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# Inter-ring communication allows the GroEL chaperonin complex to distinguish between different substrates

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## Abstract

The productive folding of substrate proteins by the GroEL complex of *Escherichia coli* requires the activity of both the chaperonin rings. These heptameric rings were shown to regulate the chaperonins' affinity for substrates and co-chaperonin via inter-ring communications; however, the molecular details of the interactions are not well understood. We have investigated the effect of substrate binding on inter-ring communications of the chaperonin complex, both the double-ring GroEL as well as the single-ring SR1 chaperonin in complex with four different substrates by using mass spectrometry. This approach shows that whereas SR1 is unable to distinguish between Rubisco, gp23, gp5, and MDH, GroEL shows clear differences upon binding these substrates. The most distinctive binding behavior is observed for Rubisco, which only occupies one GroEL ring. Both bacteriophage capsid proteins (gp23 and gp5) as well as MDH are able to bind to the two GroEL rings simultaneously. Our data suggest that inter-ring communication allows the chaperonin complex to differentiate between substrates. Using collision induced dissociation in the gas phase, differences between the chaperonin(substrate) complexes are observed only when both rings are present. The data indicate that the size of the substrate is an important factor that determines the degree of stabilization of the chaperonin complex.

**Keywords:** native mass spectrometry; bacteriophage T4; capsid proteins; substrate binding; protein folding

The GroEL–GroES chaperonin complex of *Escherichia coli* is one of the best studied chaperone assemblies. GroEL is a homo-oligomeric double-ring structure, and each ring contains seven subunits. Together with its co-chaperonin GroES, which also forms a heptameric ring, the complex assists in the folding of a variety of unrelated proteins in an ATP dependent manner, by preventing misfolding and aggregation (Fig. 1; Xu et al. 1997; Sigler et al. 1998). The central cavities of the GroEL chaperonin are hydrophobic and interact with the exposed hydrophobic surfaces of a nonnative substrate protein. After binding of the substrate to one of the GroEL rings, and subsequent binding of ATP and GroES to the same ring, a *cis*-complex is formed. Due to the binding of co-chaperonin, a conformational change in the complex occurs. The surface of the GroEL *cis*-ring becomes hydrophilic, and the substrate is released in the enclosed folding cavity. ATP hydrolysis in the *cis*-ring is followed by the binding of a new substrate and ATP to the opposite empty GroEL ring (also called *trans*-ring) (Hartl and Hayer-Hartl 2002; Ellis 2003). This binding induces the discharge of GroES and ADP molecules from the *cis*-ring so that the substrate protein is released. If the substrate protein has not yet reached its native conformation, it can rebind to the chaperonin complex and undergo another round of assisted folding (Walters et al. 2002). So for the productive
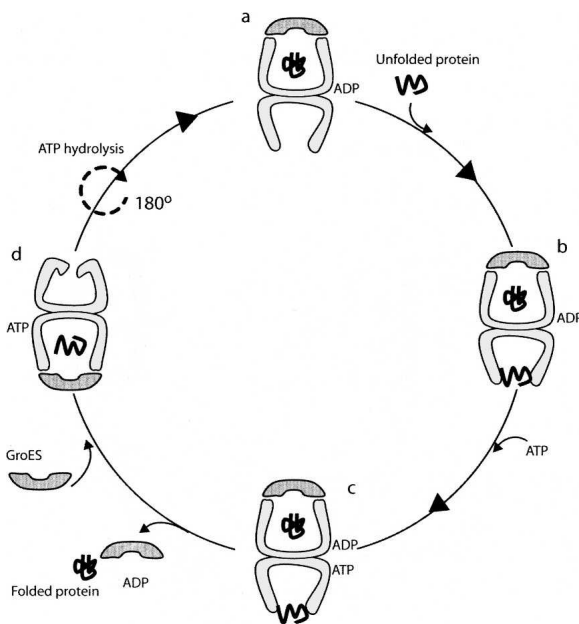
**Figure 1.** Simplified model of the functional cycle of the GroEL-GroES chaperonin complex. (*A*) An unfolded substrate binds to GroEL. (*B*) The binding of ATP to this ring increases the affinity for GroES to the same ring. (*C*) At the same time GroES dissociates from the *cis*-ring. (*D*) A new *cis*-complex is formed in which the substrate is trapped in the folding cavity.

folding of substrate proteins, the action of both rings is required, since GroES release from the *cis*-ring requires the presence of ATP in the opposite *trans*-ring (Llorca et al. 1997; Poso et al. 2004). Different allosteric routes ensure the correct intra- and inter-ring communications. Whereas the GroEL complex shows positive cooperativity with respect to ATP binding within one ring, this ATP binding results in negative cooperativity between the two rings. At the same time ATP binding promotes the binding of GroES to the ATP-containing ring, converting it from a substrate-accepting state to a folding active state. The binding of ATP to the *trans*-ring leads to the ejection of *cis*-ligands (ADP, GroES, and substrate), which is essential to allow a new cycle of substrate and GroES binding (Llorca et al. 1997; Amir and Horovitz 2004; Sewell et al. 2004). Proper inter-ring communication is thus necessary for the correct functioning of the chaperonin complex, and this is mainly controlled by ATP binding and hydrolysis. However, also the binding of substrate to the chaperonin complex can lead to long-range signals that are sent across the interface of the two heptameric rings such as the binding of substrate to the *trans*-ring, which may contribute to the accelerated ejection of *cis*-ligands (Sewell et al. 2004). Furthermore, it has been shown that the binding of a substrate protein to one ring discourages the binding of substrates in the opposite ring, also due to allosteric signaling (Wang and

Chen 2003). The binding of a substrate has been reported to induce conformational changes in the chaperonin complex, resulting in protrusion of the substrate from the cavity of the *cis*-ring and a narrowing of the opening of the unoccupied *trans*-ring. As a consequence the *trans*-ring becomes effectively inhibited from binding a second substrate. Although this may be the general notion, not all substrates might induce similar conformational changes in the GroEL chaperonin (Farr et al. 2000; Falke et al. 2005; van Duijn et al. 2006).

