4. Chaperonin-assisted folding of the bacteriophage T4 capsid protein is fast and efficient

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Abstract

Folding of bacteriophage T4 major capsid protein (gp23) is essential for phage morphogenesis, and is strictly dependent on the \textit{Escherichia coli} host chaperonin GroEL and the bacteriophage T4-encoded co-chaperonin, gp31. Gp31 is similar in overall structure to the host homologue GroES, which is unable to assist the folding of gp23 in conjunction with GroEL. To gain insight in the chaperonin-assisted gp23 folding, we determined its kinetics using the intrinsic tryptophan fluorescence of gp23 and Förster Resonance Energy Transfer (FRET) with acceptor fluorophores attached to GroEL. We find that the GroEL-gp31-mediated refolding of gp23 occurs with complex kinetics that are faster than those of previously studied substrates of the GroEL-GroES chaperonin complex. Our results suggest that this comparatively fast folding of gp23 is due to the specific way the major capsid protein has evolved to interact with the chaperonin complex.
4.1. Introduction

Bacteriophages such as λ, HK97 and T5 need the assistance of the bacterial protein machinery for the replication of their genomes and for the synthesis and folding of their proteins [86]. Their host, Escherichia coli, contains the GroEL-GroES chaperonin complex, which is required for the folding of about 3.5% of the newly-synthesized polypeptides [34,54,137]. The GroEL chaperonin is a homotetradecamer composed of two heptameric rings stacked back to back. In the presence of ATP, the dome-shaped heptameric co-chaperonin GroES binds to one GroEL ring, creating an enclosed cavity in which a substrate polypeptide can fold [30-32,127,138,139]. The inner surface of the GroEL barrel is hydrophobic and binds partly folded polypeptides with exposed hydrophobic residues [140]. Encapsulation of the polypeptide occurs after binding of ATP to the substrate-bound GroEL ring (cis-ring) and binding of GroES via the mobile loops that interact with the apical domain of GroEL. After co-chaperonin binding, the polypeptide is displaced from its binding site into the cavity, where it begins to fold [99]. The folding process is driven by ATP hydrolysis in the cis-ring. The release of GroES, ADP and partially or completely folded substrate from the cis-ring is prompted by the cooperative binding of ATP to the opposite unliganded trans-ring [141-143]. In vitro experiments, in the absence of denatured substrate, have indicated that this release of ligands is the rate-limiting step in the GroEL-GroES cycle [47]. Binding of a substrate molecule to the trans-ring accelerates the rate of release of the cis-bound co-chaperonin from GroEL [47]. It was estimated that in vivo the folding cycle could last ~8-10 s, indicating that the rate of ATP hydrolysis (0.12 s^{-1}) is rate limiting [47]. A schematic representation of the chaperonin folding cycle is presented in figure 4.1.

