Asp-52 in Combination with Asp-398 Plays a Critical Role in ATP Hydrolysis of Chaperonin GroEL*

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Ayumi Koike-Takeshita¹, Kaoru Mitsuoka⁵, and Hideki Taguchi¹

From the ¹Department of Applied Bioscience, Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi, Kanagawa 243-0292, Japan, ⁵Technology Research Association for Next Generation Natural Products Chemistry, AIST Tokyo Waterfront, 2-3-26, Aomi, Koto-ku, Tokyo 135-0064, Japan, and ⁶Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, B-56, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

Background: Chaperonin GroEL(D398A) is known to slow down the ATP hydrolysis.

Results: ATPase activity of GroEL(D52A) and GroEL(D52A/D398A) mutants are ~20% and < 0.01% of wild-type GroEL, respectively.

Conclusion: Asp-52 plays a critical role for ATP hydrolysis of GroEL.

Significance: GroEL(D52A/D398A) forms a stable symmetric GroEL-GroES complex, providing a good model to characterize the complex in detail.

Chaperonins are a subclass of double-ring chaperones that assist protein folding with the aid of GroES and ATP. Asp-398 in GroEL is known as one of the critical residues on ATP hydrolysis because GroEL(D398A) mutant is deficient in ATP hydrolysis (<2% of the wild type) but not in ATP binding. In the archaeal Group II chaperonin, another aspartate residue, Asp-52 in the corresponding E. coli GroEL, in addition to Asp-398 is also important for ATP hydrolysis. We investigated the role of Asp-52 in GroEL and found that ATPase activity of GroEL(D52A) and GroEL(D52A/D398A) mutants were ~20% and < 0.01% of wild-type GroEL, respectively, indicating that Asp-52 in E. coli GroEL is also involved in the ATP hydrolysis. GroEL(D52A/D398A) formed a symmetric football-shaped GroEL-GroES complex in the presence of ATP, again confirming the importance of the symmetric complex during the GroEL ATPase cycle. Notably, the symmetric complex of GroEL(D52A/D398A) was extremely stable, with a half-time of ~150 h (~6 days), providing a good model to characterize the football-shaped complex.

The Escherichia coli chaperonin GroEL is a double-ring chaperone that assists protein folding with the aid of GroES and ATP. Asp-398 in GroEL is known as one of the critical residues on ATP hydrolysis because GroEL(D398A) mutant is deficient in ATP hydrolysis (<2% of the wild type) but not in ATP binding. In the archaeal Group II chaperonin, another aspartate residue, Asp-52 in the corresponding E. coli GroEL, in addition to Asp-398 is also important for ATP hydrolysis. We investigated the role of Asp-52 in GroEL and found that ATPase activity of GroEL(D52A) and GroEL(D52A/D398A) mutants were ~20% and < 0.01% of wild-type GroEL, respectively, indicating that Asp-52 in E. coli GroEL is also involved in the ATP hydrolysis. GroEL(D52A/D398A) formed a symmetric football-shaped GroEL-GroES complex in the presence of ATP, again confirming the importance of the symmetric complex during the GroEL ATPase cycle. Notably, the symmetric complex of GroEL(D52A/D398A) was extremely stable, with a half-time of ~150 h (~6 days), providing a good model to characterize the football-shaped complex.

Chaperonins are a subclass of double-ring chaperones capable of assisting protein folding in an ATP-dependent manner (1–4). They have been divided into two structurally distinct classes. Group I chaperonins, such as GroEL from Escherichia coli, consist of 14 identical subunits and require a dome-shaped GroES 1:1 “bullet” shaped and symmetric 1:2 “football” GroEL-GroES complex (14–20). The GroEL-GroES complex is involved in substrate binding and an intermediate domain with a hinge region connecting the equatorial and the apical domain (5–7).

