Chaperonin-mediated Folding of Green Fluorescent Protein*

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Chaperonin-mediated folding of green fluorescent protein (GFP) was examined by real-time monitoring of recovery of fluorescence and by gel filtration high-performance liquid chromatography. Acid-denatured GFP can fold spontaneously upon dilution into the neutral buffer. When *Escherichia coli* GroEL/ES was present, folding of GFP was arrested. Folding was resumed by subsequent addition of 100 μM or 1 mM ATP, and native GFP was regenerated to 100% yield. When folding was resumed by 10 μM ATP (1.4 mol/mol GroEL subunit), about 60% of GFP recovered native structure, and one-half of them (30%) was found to be still bound to GroEL/ES, indicating the occurrence of folding in the central cavity of the GroEL ring underneath GroES (cis-folding). Because the overall rates of GroEL/ES-, ATP-mediated GFP folding were all similar to that of spontaneous folding, it was concluded that cis-folding proceeded as fast as spontaneous folding. The GroEL/ES-bound native GFP was observed only when both GroES and ATP (but not ADP) were present in the folding mixture. Holo-chaperonin from *Thermus thermophilus*, which was purified as a cpn60/10 complex, exhibited the similar cis-folding. Consistently, ATP-dependent exchange of cpn10 in the holo-chaperonin with free cpn10 was observed.

Members of the chaperonin family play an essential role in facilitating folding in the cytosol of both prokaryotes and eukaryotes (1–6). The best studied chaperonin is *Escherichia coli* GroEL. GroEL is composed of 57-kDa subunits arranged in two seven-membered rings stacked back to back, forming a central cavity ~45 Å in diameter (7–9). GroEL binds a variety of substrate polypeptides in nonnative form, and the addition of ATP is sufficient to allow the folding of some proteins in vitro. However, GroEL-mediated folding is dependent on the cochaperonin GroES in many cases, especially under the conditions where only very poor spontaneous folding can occur (10). GroES is a dome-shaped seven-membered ring of 10-kDa subunits (11) that can bind to one or both ends of the GroEL cylinder.

Binding of substrate polypeptide occurs exclusively to the GroEL ring not occupied by GroES, as observed by electron micrograph (trans-complex) (12, 13). On addition of ATP, either polypeptide or GroES is released. When polypeptide is released, it rebinds to the trans GroEL ring to regenerate trans-complex, or it completes folding by itself in the medium if conditions are suitable for spontaneous folding. When GroES is released, it rebinds to either one of two GroEL rings. If it binds to the GroEL ring not containing polypeptide, trans-complex is regenerated. If it binds to the GroEL ring containing polypeptide (cis-complex), it sequesters polypeptide in the central cavity, and productive folding can proceed there (cis-folding). ATP acts as a set timer (~15 s) to induce dissociation of the GroES from the GroEL ring, and the substrate protein is released into the medium. How much of the fraction of the substrate protein in the cis-complex has acquired the native conformations before the release differs from one protein to another (14–19).

Green fluorescent protein (GFP)1 from the jellyfish *Aequorea victoria* is a monomeric 238-residue protein that emits 508-nm fluorescent light by excitation light at 395 nm (20). The fluorophore results from autocatalytic cyclization of the polypeptide backbone between residues Ser65 and Gly67 and oxidation of the α-β bond of Tyr66 (20–23). Once formed, covalent structure of the fluorophore is stable. Denaturation of GFP by acid, base, or guanidine HCl results in loss of fluorescence, but the denatured GFP restores fluorescence after the shift of pH to neutral or dilution of guanidine HCl (24, 25). Structural bases of necessity of native protein structure for fluorescence has been provided from the recently reported crystal structures of GFPs (21, 23) in which the fluorophore interacts with many residues distant in the primary sequence. Therefore, GFP has the advantage for the study of protein folding, that is, one can readily monitor the folding in real time using fluorescence as a marker of recovery of native structure. Indeed, Weissman et al. (19) used GFP as a substrate protein of the GroEL/ES-mediated protein folding and presented a solid support for the cis-folding; GFP recovered the fluorescence while it remained bound to GroEL/ES.

