ATP-, K⁺-dependent Heptamer Exchange Reaction Produces Hybrids between GroEL and Chaperonin from *Thermus thermophilus*

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Hideki Taguchi, Kei Amada, Noriyuki Murai, Mitsuko Yamakoshi, and Masasuke Yoshida

From the Research Laboratory of Resources Utilization, R-1, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan

Chaperonin from *Thermus thermophilus* (Tcpn60₁₄, Tcpn10₀) splits at the plane between two Tcpn6₀, rings into two parts in a solution containing ATP and K⁺ (Ishii, N., Taguchi, H., Sasabe, H., and Yoshida, M. (1995) *FEBS Lett.* 362, 121–125). When *Escherichia coli* GroEL₁₄ was additionally included in the solution described above, hybrid chaperonins GroEL₁₄-Tcpn6₀, and GroEL₁₄-Tcpn6₀-Tcpn1₀₀, were formed rapidly (<20 s) at 37 °C. The hybrid was also formed from Tcpn6₀₁₄ and GroEL₁₄ but not from a mutant GroEL₁₄ lacking ATPase activity. The hybrid formation was saturated at ~300 μM ATP and ~300 mM K⁺. These results imply that GroEL₁₄ also splits and undergoes a heptamer exchange reaction with *Thermus* chaperonin under nearly physiological conditions. Similar to parent chaperonins, the isolated hybrid chaperonins exhibited ATPase activity that was susceptible to inhibition by Tcpn1₀₀ or GroES and mediated folding of other proteins. Once formed, the hybrid chaperonins were stable, and the parent chaperonins were not regenerated from the isolated hybrids under the same conditions in which the hybrids had been formed. Only under conditions in which GroEL in the hybrids was selectively destroyed, such as incubation at 70 °C, *Thermus* chaperonin, but not GroEL₁₄, was regenerated from the hybrid. Therefore, the split reaction may not be an obligatory event repeated in each turnover of the chaperonin functional cycles but an event that occurs only when chaperonin is first exposed to ATP/K⁺.

GroEL₁₄ is an *Escherichia coli* chaperonin that facilitates the proper folding of proteins in an ATP-dependent manner (1–3). As determined by x-ray crystallography, GroEL₁₄ seems to be a hollow cylinder of 14 identical 57-kDa subunits consisting of 2 heptamer rings stacked back-to-back with a dyad symmetry (GroEL₁₄-GroEL₁₄) (4). GroEL₁₄ functionally cooperates with co-chaperonin GroES₁₇ (5), a single heptameric ring of 10-kDa subunits that binds one end, or two ends in some cases, of the GroEL₁₄ cylinder (6–8). From a thermophilic bacterium, *Thermus* thermophilus, GroEL homolog Tcpn6₀₁₄ (Thermus chaperonin 60) is purified as a complex with GroES homolog Tcpn1₀. The complex, termed *Thermus* holo-chaperonin (T.holo-cpn), is composed of two heptamer rings of Tcpn6₀ and a single ring of Tcpn1₀₀ (9–12). In contrast to chaperonins from *E. coli* and *T. thermophilus*, several members of the chaperonins, including those from *Thermoanaerobacter brockii* (13), (14) and mitochondria (15, 16), are purified as a single heptamer ring. In addition, purified chaperonin from *Paracoccus denitrificans* is a mixture of a large number of tetradecamers and a small number of heptamers (10). The physiological meaning of such a divergence in the quaternary structure of chaperonin has not been understood.

Recently, we found that when T.holo-cpn is incubated with ATP and K⁺, it splits into two parts at the equator plane between the two rings of Tcpn6₀, producing cone-shaped particles (Tcpn6₀₁₄-Tcpn1₀₀) and ring-shaped particles probably corresponding to Tcpn6₀ (17). Then we observed that the products of the split reaction can reassociate to form T.holo-cpn under the appropriate conditions (18). In contrast to the split reaction, Todd et al. (14) reported that the single-ring *T. brockii* cpn6₀₁₄ dimerizes to form a double-ring structure in the presence of *T. brockii* cpn1₀₀ and ATP. These results raise the question of whether GroEL₁₄ also undergoes the tetradecamer-heptamer transition.

