Determination of the Number of Active GroES Subunits in the Fused Heptamer GroES Required for Interactions with GroEL*\textsuperscript{S}

Received for publication, December 3, 2007, and in revised form, April 21, 2008. Published, JBC Papers in Press, April 22, 2008, DOI 10.1074/jbc.M709825200

Tatsuya Nojima, Shigeto Murayama, Masasuke Yoshida\textsuperscript{1}, and Fumihiro Motojima\textsuperscript{2}

From the Chemical Resources Laboratory R1-7, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan

A double-heptamer ring chaperonin GroEL binds denatured substrate protein, ATP, and GroES to the same heptamer ring and encapsulates substrate into the central cavity underneath GroES where productive folding occurs. GroES is a disk-shaped heptamer, and each subunit has a GroEL-binding loop. The residues of the GroEL subunit responsible for GroES binding largely overlap those involved in substrate binding, and the mechanism by which GroES can replace the substrate when GroES binds to GroEL/substrate complex remains to be clarified. To address this question, we generated single polypeptide GroES by fusing seven subunits with various combinations of active and GroEL binding-defective subunits. Functional tests of the fused GroES variants indicated that four active GroES subunits were required for efficient formation of the stable GroEL/GroES complex and five subunits were required for the productive GroEL/substrate/GroES complex. An increase in the number of defective GroES subunits resulted in a slowing of encapsulation and folding. These results indicate the presence of an intermediate GroEL/substrate/GroES complex in which the substrate and GroES bind to GroEL by sharing seven common binding sites.

Chaperonins facilitate folding of nascent and stress-damaged proteins in an ATP-dependent manner; GroEL is the most extensively studied chaperonin (1–4). GroEL is found in Escherichia coli and other eubacteria and is essential for cell viability (5). It is a large cylindrical protein complex comprising two heptameric rings of identical 57-kDa subunits stacked back-to-back (6). GroEL binds a substrate protein in denatured states to form a GroEL/substrate binary complex (7–11) and then binds ATP and co-chaperone GroES to the same GroEL ring (cis-ring) to form a GroEL/substrate/GroES ternary complex (12, 13). GroES is a disk-shaped heptamer of 10-kDa subunits, and each subunit contains a mobile loop region required for binding to each of GroEL subunit in the heptamer ring (14). The binding of GroES causes encapsulation of the substrate protein into the cavity underneath GroES (cis-cavity), where substrate protein efficiently folds into the native state (cis-folding) (12, 13).

Although the overall reaction cycle of GroEL/GroES has been studied extensively, a critical encapsulation step remains unclear. Crystal structures (Protein Data Bank (PDB) number 1AON) and mutagenesis studies indicate that the amino acid residues of GroEL responsible for binding of denatured substrate protein mostly overlap with those for binding of the GroES loop region (9). Any mechanistic models of the GroEL/GroES reaction cycle must therefore explain how GroES binds to the cis-ring in which substrate protein already occupies the common binding sites. Although a heptameric ring of GroEL contains seven binding sites for substrate protein, Farr et al. (15) showed that only two or three sites (but not all seven binding sites) are required to form a GroEL/substrate binary complex. A GroEL/substrate binary complex therefore still has four or five unused binding sites that are potentially available for GroES binding. One possibility is that an intermediate GroEL/substrate/GroES ternary complex could transiently form in which the substrate protein may occupy, for example, two binding sites with GroES occupying the remaining five binding sites. In this scenario, a GroES heptamer that has two binding-defective (and five binding-active) subunits would still be capable of forming the GroEL/substrate/GroES ternary complex. To address this, we generated a single polypeptide GroES composed of seven GroES subunits fused in tandem. This tandem fused GroES heptamer (t-GroES) has already been shown to possess a very similar structure to native GroES and is fully functional as a co-chaperone of GroEL (16). Multiple t-GroES variants composed of different numbers and arrangements of the defective GroEL-binding loops among seven fused subunits were then generated and functionally characterized. The results were consistent with the notion that substrate protein and GroES are capable of sharing the seven common binding sites of the cis-ring to form the productive ternary complex.