Although cis-folding has been established as a major pathway of chaperonin-mediated protein folding, some of its important characteristics remain unclear. Is the microscopic folding process of substrate proteins in cis-folding the same as that of spontaneous folding, or does it include a different process? Is the rate of cis-folding slower, the same, or faster than spontaneous folding? Does only ATP drive cis-folding, or does ADP also do it as reported (18)? It has been known that chaperonin of a thermophilic bacterium, *Thermus thermophilus*, is purified as an apparently stable complex (holo-chaperonin) made up of chaperonin60 (cpn60, a GroEL homolog) and chaperonin10 (cpn10, a GroES homolog) (26, 35). *Thermus* holo-chaperonin is corresponding to *E. coli* GroEL/ES asymmetric complex. For cis-folding to occur, cpn10 should be released from one cpn60 ring and rebind to the cpn60 ring of the opposite side where substrate polypeptide is already bound. Can *Thermus* holo-chaperonin mediate cis-folding, and does the release-rebinding of cpn10 really happen? Here, taking advantage of GFP as a substrate protein, we have tried to answer some of these questions.

1 The abbreviations used are: GFP, green fluorescent protein; AMP-PNP, 5'-adenyl imidodiphosphate; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Reagents and Proteins—ATP, ADP, 5'-adenyl imidophosphate (AMP-PNP), bovine serum albumin (BSA), RNase A, and hexokinase were purchased from Sigma. 4-(2-Aminomethyl)benzenesulfonfonylfluoride hydrochloride was purchased from Wako (Tokyo). GroEL and GroES were purified as follows. E. coli strain JM109, bearing GroEL/E expression plasmid pRK206 (27), was cultured, collected by centrifugation, washed twice in buffer A (600 ml, 15 °C) until use. Cells were thawed in buffer A containing 0.1 M Tris-Cl, pH 7.5, 1 mM EDTA, and 1 mM diethiothreitol containing 0.2 mM 4-(2-Aminomethyl)benzenesulfonfonylfluoride-HCl and sonicated. The disrupted cells were subjected to centrifugation (20,000 × g for 20 min).

Buffer A containing the indicated additional component was used throughout the purification. The supernatant fraction was applied on 10-30% sucrose density gradient (40,000 × g for 20 h), and fractions containing GroEL (20–30%) and GroES (10–20%) were collected. The GroEL fractions were applied on a Butyl-Toyopearl column (Toyopearl) with a 5–0% saturated ammonium sulfate linear gradient, and GroEL was eluted at near 0% saturated ammonium sulfate. The fractions were then applied on a Sepharose CL-4B column (Pharmacia Biotech Inc.), equilibrated, and eluted with Buffer A containing 20% (v/v) of methanol and 100 mM Na2SO4. Fractions containing GroEL were collected, dialyzed against Buffer A, and stored as a 70% saturated ammonium sulfate suspension at 4 °C. The GroES-containing fractions of sucrose density gradient were applied successively on a Butyl-Toyopearl column (5–15% saturated ammonium sulfate gradient), an Econo-Pac column (Bio-Rad) (0–0.5 M NaCl gradient), a DEAE-Sepha- cel column (Pharmacia) (0–0.5 M NaCl gradient), a gel filtration column (G3000SWXL, Tosoh), a Butyl-Toyopearl column again (15–0% saturated ammonium sulfate gradient), and a Sephacore CL-6B column (Pharmacia). The buffer used for the last Sepharose CL-6B contained 100 mM Na2SO4. The purified GroES was stored at 4 °C as a 4% saturated ammonium sulfate suspension. Chaperonin from T. thermophilus cpn10 was purified from T. ther- mphilus cpn10 was purified from recombinant E. coli cells bearing pcpn10 gene-containing plasmid pRC70 (28). GFP was purified from recombinant E. coli BL21(DE3) cells bearing plasmid pTY58, which has no vector GFP gene after T7 promoter (29). Recombinant GFP encoded in plasmid pTY58 has unintended mutations, insertion of Ala-2 and replacement of Gua with Arg, but these mutations have no effect on the spectral properties of the protein (29). Expression of GFP was induced by the addition of 0.8 mM isopropyl-D-thiogalactopyrano- side at middle log growth phase (−0.4 absorbance at 650 nm) and cultured for another 2 h. Then, E. coli cells were harvested, suspended in Buffer A containing 0.2 mM 4-(2-Aminomethyl)benzenesulfonfonylfluoride hydrochloride, and disrupted by sonication. The sonicated mixture was then used, but later we found that inclusion of BSA in the dilution buffer influenced by whether the dilution buffer contained GroEL, and the values were taken as 100%. In the absence of supernatant, the temperature of 50 °C in the solution with components as described above, except that 0.5 µM (final) of Thermus holo-chaperonin, folding reactions were carried out at 50 °C with an on-line spectrophotometer (FS-8010, Tosoh; excitation at 398 nm and emission at 508 nm). When BSA was included in the sample solution, a protein peak appeared at 17.8 min. This was an unknown protein of Mr = 200,000 containing the commercial BSA preparation.

