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Synchronized Domain-opening Motion of GroEL Is Essential for Communication between the Two Rings*

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Escherichia coli **chaperonin GroEL consists of two stacked rings of seven identical subunits each. Accompanying binding of ATP and GroES to one ring of GroEL, that ring undergoes a large *en bloc* domain movement, in which the apical domain twists upward and outward. A mutant $GroEL_{AEX}$ (C138S,C458S,C519S,D83C,K327C) in the oxidized form is locked in a closed conformation by an interdomain disulfide cross-link and cannot hydrolyze ATP (Murai, N., Makino, Y., and Yoshida, M. (1996) *J. Biol. Chem.* 271, 28229–28234). By reconstitution of GroEL complex from subunits of both wild-type GroEL and oxidized $GroEL_{AEX}$, hybrid GroEL complexes containing various numbers of oxidized $GroEL_{AEX}$ subunits were prepared. ATPase activity of the hybrid GroEL containing one or two oxidized $GroEL_{AEX}$ subunits per ring was about 70% higher than that of wild-type GroEL. Based on the detailed analysis of the ATPase activity, we concluded that inter-ring negative cooperativity was lost in the hybrid GroEL, indicating that synchronized opening of the subunits in one ring is necessary for the negative cooperativity. Indeed, hybrid GroEL complex reconstituted from subunits of wild-type and GroEL mutant (D398A), which is ATPase-deficient but can undergo domain opening motion, retained the negative cooperativity of ATPase. In contrast, the ability of GroEL to assist protein folding was impaired by the presence of a single oxidized $GroEL_{AEX}$ subunit in a ring. Taken together, cooperative conformational transitions in GroEL rings ensure the functional communication between the two rings of GroEL.**

The *Escherichia coli* chaperonin GroEL binds to non-native proteins and facilitates their folding. GroEL is assisted by the cofactor GroES and uses the energy of ATP hydrolysis (1, 2). GroEL consists of fourteen identical 58-kDa subunits, which are arranged in two heptameric rings stacked upon one another in a back to back manner, forming a large central cavity (3–5). Each subunit of GroEL has a site for ATP hydrolysis (4), and the ATPase cycle controls binding and release of substrate protein, as well as GroES (6–8). ATP (or ADP) induces a massive upward movement of the GroEL apical domains (5, 9), which contain the binding sites for substrate proteins and for GroES (10). Horovitz and coworkers (11) have proposed a nested model describing the mixed cooperativity in ATP hydrolysis by GroEL, positive cooperativity within the same rings and negative cooperativity between the two rings. In the model, each GroEL ring is in equilibrium between two allosteric states, a tense (T) state with low affinity for ATP and a relaxed (R) state with high affinity for ATP. Binding of ATP occurs with strong positive cooperativity to one of the rings (12–15), converting the T state to the R state (11). The allosteric transitions of GroEL are most likely to correspond to the upward movement of the apical domains (16). A second level of cooperativity undergoes sequential transitions from the TT state *via* the TR state to the RR state (11). This negative cooperativity between two rings prevents ATP binding to the second ring (16).

We previously reported a mutant GroEL ($GroEL_{AEX}$; C138S,C458S,C519S,D83C,K327C)[1] in which apical and equatorial domains can be cross-linked in a reversible manner (apical-equatorial cross (X)-link) (17). Under reducing conditions, $GroEL_{AEX}$ is fully active as a chaperonin. However, under oxidative conditions, a disulfide cross-link is formed between two introduced cysteines, locking $GroEL_{AEX}$ in a closed conformation, which can bind ATP and polypeptide but is unable to hydrolyze bound ATP, release bound polypeptide, or bind GroES (17). Here, to investigate the role of each subunit in the tetradecameric GroEL complex, we prepared intersubunit hybrid complexes containing various copies of the oxidized $GroEL_{AEX}$ monomers by reconstituting the denatured GroEL monomers. Remarkably, the hybrid GroEL complex containing one or two disulfide cross-linked $GroEL_{AEX}$ monomers per ring showed larger ATPase activity at saturating ATP concentration than wild-type GroEL ($GroEL_{WT}$). This is likely caused by removal of the negative cooperativity between rings. In contrast, hybrid between $GroEL_{WT}$ and $GroEL_{D398A}$, which is ATPase-deficient but can undergo domain-opening motion, retained negative cooperativity of the ATPase activity. The coordinate closed to open conformational transition might be essential for the cooperative nature of GroEL.

