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Published on Web 03/16/2006

Tandem Mass Spectrometry of Intact GroEL−Substrate Complexes Reveals Substrate-Specific Conformational Changes in the *trans* Ring

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Abstract: It has been suggested that the bacterial GroEL chaperonin accommodates only one substrate at any given time, due to conformational changes to both the *cis* and *trans* ring that are induced upon substrate binding. Using electrospray ionization mass spectrometry, we show that indeed GroEL binds only one molecule of the model substrate Rubisco. In contrast, the capsid protein of bacteriophage T4, a natural GroEL substrate, can occupy both rings simultaneously. As these substrates are of similar size, the data indicate that each substrate induces distinct conformational changes in the GroEL chaperonin. The distinctive binding behavior of Rubisco and the capsid protein was further investigated using tandem mass spectrometry on the intact 800−914 kDa GroEL−substrate complexes. Our data suggest that even in the gas phase the substrates remain bound inside the GroEL cavity. The analysis revealed further that binding of Rubisco to the GroEL oligomer stabilizes the chaperonin complex significantly, whereas binding of one capsid protein did not have the same effect. However, addition of a second capsid protein molecule to GroEL resulted in a similar stabilizing effect to that obtained after the binding of a single Rubisco. On the basis of the stoichiometry of the GroEL chaperonin−substrate complex and the dissociation behavior of the two different substrates, we hypothesize that the binding of a single capsid polypeptide does not induce significant conformational changes in the GroEL *trans* ring, and hence the unoccupied GroEL ring remains accessible for a second capsid molecule.

Introduction

Molecular chaperones are required for the correct folding of a variety of different proteins. In *Escherichia coli*, several chaperone proteins exist, but only the GroEL−GroES chaperonin complex is essential for viability under all growth conditions. This well-studied chaperonin complex assists in the folding of approximately 10% of all the newly synthesized polypeptides. However, for only 3.5% of the proteins, the GroEL−GroES chaperonin complex is absolutely required.[1,2] The large GroEL chaperonin (M_w = 800 kDa) consists of two heptameric rings stacked back to back, each containing a distinct large central cavity. These two cavities are structurally identical in unliganded GroEL, as shown in Figure 1a.[3,4] Each GroEL subunit consists of three domains, the equatorial domain where nucleotide binding occurs, the apical domain involved in polypeptide and/or GroES binding, and a flexible intermediate domain that connects the two.[5] When the heptameric co-chaperonin GroES binds to the GroEL oligomer, in the presence of ADP or ATP and Mg^{2+} ions, an enclosed cavity is formed: the so-called "Anfinsen cage" (Figure 1b).

It is in this folding cavity (*cis* ring) that the polypeptide is well protected against aggregation or degradation that may occur in the crowded cellular environment.[6−8] The folding cycle starts when the substrate polypeptide binds to the hydrophobic residues of the apical domains of the GroEL ring (*cis* ring). Some of these hydrophobic residues also interact with specific amino

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(1) Kerner, M. J.; Naylor, D. J.; Ishihama, Y.; Maier, T.; Chang, H. C.; Stines, A. P.; Georgopoulos, C.; Frishman, D.; Hayer-Hartl, M.; Mann, M.; Hartl, F. U. *Cell* **2005**, *122*, 209−220.
(2) Houry, W. A.; Frishman, D.; Eckerskorn, C.; Lottspeich, F.; Hartl, F. U. *Nature* **1999**, *402*, 147−154.
(3) Ranson, N. A.; Farr, G. W.; Roseman, A. M.; Gowen, B.; Fenton, W. A.; Horwich, A. L.; Saibil, H. R. *Cell* **2001**, *107*, 869−879.
(4) Xu, Z.; Horwich, A. L.; Sigler, P. B. *Nature* **1997**, *388*, 741−750.
(5) Gomez-Puertas, P.; Martin-Benito, J.; Carrascosa, J. L.; Willison, K. R.; Valpuesta, J. M. *J. Mol. Recognit.* **2004**, *17*, 85−94.
(6) Ellis, R. J. *Curr. Biol.* **2001**, *11*, R1038−R1040.
(7) Hartl, F. U.; Hayer-Hartl, M. *Science* **2002**, *295*, 1852−1858.
(8) Sigler, P. B.; Xu, Z.; Rye, H. S.; Burston, S. G.; Fenton, W. A.; Horwich, A. L. *Annu. Rev. Biochem.* **1998**, *67*, 581−608.

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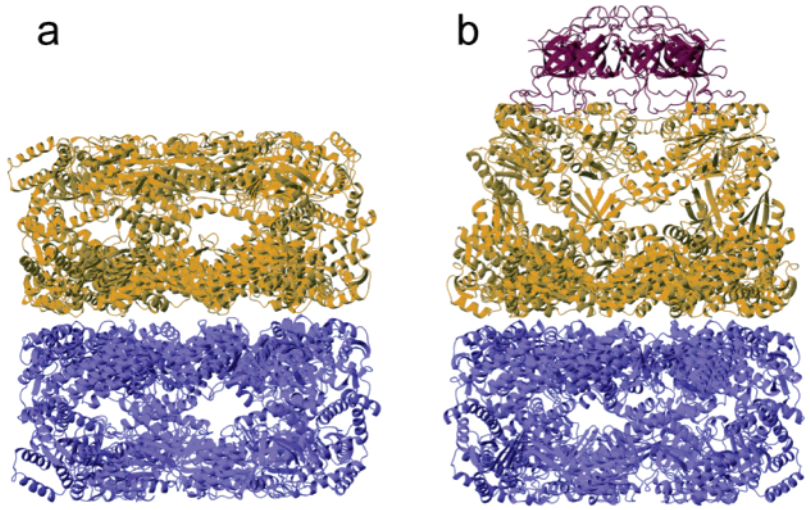

Figure 1. Crystallographic models of (a) the overall structure of GroEL and (b) the GroEL−GroES chaperonin system from *Escherichia coli*. The two heptameric rings of GroEL are indicated in yellow and blue; GroES is indicated in red (used PDB accession codes: 1AON and 1J4Z).

acids in the mobile loops of the GroES heptamer.[4,9] As a consequence of the cooperative binding of ATP and subsequently GroES to the *cis* ring, the bound substrate is displaced further into the enlarged folding cavity. Inside the GroES-capped folding chamber, the polypeptide continues to fold for about 10 s; this is the time that it takes for ATP to be hydrolyzed in the *cis* ring.[10] Binding of substrate and ATP to the unoccupied (*trans*) ring induces the release of GroES, ADP, and the substrate from the *cis* ring. In the event that the polypeptide has not completely folded, it can rebind to a chaperonin complex to undergo subsequent cycles of assisted folding.[11−13]