Chaperonin-mediated GFP Folding—GroEL (or GroEL plus GroES) was incubated for 1 min after dilution, and an indicated (final) concentration of adenine nucleotide was added to resume folding. When indicated, folding was resumed by the addition of GroES. Generation of the fluorescence at 508 nm by excitation light at 398 nm was monitored continuously with a fluorometer (FP-777, Jasco) with reaction mixture equilibrated at 4 °C for 5 min. The fluorescence intensity of the treated native GFP was not influenced by whether the dilution buffer contained GroEL, and the values were taken as 100%. In the absence of supernatant, the temperature of 50 °C in the solution with components as described above, except that 0.5 µM (final) of Thermus holo-chaperonin and 0.5 mg/ml RNase A were used instead of GroEL and BSA, respectively.

HPLC Analysis of Folded Products—Folding of GFP was carried out as described above, except that denatured GFP solution (1 µl) was diluted (0.25 µM final) into 100 µl of the dilution buffer. After 20 min of folding reaction, the solution was subjected to a brief centrifugation (17,000 × g for 5 min at 25 °C), and an aliquot (0.5 µl) was injected into a gel filtration HPLC column (G3000SWXL, Tosoh). The column was equilibrated and eluted with 50 mM 3-[N-morpholino]propanesulfonic acid-NaOH buffer, pH 7.0, 5 mM Mg(CH3COO)2, and 100 mM KCl, at a flow rate of 0.5 mL/min. Elution was monitored with an on-line spectrophotometer (280 nm) and an on-line fluorometer (FS-8010, Tosoh; excitation at 398 nm and emission at 508 nm). When BSA was included in the sample solution, a protein peak appeared at 17.8 min. This was an unknown protein of Mr = 200,000 containing the commercial BSA preparation.

Exchange of dns-cpn10 and Intact cpn10 in Holo-chaperonin—Thermus cpn10 was labeled by dansyl chloride (5-dimethylaminonaphtha- linesulfonyl chloride) as follows. To 2.5 ml of Thermus cpn10 (6.4 µM) dissolved in 50 mM sodium borate buffer, pH 9.5, and 1 mM EDTA, 11.3 µl of 10 mM dansyl chloride dissolved in acetone were added. The solution was incubated at 3 h at room temperature. Then 1.1 g of solid ammonium sulfate was added and dissolved. After centrifugation, the precipitate was dissolved in 500 µl of 50 mM Na2Pi buffer, pH 7.5, containing 1 mM EDTA and 1 mM diethiothreitol. The solution was desalted by a Sephadex G-25 column equilibrated with the same buffer. With this procedure, 1 mol of Thermus cpn10 was labeled by 3.0 mol of dansyl groups. dns-cpn10 solution (6.3 µM) was stored at 4 °C until use. To measure incorporation of dns-cpn10 into holo-chaperonin (Fig. 5B), holo-chaperonin was incubated in 100 µl of buffer A containing 0.5 µM supernatant. At 50 °C for 5 min, dns-cpn10 was added, and after 1 min, the indicated nucleotide was added. Final concentrations of the holo-chaperonin and dns-cpn10 were 0.5 and 1 µM, respectively. Incubation at 50 °C was continued for another 20 min and, after brief centrifugation (17,000 × g for 5 min at 4 °C) to get rid of dust, an aliquot of supernatant was analyzed by gel filtration HPLC (G3000SWXL). The buffer and flow rate of HPLC analysis was the same as described in the above section except that fluorescence at 528 nm with an excitation wave length at 360 nm was monitored with an on-line fluorometer. dns-cpn10 incorporated into holo-chaperonin fraction was estimated from the areas of the fluorescent peak eluted at the position of the holo-chaperonin. Chase of incorporated dns-cpn10 with free intact cpn10 (Fig. 5C) was assayed as follows. The solution (1 ml) containing 1 µM holo-chaperonin, 2 µM dns-cpn10, 50 mM Na-Pi buffer, pH 7.5, 5 mM Mg(CH3COO)2, and 100 mM KCl was preincubated for 5 min at 50 °C. Then, 1 mM ATP was added, and incubation at 50 °C was continued for another 20 min. This solution was transferred on ice, and 0.39 g of solid ammonium sulfate (final 65%) was added. After a 5-min incubation, precipitate was collected by centrifugation (17,000 × g for 5 min) and dissolved with 50 µl of the elution buffer for gel filtration HPLC described above. After brief centrifugation again (17,000 × g for 5 min), supernatant was subjected to gel filtration HPLC as described above, and fractions containing holo-chaperonin were collected. The resultant solution, which contained 0.45 µM holo-chaperonin with dns-cpn10, 42 mM [N-morpholino]propanesulfonic acid-NaOH buffer, pH 7.0, 4.2 mM Mg(CH3COO)2, and 85 mM KCl was preincubated for 5 min at 50 °C. Then, the indicated component, such as intact cpn10 and ATP, was added, and the solution was incubated at 50 °C for 5 min. After brief centrifugation (17,000 × g for 5 min at 4 °C), 50 µl of supernatant were subjected to gel filtration HPLC, and elution was monitored as described above.