Here, we report that when GroEL₁₄ and T.holo-cpn are incubated with ATP and KCl, hybrid chaperonins such as GroEL₁₄-Tcpn6₀₁₄-Tcpn1₀₀ are formed as a result of the heptamer exchange reaction. This suggests that ATP/K⁺-dependent transient dissociation of a tetradecamer into heptamers occurs not in *Thermus* chaperonin but also in GroEL.

**EXPERIMENTAL PROCEDURES**

Proteins and Materials—Isopropylmalate dehydrogenase (IPMDH) from *T. thermophilus* strain HB8 was a kind gift from Dr. T. Oshima and his colleagues (Tokyo University of Pharmacy and Life Science, Hachioji, Japan) (19). (2R*,3S*)-3-Isopropylmalic acid, a substrate of IPMDH, was purchased from Wako Pure Chemical Corp. *T. holo-cpn* was purified as described previously (9, 20). Tcpn6₀₁₄ expressed in *E. coli* was purified using modifications of procedures described previously (12). The lysate of *E. coli* cells containing expressed Tcpn6₀₁₄ was heated at 70 °C for 20 min. The supernatant containing Tcpn6₀₁₄ was recovered by centrifugation and applied to a hydrophobic interaction chromatography (Butyl-Toyopearl). The fractions containing Tcpn6₀₁₄ were pooled and concentrated by ammonium sulfate precipitation. The concentrated protein solution was applied to a gel permeation HPLC column (Tosoh) equilibrated with 25 mM Tris-HCl, pH 7.0, 100 mM Na₂SO₄, and 20% (v/v) methanol. The fractions containing Tcpn6₀₁₄ were further purified by a DEAE-5PW HPLC column. Tcpn1₀₀, expressed in *E. coli* was purified as described previously (12). GroEL₁₄ and GroES₁₇ were purified as described previously (21) from *isopropylmalate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; cpn, chaperonin.*
the lysate of E. coli cells carrying the multicopy plasmid pACYC 184 carrying groES-groEL genes, which was a kind gift from Dr. K. Ito (Kyoto University, Kyoto, Japan) (22). A mutant GroELAEX14 (C138S, C45SS, S519S, D85C, and K327C) was purified as described previously (21). Purified chaperonins were stored as a suspension in 65% ammonium sulfate at 4 °C.

Formation of Hybrid Chaperonin—T.holo-cpn or Tcpn60, (5 μg) was mixed (final volume, 10 μl) with GroEL14 (5 μg) and incubated at 37 °C for 10 min in Buffer A (25 mM Tris-HCl, pH 7.5, 300 mM KCl, and 5 mM MgCl2) containing 1 mM ATP, unless otherwise indicated. The sample solutions were applied to non-denaturing polyacrylamide gel electrophoresis (6% acrylamide), and electrophoresis was continued for about double the duration of the period required for the leading dye (bromphenol blue) to reach the front of the gel. The protein bands were stained by Coomassie Brilliant Blue. Only the regions of the chaperonin protein bands are shown in the figures.

Isolation of Hybrid Chaperonin—The ammonium sulfate precipitate of the mixture of parent and hybrid chaperonins was solubilized in a minimum volume of Buffer B (25 mM Tris-HCl, pH 7.5, and 5 mM MgCl2) and applied to a Sephadex G-25 (Pharmacia) column to remove the excessive salts. The eluted protein solution was applied to a DEAE-5PW (Tosoh) column equilibrated with Buffer B and eluted with a 0–1.0 M NaCl gradient at 1 ml/min. The chromatography was monitored by absorbance at 280 nm.

ATPase Assay—ATPase activities were assayed by measuring the amount of produced inorganic phosphate (23). Typically, the reaction was started by the addition of ATP (final concentration, 1 mM) to Buffer A containing 0.88 μM GroEL14 or Tcpn60, and, when indicated, 1.3 μM GroES, or Tcpn10. The assay solution was preincubated for 10 min at 37 °C before the addition of ATP. The reactions were terminated by the addition of perchloric acid after incubations at 37 °C for 5, 10, 15, and 20 min. The solution was treated with a malachite green reagent, and the absorbance at 630 nm was measured. One unit of activity is defined as the activity that hydrolyzes 1 μmol of ATP/min.

