubated in 0.08% (w/v) glutaraldehyde (Sigma) for 20 min at 37°C. The crosslinking reaction was stopped by adding ammonium chloride (40 mM final concentration). Electrophoresis of cross-linked proteins under native conditions was carried out in slab gels containing 4.5% polyacrylamide [17].

2.2. Electron microscopy and image processing

Samples were negatively stained with 1% uranyl acetate on thin-coated collodion grids previously glow-discharged for 15 s. Transmission electron microscopy was performed in a JEOL 1200EX-II electron microscope operated at 120 kV. Images were directly recorded using a 1024×1024 pixel GATAN ssCCD camera attached to the microscope. All the side views corresponding to symmetric complexes observed in the images were extracted without any a priori selection, and centered using a synthetic mask. Centered particles were aligned using a free-pattern algorithm [26,27]. Particles were then aligned horizontally and translated laterally to the right or to the left so that the left or right cavity, respectively, of the symmetric complexes was located inside a mask placed in the center of the GroEL ring cavity (8 pixels, 24 Å diameter). The particles thus aligned were as-

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signed to dictionary output vectors applying a 2×2 self-organizing map algorithm [28]. Dictionary output vectors were classified using multivariate statistical analysis [29]. When the coordinates of the images for the first autovector were plotted against any of the others, the particles could be separated in different populations. After classification, homogeneous populations (at the centered state) were aligned again to prevent the influence of the discarded particles in the previous alignment. Resolution of the final average images was estimated by the spectral signal to noise ratio (SSNR) method [30,31]. Average images were filtered in each case to the resolution obtained.

3. Results and discussion

3.1. Isolation of symmetric GroEL-GroES complexes containing ^{125}I-labelled rhodanese under functional conditions

To obtain functional conditions resembling those found in in vivo protein folding, the chaperonins GroEL and GroES (1:2 GroEL:GroES oligomer molar ratio) were incubated with unfolded substrate (rhodanese:GroEL 5:1 molar ratio), Mg^{2+}, K^{+} (both at physiological concentrations) and under steady state conditions for ATP. For that purpose, an ATP regenerating system was used as described previously [16]. The system was tested at different incubation times by electron microscopy and native gel electrophoresis, and it was found that the percentage distribution of the different GroEL species (GroEL, GroEL:GroES asymmetric and symmetric complexes) was maintained throughout this time (results not shown), with the symmetric complexes the majority (more than 90%) of the GroEL species (see Figs. 1 and 2).

Unfolded ^{125}I-rhodanese was used to locate the substrate-containing GroEL species. GroEL and GroES together with the substrate were incubated under functional and steady state conditions for ATP, and after 1 min incubation, aliquots of the assay were fixed as described previously [17], loaded onto a native acrylamide gel and electrophoresed (Fig. 1). Lanes c and d show respectively the mobility of GroEL, and GroEL and GroES incubated with ADP (generating homogeneous

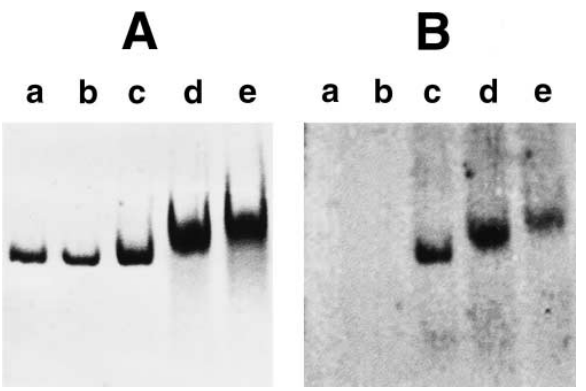


Fig. 1. Native PAGE of GroEL and GroES incubated with ^{125}I-labeled rhodanese under steady state conditions for ATP. A: Coomassie-stained native PAGE. B: Autoradiography obtained from the native PAGE. Lane a: GroEL not subjected to cross-linking; b: GroEL incubated with native ^{125}I-labeled rhodanese and cross-linked; c: GroEL incubated with denatured ^{125}I-labeled rhodanese and cross-linked; d: GroEL and GroES incubated with ADP and denatured ^{125}I-labeled rhodanese and cross-linked; e: GroEL and GroES incubated with ATP (under steady state conditions) and denatured ^{125}I-labeled rhodanese, and cross-linked. (b) is a control to show that cross-linking does not artifactually associate the substrate with GroEL [17].