The mechanism of substrate recognition and binding to the GroEL−GroES chaperonin machine has been studied for a number of polypeptides.[14−17] In addition to studies designed to identify natural substrates,[2,18,19] and to assign the hydrophobic amino acids in the apical domains of the GroEL subunits that are involved in polypeptide binding,[4] a number of investigations have been conducted to gain insight into the structure of the GroEL−substrate complexes.[20−22] However, the dynamic nature and the size of these complexes have so far hampered high-resolution structural analysis by X-ray crystallography and NMR spectroscopy. The recently reported analyses of the GroEL−GroES and a GroEL−substrate complex using NMR spectroscopy reveal that it is technically possible to study structures and interactions of such large macromolecular complexes by NMR,[23,24] which will undoubtedly further stimulate the investigation of GroEL−substrate complexes. Other lower resolution structural techniques, such as small-angle neutron scattering and cryo-electron microscopy, have provided some insight into where substrate polypeptides are localized.[22,25−27] From these studies, it appears that the GroEL chaperonin binds one substrate molecule at a time. Furthermore, it has been observed that this binding event induces significant conformational changes in the chaperonin. Specifically, the apical domains move inward, resulting in protrusion of the substrate from the cavity of the *cis* ring, while the opening of the unoccupied *trans* ring becomes narrower. As a consequence, the *trans* ring becomes effectively inhibited from binding a second polypeptide substrate,[22,26,28] a phenomenon known as negative cooperativity between the rings. Although these observations apply for the general case, the precise interaction between the substrate and unliganded GroEL is to some extent substrate-specific.[17,29] Farr et al.[17] showed, using mutated GroEL subunits, that stringent substrates (substrate proteins that are totally dependent on the chaperonin complex to reach their native conformation), such as Rubisco, bind to several apical domains of at least three different GroEL subunits, whereas a less stringent substrate, such as rhodanese, interacts with fewer subunits in the ring. Recently, we have demonstrated that electrospray ionization (ESI) mass spectrometry is an excellent method to monitor different macromolecular complexes involved in chaperonin-assisted folding of the major capsid protein of bacteriophage T4.[30] The bacteriophage T4 uses the *E. coli* host GroEL chaperonin for the folding of its major capsid protein, gp23. During infection, the host co-chaperonin GroES is replaced by the bacteriophage-encoded co-chaperonin

(9) Ellis, R. J. *Curr. Biol.* **2003**, *13*, R881−R883.
(10) Rye, H. S.; Burston, S. G.; Fenton, W. A.; Beechem, J. M.; Xu, Z.; Sigler, P. B.; Horwich, A. L. *Nature* **1997**, *388*, 792−798.
(11) Elcock, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 2340−2344.
(12) Walters, C.; Errington, N.; Rowe, A. J.; Harding, S. E. *Biochem. J.* **2002**, *364*, 849−855.
(13) Motojima, F.; Chaudhry, C.; Fenton, W. A.; Farr, G. W.; Horwich, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15005−15012.
(14) Chen, S.; Roseman, A. M.; Hunter, A. S.; Wood, S. P.; Burston, S. G.; Ranson, N. A.; Clarke, A. R.; Saibil, H. R. *Nature* **1994**, *371*, 261−264.
(15) Braig, K.; Otwinowski, Z.; Hegde, R.; Boisvert, D. C.; Joachimiak, A.; Horwich, A. L.; Sigler, P. B. *Nature* **1994**, *371*, 578−586.
(16) Houry, W. A. *Biochem. Cell Biol.* **2001**, *79*, 569−577.
(17) Farr, G. W.; Furtak, K.; Rowland, M. B.; Ranson, N. A.; Saibil, H. R.; Kirchhausen, T.; Horwich, A. L. *Cell* **2000**, *100*, 561−573.
(18) Stan, G.; Brooks, B. R.; Lorimer, G. H.; Thirumalai, D. *Protein Sci.* **2005**, *14*, 193−201.
(19) Ewalt, K. L.; Hendrick, J. P.; Houry, W. A.; Hartl, F. U. *Cell* **1997**, *90*, 491−500.
(20) van der Vaart, A.; Ma, J.; Karplus, M. *Biophys. J.* **2004**, *87*, 562−573.
(21) Shimamura, T.; Koike-Takeshita, A.; Yokoyama, K.; Masui, R.; Murai, N.; Yoshida, M.; Taguchi, H.; Iwata, S. *Structure* **2004**, *12*, 1471−1480.
(22) Falke, S.; Fisher, M. T.; Gogol, E. P. *J. Mol. Biol.* **2001**, *308*, 569−577.
(23) Fiaux, J.; Bertelsen, E. B.; Horwich, A. L.; Wuthrich, K. *Nature* **2002**, *418*, 207−211.
(24) Horst, R.; Bertelsen, E. B.; Fiaux, J.; Wider, G.; Horwich, A. L.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12748−12753.
(25) Braig, K.; Simon, M.; Furuya, F.; Hainfeld, J. F.; Horwich, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3978−3982.
(26) Falke, S.; Fisher, M. T.; Gogol, E. P. *J. Struct. Biol.* **2001**, *133*, 203−213.
(27) Thiyagarajan, P.; Henderson, S. J.; Joachimiak, A. *Structure* **1996**, *4*, 79−88.
(28) Fenton, W. A.; Horwich, A. L. *Q. Rev. Biophys*. **2003**, *36*, 229−256.
(29) Falke, S.; Tama, F.; Brooks, C. L., III; Gogol, E. P.; Fisher, M. T. *J. Mol. Biol.* **2005**, *348*, 219−230.
(30) van Duijn, E.; Bakkes, P. J.; Heern, R. M. A.; van den Heuvel, R. H. H.; van Heerikhuizen, H.; van der Vies, S. M.; Heck, A. J. R. *Nat. Methods* **2005**, *2*(5), 371−376.

gp31. In contrast to many of the model substrate proteins that have been studied so far, the capsid protein of the bacteriophage is a natural in vivo substrate of the GroEL chaperonin machine. We have observed previously that unliganded GroEL can bind up to two capsid protein molecules,[30] which seems to be in contrast to the aforementioned substrate-induced negative cooperativity between the two heptameric rings of the GroEL chaperonin.[31,32]

To further investigate and compare the binding properties of the unliganded GroEL chaperonin, we have analyzed two size-comparable substrate proteins, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, 54 kDa, dimeric under native conditions) and the bacteriophage T4 capsid protein (gp23, 56 kDa, hexameric under native conditions) by ESI mass spectrometry. In addition, we have used tandem mass spectrometry, which is a relatively new methodology in the field of structural biology. It has been demonstrated that this technique in particular may be used to generate information about the stoichiometry, structure, and stability of macromolecular protein complexes,[33−35] such as the composition of the stalk proteins of ribosomes and the stoichiometry of the heterogeneous tryptophan RNA-binding attenuation protein complex. Here we apply ESI-mass spectrometry and tandem mass spectrometry to investigate the intact 800−914 kDa GroEL−substrate complexes. On the basis of our results, we propose a model to explain the aberrant binding behavior of the bacteriophage T4 capsid protein.