Other Methods—Protein concentrations of GroEL, Thermus holo-chaperonin, and Thermus cpn10 were determined with a method by Bradford (30). Concentrations of GFP and GroES were determined...
out GroEL/ES at 0 min (spont.) was initiated by diluting denatured GFP into the dilution buffer with-ES. At 0 min, ATP at the indicated final concentration was added (open arrowhead). Time courses of folding from the GroEL/ES-bound unfolded GFP triggered by 100 µM and 1 mM ATP were very similar to that of spontaneous folding. Half-maximum times (t½) are 27 s (100 µM ATP) and 24 s (1 mM ATP), and final yields are ~100%. When folding was triggered by the addition of 10 µM ATP, which was only 1.4 mol/mol of GroEL subunit, folding of GFP started with a comparable rate to those at higher ATP concentrations (t½ = 26 s), but this phase decelerated to a much slower phase (t½ = 420 s). The yield of recovered native GFP at 20 min after the dilution was about 65%. The remaining 35% of GFP was still bound to GroEL/ES as unfolded polypeptides because further addition of 1 mM ATP (open arrow) resulted in nearly 100% of recovery of native GFP.

GroEL/ES-bound Native GFP—Analysis of the folding mixture incubated for 20 min after the addition of 10 µM ATP by gel-filtration HPLC revealed that about one-half of the native GFP was bound to GroEL/ES (Fig. 2A). The fluorescent GFP associated with GroEL/ES is fully native, as reported previously (19). Because it was confirmed that externally added native GFP did not bind to GroEL/ES in the absence or presence of ATP, the GroEL/ES-bound native GFP should be the product of cis-folding, that is, the folding in a central cavity of the GroEL ring underneath GroES. The above result, therefore, indicates that at least one-half of the productive folding triggered by 10 µM ATP proceeded through cis-folding. This amount of ATP was almost stoichiometric to the GroEL subunit and was exhausted before the release of GFP, which already completed folding in the cis-complex. Because the release of folded GFP required ATP hydrolysis, native GFP remained bound. In fact, bound native GFP generated at 10 µM ATP was released from GroEL/ES by further addition of 1 mM ATP (data not shown). At 100 µM and 1 mM ATP, native GFP in the cis-complex was all released, and there was no GroEL/ES-bound native GFP (Fig. 2B). AMP-PNP was also effective to mediate GFP folding, and a small amount of the GroEL/ES-bound native GFP was detected (Fig. 2B). We also analyzed the folding mixture at 5 µM ATP by HPLC. Total yield of native GFP at 5 µM ATP decreased to about one-half of that at 10 µM ATP, but the ratio of the bound to the free native GFP remained unchanged.

Effect of the Amount of GroES on cis-Folding—In the above experiments, GroE was added at 2:1 molar ratio to GroEL. When the amount of added GroES was changed from 1:1 to 4:1, GroES:GroEL molar ratios and folding was resumed by 10 µM ATP, the amount of the GroEL/ES-bound native GFP remained roughly equal to the amount of free native GFP, whereas the total yield of recovered native GFP varied (Fig. 2C). When the amount of GroES was decreased to 1:0.5 molar ratio, the total yield of recovered native GFP was decreased drastically, and only a trace amount of the bound GFP was detected, showing again that the cis-folding of GFP was dependent on GroES.