Chaperonin-promoted IPMDH Folding—IPMDH (16.2 μM) denatured in 6.4 M guanidine HCl was diluted 25-fold at 37 °C in Buffer A containing the components indicated in the figure legends. After a 20-min incubation at 37 °C, an aliquot was withdrawn, and the reactivated IPMDH activity was determined as described previously (9).

Other Methods—Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (24). Proteins were analyzed by polyacrylamide gel electrophoresis either on 10% polyacrylamide gels without SDS (native PAGE) (25). To obtain higher resolution on the native PAGE, electrophoresis was continued for about double the duration of the period required for the leading dye (bromphenol blue) to reach the front of the gel. Gels were stained by Coomassie Brilliant Blue R-250.

RESULTS

Formation of Hybrid Chaperonin from GroEL14 and Thermus Chaperonin—After we found the T.holo-cpn split (17), we attempted to detect the heptameric state of GroEL under various conditions but had no success. If GroEL14 splits only transiently in its ATPase cycle, we could not detect the heptamer GroEL by the usual methods. Then we used Thermus chaperonin as a trap for GroEL, that is, we incubated GroEL14 with ATP/K+ in the presence of T.holo-cpn and examined whether the hybrid chaperonin containing GroEL and Tcpn10 was formed. We took advantage of the different electrophoretic mobility of GroEL14 and T.holo-cpn in native PAGE (Fig. 1A, lanes 1–5) to identify the hybrid. As shown in Fig. 1A, lanes 5, two closely moving bands appeared between T.holo-cpn and GroEL14 when they were incubated with ATP/K+. The NH2-terminal amino acid sequences of the two bands confirmed that the upper band contained GroEL8, Tcpn60, and Tcpn10 and that the lower band contained GroEL and Tcpn60 (data not shown). Yields of phenylthiohydantoin derivatives from GroEL, Tcpn60, and Tcpn10 (upper band) were generally close to each other, indicating that the upper and lower bands corresponded to GroEL7, Tcpn60, Tcpn10, and GroEL7, Tcpn60, respectively.

As described later, an analysis of isolated hybrid chaperonins supported the structures described above. For these hybrids to be formed, heptamer exchange reactions should occur, that is, both T.holo-cpn and GroEL14 should split into heptamers that then rebind each other with a random

![Fig. 1. Hybrid formation between GroEL14 and Thermus holo-chaperonin.](image)

A, T.holo-cpn (5 μg) and GroEL14 (5 μg) were mixed (final volume, 10 μl) and incubated at 37 °C for 10 min in Buffer A containing 1 mM ATP. Modified conditions are indicated in the row other condition in the figure. Final concentrations of AMP-PNP, ADP, NaCl, and trans-1,2-diaminocyclohexanetetraacetic acid were 1, 1, 300, and 10 mM, respectively, when addition is indicated. KCl was omitted in lane 10. The sample solutions were applied to native PAGE (6% acrylamide). A component of each protein band is schematically illustrated on the right side of the figure. Shaded and white parts are those derived from T.holo-cpn and GroEL14, respectively. B, ATP concentration dependence on the formation of hybrid chaperonin. The indicated concentrations of ATP were used. The final concentration of KCl was 300 mM. C, K+ dependence. Formation of hybrid chaperonins. The indicated concentrations of KCl were used. The final concentration of ATP was 1 mM. D, the effect of excessive co-chaperonins on the formation of hybrid chaperonin. The amounts of Tcpn10 (lane 2) and GroES (lane 3) added to the mixture before ATP addition were 2 μg each. E, the rapid formation of hybrid chaperonins. Reactions were terminated at 20 s, 1 min, and 10 min by the addition of 10 mM trans-1,2-diaminocyclohexanetetraacetic acid (final concentration), and the solutions were analyzed as described above.
combination into tetradecamers. Hereafter, we term the hybrid chaperonins as follows: GroEL<sub>EL-14</sub>-Tcpn60<sub>EL-60</sub>-Tcpn10<sub>EL-60</sub>. Hybrid (EL-60–10) and GroEL<sub>EL-14</sub>-Tcpn60<sub>EL-60</sub>, Hybrid (EL-60).<sup>3</sup>

