Effect of the C-terminal Truncation on the Functional Cycle of Chaperonin GroEL

**IMPLICATION THAT THE C-TERMINAL REGION FACILITATES THE TRANSITION FROM THE FOLDING-ARRESTED TO THE FOLDING-COMPETENT STATE***

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To elucidate the exact role of the C-terminal region of GroEL in its functional cycle, the C-terminal 20-amino acid truncated mutant of GroEL was constructed. The steady-state ATPase rate and duration of GroES binding showed that the functional cycle of the truncated GroEL is extended by ~2 s in comparison with that of the wild type, without interfering with the basic functions of GroEL. We have proposed a model for the functional cycle of GroEL, which consists of two rate-limiting steps of ~3- and ~5-s duration (Ueno, T., Taguchi, H., Tadakuma, H., Yoshida, M., and Funatsu, T. (2004) Mol. Cell 14, 423–434). According to the model, detailed kinetic studies were performed. We found that a 20-residue truncation of the C terminus extends the time until inorganic phosphate is generated and the time for arresting protein folding in the central cavity, i.e. the lifetime of the first rate-limiting step in the functional cycle, to an ~5-s duration. These results suggest that the integrity of the C-terminal region facilitates the transition from the first to the second rate-limiting state.

The chaperonin GroEL is an essential molecular chaperone that mediates protein folding with its cofactor GroES in *Escherichia coli*. GroEL is composed of 14 identical 57-kDa subunits arranged in two heptameric rings stacked back-to-back, each contains a cavity. GroES consists of a dome-shaped heptameric ring of identical 10-kDa subunits and interacts with one or both GroEL rings in an ATP-regulated manner. The substrate protein is encapsulated in the GroEL cavity underneath GroES, where it folds during the time of ATP hydrolysis (1, 2). Each subunit of GroEL is divided into the following three distinct domains: apical, intermediate, and equatorial domains. The apical domain forms the entrance to the GroEL cavity and includes the residues involved in the binding to substrate proteins and GroES. The intermediate domain connects the equatorial and apical domains of each subunit. The equatorial domain, which is composed of N- and C-terminal regions, is involved in intra- and inter-ring contacts and contains the ATP-binding site (3, 4). Although the final ~20 residues in the C-terminal region are known to be structurally flexible (3), they project from the equatorial domain into the central space and form the dividing wall of each ring, as indicated by structural studies (5, 6). Recent studies have renewed interest in the functional role of the C-terminal region.

An intriguing feature is the presence of Gly-Gly-Met (GGM) repeated sequences at the C terminus of GroEL. The sequence is strongly conserved among most GroEL homologues (7), but its significance is unknown. Earlier studies have shown that up to 27 amino acids can be deleted from the C terminus without affecting *E. coli* viability in ideal growing conditions (8–10). Recently, Tang et al. (11, 12) showed that mutating the length of the C-terminal region can alter the folding rate of substrate proteins within the GroEL cavity. On the contrary, Farr et al. (13) reported no acceleration in folding rate in GroEL mutants with elongated GGM repeat(s). Elad et al. (14) suggested the C-terminal region is a potential interaction site with the substrate proteins. Machida et al. (15) mentioned that the hydrophilic residues in the C-terminal sequence are critical for the substrate folding inside the central cavity. Given that the C-terminal region is located in the central cavity, it is likely that the region contributes to maintaining the volume and the environment of the central cavity for proper protein folding. Furthermore, it should not be forgotten that the C-terminal region is closely related to the rate of ATP hydrolysis (9, 12, 13, 15, 16). Farr et al. (13) found that GGM repeat-elongated GroEL mutants exhibit an increased ATPase rate. They also suggested that disturbance of the ATPase rate can affect the folding rate of substrate proteins within the cavity. Therefore, it is important to understand what the effects of the C-terminal region are on the functional cycle of GroEL.