EXPERIMENTAL PROCEDURES

Materials—$GroEL_{WT}$ and $GroEL_{AEX}$ were purified from *E. coli* strain BL21(DE3) bearing the plasmids pET-EL (18) and pT7AEX (17), respectively, as described previously. The single-stranded DNA of the plasmid pET-EL was obtained by infecting *E. coli* CJ236 cells with

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[1] The abbreviations used are: $GroEL_{AEX}$, GroEL mutant (C138S, C458S,C519S,D83C,K327C); $GroEL_{WT}$, wild-type GroEL; $GroEL_{D398A}$, GroEL mutant (D398A); $Hybrid_{WT\text{-}AEX}$, intersubunit hybrid GroEL tetradecamer between $GroEL_{WT}$ and $GroEL_{AEX}$; $Hybrid_{WT\text{-}398}$, intersubunit hybrid GroEL tetradecamer between $GroEL_{WT}$ and $GroEL_{D398A}$; IP-MDH, isopropylmalate dehydrogenase from *T. thermophilus*; PAGE, polyacrylamide gel electrophoresis; ATPγS, adenosine 5′-*O*-(thiotriphosphate);$GroEL_{D398A/D490C}$, GroEL mutant (D398A,D490C).

helper phage M13KO7 (Amersham Pharmacia Biotech). Mutant GroEL, $GroEL_{D398A}$, and $GroEL_{D398A/D490C}$ were generated by site-directed mutagenesis using the Kunkel method. $GroEL_{D398A}$ and $GroEL_{D398A/D490C}$ were purified through the similar protocol described for $GroEL_{WT}$ (18). Purified $GroEL_{WT}$ and its mutants were stored as a suspension in 65% ammonium sulfate at 4 °C until use. Isopropylmalate dehydrogenase (IPMDH) from *Thermus thermophilus* strain HB8 was kind gift from Dr. T. Oshima (Tokyo University of Pharmacy and Life Science, Hachioji, Japan). (2R*,3S*)-3-Isopropylmalic acid, a substrate of IPMDH, was purchased from Wako Chemical Co. (Osaka, Japan). All other chemicals were the highest grade commercially available.

Preparation of Hybrid GroEL Tetradecamers—The mixture of $GroEL_{WT}$ tetradecamer and mutant GroEL tetradecamer in various ratios (total 10 mg/ml protein concentration) was denatured by addition of urea (final concentration 8 M) at 25 °C for 90 min. Reconstitution to tetradecamers was accomplished by modified protocol described in Ref 19. The urea-denatured GroEL was diluted 15-fold into buffer containing 50 mM Tris-HCl (pH 7.5) followed by addition of 10 mM $MgCl_2$, 5 mM ATP, and 0.6 M ammonium sulfate. Reconstitution was allowed to continue for 30 min at 25 °C. The reconstitution yield of the tetradecamer from the GroEL mutant subunits ($GroEL_{AEX}$ and $GroEL_{D398A}$) was as efficient as that of $GroEL_{WT}$, reaching to 90% (data not shown). The reconstituted GroEL was purified by using a gel filtration high pressure liquid chromatography column ($G3000SW_{XL}$; Tosoh) equilibrated with 25 mM Tris-HCl (pH 6.8) and 100 mM Na_2SO_4. The fractions containing the tetradecamer were collected and concentrated with ultrafiltration membrane (Ultrafree 10-kDa cut-off; Millipore). As we could distinguish the $GroEL_{AEX}$ complex from $GroEL_{WT}$ complex in native PAGE, random incorporation of $GroEL_{AEX}$ subunits into the hybrid complex was also confirmed by native PAGE. Random incorporation of $GroEL_{D398A}$ subunits into the corresponding hybrid complex was confirmed as follows. We replaced Asp-490, which is located on the outer surface of GroEL, to Cys, in addition to the D398A mutation ($GroEL_{D398A/D490C}$). The chemical modification of this surface-exposed Cys could invest the complex with a different electrophoretic mobility. The D490C mutation itself has no additional effect on the property of the $GroEL_{D398A}$ complex. After the isolation of the hybrid complex formed between $GroEL_{D398A/D490C}$ and $GroEL_{WT}$, the introduced Cys was chemically modified with (2-bromoethyl)trimethylammonium. Finally we could confirm the formation of the various hybrid complexes by native PAGE analysis (data not shown).