A mutant form of GroEL that is unable to form double-ring structures has been described. This GroEL single-ring mutant, SR1, contains four amino acid substitutions in the interface regions preventing the back-to-back stacking of the two heptameric rings (Weissman et al. 1995). Despite the lack of the second ring, the properties of SR1 are remarkably similar to those of GroEL. It is able to bind substrate molecules and enclose them in its folding cavity after nucleotide and GroES binding (Weissman et al. 1995). However, SR1 is unable to release GroES because the binding of ATP to the opposite *trans*-ring that triggers the co-chaperonin to dissociate from the complex is obviously not possible. Consequently, SR1 does not allow the release of properly folded proteins (Weissman et al. 1995; Rye et al. 1997; Sun et al. 2003).

To study the effect of inter-ring communication in the GroEL complex with respect to substrate binding, we performed (tandem-) mass spectrometric analysis on GroEL, SR1, and their complexes with different substrates. We have shown previously that the binding properties of gp23 (the major capsid protein of bacteriophage T4, 56 kDa) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, 54 kDa) to the chaperonin complex are quite distinct (van Duijn et al. 2006). Based on these original observations we hypothesized that the kind of conformational change that is induced in the chaperonin complex upon substrate binding is substrate dependent. Here we investigated the binding effects of four different substrates, namely gp5, the major capsid protein of bacteriophage P22 (47 kDa) malate dehydrogenase (MDH, 32 kDa), Rubisco, and gp23. Like Rubisco and gp23, both gp5 and MDH are also dependent on the chaperonin complex for their correct folding. By using the SR1 mutant protein we show that the second ring of the GroEL complex is essential for the chaperonin to discriminate between these four substrates. Tandem mass spectrometry experiments confirmed that only differences between the chaperonin (substrate) complexes can be observed when both rings are present. Furthermore, our results indicate that for substrates that have the ability to occupy both GroEL rings simultaneously, the size of these substrates determines most likely their stabilizing capacities of the chaperonin complex upon binding.

## Results

### Inter-ring communication is important to differentiate between substrates

We first determined the binding stoichiometries of gp23, Rubisco, gp5, and MDH to heptameric SR1 (SR1$_7$), a chaperonin complex in which inter-ring communication is evidently absent. The substrate proteins were unfolded in urea, whereafter they were diluted into a 50 mM aqueous ammonium acetate buffer containing SR1$_7$ at a ratio of 1:3 (SR1$_7$:unfolded substrate). Using electrospray ionization in combination with a buffer at physiological pH and optimal ion transmission conditions (Tahallah et al. 2001; Sobott and Robinson 2002; Sobott et al. 2002; Heck and van den Heuvel 2004; van den Heuvel and Heck 2004) it is possible to produce intact protein or protein complex ions. As ions with a range of different charges are produced for each protein complex, the mass of the complex, and in our study the binding stoichiometry of the substrates to the chaperonin complexes, can be determined accurately using mass spectrometry. Figure 2A shows the mass spectrum of free SR1$_7$ (top) with ion signals exclusively originating from the SR1 heptamer, and the mass spectrum of the SR1$_7$(gp5) complex (bottom), which also shows only one charge state distribution. From these distributions we calculated the masses of the complexes. For SR1$_7$ the mass determined from the spectrum was 399,686 ± 40 Da (theoretical mass 399,481 Da) and for SR1$_7$(gp5) we determined a mass of 447,382 ± 43 Da. This latter mass is very close to the theoretical mass of one heptameric SR1 ring with one gp5 substrate bound, namely 446,102 Da.

If we assume that the ionization efficiencies of all the chaperonin species are similar, the spectra give semi-quantitative reflections of the abundance of the different species present in solution. Upon addition of unfolded gp23, 80% of heptameric SR1 bind this substrate, whereas 20% of SR1 remained free of substrate (Fig. 2B). Rubisco and MDH both occupied 95% of the available SR1 heptamers, and gp5 completely saturated the SR1$_7$ chaperonin (Fig. 2B). In line with what was expected, SR1$_7$ could only bind one substrate molecule, and, at a ratio of heptameric SR1:unfolded substrate of 1:3, almost all of the SR1$_7$ present was occupied independent of the type of substrate.

