Discrimination of ATP, ADP, and AMPPNP by Chaperonin GroEL

HEXOKINASE TREATMENT REVEALED THE EXCLUSIVE ROLE OF ATP*

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The double ring chaperonin GroEL binds unfolded protein, ATP, and GroES to the same ring, generating the cis ternary complex in which folding occurs within the cavity capped by GroES (cis folding). The functional role of ATP, however, remains unclear since several reports have indicated that ADP and AMPPNP (5′-adenylyl-β,γ-imidodiphosphate) are also able to support the formation of the cis ternary complex and the cis folding. To minimize the effect of contaminated ATP and adenylate kinase, we have included hexokinase plus glucose in the reaction mixtures and obtained new results. In ADP and AMPPNP, GroES bound quickly to GroEL but bound very slowly to the GroEL loaded with unfolded rhodanese or malate dehydrogenase. ADP was unable to support the formation of cis ternary complex and cis folding. AMPPNP supported cis folding of malate dehydrogenase to some extent but not cis folding of rhodanese. In the absence of hexokinase, apparent cis folding of rhodanese and malate dehydrogenase was observed in ADP and AMPPNP. Thus, the exclusive role of ATP in generation of the cis ternary complex is now evident.

The bacterial chaperonin system consisting of GroEL and GroES facilitates folding of other proteins using the energy of ATP hydrolysis. GroEL is composed of 14 identical 57-kDa subunits, each containing a site for binding and hydrolysis of ATP. Seven GroEL subunits are arranged in a heptamer ring forming a central cavity, and two heptamer rings are stacked back to back. GroES is a dome-shaped, single heptamer ring of 10-kDa subunits. GroEL binds a wide range of unfolded proteins at the apical cavity surface and subsequently binds ATP and GroES to the same GroEL ring (the cis ring, a GroEL heptamer ring that binds to GroES), producing the complex consisting of GroEL, unfolded protein, and GroES (the cis ternary complex). Since the residues of the GroEL apical surface involved in GroES binding are mostly overlapped with substrate protein binding (1), GroES binding results in encapsulating unfolded protein into the enlarged cavity of the cis GroEL ring capped by GroES (the cis cavity) (2). The unfolded protein initiates folding in the cis cavity without a risk of aggregation (the cis folding). ATP hydrolysis in the cis ring and subsequent ATP binding to the opposite side of GroEL ring (the trans ring) induce the release of GroES, ADP, and substrate protein (whether folded or not) from the cis ring (3, 4). When unfolded protein is added to the GroEL-GroES complex, it binds to the trans ring, and its folding is arrested (2). Binding and release from the trans ring enable the folding for some proteins (2), especially large ones that are too large to be encapsulated in the cis cavity, by lowering the concentration of aggregation-prone folding intermediates in bulk solution (5). In contrast, the stringent substrate proteins for chaperonin fold efficiently by the cis folding in the presence of ATP (3, 6).

However, it should be noted that the functional significance of ATP for the cis ternary complex formation is unclear yet. It was reported that slow cis folding of rhodanese, a stringent substrate protein, is observed when GroES and ADP or AMP-PNP† were added to GroEL-unfolded rhodanese complex (2, 7, 8). The cis folding of dehydrofolate reductase in the presence of ADP was also reported (9). Malate dehydrogenase (MDH) and Rubisco were shown to form the cis ternary complex in ADP (10). However, these complexes did not promote folding (3). These observations have raised an intriguing question: what is the difference between the non-productive ADP-induced cis ternary complex and the productive ATP-induced cis ternary complex?

As reported previously (10), we have noticed that inclusion of hexokinase and glucose in the reaction mixtures diminished ADP-dependent cis folding of green fluorescent protein. Commercially prepared ADP and AMPPNP usually contain a trace amount of ATP. Also, if a trace amount of adenylate kinase is contaminated in purified protein, it can produce ATP from ADP constantly. Hexokinase would eliminate these unwanted ATPs during the reaction period. Another potential factor that can affect the results is heterogeneity of GroEL complexes in the reaction mixtures. For example, if two GroEL rings are not saturated by unfolded proteins and if GroES can bind preferably to free GroEL rings rather than the unfolded protein-loaded GroEL rings, two kinds of complexes would be generated: GroEL-unfolded protein complex without GroES or GroEL-GroES complex without unfolded protein in the cis cavity. Since these two complexes cannot be separated, the results may be taken as an evidence for the existence of the GroEL-GroES-unfolded protein ternary complex. Here, we reexamined the nucleotide requirement for the cis ternary complex by taking the precautions described above. The results showed that ADP is incompetent to generate the cis ternary complex, that is, ATP is stringent for the fast GroES binding to the unfolded protein-loaded GroEL rings and subsequent cis folding of substrate protein.