ATPase Assay—ATPase activity was assayed using malachite green to measure the amount of produced inorganic phosphate (20). The assay solution was preincubated for 10 min at 25 °C and then reaction was started by the addition of ATP (final concentration, 2 mM) to the assay solution containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 0.5 μM GroEL, unless otherwise stated. Assay solutions were incubated at 25 °C, and the reaction was terminated at several time points by addition of perchloric acid. The mixture was then reacted with a malachite green reagent, and absorbance at 630 nm was measured. One unit of activity is defined as the activity that hydrolyzes 1 μmol of ATP/min. Data were fit to the equation for the nested allosteric model developed by Horovitz and coworkers (11).

Folding Assay—IPMDH from *T. thermophilus* (0.33 mg/ml) was denatured in 6.4 M guanidine HCl and diluted 50-fold into the dilution buffer (100 mM potassium phosphate, pH 7.8, 1 mM $MgCl_2$) containing, when indicated, 2.0 mM ATP and 0.19 μM GroEL. The dilution buffer was preincubated for 10 min at 37 °C prior to addition of denatured IPMDH. The mixtures were incubated 60 min at 37 °C, and an aliquot was injected into the assay solution, which contained 100 mM potassium phosphate, pH 7.8, 1 mM $MgCl_2$, 1.0 M KCl, 0.9 mM NAD^+, 0.4 mM 3-isopropylmalic acid. The increasing rate of absorbance at 340 nm was monitored at 60 °C, and the activity of recovered IPMDH was normalized relative to that of IPMDH recovered by $GroEL_{WT}$.

Other Methods—Proteins were analyzed by PAGE on a polyacrylamide gel (13% (w/v) in the presence of 0.1% (w/v) SDS or 6% (w/v) without SDS). 2 μg of proteins were loaded on each lane of the gels, and the reducing reagent was always omitted from the sample solutions, the running buffer, and gels. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Protein concentrations were assayed by the Bradford method with bovine serum albumin as a standard (21). Throughout this study, the concentrations of GroEL are expressed as tetradecamers.