When we consider the double-ring GroEL$_{14}$ complex, the chaperonin assembly is able to communicate between the rings. To investigate whether the reported intercommunication has an effect on substrate binding we performed a similar experiment as with SR1$_7$. The four substrate proteins were unfolded and added to a solution containing tetradecameric GroEL at GroEL$_{14}$:substrate ratios varying from 1:1 to 1:5. Interestingly, remarkable differences were observed between the different GroEL$_{14}$(substrate) complexes (Fig. 3). In contrast to what is generally believed, three of the four substrates were able to occupy both GroEL$_{14}$ rings simultaneously. Only Rubisco could not bind to the *trans*-ring once the *cis*-ring was occupied. These results indicate that the conformation of the *trans*-ring depends on the type of substrate that binds to the *cis*-ring. The relative abundance of GroEL$_{14}$, GroEL$_{14}$(substrate), and GroEL$_{14}$ (substrate)$_2$ indicates that the binding affinities of the substrates are quite different. Whereas the affinity for each of the gp23 substrate's molecules is in the same order of
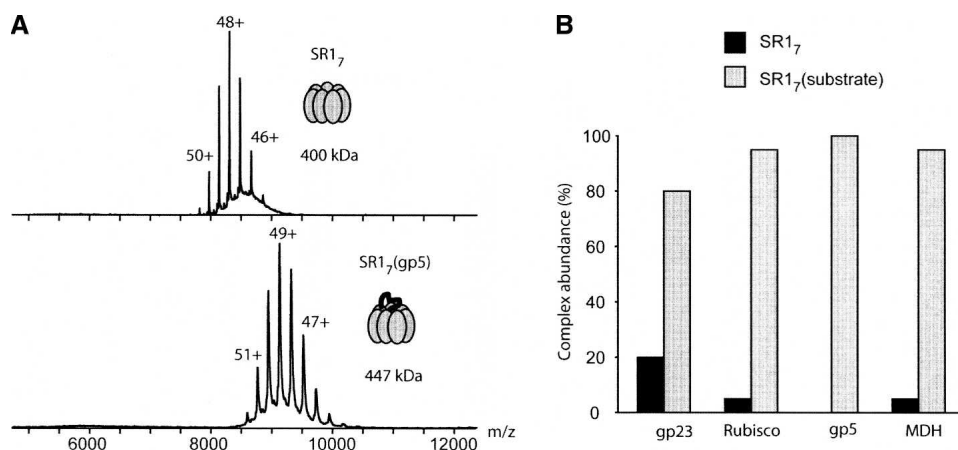


**Figure 2.** (*A*) Mass spectrum of SR1$_7$ (*top*) and SR1$_7$(gp5) (*bottom*), showing only one charge state distribution. This indicates that all the SR1$_7$, present in the 1:3 mixture of SR1$_7$ and gp5, forms a complex with unfolded gp5 substrate. (*B*) Relative abundance of free SR1$_7$ and SR1$_7$(substrate) complexes as calculated from the ion intensities present in the ESI mass spectra at a 1:3 SR1$_7$:substrate ratio. Upon the addition of unfolded gp23 80% of the heptameric SR1 complexes bind to this substrate, whereas 20% remains free. Rubisco and MDH both occupy 95% of the available heptameric SR1 complexes whereas gp5 completely saturates the mutant chaperonin complex.
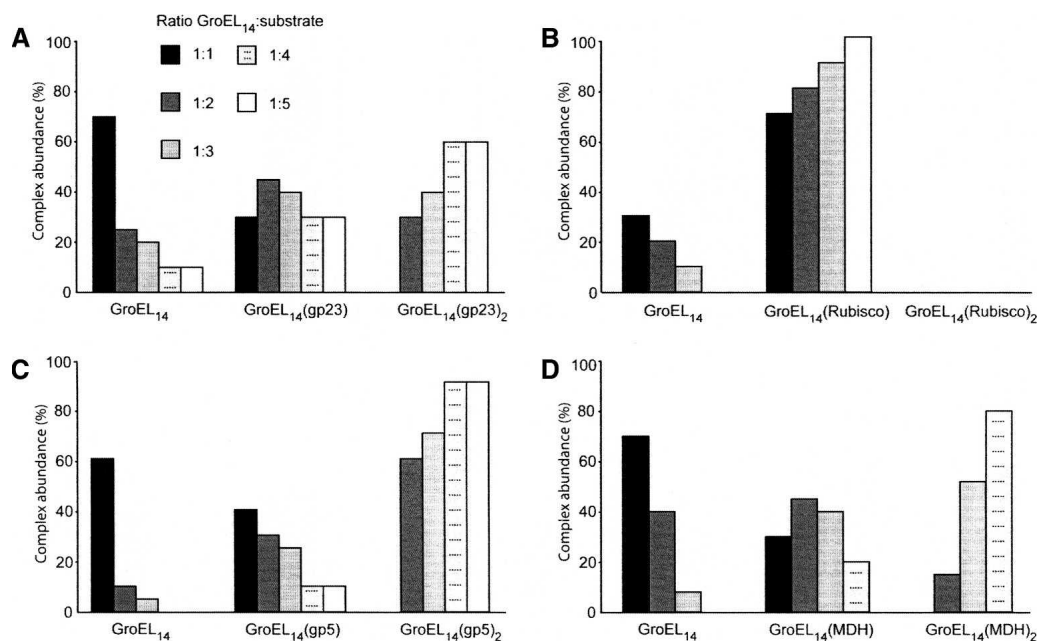
**Figure 3.** Relative abundances of the GroEL$_{14}$(substrate) complexes calculated from the ion intensities in the ESI mass spectra at varying GroEL$_{14}$:substrate ratios (from 1:1 up to 1:5). GroEL$_{14}$ in complex with (*A*) gp23, (*B*) Rubisco, (*C*) gp5, and (*D*) MDH. Whereas it is not possible to occupy both binding sites of the chaperonin complex with Rubisco at a ratio of 1:4, at the same concentration the chaperonin complex is saturated with gp5 (both rings).

magnitude, it is not very high compared to the affinity of Rubisco. At the same time the chaperonin complex has no affinity for a second Rubisco molecule. Futhermore, gp5, which, like gp23, is a major capsid protein, and MDH were able to occupy both rings of the chaperonin complex. However, gp5 and MDH bound to both rings already at significant lower concentrations than gp23. At a GroEL$_{14}$:gp5 ratio of 1:4, 90% of the available GroEL$_{14}$ had two gp5 substrates bound, while this was only 60% when gp23 was used. So even though the possible binding stoichiometries of both capsid proteins are the same, the binding affinities for both the first and the second gp5 substrate are higher. And at a ratio of 1:4 GroEL$_{14}$:MDH, there is no free tetradecameric GroEL left, and 80% of the chaperonin complex had two MDH substrate molecules bound. Thus also for MDH the binding affinities are higher than those of gp23.