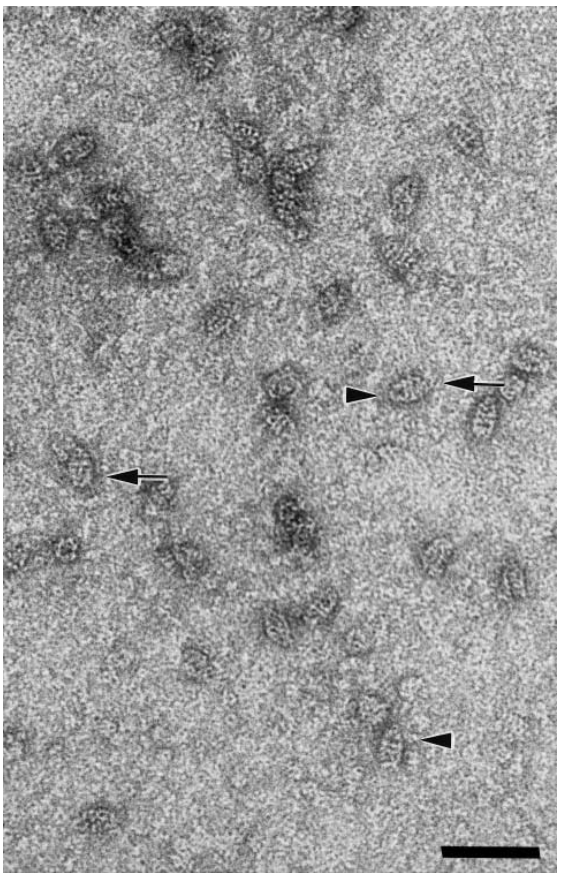
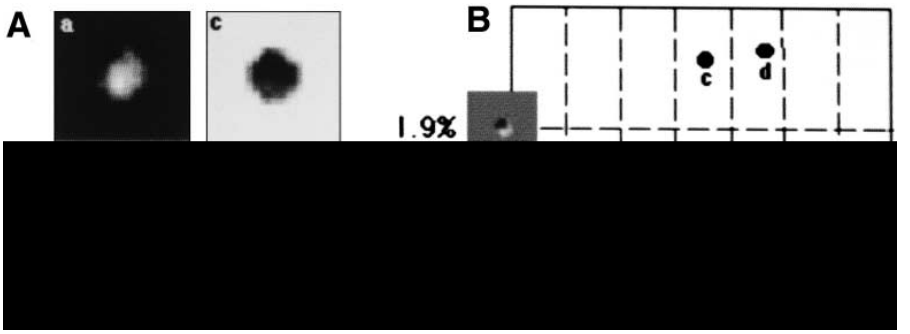


Fig. 2. Representative field of a micrograph from a negatively stained solution of non-fixed GroEL and GroES incubated with unfolded rhodanese under steady state conditions for ATP. A high percentage of symmetric complexes can be observed. The cavity formed inside the GroEL ring can be filled with stain (arrow) or unstained (arrowhead). Bar indicates 50 nm.

populations of asymmetric complexes [12,21]). The two samples were incubated in the presence of unfolded substrate. Lane e, containing the experimental sample, revealed a band with a lower mobility than the asymmetric complexes (lane d), which can be assigned to symmetric complexes [17]. When this gel was subjected to autoradiography to detect the presence of ^{125}I-rhodanese bound to GroEL (Fig. 1B), a label associated with the symmetric complexes generated during the folding assay was observed (lane e). When the solution was incubated for 30 min, no radioactive band was associated with the symmetric complexes anymore ([17], and results not shown), implying that the denatured substrate had been released from the complex and folded [17], and therefore that symmetric complexes play an active role in the protein folding cycle by holding the substrate inside the GroEL cage and discharging it once the GroES is liberated from the GroEL ring [9–11].