The conditions required for the formation of hybrid chaperonins were the same as those required for the split reaction of T.holo-cpn (17); formation was absolutely dependent on ATP and K<sup>+</sup>, and other combinations such as adenosine 5′-β, γ-imino) triphosphate + K<sup>+</sup> (Fig. 1A, lane 7), adenosine 5′-O-(thio-triphosphate) + K<sup>+</sup> (data not shown), ADP + K<sup>+</sup> (lane 8), and ATP + Na<sup>+</sup> (lane 10) were not effective in generating hybrids. When free Mg<sup>2+</sup> was removed by trans-1,2-diaminocyclohexanetetraacetic acid, no hybrid was formed (lane 9). The relatively high concentrations of ATP and K<sup>+</sup> were necessary. The hybrid formation was half-maximal at ~50 μM ATP and ~50 mM K<sup>+</sup> and saturated at ~300 μM ATP and ~300 mM K<sup>+</sup> (Fig. 1, B and C). The addition of excess Tcpn10<sub>z</sub> to the reaction mixture resulted in an increased yield of hybrid chaperonins with a simultaneous decrease of T.holo-cpn (Fig. 1D, lane 2), but the addition of GroES<sub>z</sub> had only little, if any (lane 3), effect. The hybrids were formed rapidly. In 1 min (Fig. 1E, lane 5), hybrids with an amount similar to that formed in 10 min (lane 6) were detected, and a significant amount of hybrids was formed even in 20 s (lane 4). That is as fast as a single ATPase turnover in the GroEL catalytic cycle (6).

The ATPase activity of *Thermus* chaperonin at 37 °C, the temperature at which hybrid formation was observed, is very low (see Fig. 5); hydrolysis of a single ATP molecule by Tcpn60<sub>14</sub> takes ~15 s. Nevertheless, hybrid chaperonin was formed within 20 s. This result means that only the hydrolysis of a single ATP by Tcpn60<sub>14</sub> is sufficient to form the hybrid chaperonins. This rapid formation of hybrid might be related to the observation that the formation of a tetradecamer from *T. brockii* cpn60<sub>z</sub> is also very rapid, occurring before all the cpn60 subunits could hydrolyze ATP (14). Although the real reason why the hybrid was formed in such a short period is not known, one of the possible explanations is that the initial single turnover by one cpn60 in the tetradecamer might induce a quaternary structural change in the double ring of cpn60, in the presence of a high concentration of K<sup>+</sup>.

**Cpn10 Is Not Required for Hybrid Formation**—To know whether cpn10 is required for hybrid formation or not, we used Tcpn60<sub>14</sub> instead of T.holo-cpn as one of the parent chaperonins. Tcpn60<sub>14</sub> was isolated from recombinant *E. coli* (12), and we confirmed that Tcpn60<sub>14</sub> also split in the presence of ATP/K<sup>+</sup>.<sup>4</sup> The hybrid chaperonin was formed between Tcpn60<sub>14</sub> and GroEL<sub>14</sub> in the presence of ATP/K<sup>+</sup> (Fig. 2, lane 6). Unlike the experiment using T.holo-cpn, only a single band appeared between the parent chaperonins. As expected, this band was indeed Hybrid (EL-60), because NH<sub>2</sub>-terminal amino acid sequencing showed that the band contained an almost equal amount of Tcpn60 and GroEL. It is likely that in the experiments described in Fig. 1, Hybrid (EL-60) was formed at first, and Hybrid (EL-60–10) was generated next by attaching Tcpn10<sub>z</sub> to Hybrid (EL-60).

**ATP Hydrolysis by GroEL<sub>14</sub> Is Essential for Hybrid Formation**—In spite of the fact that the condition required for hybrid formation as described above was the same condition required for the split reaction of *Thermus* chaperonin (17, 18), there was no direct evidence of a requirement for ATP hydrolysis by Tcpn10<sub>z</sub>. We used Tcpn60<sub>14</sub> instead of T.holo-cpn as one of the parent chaperonin subunits to form hybrid chaperonin. Although the real reason why the hybrid was formed in such a short period is not known, one of the possible explanations is that the initial single turnover by one cpn60 in the tetradecamer might induce a quaternary structural change in the double ring of cpn60, in the presence of a high concentration of K<sup>+</sup>.