*Tandem mass spectrometry*

To further investigate the differences in substrate binding between the single and double-ring chaperonin complex and their relative stability, gas-phase tandem mass spectrometry was used. In such experiments specific precursor ions of the chaperonin complexes are selected based on their mass and subsequently fragmented by collisional activation. Inside the collision cell the ions of the chaperonin complex collide with argon molecules, and

the internal energy of the ions increases, which can result in dissociation. The dissociation of the chaperonin complex is dependent on the protein complex itself as well as on the collison voltage that is used. In order to compare the dissociation characteristics of different noncovalent complexes, it is necessary to use the center-of-mass collision energy E$_{c.o.m.}$ instead of the laboratory collision energy (Jorgensen et al. 1999). The E$_{c.o.m.}$ takes the masses of the colliding particles as well as the initial charge state of the selected ion into account. This is required, as the internal energy of the ions increases at a higher charge state, which favors the dissociation, and also the masses of the particles involved influence the collision efficiency. Even though the dissociation process inside the collision cell concerns multiple collisions, this approach allows semiquantitative comparisons of SR1$_7$ and GroEL$_{14}$ to be made (Jorgensen et al. 1999). The E$_{c.o.m.}$ was calculated as follows:

$$E_{c.o.m.} = E_{lab} \times m_{target}/(m_{protein} + m_{target}) \qquad (1)$$

E$_{lab}$ (zeV) is the ion kinetic energy in the laboratory frame of reference, m$_{target}$ is 40 Da (the mass of the argon collision partner), and m$_{protein}$ is the mass of the selected protein complex (e.g., for GroEL$_{14}$ ~800 kDa and for SR1$_7$ ~400 kDa).

So if we now monitor the intensity of the parent ion peaks of both SR1$_7$ and GroEL$_{14}$ with increasing collision

voltages and convert these values to relative abundances of the chaperonin complexes and $E_{c.o.m.}$s we can plot breakdown graphs of the intact chaperonin complexes (Fig. 4, $SR1_7$ and $GroEL_{14}$). The maximum amount of collision voltage we can apply to our collision cell was 200 V, which corresponds to $E_{c.o.m.}$ values of 0.68 and 0.94 eV for the selected ions of $GroEL_{14}$ and $SR1_7$, respectively. Figure 4 shows that both tetradecameric GroEL as well as heptameric SR1 start to dissociate around the same center-of-mass collision energy ($\sim 0.50$ eV). At higher collison cell voltage differences started to occur. Whereas tetradecameric GroEL almost completely dissociated, still about half of the heptameric SR1 complexes were intact at the highest attainable $E_{c.o.m.}$. This indicates that in the gas phase there are possibly two different single-ring chaperonin species present, one of which is as susceptible to gas-phase dissociation as $GroEL_{14}$, whereas the other population is somewhat more resistant to gas-phase dissociation even when compared to the double-ring GroEL complex. For both $SR1_7$ and $GroEL_{14}$ the dissociating subunit is a monomer ($SR1_1$ and $GroEL_1$), resulting in SR1 hexamers ($SR1_6$) and GroEL tridecamers ($GroEL_{13}$). At a certain center-of-mass collision energy even dodecameric GroEL ($GroEL_{12}$) ions start to appear originating from the tridecamers that start to expel a second GroEL monomer.

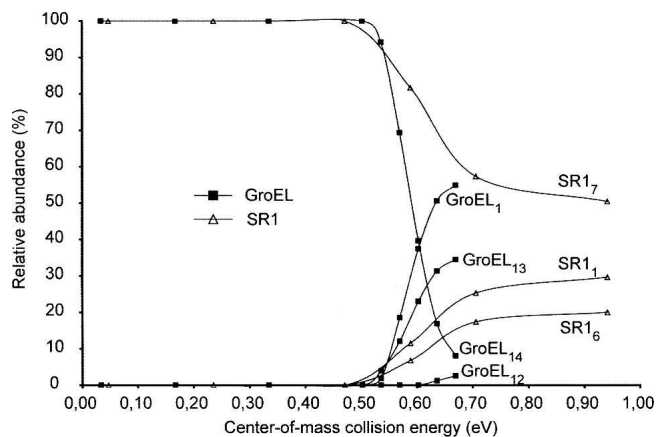Next we monitored the effect of substrate binding on the gas-phase stability of the single-ring chaperonin com-



**Figure 4.** Breakdown graphs of heptameric SR1 ($SR1_7$ open triangles) and tetradecameric GroEL ($GroEL_{14}$, black squares). Tandem mass spectrometry was performed on 47+ and 67+ ions of $SR1_7$ and $GroEL_{14}$, respectively. The curve of $SR1_7$ shows the relative abundance of the selected parent ion peak at increasing center-of-mass collision energies. As the relative abundance of $SR1_7$ decreases due to dissociation, the abundance of the dissociated products (monomers $SR1_1$ and hexamers $SR1_6$) increases. The dissociation products of $GroEL_{14}$ are monomers ($GroEL_1$) and tridecamers ($GroEL_{13}$). At higher energy values tridecameric GroEL further dissociates into dodecamers ($GroEL_{12}$) and monomers of GroEL.