GroEL is the best characterized chaperonin (1, 2, 4, 8–10); it is found in the cytoplasm of E. coli and is essential for cell viability at all temperatures (11). GroEL and GroES assist a wide spectrum of substrates in the cell (12, 13). GroEL is made of two heptameric rings that stack back-to-back with dyad symmetry (5). ATP binding to the GroEL rings induces the positive cooperative upward movement of the intermediate and apical domains, leading to the formation of a GroEL ring that binds GroES (the cis-ring), which has a cavity for the encapsulation of the substrate protein (6). The double ring GroEL permits the formation of two types of GroEL-GroES complexes: asymmetric 1:1 “bullet” shaped and symmetric 1:2 “football” GroEL-GroES₂ complexes (14–20). The GroEL-GroES complex decays with a lifetime of ~10 s, which is governed by ATP hydrolysis in the GroEL ring (e.g. 21–23). Multiple rounds of the GroEL-GroES cycle are required for the productive folding of stringent substrate proteins (e.g. 24, 25).

To investigate elementary steps in the GroEL cycle, characterizations of the key intermediate species would be necessary. To this end, the ATP hydrolysis-defective mutant GroEL(D398A), in which Asp-398 in the intermediate domain was replaced with Ala (GroEL_D398A), has been used to investigate the roles of binding and hydrolysis of ATP in the folding cycle (e.g. 26, 27). Asp-398, which is located in the intermediate domain of the unliganded GroEL, is moved into the equatorial nucleotide-binding site to position just distal to the β-phosphate of ADP (6, 26) (Fig. 1). GroEL_D398A is deficient in ATP hydrolysis (~2% of the wild type) but not in ATP binding and thus forms a long-lived ATP-bound GroEL-GroES complex (half-time, ~30 min) (26, 27) that contributes to elucidate the mechanism of GroEL.

In addition to the well-known Asp-398 in GroEL, Asp-52 of the equatorial domain has been proposed to function as a general base for the ATP hydrolysis (Fig. 1) (28, 29). In the crystal
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**FIGURE 1. Comparison of nucleotide binding site of a GroEL subunit and a thermosome α subunit.** A, the structure around the nucleotide binding site of the GroEL subunit in the cs-ring from GroEL-GroES-ADP-AlF₃ complex (PDB code 1SVT (29)). Equatorial, intermediate, and apical domains are colored blue, green, and pink, respectively. B, the structure around the nucleotide binding site of the thermosome α subunit structure from thermosome-ADP-AlF₃ complex (PDB code 1A6E (7)). Side chains of Asp-52/Asp-398 in A and Asp-63/Asp-390 in B are drawn as stick models in orange. ADP and AlF₃ are colored red and yellow as stick models, and the Mg²⁺ is green spheres.

The structure of the thermosome, the archaeal group II chaperonin from *Thermoplasma acidophilum*, binding of the transition state analog Mg-ADP-AlF₃, the water molecule at the apex of the trigonal bipyramid is fixed by hydrogen bonds to Asp-63 (Asp-52 in GroEL) of the equatorial domain and Asp-390 (Asp-398 in GroEL) of the intermediate domain. The importance of Asp-52 for ATPase activity in archaeal chaperonins has already been shown (30). The analogous residues Asp-63 (thermosome) and Asp-52 (GroEL) adopt nearly identical orientations as well (Fig. 1). However, so far the importance of Asp-52 in GroEL has not been examined.

In this study we investigated the contribution of Asp-52 in GroEL to chaperonin function and found that Asp-52 in combination with Asp-398 plays a critical role for ATP hydrolysis. GroEL(D52A/D398A) double mutant (GroEL52/398) forms an extremely stable symmetric GroEL/GroES complex in which the half-life is ~6 days, providing a good model to characterize the symmetric football-shaped complex.