§ The abbreviations used are: AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate; AMPPNP<sub>ext</sub>, AMPPNP that is always exposed to hexokinase and glucose; AMPPNP<sub>res</sub> and ADP<sub>res</sub>, commercial AMPPNP and ADP that have not been exposed to hexokinase; ADP<sub>act</sub>, ADP that is always exposed to hexokinase and glucose; ATP<sub>act</sub>, ATP exposed to hexokinase at 3 s after initiation of the reaction, by which time only a single turnover of ATP hydrolysis of GroEL can occur; MDH, malate dehydrogenase; GroES<sub>n</sub>, GroES(T19C) labeled by 5(2-iodoacetylaminoethyl) aminonaphthalene-1-sulfonic acid; GroES<sub>l</sub>, GroES(98C) labeled by 5(2-iodoacetamidinoethyl) aminonaphthalene-1-sulfonic acid; Ap5A, dideoxinosine pentaphosphate; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

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**MATERIALS AND METHODS**

**Materials**—Single-stranded DNAs of the plasmid pET-EL and pET-ES2 were obtained by infecting *Escherichia coli* C1236 cells with helper phage RKO7 (Amersham Biosciences). Mutant GroEL-GroES(T19C) was made by Kunkel methods using an oligonucleotide 5'-CAGCAGATTG-CGATTCAACTTCTTTACG-3'. GroEL, GroES mutants, and rhodanese were expressed and purified as described (11). GroEL purified by the procedures including gel permeation column chromatography in the presence of 30% methanol (11) contained only a very small amount of contaminated proteins (<0.1 Trp residues/GroEL tetradecamer). MDH from pig heart, hexokinase, ATP, ADP, and AMPPNP were purchased from Roche Diagnostics. Dihydroxyanthracene pentaphosphate (Ap5A) was purchased from Sigma. Protein concentrations were measured by BCA protein assay (Pierce) and calibrated on the basis of quantitative amino acid analysis.

GroEL Saturated with Unfolded Proteins—Rhodanese (4 μM) or MDH (4 μM) was heat-denatured at 60 °C for 15 min in the buffer (50 mM HEPES-NaOH, pH 7.2, 1 mM EDTA, and 1 mM DTT) containing 1 μM GroEL.³ By this treatment, GroEL was not impaired at all, as shown by its 100% retention of ATPase activity and chaperone activity, whereas substrate proteins were completely inactivated. Temperature was shifted down to 25 °C, and GroEL-unfolded protein complex was isolated with gel permeation HPLC. In the latter stage of this study, we used ultrafiltration (Microcon YM-100, Millipore), which was as effective as HPLC to remove unbound substrate proteins.