plex. Saturated $SR1_7$(substrate) complexes were produced with gp23, Rubisco, gp5, or MDH. For each complex a charge state was selected and subjected to increasing $E_{c.o.m.}$. Figure 5A shows some of the mass spectra obtained for the $SR1_7$(gp5) complex. We selected the 50+ charge state, and at low center-of-mass collision energies the parent ion remains intact. Once the $E_{c.o.m.}$ exceeded 0.56 eV dissociation products were formed. The dissociation products of $SR1_7$(gp5) are SR1 monomers ($SR1_1$) and hexameric SR1 complexes with gp5 still bound ($SR_6$[gp5]). But even though at increasing $E_{c.o.m.}$s the amount of dissociation products increased, still a significant amount of parent ion remains intact at 0.90 eV (this corresponds to the maximum 200-V collision voltage). Interestingly, no loss of the gp5 substrate is observed, indicating that even in the gas phase the structure of the chaperonin complex is retained to some extent (Ruotolo et al. 2005; van Duijn et al. 2006). The relative abundance of the different complexes in the spectra was calculated according to the intensities of the ions, allowing us to produce breakdown graphs as described above. Figure 5B shows the dissociation of the parent ion peaks of the different $SR1_7$(substrate) complexes as a function of the $E_{c.o.m.}$. For comparison the dissociation curve of $SR1_7$ is also shown. For reasons of clarity no curves of the dissociated products, $SR1_1$ and $SR_6$(substrate), that start to appear are shown. Overall the dissociation plots of the $SR1_7$(substrate) complexes are very similar, as they follow a similar behavior around the same $E_{c.o.m.}$s. Also no stabilizing effect of the heptameric SR1 complex upon substrate binding was observed. The single-ring chaperonin complex thus showed similar binding stoichiometries for the different substrates, and also the stabilities of the different complexes during gas-phase-induced dissociation were similar.

To investigate the gas-phase stability of the different $GroEL_{14}$(substrate) complexes, similar types of tandem mass spectrometry experiments were performed. Whereas the free $GroEL_{14}$ completely dissociated (Fig. 5), the parent ion of the $GroEL_{14}$(Rubisco) complex remained almost completely intact upon collision-induced dissociation (data not shown; van Duijn et al. 2006). In contrast to Rubisco, which stabilized the chaperonin complex significantly, binding of one gp23 showed no effect (data not shown; van Duijn et al. 2006). Gp23 had to occupy both rings of the chaperonin in order to increase the gas-phase stability of the complex (Fig. 6A). Only a very small amount of the $GroEL_{14}$(gp23)$_2$ complexes eject a monomer of GroEL at high $E_{c.o.m.}$s. When we now monitored the gas-phase dissociation of the $GroEL_{14}$(gp5) and $GroEL_{14}$(gp5)$_2$ complexes a similar behavior was found. Like with gp23, binding of two gp5 molecules increased the stability of the chaperonin complex. If only one gp5 bound to $GroEL_{14}$, the majority of the parent ions were
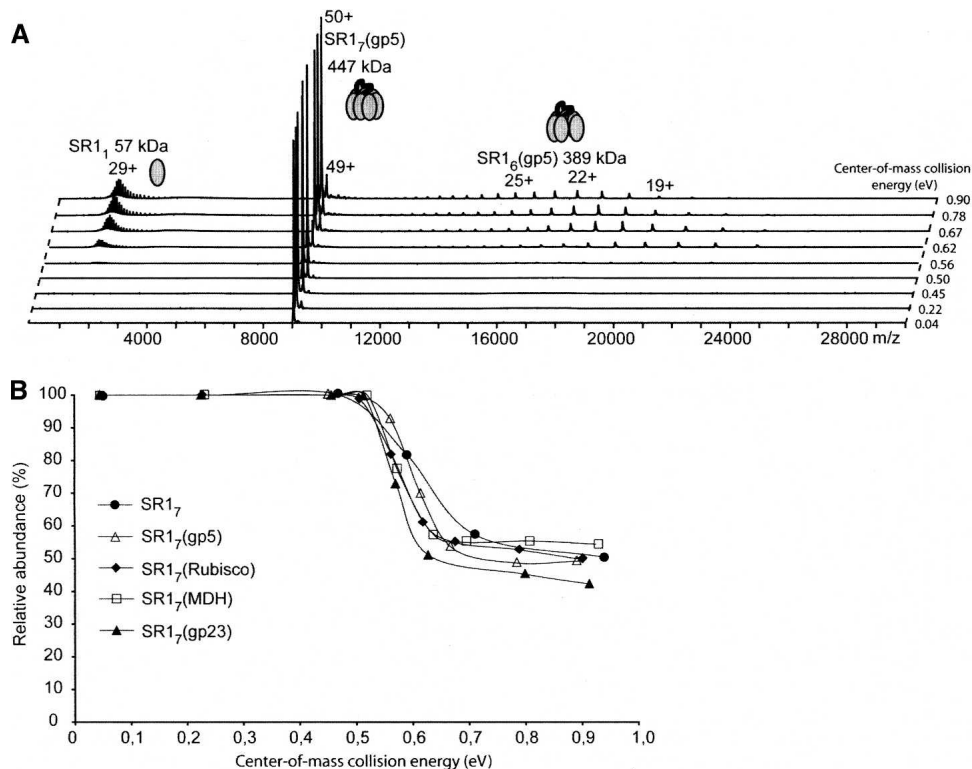
**Figure 5.** (*A*) Tandem mass spectra of the 50+ charge state of $SR1_7(gp5)$ at increasing center-of-mass collision energies. The dissociation products are SR1 monomers ($SR1_1$) and hexameric SR1 complexes with gp5 still bound ($SR1_6[gp5]$). (*B*) Breakdown graphs of different $SR1_7$(substrate) parent ion peaks as well as free $SR1_7$ (closed circles). Open triangles indicate a complex between $SR1_7$ and gp5, closed rhombus with Rubisco, open squares with MDH, and closed triangles with gp23.