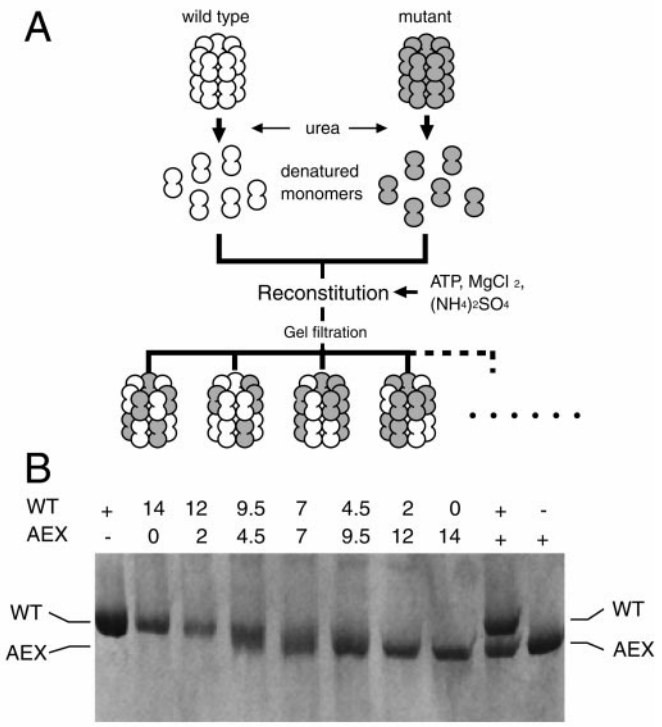

FIG. 1. **Preparation of intersubunit hybrid between $GroEL_{WT}$ and $GroEL_{AEX}$.** *A*, schematic illustration of procedures to prepare the hybrid GroEL complexes. Urea-denatured GroEL, wild-type and mutant, was mixed at various ratios and then diluted into the buffer containing ATP, $MgCl_2$, and ammonium sulfate. After the incubation at 25 °C for 30 min, the reconstituted hybrid GroEL complexes were purified by gel filtration high pressure liquid chromatography. *B*, native PAGE analysis of the isolated $Hybrid_{WT\text{-}AEX}$. Isolated $Hybrid_{WT\text{-}AEX}$ prepared in indicated molar ratios were analyzed by native PAGE. ±, +/+, and −/+ indicate the marker for the parent GroEL complexes, GroEL wild-type (*WT*), and oxidized $GroEL_{AEX}$ (*AEX*).

RESULTS AND DISCUSSION

Preparation of the Hybrid Complex between Wild-type GroEL and Oxidized $GroEL_{AEX}$—To prepare the intersubunit hybrid complexes between $GroEL_{WT}$ and oxidized $GroEL_{AEX}$, we reconstituted GroEL tetradecamers from the urea-denatured monomers in the presence of ATP and ammonium sulfate (19) with various ratios of $GroEL_{AEX}$ to $GroEL_{WT}$ (Fig. 1*A*). Regardless of the contents of $GroEL_{AEX}$, the reconstitution was very efficient, and more than 90% of the GroEL subunits were incorporated into the complex. Tetradecameric structure of the reconstituted hybrids was confirmed by gel filtration high pressure liquid chromatography (data not shown). After isolation of the reconstituted hybrid complex (termed $Hybrid_{WT\text{-}AEX}$) by gel filtration, the formation of the hybrid complex was confirmed with native PAGE (Fig. 1*B*). In native PAGE, the oxidized $GroEL_{AEX}$ tetradecamer migrated faster than $GroEL_{WT}$ (Fig. 1*B*). The $Hybrid_{WT\text{-}AEX}$ appeared as smeared bands between them, representing a mixture of GroEL hybrids containing various ratios of oxidized $GroEL_{AEX}$ and $GroEL_{WT}$. This indicated that the formation of the hybrids occurred randomly in each of the reassembled hybrid GroEL molecules in the solution. These isolated hybrid GroEL tetradecamers did not reassemble to the parent GroEL after storage at 4 °C for 1 week.

ATPase Activity of the Hybrids between $GroEL_{WT}$ and Oxidized $GroEL_{AEX}$—Oxidized $GroEL_{AEX}$ can bind ATP but cannot hydrolyze it (17), so that ATPase activities of the mixture of parent GroEL tetradecamers, *i.e.* oxidized $GroEL_{AEX}$ and $GroEL_{WT}$, are proportional to the contents of $GroEL_{WT}$ (Fig. 2*A*). In contrast, ATPase activities of the reconstituted $Hybrid_{WT\text{-}AEX}$ with various amounts of $GroEL_{AEX}$ present were not propor-