3.2. Electron microscopy and image processing of rhodanese-containing symmetric GroEL-GroES complexes

But, what is the stoichiometry of the substrate bound to the symmetric complex? Is there substrate enclosed within only one of the GroEL rings or can both GroEL rings simultaneously contain substrate during part of the folding cycle? To answer these questions, non-fixed aliquots of the samples from the functional assay (lane e in the native PAGE) were nega-



tively stained and observed under the electron microscope. Fig. 2 shows a field of GroEL obtained after 1 min incubation with substrate and under steady state conditions for ATP. In this field, besides the typical doughnut shape of the front views, side views corresponding to symmetric complexes ('footballs') were observed in a high proportion (more than 90%), in agreement with the results obtained with native electrophoresis (Fig. 1). Among them, different degrees of staining could be detected in the cavity enclosed within the GroEL ring and the capping GroES. Whereas in some cases the stain clearly contrasted with the cage by penetrating into the cavity (Fig. 2, arrow), in other cases the GroEL cage had the same density as the surrounding walls (Fig. 2, arrowhead). Although, in principle, the different staining behaviors in the cavities could be related to the presence or absence of substrate (as determined by the electrophoretic analysis of Fig. 1B, lane e), a more systematic approach was followed to distinguish between the three possible populations of symmetric complexes, those having substrate in none, one or both cavities capped by the GroES oligomer.

1564 particles corresponding to symmetric complexes were extracted from the electron microscope images, centered and aligned. A Kohonen-based self-organizing map classifier [28] was carried out to search for the variability associated with the left cavity of the symmetric complexes. The 2×2 dictionary output vectors generated by this neuronal network can be seen in Fig. 3A. Dictionary output vector a represents those particles with a stain-excluding region in the center of the mask, whereas output vector b represents those particles filled with stain in the same area. c and d represent intermediate states between those described. When the dictionary output vectors were subjected to a multivariate statistical analysis [29], the first autovector (accounting for 88.1% of the variability; inset in the horizontal axis of Fig. 3B) clearly depicted the variability associated with the presence or absence of a stain-excluding material in the central part of the mask. When the first autovector was plotted against any of the others (in this case, the third one; inset in the vertical axis of Fig. 3B), the particles were separated according to their 'positive' (a) or 'negative' (b) presence of a stain-excluding region inside the cavity. Particles represented by dictionary output vectors c and d were considered not clearly classified (coefficients close to 0). Particles corresponding to dictionary output vectors a and b were recorded in separate files (files I and II), whereas particles represented by dictionary output vectors c and d were discarded.

All the aligned particles were now subjected to a neuronal network classifier and multivariate statistical analysis focused this time in the right cavity of the symmetric complexes, and the results obtained were identical to those described above (results not shown). Again, two populations, representing those particles with a 'positive' or a 'negative' presence of a stain-excluding material inside the GroEL cavity could be clearly separated and recorded in two different files (I′ and II′).

The next step was to compare the populations having 'positive' stain-excluding material in the center of the cavity, either in the left (file I) or in the right (file I′). Those particles contained in both files would have a stain-excluding region in the cavity of both GroEL rings and could be related to symmetric particles having substrate bound to both rings (for a gallery of these particles, see Fig. 4A). The same strategy was applied to those files containing 'negative' stain-excluding matter, either in the left (file II) or in the right (file II′) cavity. Those particles represented in both files would contain a stain-filled cavity in both GroEL rings and could be related to those symmetric complexes having no substrate at all (for a gallery of these particles, see Fig. 4C). The remaining particles, not belonging to any of those two populations, would represent those particles with the stain filling only one of the GroEL cavities (either the left or the right) and could be related to those symmetric complexes having substrate bound to only one of the GroEL rings (for a gallery of these particles, see Fig. 4B).

The three populations thus obtained (1082 particles out of the original 1564 ones; 69% of the whole population) were independently processed. In the case of those particles having a stain-excluding region in both GroEL cavities, 159 particles (representing 15% of the classified symmetric complexes) were processed and an average image was obtained at 26 Å resolution (Fig. 5A). In the case of the symmetric particles having both GroEL cavities filled with stain, 281 particles (representing 26% of the classified symmetric complexes) were processed and an average image was obtained at 25 Å resolution (Fig. 5C). The third population, that of the symmetric particles having only one of their cavities filled with stain (either the left or the right one), was processed independently. In all, 642 particles (representing 59% of the classified symmetric com-