In contrast to bacteriophages λ, HK97 and T5, which depend on both GroEL and GroES, bacteriophage T4 only requires GroEL to proliferate [17,73]. The T4 genome encodes a GroES-like protein, gp31 that acts as a co-chaperonin instead of GroES [74,76,144]. Deletion of either the groEL or 31 gene leads to aggregation of gp23, T4’s major capsid protein [17,74]. Despite limited amino acid sequence identity between the two co-chaperonins (14%), gp31 can substitute for GroES in E. coli growth, indicating that the two proteins are functional homologues [15,77,78]. The major functional distinction is that GroES cannot assist the correct folding of gp23 in conjunction with GroEL, while gp31 can. It has been shown that gp23 can only be encapsulated by the GroEL-gp31 complex, raising the hypothesis that gp23 (molecular weight 56 kDa) is too large to fit into the GroEL-GroES cavity [80]. From cryo-electron microscopy images of GroEL-gp31-ADP complexes, it was estimated that the volume of the folding cavity is ~8% larger than that of the GroEL-GroES-ADP complex [84]. In vitro refolding experiments have shown that, in the presence of the GroEL, gp31 and ATP, folding and hexamerization of gp23 occurs within one minute [80]. In E. coli,
gp23 synthesis occurs late during the lytic phase of bacteriophage T4-infection, i.e. 10 minutes before cell lysis. In this short time an average of 200 progeny phages must be produced and assembled [69]. Each bacteriophage head contains 930 copies of gp23 arranged in hexamers, which means that at least ~200 000 gp23 molecules have to be folded in less than 10 minutes. Considering that an infected cell contains ~1600 GroEL double-toroids and ~7800 gp31 heptamers [125], each GroEL-gp31 complex has to complete the folding of ~125 newly-synthesized gp23 molecules. Assuming that gp23 requires one single round of folding to achieve a native conformation, the GroEL-gp31 chaperonin system would need a folding cycle rate of ~0.2 s\(^{-1}\) \textit{in vivo}, which is substantially faster than has been observed for many substrates of the GroEL-GroES chaperonin complex [54,98,145]. This notion prompted us to investigate the kinetics and mechanism of GroEL-gp31-dependent gp23 folding. Here, we have used a combination of stopped-flow kinetics, tryptophan fluorescence, and Förster Resonance Energy Transfer (FRET) to resolve the various stages of the gp23 folding in time. We find that chaperonin-assisted folding of gp23 \textit{in vitro} occurs with complex kinetics. The various steps in the folding cycle are discussed in view of the demand for rapid and efficient folding of this protein during T4 morphogenesis.

### 4.2. Materials and methods

#### 4.2.1. Production and purification of proteins

A plasmid encoding GroEL-315C under the control of an IPTG (Isopropyl \(\beta\)-D-1-thiogalactopyranoside)-inducible promoter (kind gift of Dr. Hays Rye and Dr. Art Horwich) was used to transform \textit{E. coli} strain MGM100. GroEL, GroEL-315C, SR1, GroES, and gp31 were expressed and purified as previously described [80,114]. Gp23 was expressed in \textit{E. coli} strain BL21 (DE3) using the IPTG-inducible plasmid pET2331 and purified as described earlier [88]. SR1-315C was engineered by introducing a cysteine residue at position 315 in SR1-Cys0 (kind gift of Dr. Wayne Fenton). The protein was expressed in \textit{E. coli} strain JM109 and
purified as previously described [99]. Proteins were stored at -80°C in 50 mM Tris-HCl pH 7.7, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT containing 10% (v/v) glycerol. Protein concentrations were determined by Coomassie blue-based colorimetric assay and are expressed as oligomers unless otherwise stated.

4.2.2. Fluorescent labeling

Purified GroEL-315C and SR1-315C were pre-treated with 2mM TCEP (Tris (2-carboxyethyl) Phosphine, Pierce), and incubated with a 5-fold molar excess (relative to the GroEL oligomer) of 1,5-IAEDANS (Invitrogen) in 50 mM Tris pH 7.7, 1 mM EDTA, 50 mM NaCl and 1 mM DTT (buffer A) for 1h at 25°C. Unbound fluorophores were removed by gel filtration using Sephadex G-50 equilibrated in buffer A containing 10% (v/v) glycerol. The labeling efficiency was calculated using the extinction coefficient of IAEDANS at 336nm ($\varepsilon_{336\text{nm}} = 5700 \text{M}^{-1}\text{cm}^{-1}$). Typically, the labeling efficiency was ~4.5-12 dyes/GroEL tetradecamer (GroEL$_{A}$) and ~6.9 dyes/SR1 heptamer (SR1$_{A}$).