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To this end, we constructed a C-terminal truncated mutant (termed ELtc20), which lacks the final 20 amino acid residues, and characterized it. ELtc20 was shown to have the basic functions of GroEL and the extended functional cycle (∼10 s) in comparison with that of the wild type (∼8 s). Previously, we proposed a model for the functional cycle of GroEL with two successive rate-limiting steps of 3s and ∼5 s duration (17). Detailed kinetic studies revealed that the C-terminal truncation extends the lifetime of the first rate-limiting step to ∼5 s. From these results, we suggest the notion that the integrity of the C-terminal region facilitates the transition from the first to the second rate-limiting state in the functional cycle.

**EXPERIMENTAL PROCEDURES**

**Regents and Proteins**—ATP, phosphoenolpyruvate, pyruvate kinase from rabbit muscle, lactate dehydrogenase from hog muscle, NADH, and malate dehydrogenase were purchased from Roche Diagnostics. Bovine apoa-α-lactalbumin (LA), pepsin, bovine mitochondrial rhodanese, bovine serum albumin (BSA), glucose oxidase, and catalase were obtained from Sigma. Streptavidin and tetramethylrhodamine (TMR)-5′-maleimide were from Invitrogen. IC5-maleimide and biotin-PEAC5-maleimide were purchased from Dojindo Laboratories. Cy3 monofunctional N-hydroxysuccinimide ester was from GE Healthcare.

**Preparation of GroEL, GroES, and Substrate Proteins**—ELtc20 was generated using the following primer sets: Forward, 5′-TAAATCAGCTACTATAGG-3′; Reverse, 5′-GGAAAAA-CGATTAGACTCAAT-3′ (XhoI site underlined). PCR amplification was conducted with KOD-Plus- (Toyobo) using the plasmid pET-EL as a template (18). PCR product was digested with NdeI and Xhol, and cloned into the same site of pET21c (Novagen). The construct was verified by DNA sequencing. GroEL and GroES were expressed in E. coli and purified as described previously (18). The concentrations of GroEL and GroES were determined spectrophotometrically at 280 nm (17) and were expressed as molar concentrations of tetradecamer and heptamer in this study.

ES98C, a GroES mutant with a cysteine residue added at the C terminus (19), was labeled with IC5-maleimide and biotin-PEAC5-maleimide in HKM buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl₂). The labeled ES98C (IC5bio-ES) was separated from unreacted reagents by a NAP-5 column (GE Healthcare). The extent of labeling was estimated according to the following procedure. The concentration of IC5 was determined spectrophotometrically at 645 nm with an extinction coefficient of 178,000 M⁻¹ cm⁻¹. The concentration of ES98C was determined using the Lowry method (DC protein assay; Bio-Rad). The molar ratio of IC5 to the ES98C heptamer was 1.8 throughout the study. ELwt and ELtc20 were labeled with Cy3 monofunctional N-hydroxysuccinimide ester in HKM buffer containing 20 mM sodium bicarbonate to raise the pH (∼8.7).

Labeling resulted in a stoichiometry of ∼1.1 Cy3 dye molecules per GroEL tetradecamer. Fluorescent-labeled GroEL and ES98C exhibited behavior similar to the wild-type proteins (data not shown).

LA (300 μM) was treated with HKM buffer containing 7.5 mM DTT for 15 min at room temperature. The disulfide bond-reduced form of LA (rLA) is known to bind strongly to GroEL (18, 19). Pepsin (300 μM) was dissolved in HKM buffer and was used as denatured pepsin, because it is known to lose its native conformation at a neutral pH and interact with GroEL (20, 21). Rhodanese (75 μM) was denatured in HKM buffer containing 6 mM guanidine hydrochloride for at least 1 h at room temperature. The GFP employed in this study is the S65T mutant (22). It was expressed and purified as described previously (23, 24). GFP (20 μM) was denatured in HKM buffer containing 100 mM HCl at room temperature for 2 min.