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3 The term Hybrid (EL-60–10) does not necessarily mean that Tcpn10<sub>z</sub> is attached to the Tcpn60<sub>z</sub> ring in the hybrid. Although several lines of preliminary results, such as the protease sensitivity of each ring in the hybrid and the relatively weak affinity of Tcpn10<sub>z</sub> to GroEL<sub>14</sub>, suggest that Tcpn10<sub>z</sub> is at the Tcpn60<sub>z</sub> ring side, we cannot exclude the possibility that some Tcpn10<sub>z</sub> is at the GroEL<sub>z</sub> ring side.

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**FIG. 2. Hybrid formation between GroEL<sub>14</sub> and Tcpn60<sub>EL-60</sub>.** Conditions were the same as described in the legend to Fig. 1, except that Tcpn60<sub>14</sub> was used instead of T.holo-cpn in lanes 3–6. When added, the ATP concentration was 1 mM.

GroEL<sub>14</sub> for hybrid formation. To address the question, we used a GroEL mutant called GroELAEX instead of the wild-type GroEL as a parent chaperonin (21). GroELAEX is a mutant in which apical and equatorial domains in the same GroEL subunit can be cross-linked in a reversible manner (apical-equatorial cross (X)-link) (21). In the presence of a reducing reagent, GroELAEX<sub>14</sub> retains normal functional activity as a chaperonin. In contrast, oxidized GroELAEX<sub>14</sub>, which is locked in a “closed” conformation by an interdomain disulfide bond, can bind but not hydrolyze ATP (21). Therefore, the requirement for ATP hydrolysis of GroEL<sub>14</sub> in the hybrid formation would be tested in the presence or absence of a reducing reagent. Note that the *Thermus* chaperonins have no cysteine residue (12). Just like wild-type GroEL<sub>14</sub>, the hybrid chaperonin was formed from GroELAEX<sub>14</sub> and Tcpn60<sub>14</sub> in an ATP/K<sup>+</sup>-dependent manner under reducing conditions (Fig. 3, lane 6). However, the hybrid was not formed under the oxidizing condition (lane 2), whereas the ATPase activity of GroELAEX<sub>14</sub> was completely blocked (21). Wild-type GroEL<sub>14</sub> was able to form the hybrid with Tcpn60<sub>14</sub>, irrespective of reducing or oxidizing conditions (lanes 4 and 8). The inability of oxidized GroELAEX<sub>14</sub> to form hybrid chaperonin indicates that ATP binding is not sufficient for hybrid formation and that the occurrence of ATP hydrolysis on GroEL<sub>14</sub> is essential for hybrid formation.

**Isolation and Characterization of Hybrid Chaperonins**—The hybrid between GroEL<sub>14</sub> and *Thermus* chaperonins was separated from the parent chaperonins with anion-exchange HPLC (Fig. 4, A and B). The hybrid chaperonin fraction contained Hybrid (EL-60–10) and Hybrid (EL-60) (see Fig. 7A, lane 5). In a similar manner, Hybrid (EL-60) was also purified (see Fig. 7A, lane 4). The relative staining intensities of the GroEL band and the Tcpn60 band in SDS-PAGE (Fig. 4, inset, lanes 1 and 2) were almost the same, again confirming that the hybrid chaperonins consisted of equal molar amounts of each chaperonin subunit. The molecular sizes of the isolated hybrid chaperonins were the same or very close to those of the parent chaperonins, because they were eluted from a gel-permeation HPLC column at the same retention time as that of GroEL<sub>14</sub> (data not shown).