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.[36,37] 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.[30,38,39]

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, Manchester, U.K.) equipped with a Z-spray nanoelectrospray ionization source. Needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter Instruments, Novato, CA), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, Milpitas, CA) six Pirani 501 sputter coater. To produce intact gas phase ions from large complexes in solution, it was required to cool the ions collisionally by increasing the pressure in the first vacuum stages of the mass spectrometer[40−42] to values ranging from 7.5 to 9.9 mbar. The 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 of complexes from the mass spectrum. Nanoelectrospray 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-TOF2 instrument,[41] under conditions optimized for the transmission of noncovalent complexes. Ions were isolated in the quadrupole analyzer and accelerated into an argon-filled linear hexapole collision cell. Various collision energies were used as described below, with argon at a pressure of 3.0×10^{-2} mbar. The capillary voltage was typically set at 1300 V, the cone voltage at 200 V, the ion energy at 1.5 V, and the cone gas was set at 100 L/h. Pressure conditions were 8.0×10^{-2} mbar in the analyzer and 2.3×10^{-6} mbar in the time-of-flight chamber. All spectra were mass calibrated by using an aqueous solution of cesium iodide (50 mg/mL).

Sample Preparation for Mass Spectrometry. GroEL concentrations below are given as tetradecamers, while substrate concentrations are given as monomers. The buffer of the GroEL, gp23, and Rubisco preparations was exchanged to 50 mM ammonium acetate with a pH of 6.8, by using ultrafiltration filters with a cutoff of 5000 Da (Millipore, Bedford). Lysozyme (Sigma) was directly dissolved in 50 mM ammonium acetate at pH 6.8. 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 μM. For Rubisco unfolding, also 10 mM DTT was added to prevent disulfide bridge formation. Unfolding of gp23 was confirmed by circular dichroism analysis. The unfolded substrate was added to a 50 mM ammonium acetate buffer pH 6.8, containing 2 μM GroEL at various ratios varying from 1:0 to 1:5. The resulting maximum concentration of 1.7 M urea did not affect the tetradecameric GroEL complex since negligible amounts of monomeric and heptameric GroEL were observed. The final concentration of GroEL and substrate varied between 2−1.4 and 0−7 μM, respectively, giving rise to GroEL tetradecamer-to-substrate monomer ratios of 1:0 to 1:5. Excess of urea was removed from the sample by filtration while changing the buffer to 50 mM ammonium acetate pH 6.8.

Gel Electrophoresis and Protein Detection. GroEL−gp23 and GroEL−Rubisco complexes were prepared as described above at identical ratios, except that GroEL was in 50 mM Tris-HCl buffer pH 7.5. Next, the chaperonin−substrate complexes were incubated with or without proteinase K (1.2 μg/mL) for 10 min at room temperature. Proteolysis was stopped by the addition of 1 mM PMSF, and the proteins were analyzed by 7% SDS−PAGE and western blotting. Antibodies raised in guinea pigs and rabbits were used to detect gp23 and Rubisco, respectively. Antibody binding was visualized using the electrochemiluminescence (ECL) detection system (PierceBiotechnology, Rockford, IL).

Results and Discussion

Distinct Interactions of Gp23 and Rubisco Substrates with Unliganded GroEL. As in most GroEL−substrate interaction studies, we started the experiments by unfolding the substrate protein in urea. Subsequent addition of the unfolded gp23 substrate to a GroEL containing buffer resulted in the formation of the GroEL−substrate complex. In line with what we observed previously, the addition of unfolded gp23 to GroEL in a 3:1 ratio resulted in an ESI mass spectrum that showed three

(31) Horovitz, A.; Fridmann, Y.; Kafri, G.; Yifrach, O. *J. Struct. Biol.* **2001**, *135*, 104−114.
(32) Makio, T.; Arai, M.; Kuwajima, K. *J. Mol. Biol.* **1999**, *293*, 125−137.
(33) Hanson, C. L.; Videler, H.; Santos, C.; Ballesta, J. P.; Robinson, C. V. *J. Biol. Chem.* **2004**, *279*, 42750−42757.
(34) Videler, H.; Ilag, L. L.; McKay, A. R.; Hanson, C. L.; Robinson, C. V. *FEBS Lett.* **2005**, *579*, 943−947.
(35) McCammon, M. G.; Hernandez, H.; Sobott, F.; Robinson, C. V. *J. Am. Chem. Soc.* **2004**, *126*, 5950−5951.
(36) Quaite-Randall, E.; Joachimiak, A. *Methods Mol. Biol.* **2000**, *140*, 29−39.
(37) Voziyan, P. A.; Fisher, M. T. *Protein Sci.* **2000**, *9*, 2405−2412.
(38) Pierce, J.; Reddy, G. S. *Arch. Biochem. Biophys.* **1986**, *245*, 483−493.
(39) van der Vies, S. M.; Gatenby, A. A.; Georgopoulos, C. *Nature* **1994**, *368*, 654−656.
(40) Krutchinsky, A. N.; Chernushevich, I. V.; Spicer, V. L.; Ens, W.; Standing, K. G. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 569−579.
(41) Sobott, F.; Hernandez, H.; McCammon, M. G.; Tito, M. A.; Robinson, C. V. *Anal. Chem.* **2002**, *74*, 1402−1407.
(42) Tahallah, N.; Pinkse, M.; Maier, C. S.; Heck, A. J. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 596−601.