**Steady-state ATPase Rate Measurements**—Steady-state ATPase rate of GroEL was measured at 23 °C with an ATP-regeneration system (17). The assay mixture consisted of HKM buffer containing 0.2 mM NADH, 5 mM phosphoenolpyruvate, 100 μg/ml pyruvate kinase, 100 μg/ml lactate dehydrogenase, 2.5 mM DTT, and 1 mM ATP in the presence or absence of 500 nM GroES. After a 1-min incubation, ATP hydrolysis was initiated by the injection of 100 nM GroEL into the vigorously stirred mixture. After incubation for 200 s, the denatured protein (75 μM) was diluted into the assay mixture. The decreases in the absorbance at 340 nm, because of oxidation of NADH, were monitored continuously with a spectrophotometer (V-550, Jasco). Steady-state ATPase activity was determined from the slope of the absorbance decrease with time. Measurements were repeated in triplicate.

**Sample Preparation for Microscopy**—Zero-mode waveguides (ZMWs) were fabricated in a 100-nm-thick aluminum film deposited on a concave quartz coverslip. The detailed procedures are reported elsewhere (25). The ZMW holes used in this study were ∼90 nm in diameter and ∼160 nm in depth (Fig. 2A). The coverslip with ZMWs was placed in an oxygen plasma asher to clean the surfaces before use.

A flow cell was constructed from the glass slide and coverslip with ZMWs separated by two spaces of ∼50 μm thickness. IC5bio-ES was immobilized on the bottom of ZMWs via biotinylated BSA and streptavidin as described previously (17, 26). First, 3.0 mg/ml biotinylated BSA was injected into the flow cell. The cell was washed by HKM buffer. Infusion and washing were repeated as follows: infusion of 0.33 mg/ml streptavidin, washing with HKM buffer, infusion of 25 nM IC5bio-ES, washing with HKM buffer and HKM buffer containing 10 mM Cy3-labeled GroEL, the oxygen scavenger system (25 mM glucose, 2.5 μM glucose oxidase, 10 mM catalase, and 10 mM DTT), 75 μM rLA, and 2 mM ATP.

**Microscopy and Data Analyses**—Single molecules in ZMWs were observed by using an epi-illumination configuration in an inverted microscope (IX70, Olympus). The surface-immobilized IC5bio-ES was illuminated with a red solid-state laser

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3 The abbreviations used are: ELtc20, GroEL mutant with 20-amino acid truncation from the C terminus; LA, α-lactalbumin; rLA, disulfide bond-reduced form of LA; ELwt, wild-type GroEL; ES98C, GroES mutant with a single cysteine added at the C terminus of each subunit; IC5bio-ES, ES98C modified with IC5 and biotin; ZMW, zero-mode waveguide; GFP, S65T mutant of green fluorescence protein; TMR, tetramethylrhodamine; BSA, bovine serum albumin; DTT, dithiothreitol.

4 Unless otherwise stated, wild-type GroES was used in assays.
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Fluorometric Monitoring of GFP Refolding—The denatured GFP (100 nM) was diluted 200-fold in the refolding buffer (HKM buffer plus 5 mM DTT) containing GroEL (150 nM) and GroES (1.5 μM). After a 50-s incubation, ATP (0.45 mM) was injected into the mixture using a microsyringe to initiate the refolding mediated by GroEL (time 0). Spontaneous refolding was initiated by injecting acid-denatured GFP into the refolding buffer (time 0). The fluorescence of GFP at 512 nm with excitation at 485 nm was continuously monitored with an interval of 0.01 s using a Jasco FP-6500 spectrofluorometer. The reaction mixtures were continuously stirred at 23 °C throughout the assays. The dead time of the mixing was about 400 ms. The resultant fluorescence profiles for the first 30 s in the assay were analyzed as described previously (17). The rate constants were calculated by fitting to the fluorescence profiles using the following equations (Equation 4, in the absence of GroEL and GroES; Equation 5, in the presence of GroEL and GroES) (17),

\[
I(t) = I(\infty) - \exp(-k_{\text{fold}}t)
\]

\[
I(t) = I(\infty) - \frac{k_{\text{fold}}}{k_1} \exp(-k_1t) - \frac{k_2}{k_1} \exp(-k_2t) + \frac{k_2}{k_1} \exp(-k_3t)
\]

where \(I(t)\) and \(I(\infty)\) are the fluorescence intensities of GFP at time \(t\) and the infinite time, respectively; in addition, \(k_1\) and \(k_2\) are the rate constants at each step, respectively. The rate constants were the average values from three independent experiments.