**cis Folding Assays**—To initiate *cis* folding reactions, the solution containing GroEL loaded with unfolded proteins (rhodanese or MDH) and GroES was mixed with a 3-fold volume of the solution containing the nucleotide (ATP, ADP, or AMPPNP). When indicated, hexokinase was included in the nucleotide (ADP or AMPPNP) solutions. Final concentrations of the components in the reaction mixtures were 1 mM nucleotides, 0.5 μM GroEL saturated with rhodanese or MDH, 1.0 μM GroES, 200 mM glucose, 50 mM HEPES-NaOH, pH 7.2, 200 mM CH₃COOK, 10 mM Mg(CH₃COO)₂, 1 mM DTT and, when indicated, 0.04 units/μl hexokinase. In the case of rhodanese, 20 mM Na₂S₂O₃ was additionally included in the reaction mixtures. For the single turnover ATP hydrolysis experiment, excess ATP was hydrolyzed by adding heparin (final concentration, 0.04 units/μl) to the reaction mixture at 3 s after initiation of the reaction. We confirmed that 1 mM ATP in the reaction mixture was quenched completely within next 3 s. To measure the time course of the reaction, aliquots (5 μl) were taken out at indicated times and mixed with 750 μl of the solution containing 100 mM KH₂PO₄, 150 mM Na₂SO₄, and 1 mM EDTA, and recovered rhodanese activities were measured (8, 11). In the experiments to observe the effect of the pretreatment with the nucleotide solutions, the nucleotide solutions were treated with hexokinase (0.04 units/μl) for 15 min and separated from hexokinase by ultrafiltration (Microcon YM-3). When indicated, 1 mM Ap5A was included in the nucleotide solutions. The abovementioned assays were all carried out at 25 °C. To quantitate the amount of GroES and the substrate protein in the *cis* cavity, at 2 h of incubation, released GroES and substrate protein were removed by repeated (four times) dilution-ultrafiltration procedures (Microcon YM-100). Subsequently, proteinase K (final concentration, 1 μg/ml) was added to digest non-protective unfolded protein. After a 30-min incubation at 25 °C, phenylmethylsulfonyl fluoride was added to final concentration of 1 mM, and digested products smaller than 100 kDa were removed by ultrafiltration as described above. An aliquot of the resulting solution (~20 μg) was applied to 12% polyacrylamide gel electrophoresis in the presence of SDS, and Coomassie Blue R-250-stained band intensities of substrate proteins and GroES were quantitated by using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image) and calibrating with band intensities of the known amount of proteins. Additional aliquots of proteinase K-treated, ultrafiltered samples were used for measuring the recovered activities of rhodanese or MDH that corresponded to the *cis* folding. To release all the proteins from the *cis* cavity, GroES was detached from GroEL by the addition of an equal volume of 50 mM EDTA to the aliquot. After a 90-min incubation at 25 °C (in which active dimers were formed in the case of MDH), activities of rhodanese and MDH were measured.

³ Concentrations of GroES and GroEL are all expressed as heptamer and tetradecamer, respectively, in this article.

**Kinetics of GroES Binding to and Release from GroEL**—GroES, with a fluorescence wavelength at 340 nm are shown. non, in the absence of GroEL; none/ATP, GroEL-GroES₅ formed in ATP; rholo/ATP, rhodanese-loaded GroEL-GroES₅ formed in ATP; single, Spectra of GroEL-GroES₅ formed in ATP. n.a., not applicable. Experimental details are described under “Materials and Methods.” Em, emission; A.U., arbitrary units.

**FIG. 1. Fluorescence change of GroES₅ induced by binding to GroEL.** Fluorescence spectra of GroES₅, with a fluorescence wavelength at 340 nm are shown. none, in the absence of GroEL; none/ATP, GroEL-GroES₅ formed in ATP; rholo/ATP, rhodanese-loaded GroEL-GroES₅ formed in ATP; single, Spectra of GroEL-GroES₅ formed in ATP. n.a., not applicable. Experimental details are described under “Materials and Methods.” Em, emission; A.U., arbitrary units.

- Concentrations of GroES and GroEL are all expressed as heptamer and tetradecamer, respectively, in this article.
mm ADP, 20 mm glucose, 0.01 units/µl hexokinase, 1 mm DTT, and 0.05% NaN₃. The GroES₅ fluorescence eluted in the GroEL peak was measured with an on-line fluorometer at 490 nm with excitation at 340 nm.

RESULTS AND DISCUSSION

Elimination of Contaminated ATP by Hexokinase—Trace amount of ATP in ADP and AMPPNP solutions cannot be accurately measured with the usual HPLC analysis, and we applied a luciferase assay. Commercially available ADP and AMPPNP usually are contaminated with ATP, ranging 0.1–2.0%. Purification of ADP using a Dowex ion exchange column decreased the amount of ATP to 0.02–0.05%. Treatment with hexokinase plus glucose was more effective, and ATP contamination in ADP and AMPPNP was decreased down to 0.005–0.009% (10). Taking into account that purified protein could contain trace amounts of adenylate kinase that poten-
tially generate ATP (and AMP) from ADP continuously during the reaction period, we have included hexokinase plus glucose in the reaction mixtures. Hereafter, commercial AMPPNPNP and ADP that have not been treated with hexokinase are referred to as AMPPNPraw, and ADPraw, respectively. Those treated with hexokinase plus glucose are as AMPPNPHex and ADPHex, respectively. Hexokinase was also used for the single turnover ATP hydrolysis experiment (referred to as ATPsingle); hexokinase added at 3 s after initiation of the reaction hydrolyzed all free ATP so that the second chaperonin cycle was prevented. The folding yields of rhodanese and MDH in the cis cavity under the conditions of ATPsingle were 1.1 and 0.60 mol/mol of GroEL-GroES complex, respectively, which were approximately the same as those (1.2 and 0.66 mol/mol) achieved by GroEL(D398A), a mutant deficient in ATP hydrolytic activity. Therefore, it is confirmed that the single turnover reaction of chaperonin can be measured by this procedure.