**EXPERIMENTAL PROCEDURES**

Reagents and Proteins—Pig heart mitochondrial malate dehydrogenase (MDH)\textsuperscript{3} was purchased from Roche Applied Science, apyrase was from Nacalai Tesque, ATP and ADP were from Sigma, and 5-carboxyfluorescein succinimidyl ester was from Invitrogen. GroEL mutants, wild-type GroES, and bovine mitochondrial rhodanese were purified as described (17, 18). GFP(S65T), referred to as GFP in this report, was prepared as described (19). To generate the tandem-fused GroES, two

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1 To whom correspondence may be addressed. E-mail: myoshida@res.titech.ac.jp.

2 To whom correspondence may be addressed. E-mail: fumihiro.motojima@res.titech.ac.jp.

3 The abbreviations used are: MDH, malate dehydrogenase; GFP, green fluorescent protein; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.
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blunt-end restriction sites were attached to the N- and C-terminal positions with a Gly-Gly-Gly linker. pET-ESC and its inactive mutant pET-ESN (I25S, V26S, and L27S) were initially prepared from pET-ES using the method of Kunkel. The extra N-terminal peptide stretch (MPGGG) included the Gly-Gly-Gly linker and an Smal restriction site at Pro-Gly (CCCGGG). GroES C-terminal Ala and the extra Gly contained an Nael restriction site (GCCGGG). pESC1 and pESN1 were generated by cloning the BglII-KpnI fragment of the above plasmids containing a T7 promoter and terminator into the BamHI-KpnI site of pUC19. pESC1 and pESN1 were ligated in the order required to generate various t-GroESs as described (16).

t-GroES molecules were expressed in E. coli strain BL21(DE3)pLysS cultured at 37 °C. Isopropyl-1-thio-galactoside (1 mM, and aliquots (50 μM GroES, 40 mM Na2S2O3, and 2 mM DTT) were used instead of ADP. Binding of t-GroESF to GroEL or SR1D398A in buffer A was incubated at 60 °C for 15 min. This heat-treated solution was mixed with an equal volume of 40 mM GroES, 40 mM Na2S2O3, and 2 mM DTT. In the case of SR1D398A, “trap-GroEL” (GroELN265A/D398A (17, 22)), which quickly catches unfolded protein tightly without further processing, was then added to a final concentration of 1 μM to trap any free unfolded proteins. The folding reaction was initiated by the addition of ATP (final concentration, 4 mM).

Recovery of rhodanese activity following a 1-h incubation was measured as described previously (23). For the MDH folding assay, the solution containing 60 °C for
15 min. In the case of SR1\textsubscript{D398A}, trap-GroEL was then added. GroES was added to a final concentration 4 \mu M, and ATP (final concentration, 4 mM) was added to initiate folding. After a 1-h incubation, recovery of MDH activity was measured as described previously (23). In the case of SR1\textsubscript{D398A}, GroES was detached from SR1\textsubscript{D398A} to allow dimerization of folded monomer MDH by the following procedure; ATP was eliminated by apyrase (1 unit/ml for >10 min), methanol was added (5% v/v), and the solution was treated with two cycles of freeze-thawing. This procedure allowed 95% of GroES to be detached from SR1\textsubscript{D398A} (data not shown). For the GFP folding assay, an acid-denatured GFP solution (15 \mu M) was diluted 300-fold in buffer A containing 5 mM DTT, 150 nM SR1\textsubscript{D398A}, and 2 \mu M GroES. After 10 min, trap-GroEL was added to a final concentration of 1 \mu M. 1 mM ATP was then added to initiate folding. GFP fluorescence intensity was monitored continuously with a fluorometer (excitation 485 nm/emission 512 nm, FP-6500, Jasco).