Stopped-flow Anisotropy Measurements—Rhodanese (60 μM) was mixed with a 10-fold amount of TMR-maleimide in TKE buffer (25 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.5 mM EDTA) containing 6 mM guanidine-HCl. Unreacted dye was removed by PD-10 column (GE Healthcare), and the extent of the labeling was determined by absorption spectroscopy using the following extinction coefficients (in 6 mM guanidine-hydrochloride): rhodanese, 49,000 M⁻¹ cm⁻¹ at 280 nm; TMR, 94,000 M⁻¹ cm⁻¹ at 558 nm. Approximately 0.85 mol of TMR were coupled per mol of denatured rhodanese. The denatured TMR-labeled rhodanese (27.8 μM) was diluted 100-fold in TKE buffer with 116 nM GroEL. After incubation for 15 min at room temperature, the aggregated rhodanese was removed by centrifugation. Then the supernatant was applied to a Resource Q column (GE Healthcare), and GroEL complexed with TMR-rhodanese was isolated.

Fluorescence anisotropy measurements were carried out at 23 °C using an SX.18MV stopped-flow spectrophotometer equipped with an FP.1 accessory (Applied Photophysics). A solution of 1 μM GroEL complexed with TMR-rhodanese and 5 mM DTT in HKM buffer was loaded into one syringe of the stopped-flow device. A solution of HKM buffer containing 2 μM GroES and 5 mM DTT with or without 10 mM ATP was loaded into the other syringe. Reactions were initiated by mixing equal volumes from each syringe. Excitation was done with vertically polarized light at 515 nm (bandwidth 18.6 nm). Two photomultiplier tubes, both with 550 nm long pass filters (OG550, Schott), were used to monitor the vertical and horizontal polarized emission components simultaneously. Fluorescence anisotropy data were collected with a 50-s log time base. The dead
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To examine the role of the C-terminal region in the function cycle of GroEL, we constructed a C-terminal truncated mutant (named as ELtc20), which lacks the final 20 amino acid residues. ELtc20 was expressed in E. coli, and was purified in the same manner as the wild type (termed ELwt). The truncation had no effect on the oligomer formation and slightly decreased the yield in assisted folding of GFP and malate dehydrogenase (data not shown).

We first examined the steady-state ATPase rates of ELwt and ELtc20 in the presence or absence of saturating amounts of GroES and substrate proteins (Fig. 1). GroES is known to decrease by about one-half the rate of ATP hydrolysis by GroEL (18). The presence of GroES suppressed the steady-state ATPase rate of ELwt by 37% (Fig. 1, open bar). The comparable suppression by GroES (~35%) was observed in ELtc20 (Fig. 1, filled bar). As reported previously (27–29), when substrate proteins such as rLA, denatured pepsin, and denatured rhodanese were present, the rate of ATP hydrolysis was enhanced (Fig. 1, open bar). Similarly, the presence of substrate proteins increased in the ATPase rate of ELtc20 (Fig. 1, filled bar). From these results, ELtc20 is considered to have the normal “accelerator” and “brake” for its ATPase rate. However, ELtc20 showed an ~20% reduction in its steady-state ATPase rate irrespective of the presence of GroES and substrate proteins (p < 0.01, Student’s t test). The rates of ELwt and ELtc20 in the presence of GroES and rLA or denatured rhodanese were ~0.12 and ~0.10 s\(^{-1}\), respectively. These results suggest that the truncation of the C-terminal region extends the ATPase cycle without impairing the enzyme properties.