Saturation of GroEL by Heat-denatured Proteins—At first, we prepared GroEL in which all the binding sites for substrate proteins were saturated with unfolded proteins. Dilution from the concentrated protein solutions containing chemical denaturants such as guanidium hydrochloride or urea into the reaction solution was avoided because of the inhibition effect of the denaturants on GroEL-GroES association (12). Instead, we simply heated the mixtures of GroEL and substrate proteins, either rhodanese or MDH, at 60 °C for 15 min. Rhodanese and MDH were denatured completely by this heat treatment. GroEL is a rather heat-stable protein and remained intact as ensured by the unaffected ATP hydrolysis activity and chaperone activity after the heat treatment (data not shown). Denatured substrate proteins occupied all the substrate protein binding sites of GroEL, and the GroEL loaded with a saturating amount of unfolded proteins (referred as the loaded GroEL) was separated from unbound substrate proteins with gel permeation HPLC or ultrafiltration. The molar stoichiometry of the bound substrate protein to GroEL estimated from band intensities in SDS-PAGE was 2.4 (rhodanese) and 2.5 (MDH). Saturation of substrate protein binding sites of GroEL by this procedure was confirmed as described later.

GroES Binding to the Free and Loaded GroEL—The fluorescently labeled GroESN increases its fluorescence upon binding to GroEL. The extent of increase is ~25%, regardless of nucleotides and the presence or absence of unfolded proteins (Fig. 1, data not shown for ADPHex and AMPPNPraw). Using the fluorescence increase of GroESN as a probe, we measured time courses of binding of GroES to GroEL. GroESN bound rapidly to unloaded GroEL in ATP, ADPHex, and AMPPNPHex with ATP being the fastest and ADPHex the second fastest (Fig. 2A). Binding rates of GroESN in ADPraw and AMPPNPraw were slightly faster than those treated with hexokinase (Table I). The binding reactions for all nucleotides are simulated by single exponential curves, and the calculated association rate constants (k on) are in the same order of magnitude, 10 7 M s 1 (Table I). The k on value of the GroESN binding to unloaded GroEL in ATP (7.5 × 10 7 M s 1) is consistent with the previously reported values 4 × 107 M s 1 (13) and 5 × 107 M s 1 (4), ensuring the validity to use GroESN for the binding experiments. These results show that ADP and AMPPNP are as effective as ATP in mediating GroES binding to free GroEL. However, these nucleotides have a very different effect on the GroESN binding to the loaded GroEL (Fig. 2, B–D and Table I). In ATP, GroESN binding to the rhodanese-loaded GroEL and MDH-loaded GroEL were slightly slower than that to free GroEL but still very rapid, completed within 1 s (k on > 10 7 M s 1) (Fig. 2B). In AMPPNPraw, the rates were slowed down by ~10-fold (Fig. 2C). In AMPPNPhex, the rates were slowed down further; GroES binding to the MDH-loaded GroEL and the rhodanese-loaded GroEL were reached only 40 and 5% after 5 min, respectively. Similarly, the binding of GroESN to

![Fig. 3. GroES binding to the partially loaded GroEL. Binding was monitored with the fluorescence change of GroESN. Binding reactions were initiated by mixing the solution containing GroESN and ADPHex with the solution containing the rhodanese-GroEL complexes with rhodanese-GroEL molar ratios 1:1, 2:1, and 2.5:1. Final concentrations of GroEL, GroESN, and ADPHex were 0.05 μM, 0.05 μM, and 1 mM, respectively. A.U., arbitrary units.](http://www.jbc.org/)