RESULTS

Covalent Linkage of GroES Subunits—The N and C termini of neighboring GroES subunits in the native GroES heptamer are located close to one another (average of seven pairs, 4.5\AA (C\textsubscript{α}-C\textsubscript{α})) (Fig. 1). By linking them with a Gly-Gly-Gly linker, seven members of the heptamer were fused into a single polypeptide. As shown previously (16) and in this report, t-GroES was fully functional \textit{in vitro} as compared with the native GroES. A set of triple mutations (I\textsubscript{25}S, V\textsubscript{26}S, and L\textsubscript{27}S) were then introduced in various numbers and arrangements among the seven subunits in t-GroES. These substituted hydrophobic residues in the GroES mobile loop were shown to be in contact with the GroEL apical domain H and I helices in the crystal structure of GroEL-GroES-ADP complex (PDB number 1AON) (24); in the current study, the mutations described above were confirmed to result in complete loss of the ability of GroES to bind GroEL (data not shown). In the interest of simplicity, t-GroES containing e.g. two defective subunits at the first and second positions from N terminus was referred to as t-GroES\textsubscript{2d:1,2}. Irrespective of the positions, the t-GroESs containing two binding-defective subunits were collectively referred to as t-GroES\textsubscript{2d}. The t-GroES composed of intact (binding-active) GroES subunits alone was referred to as wild-type t-GroES. It was already shown that the wild-type t-GroES has overall structural similarity to GroES and reduces ATPase activity of GroEL in the same manner as native GroES (16).

\textbf{Binding of t-GroES\textsubscript{2d} to GroEL}—The ability of t-GroES to bind GroEL in the presence of ADP was examined (Fig. 2A). It is known that ADP supports the binding of GroES to GroEL (but not to GroEL/substrate binary complex). To detect the formation of the complex, a fluorescently labeled t-GroES was mixed with 2-fold molar excess GroEL in the presence of ADP and subjected to gel filtration HPLC monitored by fluorescence in

![](https://example.com/fused-heptamer-groes.png)

**FIGURE 2. Binding of t-GroES\textsubscript{2}, to GroEL.** Binding of t-GroES\textsubscript{2}, to GroEL in the presence of ADP (A) or ATP (B) was measured. To prevent catalytic turnover, GroEL\textsubscript{D398A} was used in B. t-GroES\textsubscript{2} and GroEL were mixed at a 1:2 molar ratio in the presence of 1 mM ADP (A) or ATP (B) and were applied to gel filtration HPLC equilibrated with 0.2 mM ADP (A) or ATP (B). Elution was monitored by fluorescence of t-GroES\textsubscript{2}. GroEL/t-GroES\textsubscript{2} complexes and free t-GroES\textsubscript{2} were eluted at 12 and 17 min, respectively. Each elution pattern was normalized so that the total peak area became equal among elutions. Detailed experimental conditions are described under "Experimental Procedures." Circles with seven mini-circles illustrate the t-GroES\textsubscript{2} variants, and white and black mini-circles indicate subunits with active and defective GroEL-binding loops, respectively.
the presence of ADP. Wild-type t-GroESF co-eluted with GroEL, indicating its stable binding to GroEL. t-GroESF(1d;1) also showed stable binding. We confirmed that the positional effect of GroESF(1d) was not significant because t-GroESF(2d) also formed a complex with GroEL even if a trace fraction of free t-GroESF(2d) appeared. t-GroESF(2d;i,2), t-GroESF(2d;i,3), and t-GroESF(2d;i,4) showed the similar elution pattern. When the number of the defective subunits increased to three, only a trace amount of GroEL/t-GroESF(3d) complex was detected. When more than four defective subunits existed in t-GroESF, no complex with GroEL was detected. The same experiments were carried out in the presence of ATP (Fig. 2B). To prevent multiple turnover, a very slow (<2% of the wild-type GroEL) ATP-hydrolyzing GroEL variant, GroELD398A, was used (20). The results were very similar to those of Fig. 2A, and t-GroESF(1d) and t-GroESF(2d) formed a stable complex with GroELD398A. t-GroESF(3d) formed the complex with GroELD398A in a manner that was found to be dependent on the arrangement of the defective subunits in the t-GroESF. The apparent order of the yield of the complex formation was t-GroESF(3d;i,2,3) → t-GroESF(3d;i,3,4) → t-GroESF(3d;i,4,5) → t-GroESF(3d;i,2,5). The numbers of the longest consecutive active subunits in these t-GroESF(3d) values were 4, 3, 2, and 2, respectively, and it appeared that the yield of the complex formation decreased as the number of consecutive active subunits decreased. This may reflect a cooperative adjustment of GroEL conformation for GroES binding triggered by initial binding of a GroES subunit that is sequentially transmitted to a neighboring GroEL subunit. In general, yields of the complexes with t-GroESF(3d) were better in ATP than in ADP (Fig. 2A and B). This probably reflects the assumption that the GroEL/GroES complex is more stable in its ATP form than in its ADP form. Both complexes were too stable (half-decay time is several weeks (21)) to measure accurately, but the difference became visible by using t-GroESF(3d). In summary, at least four active subunits in a GroES heptamer are required to form GroEL/GroES complex that is stable during gel filtration.