Truncation of the C-terminal Region Extends the Duration of GroES Binding to GroEL—We previously demonstrated single-molecule imaging of the association and dissociation of the GroEL-GroES complex in the functional GroEL cycle. The dissociation of GroES from GroEL was observed to proceed as a two-step process with rate constants of ~0.3 and ~0.2 s\(^{-1}\), respectively (17, 26) (Fig. 2A). To confirm whether ELtc20 retains the same mechanism to interact with GroES as ELwt, the interaction between ELtc20 and GroES was observed at the single-molecule level (Fig. 2, B–D). In previous studies, biotinylated GroEL was immobilized to the glass surface, and the association and dissociation of fluorescently labeled GroES to and from GroES were observed (17, 26). In this study, the interaction was observed between surface-immobilized GroES, a cysteine-introduced variant (ES98C) (19) modified with IC5 and biotin (termed IC5bio-ES) and Cy3-labeled GroEL in the presence of saturating amounts of rLA5 (Fig. 2B). To this end, we used coverslips with ZMWs to prevent nonspecific binding of GroES to the surface. ZMWs consist of nanoscale holes in a metal film deposited on a fused coverslip (30) (Fig. 2B). ZMWs used in this study provide small excitation areas in diameter of ~90 nm, where IC5bio-ES molecules are immobilized; therefore, one can reduce the probability that Cy3-labeled GroES nonspecifically absorbs onto the glass surface.

The position of surface-immobilized IC5bio-ES was confirmed, and the association and dissociation of Cy3-labeled GroEL were monitored for 5 min. The repeated appearance and disappearance of Cy3 fluorescence were observed at the position of the immobilized GroES. The histograms were then constructed from the duration time of ELwt and ELtc20 interacting with IC5bio-ES (“on time”), i.e. the lifetimes of the complexes (Fig. 2, C and D). Both histograms have maximum peaks at ~5 s and do not show single exponential decays. These results indicate that the dissociation of ELwt and ELtc20 from GroES occurs through two steps, as shown in previous studies (17, 26, 31, 32). In addition, the histogram data were well fitted by the equation (Equation 1) derived from a two-step reaction (Fig. 2, A, C, and D). The rate constants obtained are summarized in Table 1. We note that the lifetime (sum of the reciprocal of the rate constants) of the ELtc20-GroES complex (~4.0 + ~5.3 s

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5 Substitution of Asp-490 for cysteine in ELtc20 showed an ~75% reduction of ATPase activity compared with ELtc20. Moreover, modification of this mutant with biotin and IC5 resulted in the 2-fold increase in its ATPase activity (data not shown). Thus, we did not use the ELtc20 mutant in a single-molecule experiment.
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We have found that the refolding of GroEL upon addition of ATP (Fig. 1). The result indicates that the truncation of the C-terminal region delays the initial burst of $P_i$ generation.

Truncation of the C-terminal Region Extends the Time for Arresting GFP Refolding—We have found that the refolding of substrate proteins is arrested in the first rate-limiting step (17) (Fig. 4A). Then the kinetic analyses of GroEL-mediated GFP refolding were carried out (Fig. 4). Acid-denatured GFP was

TABLE 1
Rate constants obtained from on-time distributions

\[ \begin{array}{ccc}
\text{GroEL} & k_1 \text{ s}^{-1} & k_2 \text{ s}^{-1} \\
\text{Elwt} & 0.31 \pm 0.13 & 0.19 \pm 0.066 \\
\text{ELtc20} & 0.25 \pm 0.15 & 0.19 \pm 0.11 \\
\end{array} \]
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FIGURE 3. Time course of P_i generation from GroEL in the absence and presence of GroES. A, schematic model for the ATPase cycle of GroEL. P_i generated from GroEL was observed to be a two-step reaction. The rate constants (k_1 and k_2) were determined by fitting the experimental data. B and C, amount of P_i generated from ELwt (B) and ELtc20 (C) was determined by the malachite green method. Open circles, in the absence of GroES; filled circles, in the presence of GroES. Data points with error bars represent the mean ± S.E. of three independent experiments. The solid lines show the fit to the data using Equation 2 and Equation 3. The values of the rate constants obtained are summarized in Table 2. Experiment details are described under “Experimental Procedures.”