**EXPERIMENTAL PROCEDURES**

**Reagents and Proteins**—Hexokinase was purchased from Sigma. Porcine malate dehydrogenase (MDH), ATP, and ADP were obtained from Roche Applied Science. The trace amount of contaminating ATP in the ADP solution was eliminated by hexokinase/glucose treatment (31). Cy3-NHS (Fluorolink Cy3 monofunctional dye) was obtained from Amersham Biosciences. The following proteins were purified and prepared as previously described: GroEL, GroES, and bovine mitochondrial rhodanese (32) and Cy3-labeled GroES (GroESCy₃) (22).

**Strains and Plasmids**—*E. coli* XL2-Blue (Stratagene) was used for site-directed mutagenesis and cloning. *E. coli* GroEL mutants were generated using QuikChange site-directed mutagenesis (Stratagene).

**ATPase Assay**—The release of ADP from GroEL was measured spectrophotometrically with an ATP-regenerating system, as described (20, 33). The assay mixture contained 0.2 mM NADH, 5 mM phosphoenolpyruvate, 100 μg/ml pyruvate kinase, 10 μg/ml lactate dehydrogenase, 5 mM dithiothreitol (DTT), and 0.2 μM GroEL in HKM buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, and 5 mM MgCl₂). Where indicated, 0.8 μM GroES and 20 μM lactalbumin reduced with 5 mM DTT for at least 1 h (reduced lactalbumin as a model substrate protein) were included. The reaction was initiated by the injection of ATP (final concentration, 1 mM) into the vigorously stirred solution. The decreases in the absorbance at 340 nm due to NADH oxidation were monitored continuously with a spectrophotometer (V-550, Jasco, Japan).

**Folding Assays**—For measurements of MDH folding, MDH was denatured in 6 M urea and 1 mM DTT for 1 h and diluted in HKM buffer containing 0.2 μM GroEL, 0.6 μM GroES, 5 mM DTT, and 1 mM ATP. The final MDH subunit concentration was 0.1 μM. At the indicated times a 25-μl aliquot was injected into 1.2 ml of the assay solution containing 0.5 mM oxalacetic acid, 0.2 mM NADH, 1 mM DTT, and 0.1 mg/ml bovine serum albumin. The rate of NADH oxidation at 25 °C was monitored at 340 nm. Rhodanese was denatured in 6 M guanidine HCl and 1 mM DTT for 1 h and diluted 40-fold into HKM buffer containing 1 μM GroEL, 2 μM GroES, 20 mM Na₂S₂O₃, and 1 mM DTT. The final rhodanese concentration was 0.5 μM. ATP was then added to a final concentration of 4 mM. At the indicated times 5-μl aliquots were added to 750 μl of a solution containing 100 mM KH₂PO₄, 150 mM Na₂S₂O₃, and 1 mM EDTA. The recovery of rhodanese activity was measured colorimetrically by the absorbance at 460 nm (34), indicating the formation of a complex between ferric ions and the thiocyanate reaction product.

**SDS-PAGE Analysis of GroEL-GroES-Substrate Ternary Complexes**—GroEL-GroES-substrate ternary complexes were prepared as described previously (27). To initiate the GroEL
ATPase cycle, the solution containing GroEL398 (or GroEL52/398), which was saturated with rhodanese, and GroES in HKM buffer was mixed with a 2-fold volume of the solution containing ATP and then incubated at 25 °C. The final concentrations of the components in the reaction mixtures were 1 mM ATP, 0.5 μM GroEL398, or GroEL52/398; saturated with rhodanese, 1.0 μM GroES, 200 mM glucose, 1 mM DTT, and 20 mM Na2S2O3. For the single turnover ATP hydrolysis experiment, the excess ATP was hydrolyzed to ADP by adding hexokinase (final concentration, 0.04 units/μl) to the reaction mixture at 3 s after the initiation of the reaction. To determine the amounts of GroES and rhodanese bound to GroEL398 or GroEL52/398, the released GroES and rhodanese were removed by ultrafiltration (Microcon YM-100, Millipore). The retained solution was analyzed by 13% SDS-PAGE. The intensity of the band staining was quantified using the ImageJ program and was calibrated using known protein concentrations.