**Binding of t-GroESF to the SR1D398A/Substrate Binary Complex**—The binding of t-GroESF to the SR1D398A/substrate binary complex was then analyzed. A single ring version of GroEL (SR1) (12) with slow ATP-hydrolyzing mutation (SR1D398A) was used to avoid the complication caused by the presence of the trans-ring (the ring opposite to cis-ring) and from multiple turnover of the reaction. Under standard experimental conditions, SR1D398A does not dissociate GroES and therefore does not release the encapsulated protein, whether

### FIGURE 3. Binding of t-GroESF to SR1D398A/substrate binary complexes.

Binding of t-GroESF to the SR1D398A/rhodanese binary complex (A) or to the SR1D398A/MDH binary complex (B) in the presence of ATP was examined. SR1D398A/substrate binary complex was generated by heat treatment. Other conditions were the same as those described in the legend for Fig. 2. The SR1D398A/substrate/t-GroESF ternary complex and free t-GroESF were eluted at 13 and 17 min, respectively, C, the ternary complex isolated from the first HPLC was reapplied to the same gel filtration HPLC under the same conditions to determine the stability of the complex. D, the SR1D398A/MDH/t-GroESF(3d;i,2,3) ternary complex isolated from the first HPLC was applied to the second HPLC. E, as a control, SR1D398A/t-GroESF(3d;i,2,3) complex isolated from the first HPLC was applied to the second HPLC. White and black minicircles indicate active and defective GroES subunit, respectively.
Folded or not, from the cavity into the medium. SR1D398A/MDH and SR1D398A/rhodanese binary complexes were prepared by heat treatment of MDH and rhodanese in the presence of SR1D398A. The binary complexes were mixed with t-GroESF, and complex formation was examined by gel filtration HPLC (Fig. 3, A and B). Wild-type t-GroESF, as well as t-GroESF(1d;1), was co-eluted with SR1D398A. t-GroESF(2d) formed the ternary complex, but the yields differed between substrate proteins; t-GroESF(2d) mostly bound to the GroEL/MDH binary complex, but binding to the GroEL/rhodanese binary complex was relatively poor. The positional effect of defective subunits was small since t-GroESF(2d;1,2), t-GroESF(2d;1,3), and t-GroESF(2d;1,4) showed similar elution patterns. As the number of defective subunits in the t-GroESF increased to more than three, t-GroESF did not form a stable ternary complex except for t-GroESF(3d;1,2,3), which formed the ternary complex with MDH, albeit at a moderate yield.

**Stability of the Ternary Complex**—To know the stability of the ternary complex, the peak fraction of the SR1D398A/rhodanese/t-GroESF ternary complex in the first HPLC was isolated and then applied to the second (same) HPLC (Fig. 3C). Ternary complexes made from wild-type t-GroESF, t-GroESF(1d;1), t-GroESF(2d;1,2), t-GroESF(2d;1,3), and t-GroESF(2d;1,4), were all recovered as the ternary complex with or without significant GroES dissociation. This fraction was then checked to confirm that rhodanese was still present (data not shown). Thus, even if the yield in the first HPLC was low in the case of GroESF(2d), the ternary complex was stable once formed. As shown later, these t-GroESF were able to mediate productive folding, and substrate proteins would already finish folding in the cis-cavity in these complexes. This was not the case when the number of defective subunits increased to three; SR1D398A/MDH/t-GroESF(3d;1,2,3) ternary complex decayed in the second HPLC (Fig. 3D). Also, SR1D398A/t-GroESF(3d;1,2,3) complex without substrate protein isolated in the first HPLC decayed in the second HPLC (Fig. 3E). The results of gel filtration HPLC suggest that at least five active GroES subunits in a GroES heptamer are required to generate stable GroEL/substrate/GroES ternary complex.