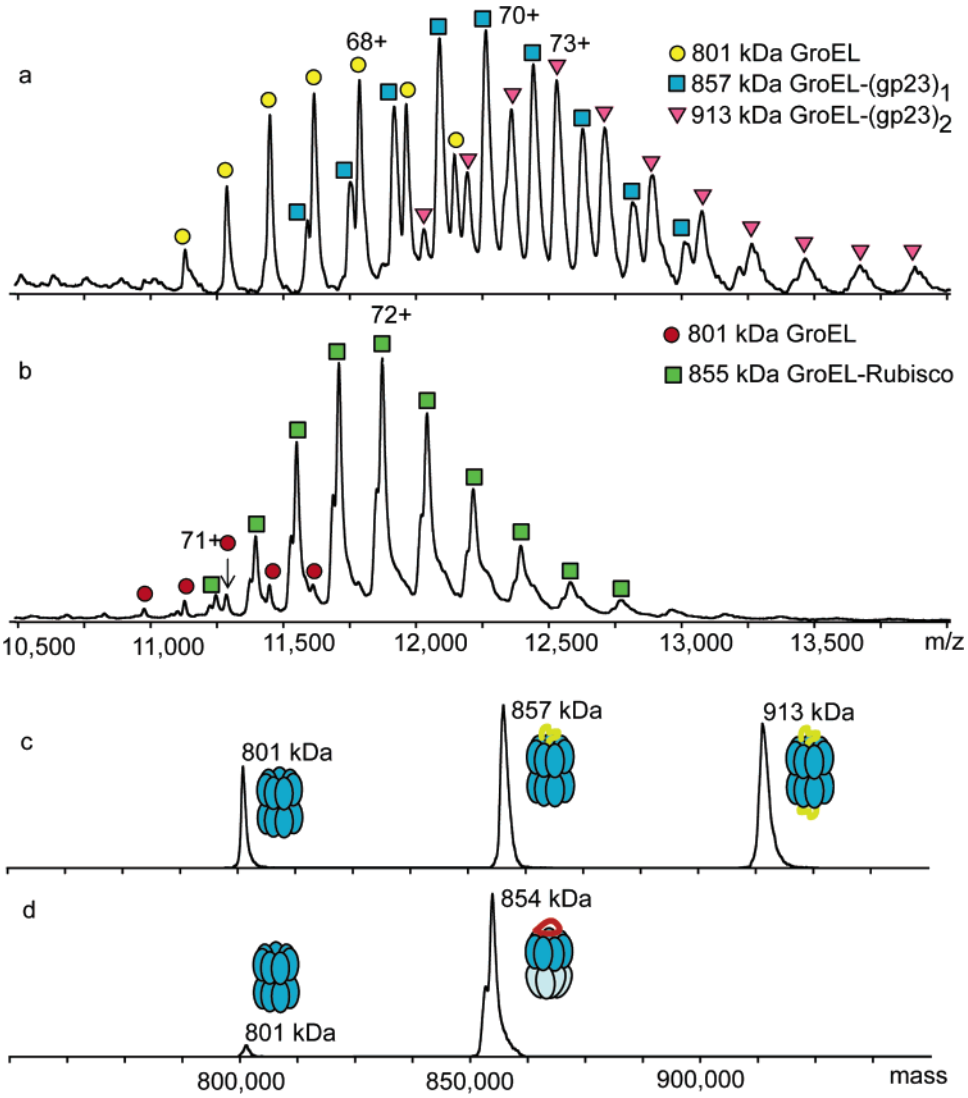

Figure 2. ESI mass spectra of GroEL−substrate complexes. Nano-ESI mass spectrum of GroEL in complex with (a) gp23 and (b) Rubisco mixed in a 1:3 molar ratio. The three different charge state distributions present in the GroEL−gp23 spectrum indicate the presence of free GroEL (circle), GroEL in complex with one gp23 (square), and GroEL in complex with two gp23 molecules (triangle). The GroEL−Rubisco spectrum shows one clear charge state distribution, representing the GroEL chaperonin in complex with one Rubisco molecule (square). (c, d) The deconvoluted mass spectra clearly show the differences in stoichiometry of the GroEL−substrate complexes.

different complexes.[30] In addition to ions originating from unliganded GroEL and GroEL with one gp23 substrate bound, GroEL complexes to which two gp23 substrates were bound were also observed (Figure 2a). Charge state distributions of these three species can be clearly distinguished in the mass spectrum as a result of the high resolution we obtained. All the ion peaks in our spectrum could be identified to a single species. Due to the small peak width we attained, some of the different charge states only slightly overlapped, and we could very accurately determine the masses of the different complexes. Free GroEL is observed distributed around the 68+ charge state. GroEL in complex with one gp23 molecule was centered around a charge state of 70+, and the charge state for the GroEL chaperonin in complex with two gp23 molecules was centered around a charge state of 73+. Deconvolution of this composite, but well-resolved mass spectrum (Figure 2c), indicated that the ratio of GroEL:GroEL−gp23:GroEL−$(gp23)_2$ was approximately 0.7:1:1. These data demonstrate that ESI mass spectrometry is a powerful method to obtain information about the stoichiometry of the different chaperonin−substrate complexes present in a complex mixture, a definite advantage over many conventional techniques. For example, analysis of the GroEL−gp23 binding stoichiometry by gel filtration chromatography (which does not distinguish between the different GroEL:substrate complexes) revealed an average binding stoichiometry of 1:1 GroEL:GroEL−gp23, without a hint of an irregular binding behavior of gp23 to GroEL (data not shown). Interestingly, when the deconvoluted mass spectrum (Figure 2c) is inspected, an average binding stoichiometry of 1:1 can be deduced, similar to what has been reported previously.[30]

If we assume that the ionization efficiencies of all the chaperonin species are similar, Figure 2a provides a semiquantitative reflection of the abundance of the different species present in solution. It is striking that we observe two gp23 substrate molecules bound to the GroEL complex, and at the same time, there is also free GroEL present. This indicates directly that the affinity for the second substrate is in the same order of magnitude as the binding affinity for the first substrate.

To investigate whether the binding behavior of the gp23 substrate is indeed unique, experiments using a well-studied GroEL model substrate Rubisco, with a molecular mass similar to that of gp23, were performed. Under identical conditions (GroEL:substrate concentrations of 1:3), only one Rubisco substrate molecule was bound to the GroEL chaperonin oligomer (Figure 2b), in agreement with the currently accepted view.[28] Note that the satellite peaks to the left of the main GroEL−Rubisco complex peak correspond to a degradation product of Rubisco, which lacks several terminal amino acids, and is approximately 1 kDa lower in mass. This degradation product does not influence the results since only ions originating from GroEL with one Rubisco substrate bound are present. Deconvolution of this mass spectrum (Figure 2d) revealed that the ratios of GroEL:GroEL−Rubisco:GroEL−$(Rubisco)_2$ were approximately 0.1:0.9:0.0.

When the GroEL−Rubisco stoichiometry was analyzed by gel filtration chromatography, an average GroEL:Rubisco binding of 1:1 was observed (data not shown), which is in agreement with the deconvoluted mass spectrum in Figure 2d. If we again assume that the mass spectrum represents a semiquantitative reflection of the relative abundances of the different species present in solution, it would indicate that, whereas the GroEL chaperonin exhibits no affinity for the second Rubisco substrate molecule, the binding constant for the first Rubisco substrate seems to be much higher than that of the first gp23 substrate since, with a GroEL:substrate ratio of 1:3, hardly any unliganded GroEL was observed.

The binding of Rubisco and gp23 to the GroEL chaperonin was further analyzed by measuring the concentration dependence of the chaperonin−substrate stoichiometries by ESI mass spectrometry. The resulting spectra were deconvoluted, and the integrated peak areas were taken as a reflection of the relative abundance of the different protein complexes. The results are summarized in Figure 3a and b and show that at equimolar GroEL:Rubisco the majority of the Rubisco is bound to GroEL. At increased substrate concentrations, all GroEL is in complex with Rubisco with a stoichiometry of 1:1. In contrast, at equimolar GroEL:gp23, less than 30% of the GroEL chaperonin is in complex with gp23. At the highest measured GroEL:gp23 ratio (1:5), the major species is clearly the doubly occupied GroEL chaperonin, although there is still unliganded GroEL present. It was not possible to further increase the relative substrate concentration, as this resulted in urea concentrations in the protein mixture that destabilize GroEL.