4.2.3. Formation of binary and ternary complexes

Purified gp23 was denatured in 6M urea for 1h at 23°C. Binary GroEL$_{A}$-gp23 and SR1$_{A}$-gp23 complexes were formed by adding aliquots of denatured gp23 to a solution containing 1 µM GroEL$_{A}$ or 1 µM SR1$_{A}$ in 50 mM Hepes-KOH pH 7.4, 5 mM KCH$_3$COOH, 10 mM Mg(CH$_3$COO)$_2$ and 2 mM DTT (buffer B), giving a final concentration of 3 µM gp23. Ternary GroEL$_{A}$-gp23-gp31 and SR1$_{A}$-gp23-gp31 complexes were formed by addition of gp31 and ATP (or ADP) to pre-formed binary complexes to final concentrations of 2 µM gp31 and 2 mM ATP. The solution was incubated for 5 minutes at 25°C.

4.2.4. Chaperonin-assisted refolding

All fluorescence experiments were performed at 25°C using a Fluoromax-3 fluorimeter (JY-Horiba) and an RX2000 Rapid-mixing Accessory (Applied Photophysics). The integration time was 50 ms per data point (slit width 5nm). Typically 5-8 traces were fitted and averaged using Origin data analysis software (Origin Lab Corporation).

Binary chaperonin-substrate complexes were formed as describe above at a final concentration of 1 µM gp23 monomer and 1 µM chaperonin (GroEL, GroEL$_{A}$, SR1 or SR1$_{A}$) in buffer B. Refolding was triggered by rapid mixing of the chaperonin-gp23 complexes with a solution containing 2 µM gp31 (or GroES) and 2 mM ATP (or ADP pre-treated with hexokinase [99]) in a 1:1 ratio. Where indicated, chaperonin-gp23 complexes were pre-incubated with 2 µM gp31, 2 mM ADP and 20 mM KF. Refolding was triggered by rapid mixing with 2 mM KAlSO$_4$ in a 1:1 ratio. Spontaneous refolding of gp23 was monitored after rapid mixing of denatured gp23 in buffer B without chaperonins. Where indicated, 1 µM urea-denatured gp23 or GroEL$_{wt}$ was additionally mixed with a third syringe immediately after the first mixing (dead time of 5 ms). The refolding of gp23 was followed by measuring
the fluorescence at 346 nm. FRET was monitored at 470 nm upon excitation at 290 nm. Changes in the environment of the IAEDANS fluorophores were determined using the fluorescence intensity at 470 nm upon excitation at 346 nm. Refolding and hexamerization of gp23 were independently monitored by electro-spray ionization mass spectrometry, using either unlabeled or labeled GroEL-315C, in combination with gp31 and ATP, as described before [88].

4.3. Results

4.3.1. Chaperonin-assisted refolding of gp23 shows complex kinetics

In order to monitor the dynamics of GroEL-assisted refolding of gp23, a GroEL variant with one unique cysteine residue positioned at the apical domain was generated (C315). In this study the FRET acceptor 1,5-IAEDANS was attached to C315 in the apical domain of GroEL giving rise to GroEL_A. The four intrinsic tryptophan residues of gp23 were used as donor. First, the ability of gp23 to bind to labeled GroEL was determined. As shown in figure 4.2, a decrease of tryptophan fluorescence intensity was observed upon addition of denatured gp23 to a solution containing GroEL_A in a ratio of 3:1. This decrease correlated with an increase of the IAEDANS signal, a clear signature of FRET. Without addition of nucleotide or co-chaperonin we noted that the GroEL_A-gp23 interaction was stable during 10 min as had been observed before for wild-type GroEL [80]. We independently confirmed that fluorescently labeled GroEL_A was able to fold gp23 in vitro in conjunction with gp31 and ATP using mass spectrometry. After two minutes of folding correctly folded and hexamerized gp23 could be clearly detected (supplemental figure S4.1) [88].