**Binding Kinetics**—The kinetics of t-GroESF binding to GroEL were measured with a rapid mixing apparatus. Binding was monitored by the fluorescence intensity of t-GroESF that decreased upon association to GroEL. The time course of the fluorescence change was well fitted by a single exponential curve (supplemental Fig. S1), and the binding rate constant ($k_{on}$) was calculated (Table 1). The $k_{on}$ value of wild-type t-GroESF, 8.29 $\times$ $10^7$ M$^{-1}$ s$^{-1}$, was similar to the value of native GroES: 7.5 $\times$ $10^7$ M$^{-1}$ s$^{-1}$ (21), 5 $\times$ $10^7$ M$^{-1}$ s$^{-1}$ (25), and 4 $\times$ $10^7$ M$^{-1}$ s$^{-1}$ (26). As the number of defective subunits in t-GroESF increased, $k_{on}$ values decreased. However, the decrement was small, and the $k_{on}$ values of all t-GroESFs with 4–7 active subunits remained in the same order of magnitude, 10$^7$ M$^{-1}$ s$^{-1}$. Thus, the binding rates of t-GroESF to GroEL appeared to be less sensitive to the presence of binding-defective subunits than the yield of the complex formation tested with gel filtration. The dissociation rate constants ($k_{off}$) might be responsible for decreasing the stability of t-GroESF containing defective subunits.

**Co-chaperone Activity of the t-GroES**—In the chaperonin reaction cycle, binding of GroES to the GroEL/substrate binary complex induces the encapsulation of substrate protein into the cis-cavity where folding follows. The co-chaperone activity of t-GroES on the folding of MDH and rhodanese was then tested. It is known that the folding of both of these proteins is dependent on both GroES and GroEL. To test the folding in the cis-cavity by a single turnover reaction, SR1D398A was used, and the binary complex with substrate protein was generated by heat treatment. Trap-GroEL (GroELN265A/D398A) was included to trap any free unfolded substrate proteins escaping from the cis-cavity. ATP was added to initiate the reaction in the presence of t-GroES, and the recovery yield of the enzyme activity was measured after the completion of folding (Fig. 4A). In the case of MDH, GroES was detached from SR1D398A by freeze-thawing to allow folded MDH monomer to form enzyme-active dimer. The results of MDH and rhodanese were very similar to each other. As compared with native GroES, wild-type t-GroES and t-GroES(1d;1) attained similar and slightly lower folding yields, respectively. GroES(2d)s showed diminished, but still significant, folding yields. GroES(3d)s had only marginal, if any, co-chaperone activity as compared with a (−GroES) control. Folding by multiple turnover of wild-type GroEL and t-GroES was also measured (Fig. 4B). In this experiment, trap-GroEL was not added to allow unfolded proteins to bind GroEL repeatedly. The results were again very similar between MDH and rhodanese but, as expected, folding yields attained by t-GroES variants were higher when compared with those of single turnover experiments. t-GroES containing five active GroES subunits showed good folding yields similar to native GroES. t-GroES(3d)s exhibited co-chaperone activity, but the effect of arrangement of active GroES subunits in the heptamer ring became obvious; t-GroES(3d;1,2,3) attained the highest yield, t-GroES(3d;1,3,5) attained the next highest, and t-GroES(3d;
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A. SR1D398A (single turnover)

B. GroEL (multiple turnover)

1,2,5) and t-GroES(3d;1,4,5) attained very poor yields. It appears that the longer consecutive arrangement of active subunits benefits the formation of the productive GroEL/substrate/GroES complex.