![Fig. 4. Release of GroES from the GroEL-GroES complex. Release of GroES was assessed by the loss of fluorescent GroESN from the GroEL-GroES complexes, which had been formed in ATPsingle, AMPPNPHex, or ADPHex. Twenty-fold molar excess of native GroES was added at time 0. Aliquots were loaded to gel permeation HPLC at the indicated times, and the remaining GroESN in the GroEL-GroES fraction was measured. Release of GroESN from the GroEL-GroES complex, rhodanese cis ternary complex formed in ATPsingle is also shown. rho/ATP single, rhodanese-loaded GroEL-GroES formed in ATPsingle.](http://www.jbc.org/)
the loaded GroEL was slow in ADP\textsubscript{raw} and even slower in ADP\textsubscript{hex} (Fig. 2D). Although these slow GroES binding to the loaded GroEL in ADP and AMPPNP have not been known previously, this can be expected because GroES and unfolded protein compete for the overlapping binding sites on the GroEL. Rather, a new mechanism is required to understand this rapid GroES binding in ATP to the loaded GroEL.

When the substrate binding sites of GroEL are not saturated with unfolded proteins, it is predicted that rapid GroES binding in ADP\textsubscript{hex} to free GroEL rings in partially loaded GroEL can be observed. Indeed, in ADP\textsubscript{hex}, GroES\textsubscript{x} bound rapidly to two-thirds of the 1:1 (molar ratio of rhodanese and GroEL) rhodanese-loaded GroEL and to a quarter of the 2:1 rhodanese-loaded GroEL (Fig. 3). The rapid binding was no longer observed for the 2:5:1 rhodanese-loaded GroEL, indicating that all the substrate binding sites of GroEL were occupied by unfolded rhodanese. These results showed that 2.5 rhodanese and MDH can bind to GroEL even if GroEL has only two rings for substrate protein binding. This discrepancy may be due to the error in estimation of protein concentration or the occasional binding of two substrate proteins to one GroEL ring as reported using citrate synthase as substrate protein (14).

**Dissociation of GroES from GroEL**—Dissociation of GroES from GroEL was measured by the exchange of fluorescently labeled GroES\textsubscript{x} bound on GroEL with an excess amount of unlabeled GroES. As shown in Fig. 4, the GroEL-GroES\textsubscript{x} complex formed in ATP\textsubscript{single} and ADP\textsubscript{hex} without substrate protein are surprisingly stable; only 10% of the GroEL-GroES\textsubscript{x} complexes released GroES\textsubscript{x} after 1 week. The GroEL-GroES\textsubscript{x} complex formed in ATP\textsubscript{single} becomes identical to that in ADP\textsubscript{hex} since the bound ATP in the complex is hydrolyzed to ADP. This result shows the extraordinary stability of the GroEL-GroES\textsubscript{x} complexes in ADP. The complex without substrate protein formed in AMPPNP\textsubscript{hex} was relatively unstable and decayed in 2 days. The GroEL-GroES\textsubscript{x}-rhodanese complex formed in ATP\textsubscript{single} was slightly less stable than the GroEL-GroES\textsubscript{x} complex in ATP\textsubscript{single} in the absence of unfolded protein. This instability in the presence of rhodanese may be due to the stimulation of GroES release by substrate protein binding to *trans* ring (4). Calculated dissociation rate constants ($k_{\text{off}}$) (Table I) are smaller than the previously reported value ($3.8 \times 10^{-5}$ s\textsuperscript{-1}) estimated from the dissociation of ADP moiety from the GroEL-GroES\textsubscript{x} complex (15). The reason of this discrepancy is not known, but there is a possibility that a trace amount of contaminated ATP would stimulate the exchange of bound ADP. The estimated dissociation constants of GroES binding to GroEL are extremely small ($\approx 10$ fM) and comparable with the dissociation constant of the biotin-avidin binding.