3Time courses of folding presented in this report were fairly well fitted by a single exponential function except folding at 10 µM ATP in Fig. 1 and at 100 µM ATP in Fig. 5A. However, it should be added that, as a matter of fact, the fitting was improved by a sum of two exponential functions by which half-times of the fast and slow components of spontaneous folding, for example, are calculated to be 29 and 137 s, respectively.
Almost all of the denatured GFP was captured by GroEL, and it was not released from GroEL in the presence of ADP as shown in Fig. 4B. At 0 min, GroES was added (arrow) to initiate folding of GFP. Spontaneous folding of GFP initiated by dilution at 0 min was also shown. The inset shows the yield and distribution of native GFP recovered after 20 min of folding reaction analyzed by HPLC. The column signified by 1 mM ADP was a result of an experiment carried out with the same procedure as described in Fig. 1 except that ADP without treatment to remove ATP was used instead of ATP.

ADP-triggered Folding from GroEL/ES-bound Unfolded GFP—Native GFP was also generated when 1 mM ADP was added to the solution of the GroEL/ES-bound unfolded GFP complex. The final yield of recovered native GFP was nearly 100% (Fig. 3). However, this ADP-triggered folding proceeded slowly ($t_{1/2} = 64$ s). This slow generation of native GFP was more pronounced ($t_{1/2} = 212$ s) when folding was initiated by 100 μM ADP. ADP at 10 μM even failed to initiate folding at a measurable rate. HPLC analysis showed that all of the native GFP produced was free in the solution (Fig. 3, inset). It should be noted that the procedure of this experiment was different from that of the ATP-triggered folding experiment. Hexokinase, glucose, and ADP were added to the dilution buffer and incubated for 5 min to eliminate contaminating ATP in the ADP preparation. Then denatured GFP was diluted, and folding was triggered by the addition of GroES (see the legend of Fig. 3).

Commercially available ADP usually contains a small amount of ATP. When we used an ADP preparation containing 2.2% or ATP without hexokinase treatment, folding kinetics were fast ($t_{1/2} = 27$ s at 1 mM ADP and $t_{1/2} = 260$ s at 100 μM ADP, data not shown), and more significantly, a small amount of the GroEL/ES-bound native GFP was detected by HPLC analysis in the folding mixture at 1 mM ADP (Fig. 3, inset, 1 mM* and 100 μM ADP, data not shown). Therefore, a small amount of contaminated ATP in the ADP preparation was critical in this experiment.

Folding from GroEL-bound Unfolded GFP—When acid-denatured GFP was diluted into the buffer containing GroEL (but not GroES), unfolded GFP was captured by GroEL, and subsequent addition of 100 μM or 1 mM ATP to the solution triggered the folding (Fig. 4A). Folding at 1 mM ATP was slow ($t_{1/2} = 250$ s) but reached 100% yield after 900 s. Folding at 100 μM ATP proceeded at a slightly slower rate but stopped suddenly at 350 s when about 60% of fluorescence was recovered. Be-
cause further addition of 1 mM ATP induced folding again (Fig. 4A, open arrow), this stop was due to shortage of ATP that was exhausted by this time point. Folding was not initiated by 10 μM ATP. Multiple turnover of ATPase appeared to be required to generate native GFP from GroEL-bound unfolded GFP in the absence of GroES. ADP did not or only very poorly supported the folding (Fig. 4B). The GroEL-bound native GFP was not detected in any samples (Fig. 4, insets).

**Thermus Holo-chaperonin-dependent Folding of GFP**—As reported previously (26, 35), chaperonin of *T. thermophilus* is purified as a stable complex (holo-chaperonin) made up from 14 cpn60 subunits and 7 cpn10 subunits. At the temperature of the folding assay of holo-chaperonin (50 °C), the yield of spontaneous folding of GFP was about 19% of that at 25 °C. However, holo-chaperonin captured unfolded GFP upon dilution of acid-denatured GFP into the buffer, and subsequent addition of 100 μM or 1 mM ATP resumed the folding, which finally reached nearly double of that of spontaneous folding (Fig. 5A). ATP at 10 μM was also effective to trigger the folding, but the final yield was about one-half of that of folding at 100 μM ATP. It is interesting to examine if holo-chaperonin can mediate cis-folding, because if the holo-chaperonin is so stable that the cpn10 ring is coupled to the cpn60 ring during the chaperonin catalytic cycle, then substrate protein cannot have an opportunity to be located in the cis-position. The result, however, was similar to those observed for *E. coli* GroEL/ES, i.e. holo-chaperonin-bound native GFP was detected in the folding mixture initiated by 10 μM ATP, and its amount was about equal to the free native GFP (Fig. 5A).