dissociated into $GroEL_{13}(gp5)$ and $GroEL_1$. It is even possible for the complex to eject a second GroEL monomer at the highest collision voltage ($E_{c.o.m.}$ of 0.65 eV, Fig. 6B), similar to free $GroEL_{14}$. The amount of parent ion that remains is much larger when both rings contain a gp5 substrate (Fig. 6C). However, the effect was still somewhat smaller than when two gp23 substrates were bound (Fig. 6A), and this might be because gp5 is smaller than gp23 (47 kDa vs. 56 kDa).

The number of interactions that a smaller substrate makes with the heptameric GroEL ring might be expected to be less. If these interactions contribute to the stability of the chaperonin complex, the stability of the complex decreases when the substrate makes fewer interactions. This realization inspired us to investigate the stabilizing effect of an even smaller substrate, MDH (32 kDa), to the $GroEL_{14}$ complex. Both $GroEL_{14}(MDH)$ and $GroEL_{14}(MDH)_2$ dissociated completely into monomeric GroEL and $GroEL_{13}(MDH)$ or monomeric GroEL and $GroEL_{13}(MDH)_2$, respectively, and at higher energy values also $GroEL_{12}(MDH)$ or $GroEL_{12}(MDH)_2$ was formed. So, none of these chaperonin complexes gained any extra stability due to the binding of one or two MDH substrates (data not shown). This is in line with our hypothesis that

the size of the substrate is an important factor for substrate-dependent stabilization upon binding to the chaperonin complex, assuming that an increased size means a larger number of interactions. Figure 6D schematically summarizes the observed gas-phase stabilities of the different $GroEL_{14}$(substrate) complexes.

## Discussion and Conclusion

Here we have investigated the effect of inter-ring communication in the chaperonin complex upon substrate binding. Whereas $SR1_7$ responds similarly to the binding of gp23, Rubisco, gp5, and MDH, their binding induces different effects in the $GroEL_{14}$ complex. In line with what was expected, $SR1_7$ could only bind one substrate protein, and at a 1:3 ratio of heptameric SR1:unfolded substrate almost all of the single-ring chaperonin complex present was occupied by a single substrate, independent of the substrate that was used. It was already known that the binding of a substrate to one of the GroEL rings could have an effect on the binding characteristics of the opposite GroEL ring. However, so far this always showed to be a negative cooperative effect, i.e., the binding of the first substrate effectively inhibited the binding of a
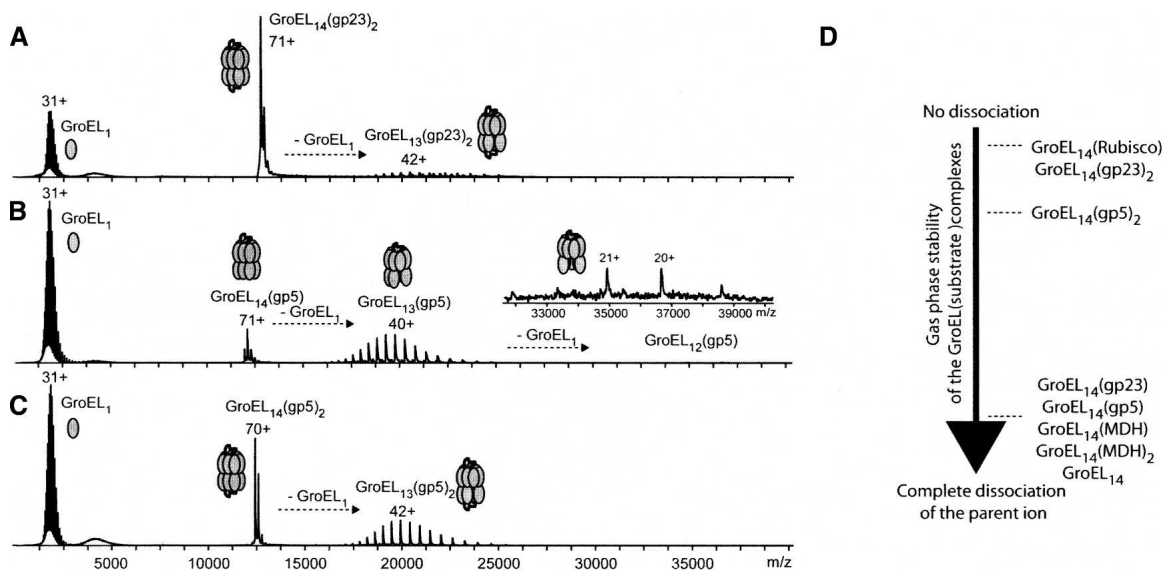
**Figure 6.** Tandem mass spectra of (*A*) the 71+ charge state of GroEL$_{14}$(gp23)$_2$ at a center-of-mass collision energy of 0.62 eV; (*B*) the 70+ charge state of GroEL$_{14}$(gp5) at a center-of-mass collision energy of 0.65 eV; (*C*) the 71+ charge state of GroEL$_{14}$(gp5)$_2$ at a center-of-mass collision energy of 0.63 eV. Even though gp5 is able to stabilize the tetradecameric GroEL complex when it occupies both rings, this effect is smaller than when two gp23 substrates bind to the complex; compare *A* and *C*. (*D*) Summary of the gas-phase stability of the different GroEL$_{14}$(substrate) complexes, determined from the collision-induced dissociation experiments.