**Discrimination of ATP, ADP, and AMPPNP by Chaperonin GroEL**

![Discrimination of ATP, ADP, and AMPPNP by Chaperonin GroEL](http://www.jbc.org/Downloaded from)

**FIG. 5. Recovery of rhodanese activities assisted by GroEL and GroES in various nucleotides.** Recovery yields were expressed as mol of recovered enzyme per mol of GroEL. A, recovery in ATP, ATP\textsubscript{single}, AMPPNP\textsubscript{raw}, ADP\textsubscript{raw}, AMPPNP\textsubscript{hex}, and ADP\textsubscript{hex}. B, recovery in AMPPNP\textsubscript{pre-hex} and ADP\textsubscript{pre-hex} that had been pretreated with hexokinase and effect of 1 mM Ap5A, an inhibitor of adenylate kinase. Ap5A was also added to the reaction mixture for ATP as a control. Note that hexokinase was included in the reaction mixtures for ATP\textsubscript{single} (after 3 s), AMPPNP\textsubscript{hex}, and ADP\textsubscript{hex} during the reaction period (A) but was removed from the reaction mixtures of AMPPNP\textsubscript{pre-hex} and ADP\textsubscript{pre-hex} prior to the initiation of the reaction (B). No rhodanese recovery was observed in the absence of nucleotide (data not shown).
This strong GroES binding may enable GroES to bind the loaded GroEL, the GroES binding site of which is covered by substrate protein.

*cis* Folding of Rhodanese—From the slow binding of GroES to the rhodanese-loaded GroEL in ADP$_{\text{raw}}$ and AMPPNP$_{\text{raw}}$, it can be predicted that formation of the *cis* ternary complex and the *cis* folding cannot occur in these nucleotides. To examine this, we compared the effect of these nucleotides on the chaperonin-assisted folding of rhodanese. Since it has been known that rhodanese folds in the *cis* cavity but does not fold spontaneously (16), all the recovered rhodanese activity can be attributed to the result of the *cis* folding. The reaction was started by mixing the rhodanese-loaded GroEL, GroES, and nucleotides (Fig. 5A). In ATP, the yield of recovered rhodanese reached to more than 80% of the total bound rhodanese in 60 min. In ATP$_{\text{single}}$, the recovered rhodanese was nearly 50%, showing that unfolded rhodanese bound in one GroEL ring is folded in the *cis* cavity after single ATP hydrolytic cycle was terminated. We observed significant recovery of rhodanese activity also in ADP$_{\text{raw}}$ (~50%) and AMPPNP$_{\text{raw}}$ (~20%). The same results were reported previously by others (7–9), and it has been thought that ADP and AMPPNP can substitute ATP to some extent for the chaperonin function. However, hexokinase treatment diminished rhodanese activity in ADP$_{\text{hex}}$ and AMPPNP$_{\text{hex}}$ (Fig. 5A). To determine which is the cause of ATP contamination, contaminated ATP or ATP production by contaminated adenylate kinase, rhodanese activity was measured using pre-hexokinase-treated ADP and AMPPNP (ADP$_{\text{pre-hex}}$ and AMPPNP$_{\text{pre-hex}}$) that were treated with hexokinase and separated from hexokinase by ultrafiltration (Fig. 5B). Approximately 50% of rhodanese was recovered in ADP$_{\text{pre-hex}}$. The initial lag period in the rhodanese recovery in ADP$_{\text{pre-hex}}$ can be interpreted as the time required for the accumulation of ATP by adenylate kinase. Indeed, in the presence of Ap5A, a potent inhibitor of adenylate kinase (17), rhodanese reactivation disappeared in ADP$_{\text{raw}}$, whereas it was not affected in ATP. The reason for no rhodanese recovery in AMPPNP$_{\text{pre-hex}}$ may be explained by the low concentration of contaminated ADP that is not enough for adenylate kinase to produce ATP. These results lead to conclusion that the causes for rhodanese recovery in ADP$_{\text{raw}}$ and AMPPNP$_{\text{raw}}$ are trace amounts of contaminated adenylate kinase and contaminated ATP, respectively.