TABLE 2
Rate constants obtained from profiles of P_i generation
Values are reported with the error of the fit.

| GroEL   | Without GroES, k | With GroES |
|---------|------------------|------------|
|         | k_1, s^{-1}      | k_2, s^{-1}|
| ELwt    | 0.18 ± 0.0039    | 0.30 ± 0.023|
| ELtc20  | 0.15 ± 0.0017    | 0.21 ± 0.023|

FIGURE 4. Kinetic assay of GFP refolding mediated by GroEL and GroES. A, schematic model for the folding reaction of GFP mediated by GroEL and GroES. The reaction was observed to be a two-step reaction. The rate constants (k_1 and k_2) were determined by fitting the experimental data. B, refolding of GFP was monitored by its fluorescence recovery. Spontaneous refolding was initiated by injecting acid-denatured GFP into the refolding buffer (green line). Acid-denatured GFP was diluted in the refolding buffer containing GroEL and GroES. After a 50-s incubation, the refolding was initiated by injecting 0.45 mM ATP into the mixture (ELwt, red line; ELtc20, blue line). The profiles were fitted by a single exponential (spontaneous refolding), and the convolution of two exponentials (GroEL-mediated refolding). The corresponding fits are shown in light colored lines. Spontaneous refolding, light green line; ELwt, faint pink line; ELtc20, light blue line. Results are representative of three independent experiments. The values of the rate constants obtained are summarized in Table 3. Experiment details are described under “Experimental Procedures.”

TABLE 3
Fitting parameters obtained from refolding curves of GFP
Values represent the mean ± S.E. of three independent experiments. The fitting errors are sufficiently smaller than the experimental errors.

|                  | k_1, s^{-1} | k_2, s^{-1} |
|------------------|------------|------------|
| Without          | 0.018 ± 0.00065 |
| ELwt             | 0.30 ± 0.014         | 0.046 ± 0.0013 |
| ELtc20           | 0.21 ± 0.0035*       | 0.044 ± 0.022 |

* p < 0.01 is a significant difference from the value in ELwt (Student’s test).

diluted in the refolding buffer containing GroEL (ELwt or ELtc20) and GroES, and ATP was injected into the mixture to initiate the refolding of GFP. In each case, an initial lag phase was observed before the exponential increase in GFP fluorescence (Fig. 4B, red and blue lines). No such lag phase was detected in the spontaneous refolding (Fig. 4B, green line), reflecting that the lag phase corresponds to the state where the refolding is arrested by GroEL. Thus, the resulting profiles were fitted by Equation 4 and Equation 5 described under “Experimental Procedures,” and then the fitted lines were shown by the light colored lines in Fig. 4B. The values of the rate constants are summarized in Table 3. The values of the rate constants (k_1)
corresponding to the lag phases of ELwt and ELtc20 were found to be 0.30 ± 0.014 and 0.21 ± 0.0035 s⁻¹, respectively (Table 3). The values of the rate constants (kₙ) corresponding to the refolding phases of ELwt and ELtc20 were determined to be 0.046 ± 0.0013 and 0.044 ± 0.022 s⁻¹, respectively (Table 3). Note that the k₁ value of ELtc20 was smaller than that of ELwt. Besides, the first-order rate constant (k_folding) of spontaneous folding was estimated as 0.040 ± 0.00065 s⁻¹ (Table 3), and the value was in good agreement with the k_folding values of ELwt and ELtc20. Namely, GFP refolding is assumed to occur in the presence of ELwt and ELtc20, at rate constants (k_folding) equivalent to spontaneous refolding. These results indicate that truncation of the C-terminal region extends the time for arresting GFP refolding.