second substrate in the opposite ring (Wang and Chen 2003; Falke et al. 2005). In contrast to these studies we show that the stoichiometry of substrate binding to the chaperonin complex is dependent on the type of substrate. The capsid proteins gp23 and gp5 as well as MDH are able to occupy both GroEL rings simultaneously, whereas Rubisco only binds to one heptameric ring of the chaperonin complex at the time. This suggests that the inter-ring communications in the chaperonin complex occur already without ADP or ATP present and are substrate dependent. Since the possibility of inter-ring communication is absent for SR1$_7$, this complex reacts similarly to all four substrates. To further investigate the differences between GroEL$_{14}$ and SR1$_7$ upon substrate binding tandem mass spectrometry was used. This method allows the investigation of the gas-phase stability of protein complexes, as several studies have shown that it is very well possible that the three-dimensional structure of protein complexes may be retained in the gasphase to some extent (Ruotolo et al. 2005; van Duijn et al. 2006; R.H.H. van den Heuvel, H. Mazon, E. van Duijn, S.A. Synowsky, K. Lorenzen, C. Versluis, S.J.J. Brouns, J. van der Oost, J. Hoyes, and A.J.R. Heck, in prep.).

Using tandem mass spectrometry we observed that both SR1$_7$ and GroEL$_{14}$ start to dissociate around the same center-of-mass collision energy. This indicates that the chaperonin complexes are equally stable in our gas-phase-induced dissociation experiments. This is in contrast to an earlier study by Panda et al. (2002), who monitored the dissociation characteristics of GroEL$_{14}$ and SR1$_7$ by high hydrostatic pressure. Their results indicated that the presence of the second ring in the chaperonin complex is important for its stability. However, this approach is very different from the one we used here. First of all, high hydrostatic pressure study was performed in the solution phase. Furthermore, much more energy can be put into the protein complex by high hydrostatic pressure than by tandem mass spectrometry, even though pressures were used at which oligomeric proteins generally only undergo reversible dissociation and no denaturation (<3 kbar). Under these conditions complete dissociation of SR1$_7$ and GroEL$_{14}$ into monomers was observed. By using tandem mass spectrometry we were only able to dissociate, at the most, two subunits from the chaperonin complexes, leaving the rest of the complex intact. In addition, the dissociated monomers originating from the SR1$_7$ complex were unsuitable for reassociation, indicating that they did undergo some conformational drift.

In addition to the fact that the single-ring chaperonin complex showed similar binding stoichiometries for the different substrates, the stability of the different SR1$_7$(substrate) complexes during gas-phase-induced dissociation was also the same. It was the presence of the second heptameric ring in the chaperonin complex that made a clear difference. Interesting to mention here is that previously we have shown that, upon the binding of the cochaperonin GroES to GroEL, the chaperonin complex

does not gain any stability (R.H.H. van den Heuvel, H. Mazon, E. van Duijn, S.A. Synowsky, K. Lorenzen, C. Versluis, S.J.J. Brouns, J. van der Oost, J. Hoyes, and A.J.R. Heck, in prep.). Hence binding a substrate or GroES appears to have a very distinct impact on the stability of the chaperonin complex. The binding of two capsid proteins to the chaperonin significantly stabilized the complex. However, we observed that the stabilizing effect of the somewhat smaller capsid protein gp5 (47 kDa vs. 56 kDa) was slightly smaller. It is known that substrates are able to bind multivalently to the heptameric ring of the chaperonin complex (Farr et al. 2000; Wang and Chen 2003). A study by Farr et al. (2000), showed that, for the productive folding of a stringent substrate like Rubisco, a minimum of three consecutive GroEL monomers were required. Stringent substrates are dependent on the complete chaperonin system to reach their native conformation, like our substrate proteins gp23, Rubisco, gp5, and MDH all are. However, for a larger substrate it becomes more likely that in addition to the interactions it makes with the three adjacent GroEL monomers, it can also bind to another GroEL monomer that is not necessarily directly next to the others. As the substrate is large enough to interact with other surfaces within the heptameric ring as well, the total number of interactions between the substrate and the GroEL ring are likely to increase. We suggest that if the number of interactions increases, the interactions with $GroEL_{14}$ strengthen, and it thereby becomes more difficult to dissociate the chaperonin complex. This hypothesis is confirmed by our observations with the smallest substrate of our studies, MDH, which is only 32 kDa. The $GroEL_{14}$ complex with two MDH substrates bound was as susceptible to dissociation as the free $GroEL_{14}$ complex. Very recently it was also shown by Tang et al. (2006), that the efficiency of the chaperonin complex is different for differently sized substrates. In their approach they showed that the volume of the GroEL cavity influences the folding reaction. Wheareas for larger substrates (40–50 kDa) the size of the GroEL cavity was optimal for the folding reaction, for smaller substrates (30 kDa) it was benificial to decrease the volume of the cage. It thus could be very likely that the ability for a substrate to interact with more GroEL subunits at the time favors the folding reaction and at the same time stabilizes the complete chaperonin complex.