**Electron Microscopy**—The solution containing 1 mM ATP, 5 mM DTT, 0.5 μM GroEL52/398, and 1.5 μM GroES in HKM buffer was subjected to ultrafiltration (Microcon YM-100) to remove free GroES. An aliquot of the solution was applied on an electron microscope specimen grid covered with a carbon support film. The specimen was immediately stained with 2.0% uranyl acetate and observed with an electron microscope (JEM-1230, JEOL) at an accelerating voltage of 100 kV. Images were recorded onto a CCD camera (FastScan-T114T, TVIPS) at a direct magnification of ×50,000.

**GroES and Nucleotide Binding Assay Using Gel Filtration**—The ATP football complexes of GroEL52/398 were formed by mixing 3 μM GroEL52/398 with 6 μM GroES in 20 mM HEPES-KOH, pH 7.4, 1 mM DTT, 1 mM ATP, 50 mM KCl, and 5 mM MgCl2 to initiate the ATP hydrolysis reaction (t = 0). After an incubation for 5 min at 25 °C, the complexes were isolated by gel filtration using three TSK-gel guard columns (Tosoh, Tokyo, Japan) in a buffer containing 25 mM HEPES-KOH, pH 7.0, 100 mM Na2SO4, and 5 mM MgSO4. The binding capacity of the complexes or the unliganded GroEL52/398 to GroES was then examined by mixing 0.3 μM complexes or the unliganded GroEL52/398 with 0.6 μM GroES-Cy3 in 1 mM ATP at the indicated times after the initiation of ATP hydrolysis. After a 5-min incubation at 25 °C, the samples were analyzed with a gel filtration HPLC column (G3000SWXL, Tosoh). Aliquots were loaded onto the column, which was equilibrated with a buffer containing 20 mM HEPES-KOH, pH 7.4, 10 mM KCl, 5 mM MgCl2, and 100 mM Na2SO4. The flow rate was 0.5 ml/min, and the elution profile was monitored for the Cy3 fluorescence by an in-line fluorometer (excitation at 550 nm, emission at 570 nm).

**Quantification of Bound Nucleotides**—To analyze the bound nucleotides in the GroEL52/398-GroES complex, we mixed 3 μM GroEL398 with 6 μM GroES in 20 mM HEPES-KOH, pH 7.4, 1 mM DTT, 1 mM ATP, 50 mM KCl, and 5 mM MgCl2. After 5 min, aliquots were rapidly subjected to gel filtration using three TSK-gel guard columns (Tosoh), connected in series, and equilibrated with a buffer containing 25 mM HEPES-KOH, pH 7.0, 100 mM Na2SO4, and 5 mM MgSO4. The isolated GroEL52/398 complexes were treated with perchloric acid (final, 1.0%), and the supernatant was neutralized with K2CO3. The supernatants were applied to a reverse-phase HPLC column (ODS-80Ts, Tosoh) for the separation of ATP and ADP while monitoring the absorbance at 260 nm. The amounts of nucleotides were calculated by the integrated peak areas and were calibrated using known nucleotide concentrations.

**RESULTS**

**Novel GroEL Residue That Is Critical for ATP Hydrolysis**—According to the proposal that Asp-52 in GroEL might affect ATP hydrolysis activity (28), we constructed and purified a GroEL mutant in which Asp-52 was replaced with Ala (GroEL52). GroEL52 was defective in ATP turnover and exhibited ~20% of the wild-type GroEL (GroELWT) activity (Fig. 2), indicating that Asp-52 in GroEL is important for ATP hydrolysis. The reduction of the ATPase activity of GroEL52 was not as drastic as compared with that of GroEL398, which has ~2% of GroELWT activity. We then constructed a double mutant in which both Asp-52 and Asp-398 were replaced with Ala (GroEL52/398). Under conventional steady state conditions, only a background level of ATPase activity was observed in GroEL52/398, which was lower than that of GroEL398 (Fig. 2).