To measure the kinetics of the chaperonin-assisted folding of gp23, GroEL_A-gp23 complexes were formed by adding denatured gp23 to GroEL in a 1:1 ratio (relative to tetradecamers), resulting in ~30 % of GroEL_A being occupied by one gp23 molecule (data not shown) [146]. Upon initiation of the folding reaction by rapid mixing of gp31 and ATP, complex transients in both tryptophan and IAEDANS fluorescence intensity were observed (Figure 4.2 C and D). The tryptophan fluorescence time trace (Figure 4.2 C) directly revealed that the chaperonin-assisted gp31 folding process consists of at least three kinetic phases: phase 1 on the sub-second time scale; phase 2 in the range of 1 to 5 seconds; phase 3 on a time scale of more than 5 seconds. In the IAEDANS trace (Figure 4.2 D), at least phases 1 and 3 appear to be present. To determine the kinetic rates of the three components, we performed a global fit to both donor and the acceptor trace. Both traces could be well fitted with a sum of three exponentials with rates $k_1 = 6.25 \pm 1.18 \text{s}^{-1}$, $k_2 = 0.25 \pm 0.03 \text{s}^{-1}$, and $k_3 = 0.089 \pm 0.007 \text{s}^{-1}$. In order to determine the molecular nature of the three kinetic components, we performed additional experiments and analyses, described in the following sections, phase by phase.
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4.3.2. Phase 1: fast encapsulation of gp23 in GroEL-gp31

Rapid mixing of the GroELA-gp23 binary complexes with gp31 and ATP, resulted in an initial increase of tryptophan fluorescence with a rate of $k_1 = 6.25 \pm 1.18 \text{ s}^{-1}$ (Figure 4.3 B). The increase of tryptophan fluorescence coincided with a decrease of the IAEDANS fluorescence, indicating a decrease of FRET, which could be caused by release of gp23 in the GroEL-gp31 folding cavity after encapsulation. To test this, the same experiment was performed with ADP instead of ATP. Under these conditions, gp31 is known to encapsulate gp23 [80]. Upon mixing of GroELA-gp23 complexes with gp31 and ADP, the tryptophan fluorescence intensity also increased, at a rate of $k_{1-ADP} = 3.70 \pm 0.47 \text{ s}^{-1}$ (Figure 4.3 B). When GroES was used instead of gp31, no increase in tryptophan fluorescence was observed on this time scale (Figure 4.3 B), in agreement with the notion that GroES cannot encapsulate gp23 bound to GroEL [80]. A decrease in IAEDANS fluorescence was observed, which is likely the result of GroES binding to empty GroEL, since a similar signal was observed in the absence of gp23 (Figure 4.3 C). No change in tryptophan fluorescence was observed when either gp31 or GroES was omitted from the reaction mixture (data not shown).
Figure 4.3: Phase 1: encapsulation of gp23.

(A) Schematic representation of the experimental procedure. (B) Changes in tryptophan emission upon excitation at 290 nm in the presence of ATP (solid squares) or ADP (open squares) occurred with rate constants of $k_{\text{ATP}} = 6.25 \pm 1.18 \text{s}^{-1}$ and $k_{\text{ADP}} = 3.70 \pm 0.47 \text{s}^{-1}$ (superimposed gray line). In the presence of GroES and ATP (solid circles), no change of fluorescence was observed. (C) Changes in IAEDANS emission upon excitation at 290 nm (solid squares) or GroEL-GroES (solid circles) in the presence of ATP. In the case of GroES, it is likely that the changes in IAEDANS fluorescence reflect the binding of GroES in trans of the GroEL-gp23 binary complex, or the binding of GroES to empty GroEL molecules. Note that the data obtained with gp31 and ATP is the same as that shown in figure 4.2.

Taken together, these results suggest that the fast FRET decrease corresponds to the encapsulation of gp23 upon binding of gp31 and subsequent release of gp23 into the folding cavity, leading to a larger distance between the IAEDANS fluorophores on GroEL and gp23’s tryptophan residues (Figure 4.1, phase 1). The rate we observed is in agreement with rates observed for the release of various substrates into the GroEL-GroES cavity [93,141,147].