In conclusion we show that inter-ring communication is important for the chaperonin complex for its response to ligand binding even in the absence of nucleotides. In the absence of the second GroEL ring the chaperonin is unable to differentiate between different substrates. Two different GroEL complexes have been described, both containing a single point mutation at the contact surface between the rings (E434K and E461K) (Sot et al. 2002, 2005). The initial glutamic acid residues are involved in salt-bridge formation, which contributes to the ring–ring interactions. Distorting these salt bridges strongly affects the inter-ring communications of the chaperonin system, and these mutant double-ring GroEL complexes are thought to lack the negative inter-ring cooperativity for substrate binding (Sot et al. 2005). It would be interesting to determine the behavior of these mutants upon binding of different substrates by using our approach.

## Materials and Methods

### Protein preparations

GroEL was overexpressed in *Escherichia coli* strain MC1009 containing plasmid pSL6. Cells were grown in Luria-Bertani (LB) medium with 100 μg/mL ampicillin and 0.0005% (w/v) arabinose at 37°C under vigorous aeration. GroEL was purified according to a previously described protocol, slightly modified by the introduction of an acetone precipitation step (Quaite-Randall and Joachimiak 2000; Voziyan and Fisher 2000). SR1 was overexpressed in *E. coli* strain MC1009 containing plasmid pET11a-SR1 (a kind gift from N. Strippel, Max Planck Institute, Germany), and purified as described previously (Horwich et al. 1998). The major capsid protein gp23 was expressed from the IPTG inducible plasmid pET2331 in *E. coli* strain BL21(DE3), a generous gift from L. Black (University of Maryland, Baltimore, USA). Dimeric Rubisco from *Rhodospirillum rubrum* was expressed in *E. coli*. Gp23 and Rubisco were purified as described previously (Pierce and Reddy 1986; van der Vies et al. 1994; van Duijn et al. 2005). Purified gp5 was a kind gift from C. Teschke (University of Connecticut, USA). Mitochondrial malate dehydrogenase was purchased from Sigma (M2634).

### Mass spectrometry

Mass spectrometry (MS) measurements were performed in positive ion mode using an electrospray ionization time-of-flight (ESI-TOF) instrument (LC-T; Micromass) equipped with a Z-spray nano-electrospray ionization source. Needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments) on a P-97 puller (Sutter Instruments), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories) six Pirani 501 sputter coater. To produce intact gas-phase ions from large complexes in solution the pressure in the first vacuum stages of the mass spectrometer was increased (to values ranging from 7.5–9.9 mbar) to cool the ions collisionally (Krutchinsky et al. 1998; Tahallah et al. 2001; Sobott et al. 2002). The exact pressure was selected to facilitate preservation of noncovalent interactions and promote efficient ion desolvation in the interface region of the instrument. This, in turn, resulted in adequately sharp ion signals to allow for confident determination of the stoichiometries. Nano-electrospray voltages were optimized for generation and transmission of the macromolecular protein complexes; the needle voltage varied between 1300 and 1500 V, and the sample cone voltage was set at 200 V.

Tandem mass spectra were acquired on a modified Q-TOF 1 instrument under conditions optimized for the transmission of noncovalent complexes (R.H.H. van den Heuvel, H. Mazon,

E. van Duijn, S.A. Synowsky, K. Lorenzen, C. Versluis, S.J.J. Brouns, J. van der Oost, J. Hoyes, and A.J.R. Heck, in prep.). Ions were isolated in the quadrupole analyzer and accelerated into an argon-filled linear hexapole collision cell. Various collision energies were used, with argon at a pressure of $2.0 \times 10^{-2}$ mbar. The capillary voltage was typically set at 1500 V and the cone voltage at 150 V. The pressure in the first vacuum stage of the instrument was increased by reducing the pumping efficiency of the rotary pump. Since the first Pirani gauge is located between the speedivalve and the rotary pump we could not measure the actual pressure in the first vacuum stage. In the second hexapole chamber the pressure was $1.0 \times 10^{-2}$ mbar, the third vacuum chamber, containing the quadrupole, had a pressure of $6.7 \times 10^{-4}$ mbar. Pressure conditions in the collision cell were $2 \times 10^{-2}$ mbar and $2.0 \times 10^{-6}$ mbar in the time-of-flight chamber.

## Sample preparation for mass spectrometry

SR1 and GroEL concentrations below are given as heptamers and tetradecamers, respectively, while substrate concentrations are given as monomers. The buffer of the all the proteins was exchanged to 50 mM ammonium acetate with a pH of 6.8, by using ultrafiltration filters with a cutoff of 5000 Da (Millipore) before spectroscopic analysis. SR1(substrate) and GroEL(substrate) complexes were formed by first unfolding the substrate in 8 M urea for 1 h at room temperature at a final substrate concentration of 25 $\mu$M. For Rubisco unfolding also 10 mM DTT was added, to prevent disulfide bridge formation. The unfolded substrate was added to a 50 mM ammonium acetate buffer (pH 6.8), containing 1 $\mu$M SR1 or 2 $\mu$M GroEL at various ratios varying from 1:0 to 1:5. The resulting maximum concentration of 1.7 M urea did not affect the oligomeric SR1 or GroEL structures. The final concentration of SR1(substrate) and GroEL(substrate) complexes varied between 1.4 and 2 $\mu$M. Excess of urea was removed from the sample by filtration while changing the buffer to 50 mM aqueous ammonium acetate (pH 6.8).

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