**Truncation of the C-terminal Region Extends the Time for Arresting the Bound Substrate within the GroEL Cavity**—To confirm that the substrate protein mobility is restricted during GroEL-mediated folding reaction, we examined the time-resolved fluorescence anisotropy of a fluorescently labeled substrate protein bound to GroEL. Denatured TMR-labeled rhodanese, used as a substrate protein, was incubated with ELwt and ELtc20 to form the binary complex as described under “Experimental Procedures.” The reactions were initiated by mixing the binary complexes with GroES in the presence or absence of ATP using a stopped-flow apparatus. The changes in anisotropy were recorded in log time-base to improve the time resolution in the initial change. In the absence of ATP, no anisotropy changes occurred throughout the measurements (Fig. 5, A and B, gray). When the binary complex was mixed with ATP and GroES, subsequent stepwise decays were observed (Fig. 5, A and B, black).

The time trajectories of anisotropy in the presence of ATP were well fitted by assuming three-step reactions (Equation 6). The kinetic parameters estimated are summarized in Table 4. The values of the rate constants (kᵢ) corresponding to the first phase (A₁ → A₂) of ELwt and ELtc20 were 29 and 30 s⁻¹, respectively (Table 4). Judging from the association rate constant (kₐ = 3.0 × 10⁷ s⁻¹ M⁻¹) of GroES to the GroEL-bound rhodanese (33) and the concentration (1 μM) of GroES, the first phase seems to occur upon binding of GroES to GroEL. In addition, the anisotropy decay of the first phase was the largest in the reaction as follows: ELwt, 54% of the total decay; ELtc20, 53% of the total decay (Table 4). Therefore, we assigned the first phase to the displacement of bound TMR-rhodanese into the cavity. FRET measurements between the apical domain of GroEL and substrate proteins support this notion that bound proteins are released into an enclosed cavity immediately upon binding of GroES to GroEL (17). In the second phase (A₂ → A₃), the anisotropy showed slight decay as follows: ELwt, 14% decay; ELtc20, 13% decay (Table 4). In this phase, there was a striking difference between the rate constants (kᵢ) of ELwt and ELtc20. The k₂ values of ELwt and ELtc20 were estimated to be 0.35 and 0.18 s⁻¹, respectively (Table 4). The values are similar to those of k₁ in GFP refolding (Table 3). Consequently, this phase might correspond to the state in which TMR-rhodanese interacts with the walls of the central cavity and in which its mobility is restricted. The anisotropy changes in the third phase (A₃ → A₄) were relatively large as follows: ELwt, 31% decay; ELtc20, 33% decay. The third phase could be the process in

**FIGURE 5. Changes in the fluorescence anisotropy of GroEL-bound rhodanese upon the addition of ATP and GroES.** A, schematic model for the reaction of protein encapsulation and release mediated by GroEL and GroES. The reaction was assumed to be a three-step reaction. The rate constants (k₀, k₁, and k₂) were determined by fitting the experimental data. B and C, binary complexes between denatured TMR-rhodanese and either ELwt (B) or ELtc20 (C) were prepared and rapidly mixed in a stopped-flow apparatus with GroES in the absence (gray) and presence of ATP (black). Changes in fluorescence anisotropy were recorded in log time-base. Each profile was the average of 29–34 individual shots. The profiles in the presence of ATP were fitted by assuming a three-step reaction (Equation 6). The corresponding fits are shown in light gray lines. The values of the fitting parameters are summarized in Table 4. Experiment details are described under “Experimental Procedures.”

**TABLE 4**

| GroEL   | A₁   | k₀      | A₂   | k₁      | A₃   | k₂      | A₄   |
|---------|------|---------|------|---------|------|---------|------|
| ELwt    | 0.265 | 29      | 0.246| 0.35    | 0.241| 0.10    | 0.230|
| ELtc20  | 0.287 | 30      | 0.271| 0.18    | 0.267| 0.107   | 0.257|
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which TMR-rhodanese is released from the inside to outside of the GroEL cavity and/or that TMR-rhodanese folds within the central cavity. The rate constants corresponding to the third phase ($k'_3$) had approximately the same values (Table 4). Taken together with the above results (Fig. 4 and Table 3), the difference between the rate constants of ELwt and ELtc20 in the second phase may indicate that truncation of the C-terminal region extends the folding-arrest state in the functional cycle.