The chaperone activity of GroEL52/398 was tested using two stringent substrate proteins, rhodanese and MDH (27, 35). For rhodanese, all mutants, GroEL52, GroEL398, and GroEL52/398, retained the same chaperone activity as GroELWT (Fig. 3A). We further confirmed that the kinetics of GroEL-assisted rhodanese folding were essentially the same for all GroEL variants (Fig. 3B). These results suggest that the ATP-mediated ability of GroEL52 and GroEL52/398 to encapsulate the substrate protein was normal.

On the other hand, the yields of folded MDH after 60 min differed among the mutants (Fig. 3A). Although GroEL52 mediated efficient folding of MDH, the recovered MDH activity was ~10% in GroEL52/398, which is less than that in GroEL398 (Fig. 3A). Because MDH is a homodimeric enzyme, the recovery of the enzyme activity requires an assembly process after the
release from the GroEL-GroES complex. This result suggested that GroEL52/398 did not release the MDH subunit even after 60 min.

**ATP-dependent Formation of Symmetric GroEL-GroES Complex Using GroEL52/398**—We next characterized the complex between GroEL52/398 and GroES upon ATP binding. The isolation of a GroEL52/398-GroES complex encapsulating rhodanese by an ultrafiltration method revealed that the complex held rhodanese even after 120 min (Fig. 4), confirming again that the GroEL52/398-GroES complex is more stable than GroEL398.

Quantification of GroES based on the band densities revealed that 1.7–2.2 mol of GroES heptamer/(mol of GroEL52/398 tetradecamer) was retained in the isolated GroEL52/398-GroES complex, suggesting the formation of the symmetric GroEL-GroES complex using GroEL52/398 (Fig. 4, lanes 4–6).

**Electron Micrograph of the Symmetric GroEL-GroES Complex**—We observed the images of the GroEL52/398-GroES complexes under an electron micrograph. The images showed a football-shaped complex in which GroES associated to both ends of the GroEL molecules (Fig. 5), confirming the formation of symmetric complex using GroEL52/398.

**Persistence of the Symmetric Complex for a Week Using GroEL52/398**—To determine how long the symmetric complex produced from GroEL52/398 persists, we analyzed the complex by gel filtration and found that the symmetric complex is extremely stable, and full release of GroES required ~12 days (Fig. 6A). The time course of the ATP hydrolysis by GroEL52/398 in the isolated GroEL52/398-GroES complex was quantitated (Fig. 6B) (27). Extrapolation of the bound ATP to 0 h yielded a value of around 14 mol of ATP per GroEL52/398 tetradecamer (Fig. 6B), confirming again that GroEL52/398 and GroES formed a symmetrical football complex containing ATP. The time course of the ATP hydrolysis was very slow; half-time of ATP hydrolysis was around ~150 h (Fig. 6B), which corresponds to more than ~60,000-fold of that of wild-type GroEL. Also, the kinetics of ATP hydrolysis well correlated with the increased rebinding of GroES, as assessed by the binding of Cy3-labeled GroES (GroESCy3) to the symmetric complex (20, 22, 27). These results suggested that GroEL52/398 releases GroES from the ATP football complex upon ATP hydrolysis, leading to the exchange of GroES, and the ternary complex remained in the ATP-bound form with a half-life of ~150 h (~6 days).
DISCUSSION

The existence of the symmetric football GroEL-GroES complex was well recognized even in the early stages of GroEL studies (e.g. Refs. 14–17). However, its transient nature as an intermediate during the ATPase cycle of GroEL has made it difficult not only to characterize the importance of the complex in the GroEL function but also to prepare the homogeneous population for detailed structural analyses. In this report we identified a novel GroEL residue, Asp-52, that is critical for the ATP hydrolysis of GroEL.