4.3.3. Phase 2: folding of gp23 within the cavity of the GroEL-gp31 complex

In the second phase of the gp31-GroEL assisted folding reaction (Figure 4.2 C), a decrease of the gp23 tryptophan signal was observed with a rate of $k_2 = 0.25 \pm 0.03 \text{s}^{-1}$, while the IAEDANS signal was largely unaltered (Figure 4.2 C and D). We speculated that this phase is due to the folding of gp23 in the gp31-GroEL cavity, leading to an altered environment of the tryptophan residues, resulting in decreased tryptophan fluorescence intensity.
Figure 4.4: Phase 2: folding of gp23 within the GroEL-gp31 complex.

(A) and (C) Schematic representation of experimental procedures. (B) Changes in tryptophan emission upon excitation at 290 nm during GroEL-gp31-assisted folding of gp23 in the presence of ATP (top panel), ADP (bottom panel) or (D) ADP + AlF₃. Note that the data obtained with ATP is the same as that shown in figure 4.2.

To test this hypothesis we performed the same experiments with ADP instead of ATP, since it has been shown that ADP is not capable of inducing the large conformational changes in the GroEL-co-chaperonin complex required to assist folding [99]. As can be seen in figure 4.4 B, no decrease of the tryptophan fluorescence intensity was observed on the seconds time scale, consistent with our hypothesis that folding of gp23 in GroEL-gp31-ATP complexes occurs with a rate of 0.25 ± 0.03 s⁻¹. To further test this, we used the non-hydrolysable ATP analogue ADP•AlF₃, which has been shown to produce folding-active ternary complexes by mimicking the transition state of ATP hydrolysis [98,99,148]. Folding-active GroELₐ-gp23-gp31-ADP•AlF₃ complexes were generated by incubating GroELₐ-gp23 complexes with gp31 and KF, prior to rapid mixing with KAlSO₄ (Figure 4.4 C). We did not observe the sub-second increase of tryptophan fluorescence (Figure 4.4 D), since encapsulation had already occurred prior to stopped-flow mixing. We did, however, observe a decrease of the tryptophan fluorescence intensity, with a rate constant of k_{2-AlFx} = 0.11 ± 0.02 s⁻¹ (Figure 4.4 D). While the amplitude of the decrease in tryptophan fluorescence is comparable to that in the presence of ATP, the rate is substantially slower (Figure 4.4 B and D). This slower rate might reflect the different starting conditions of the two
experiments (Figure 4.4 A and C) and is in line with the slower rate of conformational changes observed for GroEL-GroES complexes in the presence of ADP•AlF₃, compared to ATP [99]. Taken together, these results suggest that the ATP-driven folding of gp23 in the cis-cavity of a GroEL-gp31 complex occurs rapidly with a rate of 0.25 ± 0.03 s⁻¹.

4.3.4. Phase 3: release of folded gp23 from the GroEL-gp31 chaperonin complex

Phase 3 of GroEL-gp31-assisted folding of gp23 occurs on the tens of seconds time scale (Figure 4.2 B). During this phase we observed an increase of the gp23 tryptophan fluorescence intensity, correlated to a decrease of that of IAEDANS, both with a rate of k₃ = 0.089 ± 0.007 s⁻¹ (Figure 4.2 C and D). Interestingly, this rate is similar to the rate of ATP hydrolysis of GroEL in the presence of gp31 [149], as well as the rate of gp31 dissociation from GroEL during steady-state hydrolysis (chapter 3 of this thesis). We concluded that phase 3 corresponds to a decrease in FRET and hypothesized that it is due to release of folded gp23 from GroEL (see figure 4.1, phase 3). To test this hypothesis, the same experiment with an ejection-deficient single-ring mutant of GroEL, SR1, was performed [99]. With SR1, no FRET decrease was observed on the tens-of-seconds time scale (Figure 4.5 B and C). Also, when gp23 refolding is triggered with ADP•AlF₃ (see above and figure 4.4 C), the GroELₐ-gp23-gp31 complex is “locked” and gp31 and gp23 cannot be released from the cis-cavity [150]. Consistent with the SR1 experiment, we did not observe a FRET decrease on the tens-of-seconds time scale, in the presence of ADP•AlF₃ (Figure 4.5 B). Taken together, these results suggest that phase 3 of the GroEL-gp31-assisted refolding of gp23 is due the release of folded gp23 from the chaperonin with a rate of 0.089 ± 0.007 s⁻¹.