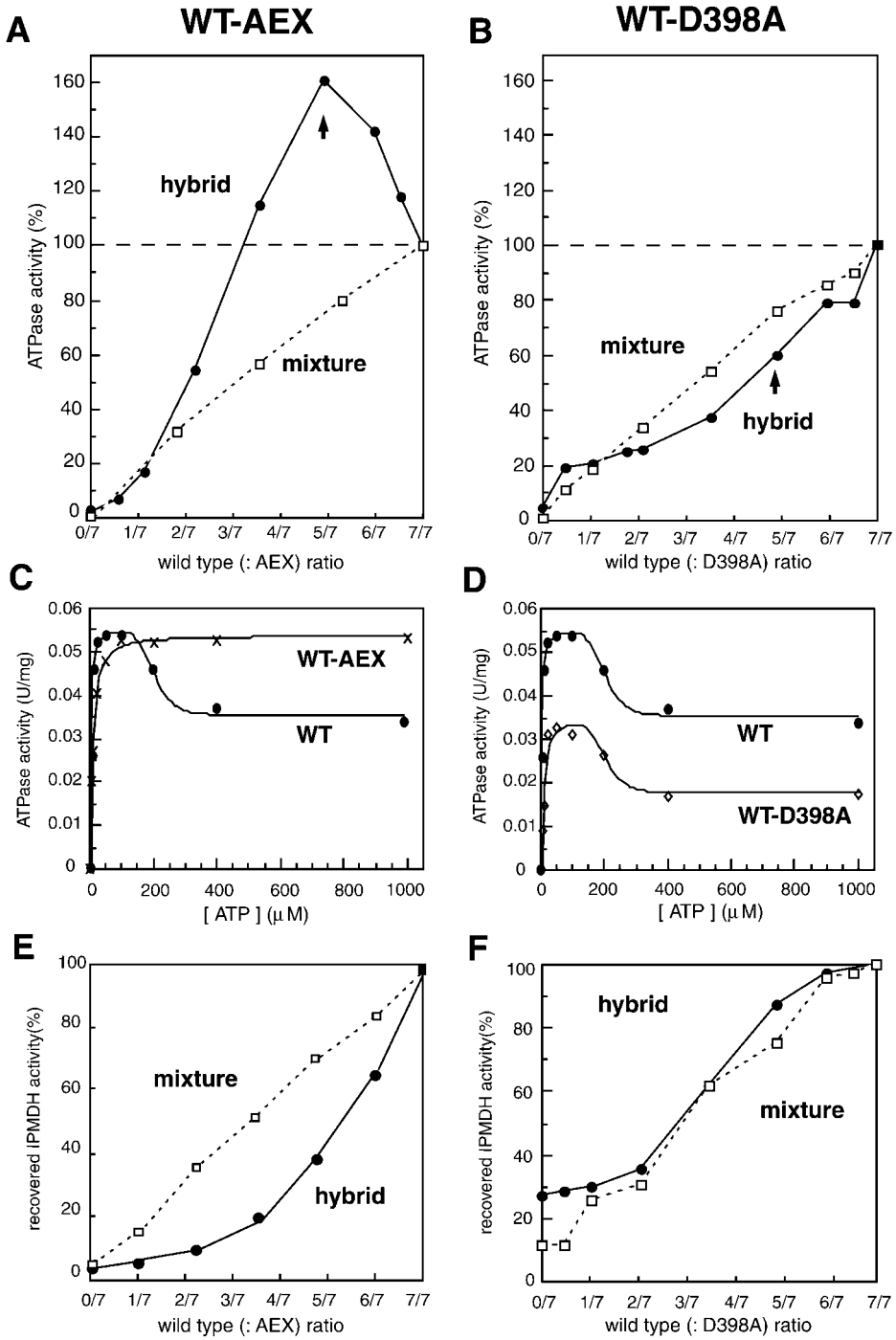

FIG. 2. **Properties of hybrid GroEL, the Hybrid$_{WT-AEX}$, and the Hybrid$_{WT-398}$.** The *left three panels* (*A*, *C*, and *E*) contain the Hybrid$_{WT-AEX}$; *right three panels* (*B*, *D*, and *F*) contain the Hybrid$_{WT-398}$. *A* and *B*, ATPase activities of hybrid GroEL complexes in various ratios. *mixture* indicates the minimal mixtures of parent GroEL complexes in the indicated ratios. Assays were carried out at 25 °C for 10 min. Activity of GroEL$_{WT}$, 0.036 μmol/mg/min, was set as 100%. *Arrows* indicate the activities of 5:2 (wild-type:mutant) hybrid GroEL. *C* and *D*, effect of ATP concentration on the ATP hydrolysis by the hybrid GroEL. 5:2 hybrid GroEL (80 nM), indicated by *arrows* in *A* and *B*, was subjected to the ATPase assay for 3 min. As a control, results obtained from wild-type GroEL (*WT*) are also shown. Data were fit to the equation for the nested allosteric model (11). *E* and *F*, effect of hybrid GroEL tetradecamers on folding of IPMDH. IPMDH denatured in 6.4 M guanidine HCl was diluted 50-fold into the buffer containing the hybrid GroEL tetradecamers at 37 °C and then followed by the addition of ATP. After a 60-min incubation, recovered IPMDH activity was measured. The extent of recovered IPMDH activity was expressed by the activity recovered by GroEL$_{WT}$. Under these conditions, ~70% of denatured IPMDH spontaneously, *i.e.* without GroEL and ATP, folds.