**FIG. 4. Time courses of GFP folding mediated by GroEL plus ATP or ADP.** All experiments were performed in the absence of GroES. A, denatured GFP was diluted at 1 min (black arrowhead), and folding was initiated at 0 min by the addition of the indicated amount of ATP (open arrowhead). At 10 min, 1 mM ATP was added to the solution of 10 μM ATP (open arrow). The inset shows the yield and distribution of native GFP recovered after 20 min of folding reaction analyzed by HPLC. B, GroEL, hexokinase, glucose, and indicated concentrations of ADP were incubated at 25 °C for 5 min. Then, denatured GFP was diluted into the solution at 0 min (filled arrowhead). The inset shows the yield and distribution of native GFP recovered after 20 min of folding reaction analyzed by HPLC.

**FIG. 5. Folding of GFP mediated by holo-chaperonin from *T. thermophilus* and exchange of cpn10.** A, the yield of the holo-chaperonin-bound native GFP (■) and the free native GFP (□) after 20 min of the folding reaction at 50 °C. Folding was initiated by the addition of indicated concentration of ATP, 1 mM ADP, or 1 mM AMP-PNP. The peak areas of fluorescence of the holo-chaperonin-bound native GFP and free native GFP eluted from gel filtration HPLC were expressed as a percentage of the peak area of the free native GFP produced by the spontaneous folding of the same amount of GFP. B, ATP-dependent incorporation of dns-cpn10 into holo-chaperonin. After a 1-min incubation of *Thermus* holo-chaperonin with dns-cpn10, the indicated nucleotide (none, 1 mM ADP, or 1 mM ATP) was added, and the incubation at 50 °C was continued another 20 min. In the experiment of the rightmost column, *Thermus* holo-chaperonin was preincubated for 5 min at 50 °C with intact cpn10 (0.5 μM), and then the above procedures were carried out. The reaction mixture was analyzed by gel filtration HPLC. Elution was monitored with fluorescence at 528 nm (a peak wavelength of dansyl group), and an area of the fluorescent peak eluted at the position of *Thermus* holo-chaperonin was obtained. Background fluorescence of *Thermus* holo-chaperonin alone (the value was 4.01) was subtracted. The axis is arbitrary units. C, ATP-dependent exchange of dns-cpn10 contained in holo-chaperonin with free cpn10. *Thermus* holo-chaperonin containing dns-cpn10 was washed as described under “Experimental Procedures.” *Thermus* holo-chaperonin (0.45 μM) containing dns-cpn10 was incubated at 50 °C for 5 min. Then, none, cpn10 (intact one, 0.9 μM), 1 mM ATP, or 1 mM ATP plus cpn10 (intact one, 0.9 μM) was added, and the solutions were incubated at 50 °C for 20 min. An aliquot of the solution was analyzed by gel filtration HPLC, and the area of fluorescence (528 nm) peaks of the holo-chaperonin (■) and the free cpn10 (□) were obtained. The axis is an arbitrary scale. The background fluorescence of holo-chaperonin alone (the value is 21.3) was subtracted. Other experimental procedures are described under “Experimental Procedures.”
Chaperonin-mediated GFP Folding

...erolin-bound native GFP was detected, even in the case when the folding was initiated by 100 μM or 1 mM ATP. ADP at 1 mM also supported the folding. ADP pretreated with hexokinase was used instead of its inclusion into the dilution buffer, but the holo-chaperonin-bound native GFP was hardly detected. AMP-PNP, which is efficient in GroEL/ES-mediated folding, supported the folding only poorly, but one-half of the generated native GFP was found in the holo-chaperonin fraction (Fig. 5A).