Asp-398 in the intermediate domain is known to be involved in ATP hydrolysis, and the GroEL398 mutant has contributed significantly toward the elucidation of the mechanism of GroEL (e.g. 21, 26). In addition to the well known GroEL398, we demonstrated that Asp-52 participates in the ATP hydrolysis, thus providing evidence supporting a previous proposal that Asp-52 is one of the catalytic residues that traps the water molecule attacking the γ-phosphate of ATP (28).

Although the ATPase deficiency of the single mutation of Asp-52 to Ala (GroEL52) is not drastic as compared with that of GroEL398, the double mutant GroEL52/398 has significantly reduced ATP hydrolysis activity, even less than GroEL398, indicating that both Asp-398 and Asp-52 are involved in the ATP hydrolysis. Because it was previously shown that the corresponding two aspartic acid residues are critical for the ATPase activities of the group II chaperonins (Fig. 1B) (7, 30, 36), the catalytic mechanism using two aspartic acids would be conserved among all chaperonin groups.

The extremely slow ATP hydrolysis activity of GroEL52/398 enables the formation of stable symmetric GroEL-GroES complex (football-shaped complex). Because the introduction of D52A mutation is novel in group I chaperonins, we must evaluate whether the symmetric complex using GroEL52/398 is the mimic of the real intermediate during the functional GroEL cycle. The yields and kinetics of rhodanese folding assisted by GroEL52/398 were indistinguishable from those by GroELWT or GroEL398 (Fig. 3). In addition, the symmetric complex formed by GroEL52/398 normally released GroES after the ATP hydrolysis (Fig. 6). Taken together, we concluded that GroEL52/398 mimics the functional intermediate of the GroEL cycle.

The formation of the symmetric complex requires ATP-bound GroEL rings. In this respect it is of interest to consider nonhydrolyzable ATP analogs such as ATPγS or AMPPNP to prepare the stable symmetric complex using wild-type GroEL. Indeed, it has been shown that those ATP analogs can be used to form the symmetric complex (16). We also tested the formation of the symmetric complex using the ATP analogs. ATPγS was relatively efficient to form the symmetric complex (~1.7
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GroES heptamer/GroELWT tetradecamer, whereas AMPPNP was not effective to form the symmetric complex (data not shown). Importantly, Mendoza et al. (37) showed that ATPyS or AMPPNP were unable to assist the GroE-dependent folding of rhodanese. Taken together, ATP analogs such as ATPyS or AMPPNP are not perfect ATP analogs to mimic the functional intermediate of wild-type GroEL cycle, as already suggested before (26). Rather, we note that the symmetric complex using ATP and fluoroberyllate (ATP-BeFx) (20) would be almost the same character with the symmetric complex using GroEL.\(^{52/398}\).

We would like to emphasize that the half-life of the football complex with GroEL\(^{52/398}\) is extremely long. The week-long persistence of the football structure facilitated its crystallization. In fact, we have determined a crystal structure of symmetric football complex with GroEL\(^{52/398}\) (Ref. 38; PDB code 3WVL). Briefly, the overall structure of the football complex resembled the GroES-bound GroEL-ring of the asymmetric 1:1 GroEL-GroES complex (the bullet complex). However, the two GroES-bound GroEL rings form a modified interface by an \(\sim 7^\circ\) rotation about the 7-fold axis (38). As a consequence of the rotation, the inter-ring contacts between the two GroEL rings in the football complex differed from those in the bullet complex. The differences provide a structural basis for the apparently impaired inter-ring negative cooperativity that permits the formation of the symmetric complex (38).

In summary, because GroEL\(^{52/398}\) is only defective in the ATP hydrolysis and the function to encapsulate the substrate proteins is normal, the long-lived football complex using GroEL\(^{52/398}\) will pave the way for characterizations of the chaperonin encapsulating the substrate protein by other methods, such as NMR.

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