tional to the amounts of oxidized GroEL$_{AEX}$. They showed almost proportional activity until the ratio of WT:AEX was 1:6 but showed higher activity than the proportional one when the ratio of WT:AEX was between ~2:5 and 6.5:0.5. The ATPase activity of the hybrid complex was at the maximum when the ratio of WT:AEX was 5:2. This result was unexpected, because it has been believed that ATP hydrolysis by GroEL is highly cooperative within each GroEL ring (11, 12), predicting that incorporation of a single inactive subunit into the ring may be sufficient to abolish the entire ATPase activity.

Dependence of ATPase Activity on ATP Concentration—The ATPase activity of GroEL$_{WT}$ exhibits an apparent substrate inhibition, which has been explained by negative cooperativity between two rings (11). As the ATP concentration increases, ATPase activity of GroEL$_{WT}$ also increases, reaching the maximum activity at 0.1 mM ATP and then decreasing to about 65% of the maximum value (Fig. 2*C*, *closed circles*). However, ATPase activities of the 5:2 Hybrid$_{WT-AEX}$, which has 1.6-fold higher ATPase activity than that of GroEL$_{WT}$, did not show the substrate inhibition (Fig. 2*C*, *crosses*). The inhibition of ATPase at ATP concentrations above 0.1 mM ATP is caused by the negative cooperativity between rings; at low ATP concentrations, one of two rings of GroEL is involved in ATP hydrolysis at a given moment, but at concentrations >0.1 mM ATP, the other ring also binds ATP, resulting in inhibition of ATP hydrolysis of the first ring. According to this scenario, Hybrid$_{WT-AEX}$ is likely impaired in communication between rings because of the presence of the GroEL$_{AEX}$ subunits, which are locked in closed conformation.

Hybrid GroEL between GroEL$_{D398A}$ and GroEL$_{WT}$—It is

likely that the presence of one or two closed GroEL subunits in the same ring prevents the suppressive interaction between rings, and uninhibited ATPase activity occurs in the wild-type subunits. Regarding this hypothesis, another ATPase-deficient mutant, $GroEL_{D398A}$, was used to investigate the role of conformational change in ATPase activity of hybrid GroEL. $GroEL_{D398A}$ is mostly deficient in the ATPase activity because of the lack of carboxyl group required for the ATP hydrolysis but can undergo domain-opening motion (7). Hybrid GroEL composed of $GroEL_{D398A}$ and $GroEL_{WT}$ (termed $Hybrid_{WT\text{-}398}$) were prepared in a similar procedure as that described for the $Hybrid_{WT\text{-}AEX}$. Random incorporation of the GroEL subunits into the $Hybrid_{WT\text{-}398}$ was confirmed by native-PAGE (data not shown; see "Experimental Procedures"). ATPase activity of the $Hybrid_{WT\text{-}398}$ was nearly proportional to the content of $GroEL_{WT}$ (Fig. 2*B*), indicating that the incorporated ATPase-deficient $GroEL_{D398A}$ subunit did not interfere the ATP hydrolysis of competent $GroEL_{WT}$ subunits. This result is in contrast to that of the $Hybrid_{WT\text{-}AEX}$, suggesting that the $Hybrid_{WT\text{-}398}$ retained negative cooperativity of ATPase function. Indeed, dependence of ATPase activity of the 5:2 $Hybrid_{WT\text{-}398}$ on ATP concentration showed substrate inhibition derived from the negative cooperativity of ATPase even though the specific activity of the complex was lower than that of $GroEL_{WT}$ tetradecamer (Fig. 2*D*).