4.3.5. Effect of trans-binding substrate on folding and release rates

It has been shown that binding of denatured substrate to the trans-ring of cycling GroEL-GroES [47] and GroEL-gp31 (chapter 3 of this thesis) complexes accelerates the dissociation of co-chaperonin from the cis-ring, in the absence of encapsulated substrate. In order to determine whether substrate binding in trans has an effect on the rates of folding and release of cis-encapsulated gp23, GroELₐ-gp31 complexes were mixed with gp31 and ATP, immediately followed by addition of excess denatured substrate using a double-mixing stopped-flow apparatus (Figure 4.6 A). As can be seen in figure 4.6 B, a rapid increase of tryptophan fluorescence occurred, followed by a slow decrease, similar to what was observed in the absence of excess denatured substrate (Figure 4.4 B). We could not exclude that a difference in the signals was masked by the binding of additional gp23 to the trans-ring of GroELₐ-gp31 complexes. To test this, we used a trans-binding substrate that contains no tryptophans, i.e. wild-type GroEL itself.
Also when excess denatured GroEL was added to the cycling complexes, no acceleration of the cis-folding rate of gp23 was observed (Figure 4.6 B). These results suggest that the binding of substrate to the trans-ring does not influence the folding rate of cis-encapsulated gp23 (Phase 2). In order to probe the effect of substrate trans-binding on the rate of gp23 release from the cis-ring (Phase 3), tryptophan fluorescence was monitored for 50s after the addition of denatured GroEL to cycling GroELA-gp23-gp31 complexes. The binding of denatured GroEL in trans also had no accelerating effect on the release rate of folded gp23 from the chaperonin complex (Figure 4.6 C). A key difference between the experiments presented here and the ones focusing on co-chaperonin dissociation (Chapter 3 of this thesis and [47]) is that in the latter, no substrate was encapsulated in the cis-ring. It could very well be that encapsulated gp23, which tightly fits in the GroEL-gp31 complex [150], prevents transmission of allosteric signals from the trans-ring. It is unknown whether
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Figure 4.6: Effect of trans-bound substrate on gp23 folding and release rates.

(A) Schematic representation of the experimental procedure. (B) Top panel: control experiment in which urea was added to cycling GroEL-gp31 complexes. Bottom panel: effect of added denatured gp23 (light gray) and denatured GroEL (black) on the folding rate of gp23 by GroEL-gp31. (C) Effect of added denatured GroEL (light gray) on the rate of ligand release during the folding cycle of GroEL-gp31 (in black, additional urea was used as a control).

4.4. Discussion

During bacteriophage T4 infection of E. coli, the phage major capsid protein, gp23, is folded by the host chaperonin GroEL in conjunction with the phage-encoded co-chaperonin gp31. Why gp31 is required instead of GroES has been the subject of research for over a decade [17,86,144]. In the experiments presented here, we have monitored the folding of gp23 by the GroEL-gp31 chaperonin complex in real-time using FRET between the intrinsic tryptophan residues of gp23 and IAEDANS fluorophores positioned on the apical domain of GroEL. We have found that after rapid encapsulation and release of gp23 into the GroEL-gp31 cavity, folding occurs with a rate of $0.25 \pm 0.03 \text{ s}^{-1}$. The rate of gp23 release from the complex is 2.8-fold slower ($0.089 \pm 0.007 \text{ s}^{-1}$), which indicates that the efficiency of a
single gp23 folding cycle is very high. Assuming substrate folding and co-chaperonin release to be independent stochastic processes, we can estimate that in ~74% of the folding cycles \((2.8/(1+2.8))\), gp23 folding is complete before dissociation of the substrate-chaperonin complex. Both this high efficiency and the fast folding rate are in agreement with previous, more qualitative measurements [80]. The folding rate determined here also agrees very well with our estimate of the required rate of gp23 folding in vivo \((0.2 \text{ s}^{-1})\).