When hybrid chaperonins formed from GroEL<sub>14</sub> and T.holo-cpn were examined by electron micrograph, two kinds of particles, GroEL<sub>14</sub>-like rectangular particles and bullet-shaped particles similar to T.holo-cpn, were observed (data not shown). Hybrid (EL-60) hydrolyzed ATP at 0.09 unit/mg<sup>−1</sup> at 37 °C (Fig. 5). Because T.holo-cpn and Tcpn60<sub>EL-60</sub> hydrolyzed ATP very slowly

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<sup>3</sup> Unpublished observations.

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Incubated for 10 min at 37 °C in Buffer A in the absence or presence (confirmed separately in 15% SDS-PAGE (data not shown).

2 Hybrid (EL-60), exhibited ATPase activity at 0.08 unit/mg.

GroEL14 and Tcpn107 inhibited the ATPase activity of GroEL14 (35–40% inhibition). The ATPase activity of the hybrid chaperonins was also inhibited by GroES7 and Tcpn10, (55–60% inhibition for Hybrid (EL-60), and ~30% inhibition for the mixture of Hybrid (EL-60–10) and Hybrid (EL-60)).

Chaperone Activity of Isolated Hybrid Chaperonins—We examined the effect of hybrid chaperonins on the folding of IP-MDH from T. thermophilus under a condition in which the yield of spontaneous folding was only ~10% (Fig. 6). The following experiments were carried out in the presence of ATP. Under this condition, GroEL14 alone hardly promoted reactivation (less than 10% reactivation of IPMDH activity), and GroES7 was required for effective GroEL-promoted folding. Tcpn10 was as effective as GroES7 in this GroEL-promoted folding assay. In contrast to GroEL14, Tcpn6014 alone was able to promote folding of IPMDH (~35%). Further addition of Tcpn10 increased the yield of reactivation about twice, whereas the effect of GroES7 was only marginal. Similar to GroEL14, Hybrid (EL-60) alone had almost no effect on folding (less than 10%), and the addition of GroES7 or Tcpn10 was required for effective folding (80–90%). In the case of the mixture of Hybrid (EL-60–10) and Hybrid (EL-60), the folding of IPMDH was promoted moderately (~35%) even in the absence of GroES7, or Tcpn10, probably due to the endogenous presence of Tcpn10. The inclusion of GroES7 or Tcpn10 in the solution caused additional promotion of folding (50–60%). Thus, it is clear that both Hybrid (EL-60) and Hybrid (EL-60–10) are active in promoting protein folding.

Parent Chaperonins Were Not Regenerated from Isolated Hybrid Chaperonins—The isolated hybrid chaperonins were very stable. After storage at 4 °C for 3 weeks, about 90% of the hybrid chaperonins were still in the hybrid forms (data not shown). To investigate whether the two heptamer rings of hybrid chaperonins could reexchange each other in the presence of ATP/K1, we incubated the hybrid chaperonins with ATP/K1 and analyzed them by native PAGE. As shown in Fig. 7B, regeneration of the parent chaperonins, GroEL14 and Tcpn60 (or Tcpn6014), was not observed, irrespective of whether Hybrid (EL-60) or Hybrid (EL-60–10) was used as a starting hybrid chaperonin. Further addition of either GroES7 or Tcpn10 did not change the result (data not shown). This result was unexpected, because if the hybrid chaperonin split in the presence of ATP/K1, as observed for Thermus chaperonin, parent chaperonins should be regenerated more or less as a result of random reassociation of heptamers. Then we examined the effects of heat (70 °C for 10 min) or proteinase K treatment on the stability of the hybrid chaperonins. As shown in Fig. 7C and D, Thermus chaperonin was resistant to both treatments under the conditions tested, whereas GroEL was destroyed. After the isolated hybrid chaperonins were incu-
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**DISCUSSION**

**Hybrids Are Formed as a Result of Heptamer Exchange**—In this report, we demonstrated ATP/K⁺-dependent formation of the hybrid between GroEL₁₄ and Thermus chaperonin. This is a result of the heptamer exchange reaction between both chaperonins. One can argue the possibility that the hybrids are made up from three stacked heptamers (GroEL₁₄ Tcpn6₀₁₄ T.holo-cpn, and hybrid chaperonins) was 1.3 μm each (as an oligomer). The column labeled spontaneous represents the reaction without chaperonin. After dilution, ATP was added to the solution (final concentration, 1 mM) to initiate the folding reaction. When added, the concentration of co-chaperonins (GroES, and Tcpn₁₀₁₀) was 1.7 μm each (as an oligomer). After a 20-min incubation in the presence of ATP, recovered IPMDH activity was measured. An activity of the same amount of native IPMDH was taken as 100%.