Folding Kinetics of GFP—Folding kinetics in the cis-cavity were investigated by using GFP as a substrate protein. SR1D398A/GFP binary complex was generated by the dilution of acid-denatured GFP into the SR1D398A solution. Following the addition of trap-GroEL, ATP was added to initiate the reaction in the presence of excess t-GroES, and the recovery of GFP fluorescence was monitored. The final yield of GFP folding was similar to those of rhodanese and MDH attained by SR1D398A single turnover (Fig. 5A). Setting the yields at 5 min as 100%, we compared folding time courses (Fig. 5B). In all cases, a slow (lag) phase, probably corresponding to encapsulation, was followed by an accelerated phase, probably corresponding to folding in the cis-cavity. The time courses were simulated well with equations assuming two rate constants: native GroES (0.41 s⁻¹, 0.05 s⁻¹), wild-type t-GroES (0.40 s⁻¹, 0.05 s⁻¹), t-GroES(1d;1) (0.32 s⁻¹, 0.037 s⁻¹), t-GroES(2d;1,2) (0.34 s⁻¹, 0.040 s⁻¹), t-GroES(2d;1,3) (0.16 s⁻¹, 0.025 s⁻¹), and t-GroES(2d;1,4) (0.12 s⁻¹, 0.022 s⁻¹). Both phases were slowed down as the number of binding-defective GroES subunits increased, except t-GroES(1d;1), which was slower than t-GroES(2d;1,2). We speculate that slow encapsulation allows more unfolded GFP molecules to escape to the medium. They are immediately caught by trap-GroEL and cannot fold. Thus, slow folding kinetics can result in low final folding yield.

DISCUSSION

Numbers of Active GroES Subunits Necessary for Functions as Co-chaperone—Binding experiments of t-GroES suggest that at least four GroES subunits in a GroES heptamer are involved in the formation of GroEL/GroES complex (Fig. 2). Based on the experiments using tandem-fused GroEL heptamers containing various numbers and arrangements of GroELV263S, Farr et al. (15) indicated that only one active GroEL subunit in a GroEL ring was sufficient for binding of GroES. However, GroELV263S is a leaky mutant in terms of GroES binding, and even the tandem GroEL made of seven GroELV263S subunits still retained significant ability to bind GroES (~20% of wild-type GroEL). Therefore, direct comparison with the results presented here may not be appropriate. For efficient formation of the GroEL/substrate/GroES ternary complex, the requirement is more stringent; at least five GroES subunits were found to be necessary for efficient folding of MDH and rhodanese by single turnover of the chaperonin reaction (Fig.

FIGURE 4. Co-chaperone activity of t-GroES. A, SR1D398A-assisted rhodanese and MDH folding in the presence of trap-GroEL, representing single turnover of chaperonin reaction cycle. SR1D398A/substrate binary complex was generated by heat treatment. The recovery of activity was measured at 60 min after the addition of ATP. In the case of MDH, GroES was detached from SR1D398A for folded MDH monomers to form an enzyme-active dimer in the medium. The recovered activities were plotted relative to the activity of the same amount of untreated MDH or rhodanese. B, wild-type GroEL-assisted rhodanese and MDH folding in the absence of trap-GroEL, representing multiple turnover of chaperonin reaction cycle. GroEL/substrate binary complex was generated by heat treatment. Leftmost columns (seven mini-circles), native GroES; rightmost columns (−ES), none (without GroES). Detailed experimental conditions are described under “Experimental Procedures.”
The very poor activity of t-GroES containing four active GroES subunits observed in single turnover experiments was apparently magnified more or less by multiple turnover of the chaperonin reaction using wild-type GroEL, especially for t-GroES(3d;1,2,3) (Fig. 4B).