**DISCUSSION**

The functional cycle of GroEL proceeds at a rate constant of 0.12 s$^{-1}$ (lifetime, ~8 s) in the presence of saturating amounts of GroES and substrate proteins (28, 34) (Fig. 1). Steady-state ATPase rate and release kinetics of GroES showed that ELtc20 has an extended functional cycle of ~10 s, without interfering with the basic functions of GroEL (Figs. 1 and 2). Thus, the extension of the functional cycle is likely to reflect an increase(s) in the lifetime of intermediate(s) in the cycle. We previously proposed that the functional GroEL cycle consists of two rate-limiting steps of ~3 and ~5 s duration. As observed previously, in the first rate-limiting step (~3 s), ATP hydrolysis occurs, and the folding of substrate proteins is arrested in the GroEL-GroES cavity. In the second rate-limiting step (~5 s), by contrast, protein folding occurs (17) (Fig. 6A). To address whether the C-terminal truncation affects the first and/or second rate-limiting steps, detailed kinetic analyses were performed. The rate constants ($k_1$) of the burst phase corresponding to the first round of ATP hydrolysis of ELwt and ELtc20 were observed to be 0.30 (lifetime, ~3.3 s) and 0.21 s$^{-1}$ (lifetime, ~4.8 s), respectively (Fig. 3 and Table 2). The rate constants ($k_2$) corresponding to the lag phase in GFP refolding in the presence of ELwt and ELtc20 were 0.30 (lifetime, ~3.3 s) and 0.21 s$^{-1}$ (lifetime, ~4.8 s), respectively (Fig. 4 and Table 3). Besides, the rate constants ($k_3$) corresponding to the lag phase in the anisotropy change in the presence of ELwt and ELtc20 were estimated to be 0.35 (lifetime, ~2.9 s) and 0.18 (lifetime, ~5.6 s), respectively (Fig. 5 and Table 4). Both the burst phase and the lag phase are thus assumed to be the first rate-limiting step in the functional cycle (17). Therefore, we concluded that the lifetime of the first rate-limiting step in the ELtc20 functional cycle is extended to ~5 s, namely the cycle of ELtc20 is defined by ~5 and ~5 s duration (Fig. 6B). These results suggest that the truncation of the C-terminal region extends the lifetime of the first rate-limiting step in the functional cycle. Interestingly, the time given to substrate protein to fold was ~5 s in the ELtc20 cavity, although the C-terminal truncation led to increased encapsulation time of the substrates. This finding will help to examine the effect of the C-terminal region on the rate of protein folding within the GroEL-GroES cavity.

From the finding that the lifetime of the first rate-limiting step is extended in ELtc20, it is likely that the integrity of the C-terminal region facilitates the transition from the first to the second rate-limiting state, i.e. from the folding-arrested to the folding-competent state. Recently, Tang et al. (11, 12) showed that the chemical environment of the GroEL cavity is critical for the proper folding of proteins. We reconfirmed that folding of substrate protein is arrested in the first rate-limiting step (Figs. 4 and 5). Cliff et al. (35) also showed that substrate proteins remain bound to the inner walls of the initially formed GroEL-GroES cavity upon initial association of GroES. Taken together with these findings, the cavity surface in the first rate-limiting step is assumed to be lined with hydrophobic residues, which bind non-native substrates that expose regions of a hydrophobic surface. On the other hand, in the second rate-limiting step, where protein folding occurs, the walls of the cavity will be hydrophilic, favoring burial of a nonpolar surface in the substrate protein and promoting folding to the native state, just like the cis-cavity in the crystal structure of the GroEL-GroES complex formed in ADP (4). It can be predicted that the GroEL cavity is switched from a hydrophobic to a hydrophilic environment associated with the transition from the first to the second rate-limiting step, and that the truncation of the C-terminal region interferes with such an environmental change. Further studies will be needed to verify this hypothesis.

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