To our knowledge, the 20 kDa dihydrofolate reductase (DHFR) is the only other GroEL substrate for which double occupancy has been suggested.[25,43] One might argue that the structural changes that occur upon binding of DHFR may influence the conformation of the *trans* ring, similar to that observed for other substrates, but that the opening in the *trans* ring remains large enough for the small DHFR molecule to enter and bind to the hydrophobic apical domain binding sites. However, given the large mass difference between DHFR (20 kDa) and the capsid protein gp23 (56 kDa), it is unlikely that this argument could account for the double occupancy observed for the latter. We therefore hypothesize that gp23 has a significantly different effect on the conformation of the *cis* and

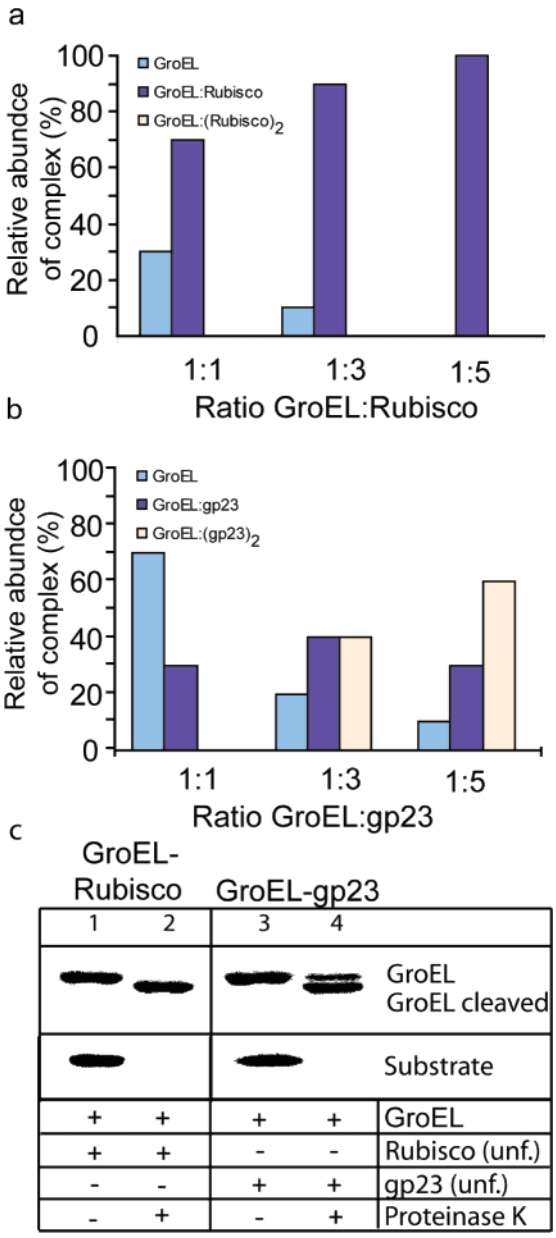

Figure 3. Relative abundance of the GroEL−substrate complexes in the ESI mass spectra at varying GroEL:substrate ratios. (a) At a 1:1 GroEL:Rubisco ratio, the majority of GroEL has one Rubisco molecule bound. At ratios of 1:3 and 1:5, GroEL is in complex with one Rubisco molecule for 90 and 100%, respectively. (b) Only 30% of the GroEL oligomer is in complex with one gp23 molecule at a GroEL:gp23 ratio of 1:1. At a GroEL:gp23 ratio of 1:3, 40% of the GroEL is in complex with one gp23 molecule, 40% of the GroEL complexes originates from GroEL in complex with two gp23 molecules, while only 20% of the GroEL remains unliganded. At the highest GroEL:gp23 ratio (1:5), the major species (60%) is the doubly occupied GroEL chaperone, 30% of the GroEL complexes have one gp23 molecule bound, and still 10% of the GroEL is unliganded. (c) Gp23 or Rubisco binding results in different effects on GroEL protection against degradation by proteinase K (compare to 1:3 ratios in the bar diagrams shown in a and b). Whereas gp23 confers some protection of the GroEL against proteolysis, Rubisco does not (compare lanes 2 and 4).

trans rings of the tetradecameric GroEL than the well-studied Rubisco substrate. It is generally accepted that considerable narrowing of the opening of the *trans* ring occurs upon substrate binding, thereby effectively inhibiting the entrance of a second substrate.[22] Our data suggest that such a process may indeed occur when Rubisco is used as the substrate; however, it seems that gp23 does not induce similar conformational changes. As

(43) Martin, J.; Langer, T.; Boteva, R.; Schramel, A.; Horwich, A. L.; Hartl, F. U. *Nature* **1991**, *352*, 36−42.

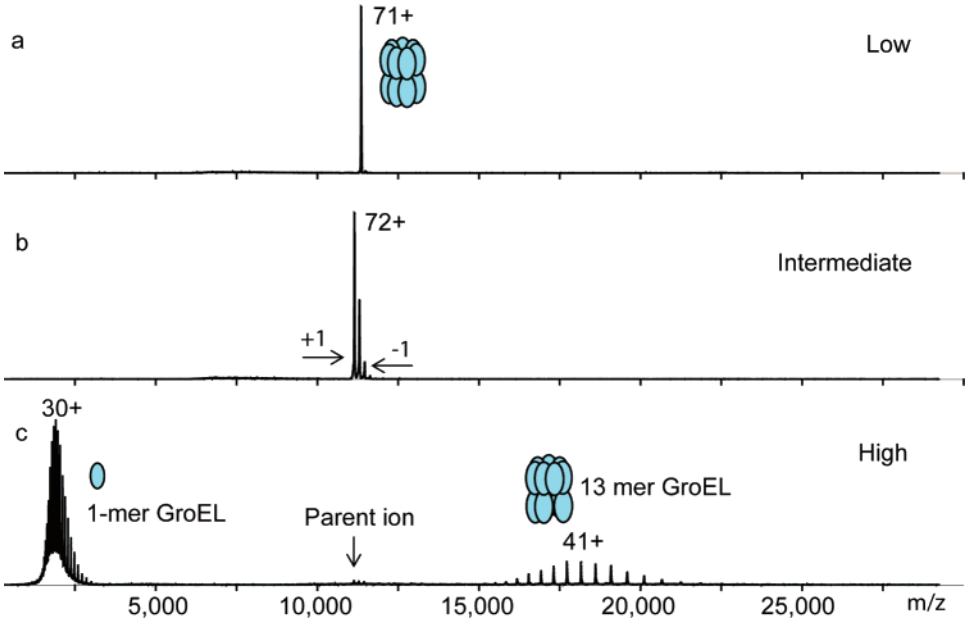

Figure 4. Tandem mass spectra of tetradecameric GroEL at (a) low, (b) intermediate, and (c) high collision energies. At the intermediate collision energy, the charge increases and reduction is indicated by arrows. At a high collision energy, tetradecameric GroEL is completely dissociated into tridecameric and monomeric GroEL.

a result, the GroEL−gp23 *trans* ring still may have the "larger" opening, allowing the entrance and binding of a second polypeptide substrate.

