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Characterisation of mutations in GroES that allow GroEL to function as a single ring

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ABSTRACT

The chaperonin GroEL contains two seven-subunit rings, and allosteric signals between them are required to complete the GroEL reaction cycle. For this reason SR1, a mutant of GroEL that forms only single rings, cannot function as a chaperone. Mutations in SR1 that restore chaperone function weaken its interaction with the cochaperonin GroES. We predicted that GroES mutants with reduced affinity for GroEL would also restore function to SR1. To test this, we mutated residues in GroES in and near its contact site with GroEL. Nearly half of the mutants showed partial function with SR1. Two mutants were confirmed to have reduced affinity for GroEL. Intriguingly, some GroES mutants were able to function with active single ring mutants of GroEL.

1. Introduction

GroEL is the only molecular chaperone which is known to be essential for growth of *Escherichia coli* at all temperatures [1]. GroEL binds a subset of *E. coli* proteins, including several which are essential for cell growth, and allows them to fold under conditions where their aggregation is minimised. The details of this process, including the structural changes undergone by the GroEL protein and the identity of the proteins which require GroEL for their folding, have both been the subject of intense research for a number of years (reviewed recently in Refs. [2–5]). Although the majority of attention has focussed on GroEL, it is only one part of a two-component machine, since the function of GroEL depends on the action of the cochaperonin GroES, which is also essential for growth [1].

GroEL is a double ring complex with seven GroEL protomers in each ring [6]. GroEL sequesters unfolded or partially folded proteins in the cavity at the centre of one of the rings, where they are able to fold without interacting with other unfolded proteins [7–10]. Initially, proteins bind to a hydrophobic patch which is present on the apical domain of each subunit of GroEL, and is hence present as a hydrophobic band around each end of the two rings [11,12]. Binding of ATP to GroEL causes large domain movements, which in some cases partially unfolds the bound substrate, which may assist its subsequent search for the correct folded conformation [2,4,13,14]. Bound proteins are displaced into the central cavity by the binding of the cochaperonin GroES, which caps the cavity for most of the reaction cycle. The bound ATP is then hydrolysed, which weakens the binding between GroEL and GroES, but GroES is not displaced until ATP binds the opposite ring. Loss of GroES uncaps the protein folding cavity, and allows the egress of the substrate, which may bind again to the same or to a different GroEL complex if it has not been completely folded [9,10,15,16].

Structural studies of GroES show that it possesses a flexible loop (generally referred to as the "mobile loop") of amino-acyl residues which is undefined in the free protein but which anchors GroES to GroEL when the two form a complex [17,18]. Three amino-acids at the base of this flexible loop form contacts with the GroEL apical domain, and the formation of these contacts requires a large change in the conformation of the GroEL–ATP complex. This change in conformation leads to the burying of the hydrophobic regions in GroEL, which leads to loss of binding of the substrate protein. Because GroES now caps the GroEL ring, the substrate protein is displaced into the cavity in the GroEL ring, where it remains for the duration of the ATP hydrolysis step, the longest step in the reaction cycle. The protein can thus fold in the cavity without interacting with any other unfolded proteins, the folding possibly being favoured by the limited size of the cavity and the hydrophilicity of the cavity walls [4,5].

The double ring structure of GroEL is needed for completion of the reaction cycle, since information has to pass between the rings

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to signal the progress of the ATP hydrolytic cycle, which takes place on one ring and causes conformational changes in the other [19,20]. Mutants of GroEL which cannot form double rings are incapable of completing the reaction cycle, not because they cannot fold protein, but because the bound GroES cannot be released without an allosteric signal from the other ring [16], thus trapping the folded protein in the GroEL cavity. However, Hsp60 (the mitochondrial homologue of GroEL) has very weak ring–ring interactions but can nevertheless function as a chaperone in vitro and can replace GroEL in *E. coli* as long as it is expressed with its cognate cochaperonin, Hsp10 [21–24]. Moreover, mutations in the inactive single ring form of GroEL can be found which partially restore its function [25,26]. In both cases, the ability of these forms of chaperonin to complete their reaction cycles is due to weakening of their interaction with the cochaperonin, obviating the need for an allosteric signal from the second ring.

The interaction between GroEL and GroES is determined in part by the mobile loop of the cochaperonin [27]. It follows that it should be possible to obtain mutations in GroES that also weaken its binding to GroEL and allow GroEL to function as a single ring, by changing the interaction of the mobile loop with GroEL. In this study, we have explicitly tested this prediction using site-directed mutagenesis of GroES residues at or close to the interaction site, and have tested the properties of these mutants with wild-type GroEL, with an inactive single ring mutant of GroEL (SR1), and with several active single ring mutants. We find that many of these mutations enable the in vivo function of a normally inactive single-ring form of GroEL.