The concentration of chaperonins (GroEL₁₄ Tcpn6₀₁₄ T.holo-cpn, and hybrid chaperonins) was 1.3 μm each (as an oligomer). The column labeled spontaneous represents the reaction without chaperonin. After dilution, ATP was added to the solution (final concentration, 1 mM) to initiate the folding reaction. When added, the concentration of co-chaperonins (GroES, and Tcpn₁₀₁₀) was 1.7 μm each (as an oligomer). After a 20-min incubation in the presence of ATP, recovered IPMDH activity was measured. An activity of the same amount of native IPMDH was taken as 100%.

**FIG. 6.** The effect of hybrid chaperonins on the folding of IPMDH. IPMDH denatured in 6.4 M guanidine HCl was diluted 25-fold to 0.65 μM at 37 °C by injecting Buffer A containing the indicated components. The concentration of chaperonins (GroEL₁₄ Tcpn6₀₁₄ T.holo-cpn, and hybrid chaperonins) was 1.3 μm each (as an oligomer). The column labeled spontaneous represents the reaction without chaperonin. After dilution, ATP was added to the solution (final concentration, 1 mM) to initiate the folding reaction. When added, the concentration of co-chaperonins (GroES, and Tcpn₁₀₁₀) was 1.7 μm each (as an oligomer). After a 20-min incubation in the presence of ATP, recovered IPMDH activity was measured. An activity of the same amount of native IPMDH was taken as 100%.

**FIG. 7.** The stability of hybrid chaperonins under various conditions. A and B, isolated hybrid chaperonins (4 μg of each) were incubated at 37 °C for 10 min in Buffer A in the absence (A) or presence (B) of 1 mM ATP. C, isolated hybrid chaperonins (4 μg of each) were incubated at 70 °C for 10 min in Buffer A. D, isolated hybrid chaperonins (4 μg of each) were treated with 0.2 μg of proteinase K at 25 °C in Buffer A. After a 30-min incubation, phenylmethylsulfonyl fluoride (final concentration, 5 mM) was added to stop the proteolysis. Sample solutions were analyzed with 6% native PAGE as described in the legend to Fig. 1.
should be formed as a result of a heptamer exchange reaction. Two explanations are possible: (a) split reaction occurs only once before the chaperonin starts the first turnover of the functional cycle; or (b) split reaction occurs in each of the reaction cycles, but reassociation always happens to heterologous combinations (GroEL-Tcpn607) rather than homologous combinations (GroEL-GroEL or Tcpn607-Tcpn607), thus producing the hybrid again. However, the latter possibility is unlikely, if not impossible, because the parent GroEL14 is not regenerable from the hybrid GroEL7-MR1-GroEL7 in the presence of ATP/K+ (27), and it is not easy to assume that wild-type GroEL7 has a much higher affinity to mutant MR1-GroEL7 than it does to wild-type GroEL7.

A requirement for high concentrations of ATP and K+ is also contradictory to the notion that the split is one of obligatory steps in the chaperonin functional cycle. Steady-state ATPase activity of GroEL14 is inversely dependent on K+; it is saturated at ~5 mM K+ in the presence of 50 μM ATP and at ~300 mM K+ in the presence of 2 μM ATP (26, 30). On the contrary, hybrids were formed only when concentrations of both K+ and ATP were high, and the yield of hybrids was saturated at ~300 mM K+ and ~300 μM ATP (Fig. 1, C and D). If either one of the concentrations was reduced, the yield of hybrids decreased, and no hybrid formation was observed at 5 mM K+/1 mM ATP or at 300 mM K+/2 μM ATP. Therefore, the requirement of K+ for hybrid formation is a different phenomenon than the K+ requirement for steady-state ATPase activity. Although the occurrence of the heptamer exchange reaction has been established, understanding of its functional and physiological significance awaits further study.

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