That the effect on the GroEL chaperonin complex is substrate-dependent can also be demonstrated using protease digestion experiments. It has been shown previously that limited proteolysis can be used to determine the presence of asymmetric GroEL−GroES−ADP complexes. Proteinase K treatment of such complexes results in the removal of 16 amino acids from the C-terminus of each of the GroEL subunits in the *trans* ring.[44−46] We applied the same approach on the different GroEL−substrate complexes. When unfolded gp23 or Rubisco was bound to GroEL, they were both susceptible to degradation by proteinase K. However, binding of gp23 to GroEL afforded some limited protection of the GroEL subunits against proteolysis, while Rubisco binding did not have this effect (Figure 3c). Consequently, the difference in protection is most likely due to the differences in binding stoichiometry. Binding of gp23 to GroEL, at a 1:3 ratio, resulted in three different complexes, out of which approximately 40% of the GroEL population was loaded with two gp23 molecules. Since both rings in this complex are occupied by substrate, the protease prevents access to the C-terminus of the subunits and GroEL is therefore protected from proteolysis. In contrast, Rubisco binds exclusively to GroEL in a 1:1 ratio; consequently, one of the heptameric rings always remains unoccupied and therefore unprotected from C-terminal cleavage.

It is intriguing to speculate about the possible biological significance of the observed substrate-dependent conformational changes in GroEL. However, we have to keep in mind that our experiments are all in vitro and that inside the cell GroEL is unlikely to be present without GroES and/or substrate bound. The remarkable ability of the GroEL chaperonin complex to recognize a diverse range of small and large substrate proteins is under continuous investigation.[1] It is thus not very likely that a very small substrate (i.e., DHFR) would interact identically with GroEL as would a large substrate (Rubisco), as the latter is likely to have a larger interaction surface which may result in binding to more subunits in the heptameric ring. We note that gp23 is a natural substrate of GroEL, whereas Rubisco is not, making it plausible that the two-substrate binding model we observe here is possibly correct for natural substrates of GroEL, something we will pursue to study in the near future. In addition, the observation that binding of the gp23 capsid protein induces unique conformational changes in GroEL might be one of the reasons why the folding of this protein requires the unique phage-encoded co-chaperonin gp31.[39]

Tandem Mass Spectrometry on the Intact GroEL−Substrate Complexes. To further investigate the effect of substrate binding on GroEL, intact GroEL−substrate complexes were analyzed by tandem mass spectrometry. First the dissociation pathway of unliganded GroEL was studied, following a similar approach to that described by Sobott and Robinson.[47] During collision-induced dissociation (CID), the gas-phase ions of the 800 kDa GroEL complex were accelerated in the collision cell, which was filled with argon gas, using voltages up to 200 V. When ions of the 71+ charge state of GroEL (Figure 4a) at an *m*/*z* value of 11 290 were isolated, the accelerating voltage led to an ion kinetic energy of 14.2 keV. This value may seem excessive; however, one has to keep in mind that the collision energy in the center-of-mass frame is reduced to less than 0.01% of the laboratory-frame energy (due to the high mass of the GroEL and the relative low mass of the collision partner), which effectively means that less than 1 eV per collision event will be converted into internal energy for the GroEL ions. This small amount of energy transfer demonstrates that numerous collisions are essential for fragmentation of the protein ions. This number

(44) Bakkes, P. J.; Faber, B. W.; van Heerikhuizen, H.; van der Vies, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 8144−8149.
(45) Langer, T.; Pfeifer, G.; Martin, J.; Baumeister, W.; Hartl, F. U. *EMBO J.* **1992**, *11*, 4757−4765.
(46) Weissman, J. S.; Hohl, C. M.; Kovalenko, O.; Kashi, Y.; Chen, S.; Braig, K.; Saibil, H. R.; Fenton, W. A.; Horwich, A. L. *Cell* **1995**, *83*, 577−587.
(47) Sobott, F.; Robinson, C. V. *Int. J. Mass Spectrom.* **2004**, *236*, 25−32.