**Heterogeneous GroEL/Substrate Binary Complexes**—Note-worthy is the observation that the isolated ternary complexes containing rhodanese and GroES(2d) were stable in the second gel filtration HPLC, although their yields in the first gel filtration HPLC were low (Fig. 3C). As Farr et al. (15) demonstrated, two or three binding sites in the cis-ring of GroEL are sufficient for binding of a denatured substrate protein. If so, four or five binding sites remain unused. The number and distribution of the used and the unused sites in the cis-ring would be different from one GroEL/substrate protein binary complex to another, making the population of binary complexes heterogeneous. Active GroES subunits in t-GroES(2d) may bind to some (or all) of the unused binding sites in the cis-ring of the binary complex, producing the mixture of ternary complexes with different positional patterns of binding site occupancy. They have different stability, and only sufficiently stable complexes, in which five GroES subunits are recruited in binding, can accomplish folding of MDH and rhodanese in the cis-cavity. If ATP hydrolysis is delayed as in the case of the D398A mutant, release of GroES is also delayed and the complex (corresponding to Intermediate II in the next paragraph) can withstand HPLC procedures (Fig. 3C). This may explain why the ternary complexes containing MDH were formed at higher yield than those containing rhodanese (compare results of t-GroES(2d) in Fig. 3A and B). It has been reported that denatured MDH has the tendency to occupy consecutive binding sites in the cis-ring, but rhodanese does not (15, 27, 28). As a consequence, the GroEL/MDH binary complex may leave consecutive unused binding sites more often than the GroEL/rhodanese binary complex. As the binary complex with more consecutive binding sites produces more stable ternary complexes, the GroEL/MDH/t-GroES(2d) ternary complex could be formed at a higher yield than GroEL/rhodanese/t-GroES(2d) ternary complex.

**Substrate Protein and GroES May Share the Seven Common Binding Sites in the Intermediate State**—Based on the results presented here, we propose a model to explain how substrate protein and GroES bind the common binding sites on the cis-ring of GroEL (Fig. 6). At first, denatured substrate protein binds to GroEL by using several binding sites on the ring of GroEL with various positional combinations, generating the heterogeneous populations of binary complexes. Given that one molecular species is providing substrate protein two binding sites (Intermediate I), GroES can bind to this complex through the remaining five binding sites, generating Intermediate II. The two/five sharing of seven binding sites between

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**FIGURE 5.** GFP folding assisted by SR1D398A and t-GroES variants. Denatured GFP was diluted in buffer containing SR1D398A and t-GroES. Trap-GroEL was added prior to ATP addition to prevent spontaneous folding in the medium. A, final folding yield of GFP at 10 min after the addition of ATP. The yields are expressed as value relative to that of the same amount of native GFP. Leftmost column, native GroES; B, time courses of GFP folding. GFP folding was initiated by adding ATP at 0 s. Recovery of GFP fluorescence was fitted by the convolution of two exponentials, $C/(k_2 - k_1) (-k_1 e^{-k_1 t} + k_2 e^{-k_2 t}) + k_0$ (overlaid with white lines) (29). The fluorescence intensity of each curve was normalized as the percentage of the recovered fluorescence intensity at 5 min. White and black minicircles indicate active and defective GroES subunit, respectively.

**FIGURE 6.** This model shows that substrate protein and GroES share the seven binding sites on GroEL. Denatured substrate protein (red) occupies some, for example two, of the binding sites on GroEL (Intermediate I). In the presence of ATP, GroES binds to GroEL by occupying the remaining five binding sites to form a transient intermediate (Intermediate II). After competition for binding sites (Intermediate II-1 and Intermediate II-2), substrate protein is deprived of all binding sites by GroES and is discharged into the cis-cavity (Intermediate III).
substrate protein and GroES subunits in this case can be one/six or three/four depending on the substrate protein. Finally, GroES occupies all seven binding sites in the cis-ring of GroEL, and substrate protein is discharged into the cis-cavity where the folding starts (Intermediate III). In this model, folding does not start immediately upon GroES binding but after a lag period that corresponds to the lifetime of the intermediate II, as we observed for GroEL/GroES-assisted GFP folding in the single molecule study (29, 30). If there are one or two defective GroES subunits in the GroES heptamer, deprivation of the binding sites from substrate protein by the GroES heptamer should take a longer time, and the lag period will be extended. Indeed, the lifetime of the lag in GroEL/GroES-assisted GFP folding was 2.6 s for wild-type t-GroES and 6.2 s for t-GroES(2d;1,4) (Fig. 5B). The folding following the lag also appeared to be slowed down by the presence of defective GroES subunits in the GroES heptamer, probably because of occasional rebinding of substrate protein to the unused binding sites (corresponding to the positions of defective GroES subunits) on the cis-ring before completion of folding. The model proposed here requires further testing.

Acknowledgment—We thank A. Koike-Takeshita for valuable discussions.

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