*Formation of the cis Ternary Complex*—We assessed the formation of the *cis* ternary complexes by using protease treatment; the substrate proteins entrapped in the *cis* cavity are protected from the protease digestion, whereas those bound to the *trans* ring are readily digested as well as free unfolded proteins (2, 16). The samples incubated for 120 min as in Fig. 5A were ultrafiltrated to remove free GroES, digested by proteinase K, and ultrafiltrated to remove proteinase K and digested polypeptide. Three kinds of GroEL complexes, GroEL, GroEL-GroES, and the GroEL-GroES-rhodanese *cis* ternary complex, should exist after this procedure. The relative populations of GroEL, GroES, and rhodanese were estimated from the band intensities in the CBB-stained SDS-PAGE (Fig. 6A). The rhodanese-loaded GroEL contained 2.4 mol rhodanese/mol of GroEL (lane 1), and all rhodanese molecules were digested by proteinase K (lane 2). In ATP, the folded rhodanese molecules had been removed by ultrafiltration, and GroEL-GroES complexes remained (lane 3). In AMPPNP$_{\text{raw}}$, ADP$_{\text{raw}}$, and ATP$_{\text{single}}$, significant amount of proteinase K-resistant rhodanese were observed (lanes 4–6). Molar ratios among GroEL, rhodanese, and GroES in these three lanes were 1.0:0.8–1.4.
0.7–1.1, indicating that the GroEL-GroES-rhodanese cis ternary complex was formed in these nucleotides. The rhodanese molecules held in the cis cavity of these complexes had finished folding because rhodanese activity of these complexes is equivalent to the estimated amount of rhodanese from SDS-PAGE (Fig. 6A, lower panel). In contrast, in AMPPNP
\textsubscript{hex} and ADP
\textsubscript{hex}, no rhodanese and a faint amount of GroES were observed (lanes 7 and 8), suggesting that GroES cannot bind to the rhodanese-loaded GroEL or can form only unstable GroEL-GroES-rhodanese ternary complex. Slow GroES binding to rhodanese-loaded GroEL in AMPPNP
\textsubscript{hex} and ADP
\textsubscript{hex} (Fig. 2, C and D) can be due to the formation of such unstable complex. Next, we used MDH as a substrate protein (Fig. 6B). Molar ratios among GroEL, MDH, and GroES in AMPPNP
\textsubscript{row}, ADP
\textsubscript{row} and ATP
\textsubscript{single} (lanes 4–6) were 1.0:0.8–1.1:1.0–1.1, indicating the stoichiometric formation of the cis ternary complexes as well as rhodanese. The results of AMPPNP
\textsubscript{hex} (lane 7) and ADP
\textsubscript{hex} (lane 8) are different from those of rhodanese. In AMPPNP
\textsubscript{hex}, smaller amounts of MDH than ATP
\textsubscript{single} were encapsulated in the cis cavity, whereas rhodanese was not encapsulated. In ADP
\textsubscript{hex}, no MDH band was observed as rhodanese; however, GroES was bound to GroEL in contrast to rhodanese. Probably, MDH was gradually released from GroEL, and then GroES bound to these newly available binding sites. Folding of MDH in the cis cavity was assessed by measuring MDH activity after recovery occurred much more slowly in AMPPNP
\textsubscript{hex} than in ADP
\textsubscript{hex}, indicating higher affinity of MDH to GroEL in AMPPNP
\textsubscript{hex} than in ADP
\textsubscript{hex}, which is consistent with slower GroES\textsubscript{B} binding to MDH-loaded GroEL in AMPPNP
\textsubscript{hex} than in ADP
\textsubscript{hex} (Fig. 2, C and D). The small cis folding of MDH in AMPPNP
\textsubscript{hex} detected in Fig. 6B may be related to this relatively strong affinity of MDH to GroEL in AMPPNP
\textsubscript{hex} and accidental entrapping of MDH in the cis cavity, whereas GroES competes for its binding sites.

\textsuperscript{2} In AMPPNP
\textsubscript{hex} and ADP
\textsubscript{hex}, MDH in the MDH-loaded GroEL appeared to be gradually released from GroEL, and then GroES bound tightly to the newly available binding sites, because recovery of some MDH activity was observed even before the addition of EDTA (data not shown). In this case, recovery occurred much more slowly in AMPPNP
\textsubscript{hex} than in ADP
\textsubscript{hex}, indicating higher affinity of MDH to GroEL in AMPPNP
\textsubscript{hex} than in ADP
\textsubscript{hex}, which is consistent with slower GroES\textsubscript{B} binding to MDH-loaded GroEL in AMPPNP
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\textsuperscript{3} Discrimination of ATP, ADP, and AMPPNP by Chaperonin GroEL

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