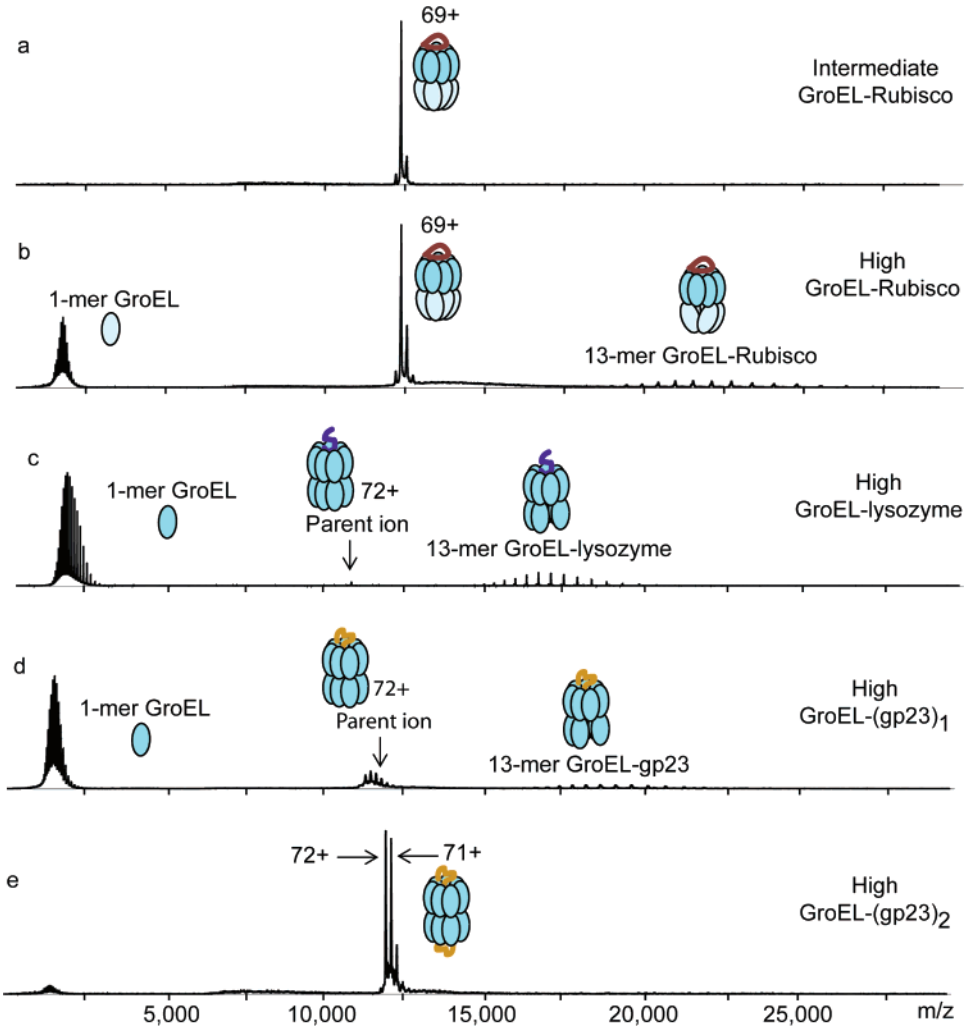

Figure 5. Tandem mass spectra of GroEL−substrate complexes. (a) Tandem mass spectrum of the 1:1 GroEL−Rubisco complex, showing only product ions that are generated via charge increase and reduction of the precursor GroEL ions obtained at intermediate collision energies and (b) at the highest attainable collision energy (i.e., 200 V). (c) Dissociation of the 1:1 GroEL−lysozyme complex at the highest attainable collision energy (200 V), showing the exclusive formation of monomeric GroEL subunits and complexes of tridecameric GroEL with the lysozyme substrate. (d) Tandem mass spectrum of GroEL in complex with a single gp23 substrate at the highest collision energy. The only product ions are monomeric GroEL and tridecameric GroEL in complex with gp23, indicating no increased stability relative to unliganded GroEL. (e) Tandem mass spectrum of GroEL in complex with two gp23 substrates at the highest collision energy. GroEL in complex with two gp23 substrate molecules shows nearly no dissociation, indicating a major increase in gas-phase stability of the chaperonin complex upon the binding of two gp23 substrate molecules.

of collisions that the protein ions encounter in the collision cell can be estimated, using the dimensions of the ions, the length of the collision cell, and the argon pressure in the collision cell. This tells us that our chaperonin ions experience more than thousands of collisions during their flight through the collision cell,[47] and only the energy that is converted from translational energy into internal energy during gas-phase collisions will lead to CID, which is why achieving significant fragmentation of some large macromolecular complexes can be problematic.

At intermediate collision voltages (100−150 V laboratory frame), no fragmentation of GroEL was observed, but ions of both lower and higher charge states of the intact tetradecamer appeared (Figure 4b). Lower charge states may originate from stripping of protons or small positively charged buffer ions adducted to the protein surface. Conversely, charge states higher than that of the precursor ion likely result from the loss of small negatively charged ions.[47] At the highest attainable collision voltage (200 V), tetradecameric GroEL dissociated completely into tridecameric and monomeric GroEL particles (Figure 4c), in full agreement data previously reported.[47] Our data show a highly asymmetric charge distribution, whereby the monomeric collision product retains 30 charges (on average) and the tridecameric species no more than 41 charges. This asymmetric partitioning of charge is a common feature during gas-phase dissociation of large noncovalent complexes.[48,49]

The CID behaviors of the GroEL−substrate complexes were compared to those of unliganded GroEL, and any change in dissociation behavior was assumed to be a result of substrate binding. At intermediate voltages, the GroEL−Rubisco (1:1) complex behaved much like unliganded GroEL, that is, displaying both lower and higher charge states of the precursor ions (Figure 5a). However, at a collision energy of 200 V, this chaperonin−substrate complex showed a clear difference in dissociation behavior compared to unliganded GroEL. Whereas free GroEL was completely dissociated into monomers at this collision potential, the GroEL−Rubisco complex remained largely intact. Even though small amounts of dissociation

(48) Benesch, J. L.; Sobott, F.; Robinson, C. V. *Anal. Chem.* **2003**, *75*, 2208−2214.
(49) Versluis, C.; Heck, A. J. R. *Int. J. Mass Spectrom.* **2001**, *210*, 637−649.

products were observed, the precursor ion peak clearly remained the major species present (Figure 5b). It should be noted that the differences were unlikely due to the slightly different masses of the GroEL and GroEL−Rubisco complexes since the difference in the center-of-mass collision energies of these species is negligible. We therefore conclude that, even in the gas phase, the binding of Rubisco has a stabilizing effect on the chaperonin complex. Interestingly, the fragmentation products were free GroEL monomeric ions, concomitant with ions that originated from intact complexes of Rubisco and tridecameric GroEL. It therefore appears that, upon binding of Rubisco to GroEL, the occupied (*cis*) ring gains stability, presumably through direct interactions with the substrate. Since substrate binding to GroEL induces conformational changes in the unoccupied (*trans*) ring, it is very well possible that the more compact *trans* ring has also gained some stability. Consequently, the whole GroEL−Rubisco complex is less prone to dissociate even under the most extreme conditions used during tandem mass spectrometric analysis in the gas phase.

The dissociation behavior of multi-protein complexes is not fully understood but has been studied extensively. From all these studies, it has become clear that there is a strong tendency of multi-subunit complexes to decompose via a similar pathway; that is, first the smallest subunit dissociates, taking away a major part of the charges.[47,50] Therefore, our observation of GroEL (57 kDa monomer) dissociation from the GroEL−Rubisco complex suggests that Rubisco (54 kDa) is still in the folding cavity. Consequently, it seems to be energetically favorable to dissociate a surface-exposed GroEL subunit, rather than the internally protected Rubisco substrate. Our data thus imply that the solution-phase structure of the chaperonin complex is to some degree preserved in the gas phase.

Since the mass difference between the unfolded Rubisco subunit and one monomer of GroEL is very small, we further investigated the dissociation behavior of the chaperonin, a 1:1 complex of GroEL and lysozyme. This is another well-studied, and rather small (14 kDa), GroEL model substrate. As can be seen from the spectrum shown in Figure 5c, the dissociation product of GroEL−lysozyme, at a high collision energy, was monomeric GroEL (57 kDa) and tridecameric GroEL in complex with lysozyme. Dissociation of lysozyme (14 kDa) from the chaperonin−substrate complex was not observed, thereby supporting our previous hypothesis that the substrate is still bound inside the folding cavity formed by the GroEL subunits in the gas phase.

Next, the GroEL chaperonin with one gp23 substrate molecule bound was analyzed by tandem mass spectrometry. We carefully selected the charge states of the ions, and they showed minimal overlap with ions of other chaperonin−gp23 stoichiometries. Although different charge states were used as a precursor ion (Figures 4 and 5), the dissociation patterns were not significantly different (in the range of 69−72 charges). No dissociation of the precursor ion peak was observed for the 1:1 GroEL−gp23 complex at intermediate collision voltages (data not shown), a behavior similar to that of the 1:1 GroEL−Rubisco complex. However, at the highest collision voltage, the 1:1 GroEL−gp23 complex gave rise to a very different pattern. Again, product ions corresponding to monomeric GroEL and tridecameric GroEL in complex with gp23 were observed. In contrast to the data obtained with Rubisco, nearly no precursor ions remained (Figure 5d), and the dissociation spectrum at this collision voltage appeared to be very similar to that observed for unliganded GroEL (Figure 4c). Thus binding of a single gp23 molecule does not seem to have a stabilizing effect on the chaperonin complex. This finding is in agreement with our hypothesis that binding of a single gp23 substrate molecule does not induce major conformational changes, especially not in the unoccupied GroEL *trans* ring. Consequently, the structure and conformation of the *trans* ring is still similar to that in the unliganded GroEL ring and, therefore, displays similar dissociation behavior in the gas phase. This also explains the double occupancy we observed when gp23 was used as the GroEL substrate. If the conformation of the *trans* ring is not significantly affected by the binding of the first gp23 substrate, it would presumably still be accessible for a second gp23 substrate molecule.