Chaperone Activity of Hybrid GroEL—Chaperone activities of the hybrid GroEL complexes were examined using guanidine HCl-denatured IPMDH (22), which has no cysteine (23). All of the GroEL complexes used, *i.e.* $GroEL_{WT}$, $GroEL_{AEX}$, $GroEL_{D398A}$, $Hybrid_{WT\text{-}AEX}$, and $Hybrid_{WT\text{-}398}$, were able to bind denatured IPMDH and arrest the spontaneous folding of IPMDH (data not shown). When ATP was added, $GroEL_{WT}$ released the arrested IPMDH into the medium where IPMDH accomplished folding, and enzymatic activity was recovered. The yield of recovered IPMDH thus measured for $Hybrid_{WT\text{-}AEX}$ and mixtures of parent GroEL tetradecamers are shown in Fig. 2*E*. In contrast with the ATPase activity, chaperone activity was severely inhibited by the presence of the oxidized $GroEL_{AEX}$ subunits in the hybrid complex independent from the number of $GroEL_{AEX}$. This result indicates that incorporation of oxidized $GroEL_{AEX}$ subunit of the heptameric ring is sufficient to inhibit the chaperone activity. Although the suppression of the chaperone activity was not complete, the result clearly shows that the negative cooperativity between two rings transmitted by conformational changes of the subunits is important for the ability to release the non-native proteins.

In contrast to the $Hybrid_{WT\text{-}AEX}$, the chaperone activity of the $Hybrid_{WT\text{-}398}$ was similar to that of mixture of parent GroEL tetradecamers, indicating that there is no additional inhibitory effect of $GroEL_{D398A}$ subunits in the $Hybrid_{WT\text{-}398}$ (Fig. 2*F*).

Importance of Cooperative Conformational Transition in GroEL Rings—The most compelling result in this report is that the incorporation of even one or two cross-linked $GroEL_{AEX}$ mutants into the GroEL ring results in the stimulation in the ATPase activity, but it suppresses the chaperone activity. Dependence of the ATPase activity of the $Hybrid_{WT\text{-}AEX}$ on ATP concentration reveals that there is no apparent substrate inhibition, which can be explained by a disruption of the inter-ring negative cooperativity with respect to ATP. This indicates that the double rings of the $Hybrid_{WT\text{-}AEX}$ forms the RR state upon ATP binding. Disruption of the negative cooperativity has been also observed in the GroEL (R13G,A126V) double mutant (24), which was used for determination of the GroEL crystal structure (3, 4). Notably, the crystal structure with bound nucleotides has revealed that the double mutant unusually bound 14 ATPγS per tetradecamer (4), suggesting that a symmetric RR state was achieved upon ATP binding (24). The structural basis for the disruption of the negative cooperativity in the double mutant is not clear. In contrast, disruption of the negative cooperativity in the $Hybrid_{WT\text{-}AEX}$ is because of its inability to undergo the required conformational change in the incorporated $GroEL_{AEX}$ subunits. On the other hand, the hybrid GroEL complex containing $GroEL_{D398A}$, which can undergo domain-opening motion (7), retained the negative cooperativity like wild-type GroEL. We therefore conclude that the negative cooperativity between rings requires a coordination of cooperative conformational transition within rings.

Asymmetric behavior of GroEL double rings upon ATP binding is very important in the chaperone function. Indeed, alternation of active rings during the ATPase cycle is essential for the productive GroEL-promoted folding (6–8). It is plausible that release of substrate protein was inhibited in the $Hybrid_{WT\text{-}AEX}$, because the hybrid GroEL tetradecamer remains symmetric upon ATP binding. Taken together, these results suggest that cooperative conformational transitions within one GroEL ring ensure proper communication between the two rings, which are necessary for maintenance of the asymmetric GroEL reaction cycle.

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