How does the chaperonin-assisted folding rate of gp23 compare to that of other substrates? The GroEL-GroES folding rate of many proteins (e.g. MDH [151], Rubisco [152] and Rhodanese [29,141]) have been determined using assays based on regain of substrate enzymatic activity as a measure of folding. These experiments, however, do not probe the folding rate during only one single cycle of the chaperonin complex, but might also probe the action of multiple cycles, on a time scale of several minutes. This makes them hard to compare to our measurements. Real-time measurements, using fluorescence as a measure of folding, have been performed on a smaller number of substrates including Rubisco, the Green Fluorescent Protein (GFP), and the Maltose Binding Protein (MBP). Folding of Rubisco in the GroEL-GroES folding cavity takes place with a rate of ~0.004 s\(^{-1}\), as determined from the fluorescence anisotropy of the intrinsic tryptophans [141] and FRET between a donor and acceptor attached to Rubisco [153]. GFP folds in the GroEL-GroES cavity at a rate of 0.025 s\(^{-1}\), as determined from the recovery of its intrinsic fluorescence [95]. Finally, the chaperonin-assisted folding rate of MBP was determined using intrinsic tryptophan fluorescence intensity. Three variants of the enzyme were tested, yielding substantially different folding rates, the fastest of which being 0.0029 s\(^{-1}\) (for wild-type MBP) [154]. It is clear that the rate of chaperonin-assisted folding varies and depends on the nature of the substrate. Evidently, chaperonin-assisted folding of gp23 is significantly faster than that of any of the other substrates studied so far.

The question now arises whether the fast chaperonin-assisted folding of gp23 is caused by the co-chaperonin gp31 or gp23 itself. We have no indications that the intrinsic cycling and folding kinetics of GroEL-gp31 are different from those of GroEL-GroES (chapter 3 of this thesis and [79,144]). It thus appears more likely that gp23 has unique properties that allow it to be folded fast and efficiently by GroEL. Several experimental approaches have revealed that different substrates interact differently with GroEL [59,64]. There are strong indications that stringent substrates such as Rubisco and MDH bind to more GroEL domains than less stringent substrates such as rhodanese [59]. Cryo-EM of gp23 in complex with GroEL has revealed that gp23 is bound to even more GroEL domains (at least five) than MDH (three) [60,61]. Furthermore, gp23 appears more structured and occupies more of the GroEL cavity. After encapsulation with gp31 and subsequent folding, gp23 in its native conformation is oriented very specifically and seems to exert pressure on the walls of
the cavity, suggesting that interactions of gp23 with the GroEL-gp31 cavity are needed for correct folding and release from the cavity [60]. In the cell, newly synthesized gp23 folds into a form that is prone to aggregation. We speculate that this form of gp23 binds efficiently to GroEL in a very specific way, while adopting a rather structured conformation. After encapsulation by the co-chaperonin, the specific interactions between substrate and chaperonin could help gp23 to overcome the energy barrier necessary for folding into its native state more quickly and efficiently than other substrates.

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**Figure S4.1:** *In vitro* refolding activity of fluorescently labeled GroEL monitored by mass spectrometry.

(A) Schematic representation of the experimental procedure. (B) Nano-electrospray ionization mass spectrum of a solution containing hexameric gp23 after gp31 and ATP were added to binary GroEL-gp23 complexes.
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