If the hypothesis made above is correct, the GroEL chaperonin in complex with two gp23 substrate molecules (one bound to the *cis* and one to the *trans* ring) should show an increased stability, as both rings are expected to be stabilized by interactions with the substrate. Figure 5e shows the tandem mass spectrum of GroEL in complex with two gp23 substrate molecules at the highest available collision voltage. Even though small amounts of dissociation products were observed, the GroEL−$(gp23)_2$ precursor complexes remained largely intact. The minor dissociation products are monomeric GroEL ions and tridecameric GroEL ions, which retains the two gp23 substrate molecules. Thus, binding of two gp23 substrate molecules induces a significant increase in chaperonin complex stability in the gas phase, which is in support of our hypothesis. This observation also suggests that the second substrate molecule is not loosely attached at random to GroEL, but indeed is bound inside the GroEL *trans* ring. If it would have bound nonspecifically to the outside surface of GroEL, most likely the gp23 substrate would dissociate first before a monomer of GroEL is ejected from the complex.

Conclusions

We have demonstrated that binding of the substrates Rubisco and gp23 to the GroEL chaperonin has a strikingly different effect on the conformation and stability of the chaperonin complex, resulting in different binding stoichiometries. A summary of our findings is shown schematically in Figure 6. Using a mass spectrometric approach, we show that gp23 does not behave as a typical GroEL model substrate since it is able to occupy simultaneously the inside of the GroEL *cis* and *trans* rings. Until now, the generally accepted idea about GroEL−substrate interaction has been that the chaperonin binds only one substrate molecule at a time. Once the first GroEL cavity is occupied, the conformation of the empty *trans* ring is altered, such that a second substrate is unable to bind. Accordingly, GroEL−Rubisco complexes with an exclusively 1:1 stoichiometry were observed. The clear difference between the binding behaviors of gp23 and Rubisco, however, tempted us to hypothesize that gp23 does not have the same effect on the conformation of GroEL as Rubisco, that is, gp23 does not influence the structure of the unliganded GroEL *trans* ring. This aberrant binding behavior of the gp23 substrate was investigated in more detail by analyzing intact 800−914 kDa GroEL−

(50) Heck, A. J.; Van Den Heuvel, R. H. *Mass Spectrom. Rev.* **2004**, *23*, 368−389.

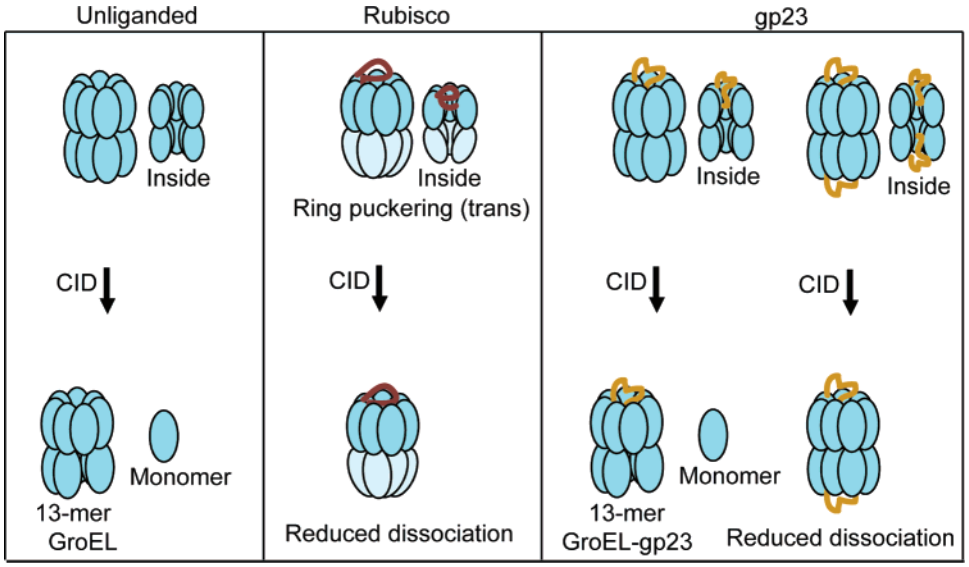

Figure 6. Representation of substrate-specific GroEL−substrate interactions. Upon binding to Rubisco, the GroEL *cis* ring is stabilized due to multiple interactions with the substrate. Concomitant conformational changes are induced in the *trans* ring that results in a narrower opening (light blue ring vs dark blue ring), inhibiting the entrance and binding of a second substrate. These conformational changes tighten the interactions between the GroEL subunits, making it less amenable to dissociation. The gp23 substrate does not induce similar conformational changes in the *trans* ring when it occupies the *cis* ring, thereby still allowing the entrance and binding of a second gp23 substrate. In case of gp23 binding, the *trans* ring conformation does not change significantly and is therefore more susceptible to gas-phase dissociation. If both the *cis* and *trans* rings of GroEL have a gp23 substrate molecule inside, the complex remains intact even under the most harsh collision-induced dissociation (CID) conditions, revealing that both rings are stabilized by substrate interactions.

substrate protein complexes using tandem mass spectrometry. Our data reveal that the binding of one Rubisco molecule to GroEL oligomer is sufficient to stabilize the chaperonin complex against gas-phase fragmentation, in contrast to gp23, which has to bind to both GroEL rings in order to generate a similar effect. These results hence suggest that the physiological GroEL substrate, gp23, has a different effect on the GroEL conformation than does typical model substrates. It would thus be interesting to determine whether this behavior is also displayed by other physiological GroEL substrates.

Normally upon substrate binding to the GroEL *cis* ring, a conformational change occurs in the *trans* ring.[31] A conformational change induced in this ring results in a narrowed opening, which effectively reduces its accessibility for a second protein substrate. The tighter interaction of the GroEL subunits in the *trans* ring may possibly confer an increased stability and is hence less prone to dissociation by tandem mass spectrometry. This is consistent with our observations for the 1:1 GroEL−Rubisco complex, which remained stable even under the most extreme conditions of collision-induced dissociation. We propose that the natural gp23 substrate does not follow this allosteric theory. It appears that the conformation of the unoccupied GroEL *trans* ring is hardly affected, and thus a second gp23 molecule is able to bind. As the *trans* ring conformation has not significantly changed in the 1:1 GroEL−gp23 complex, it is equally prone to dissociation as unliganded GroEL in CID experiments. Finally, the major increase in stability of the doubly occupied GroEL−gp23 complex comes from the interactions of both heptameric rings, with substrates that are present in the folding cavities of the chaperonin complex.

Acknowledgment. This research was supported by FOM, Project Number 01FB12, and a fellowship (E.D.) from the European Molecular Biology Organisation (EMBO), and the Natural Sciences and Engineering Research Council of Canada (D.A.S.).

JA056756L