2. Materials and methods

2.1. Strains and plasmids

E. coli K-12 TG1 [28] was used for routine molecular biology procedures. Complementation studies were done in strain MGM100, in which the native promoter of the *groE* operon has been replaced by the tightly glucose-repressible pBAD promoter of the *ara* operon [29]. P1 transduction to delete the chromosomal *groES* and *groEL* genes was from stain NL192Ω [30]. All protein expression for complementation experiments was done from derivatives of the expression plasmid ptrc99A [31]. Protein expression for protein purification was either from the same plasmid, or from pET22b in *E. coli* BL21 (DE3) (Novagen).

2.2. Growth conditions and complementation assays

Bacteria were grown in L-broth or on L agar plates at 37 °C unless otherwise indicated. P1 transductions were done as described in [26]. For complementation experiments, overnight cultures were adjusted to an OD600 of 0.8–0.9 with L-broth, then diluted in L-broth from 10^{-1} to 10^{-6}. Each dilution was spotted onto LB agar plates (containing 0.2% arabinose or glucose, 50 μg/ml kanamycin, 100 μg/ml ampicillin, with or without 0.1 mM IPTG for MGM100 strains). Plates were incubated at the experimental temperature overnight and scored for growth the following day. All assays were done with a minimum of three independent biological replicates. Growth equivalent to wild-type was assigned a value of 4, and for each 10-fold reduction in growth (as judged by colony number and size), this value was reduced by 1. Thus, reduction in growth by 10^4-fold or more was scored as zero growth. To convert these values to "low", "moderate" or "high", values were summed for each mutant under all conditions and expressed as a percentage of the wild-type. Values greater than 67% were recorded as high, 33–66% as moderate, and less than 33% but greater than zero as low.

2.3. Molecular biology methods

Enzymes for DNA manipulation were purchased from New England Biolabs and used according to the manufacturer's instructions. Plasmid preparations were done using either the Qiagen QIAprep Spin Miniprep kit (Catalogue No. 27104) or the Sigma GenElute™ Plasmid Maxi-prep Kit (Product No. PLX15) according to the manufacturer's instructions. Oligonucleotides for site-directed mutagenesis were synthesized with all possible combinations of bases at the appropriate positions, by Alta Biosciencs (University of Birmingham). Site-directed mutagenesis was done using the Quikchange Site-directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutants were identified by sequencing. DNA sequence analysis was done using the BigDye version 3.1 sequencing kit (Applied Biosystems), the products of sequencing reactions were run by the Functional Genomics Laboratory at the University of Birmingham.

2.4. Protein purification

Wild-type and mutant GroEL was purified as previously described [26]. GroES proteins were his-tagged with six additional histidine residues at their C-terminus, and purified as follows: 20 ml of an overnight culture was diluted into 1 l of fresh medium and grown at 37 °C for 2 h with shaking, and expression was induced (1 mM IPTG, 3 h). Cells were harvested by centrifugation and the pellet was resuspended in 40 ml 5% glycerol/1 mM PMSF, recentrifuged, resuspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4, 1 mM PMSF) and lysed by sonication. The supernatant was cleared by centrifugation and passage through a 0.2 μm filter and loaded onto an equilibrated 1 ml Amersham HisTrap FF column by an Amersham AKTA explorer HPLC system. The column was washed with binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 7.4, 1 ml/min) until the absorbance reached a steady baseline. Proteins were eluted with elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4 with H_3PO_4) for 5 minutes. Fractions were analysed using 12% SDS–PAGE, pooled, and dialyzed overnight against buffer G (20 mM MOPS, 100 mM KCl, 10 mM $MgCl_2$, pH 7.4). Some GroES mutant proteins were found to precipitate after dialysis, so these were prepared in the presence of 50% glycerol. Dialyzed samples were applied to an equilibrated Amersham Sephacryl S300 column. Proteins were eluted with buffer G2 or G5 (20 mM MOPS, 100 mM KCl, 10 mM $MgCl_2$, 20% or 50% glycerol, pH 7.4). Collected fractions were analysed on 12% SDS–PAGE and pooled as appropriate.

2.5. ATPase assays

The ATPase activity assays were done using EnzCheck phosphate assay kit (Molecular Probes). Reactions were set up according to the manufacturer's instructions except that a different buffer was adopted (10% or 50% glycerol, 25 mM Tris pH7.5, 5 mM KCl, 5 mM $MgCl_2$, and 2 mM DTT). Final concentration for GroES and GroEL proteins in the reaction mix were 0.4 μM and 0.2 μM, respectively (oligomer). ATP concentration was 1 mM. The reaction mix was incubated at 22 °C for 10 min before GroEL was added, after which absorbance at 360 nm was measured continuously for 180 s. ATPase rates were calculated according to a standard curve generated with inorganic phosphate.

3. Results and discussion

The structure of GroES–GroEL–ADP_7 complex [18] shows that the major contacts between GroES and GroEL are mediated by