**ATP-dependent Exchange of dns-cpn10**—As described above, for cis-folding to occur, the cpn10 ring of the holo-chaperonin should reversibly dissociate from the cpn60 cylinder during the chaperonin functional cycle. To examine this by experiment, we prepared fluorescently labeled cpn10 with covalent modification by dansyl chloride (dns-cpn10), and incorporation of dns-cpn10 into the holo-chaperonin was measured with gel filtration HPLC (Fig. 5B). Some background incorporation into the holo-chaperonin fraction was observed, even when adenine nucleotide was not added. The addition of 1 mM hexokinase-treated ADP did not improve the incorporation, but 1 mM ATP stimulated the incorporation significantly. The amount of incorporated dns-cpn10 at 1 mM ATP was 0.31 mol per mol of the cpn60 14-mer. The addition of nonlabeled (intact) cpn10, at the same concentration as holo-chaperonin (0.5 μM), suppressed the incorporation of dns-cpn10. The amount of incorporated dns-cpn10 was less than the amount predicted from the assumption that whole cpn10 in the holo-chaperonin is readily exchangeable in the presence of ATP. The reason for this discrepancy is not known, but it is possible that binding of dns-cpn10 to the cpn60 ring is less efficient than intact cpn10. Most of the incorporated dns-cpn10 was retained during incubation with nonlabeled cpn10, but when ATP was further included in the solution, about one-half of the dns-cpn10 was lost from the holo-chaperonin fraction (Fig. 5C). Apparently, ATP alone did not stimulate the release of dns-cpn10; probably the rebinding of dns-cpn10 occurred simultaneously, which compensated the release. These results show that the cpn10 moiety of the holo-chaperonin becomes exchangeable with free cpn10 in the presence of ATP. Thus, although the holo-chaperonin of *T. thermophilus* is a stable complex when it is not working, but when ATP is provided, cpn10 can readily dissociate from and rebind to the cpn60 ring.

**DISCUSSION**

**Folding of GFP**—GFP is widely used as a very convenient tool for studying gene expression and protein localization. This report shows that GFP is also a convenient protein for the study of *in vitro* protein folding. Folding of GFP can be easily and directly monitored in real time by measuring fluorescence. This is of great advantage over other proteins, the recovery of native structure of which is usually measured by their recovered enzyme activities assayed in different test tubes. It has been generally accepted that the local structure around the catalytic site of the enzyme is generated by the global structure of the protein. Similarly, the local protein folding of GFP around the fluorophore should require the correct folding of the whole protein, and the fluorescent property of GFP is sensitive to the tertiary structure of the protein. As reported by Bekman and Ward (24), recovered fluorescence of GFP was indistinguishable from that of the native GFP. This is also the case for the chaperonin-mediated folding of GFP. Weissman et al. (19) reported that excitation and emission spectra and fluorescence lifetime of the GFP folded in the cavity of the single-ring mutant GroEL capped with GroES were exactly identical to those of the native GFP free in the solution, indicating GFP bound to GroEL/ES is fully native.

Bekman and Ward (24, 25) reported that folding of GFP from an acid-, base-, or guanidine HCl-denatured state proceeded at a halftime of −5 min. We observed that spontaneous folding of GFP from an acid-denatured state proceeded much faster (t½ = 24 s). Under the conditions we used, folding from base- or guanidine HCl-denatured state also proceeded quickly. We repeated experiments of folding of acid-denatured GFP under the conditions described by Ward and Bekman (25) and found that the fluorescence recovery was fast. The real reason for the discrepancy between their result and ours is not known but is possibly the purer preparation of the recombinant GFP. A drawback of GFP as a tool of gene expression is that it takes from 90 min to 4 h for newly synthesized GFP to be fluorescent (22, 31, 36). The folding of the nascent polypeptide chain without the fluorophore is probably as fast as that of the denatured GFP with the preformed fluorophore, and slow formation of the fluorophore by oxidative cyclization in the folded protein might be responsible for the slow time course for the fluorophore formation in *vivo*, as postulated by Heim et al. (22).

**cis-Folding of GFP Is Dependent on GroES and ATP**—Fig. 6 illustrates a schematic model of chaperonin-mediated protein folding based on the recent reports (15, 17–19). Consistent with this model, cis-folding is absolutely dependent on GroES because ATP-triggered folding of GFP captured by GroEL in the absence of GroES did not generate the GroEL-bound native GFP (Fig. 4A). This model can also explain the reason why the GroEL/ES-bound native GFP was detected predominantly at low concentrations of ATP (Fig. 2). The GroEL/ES-bound native GFP should correspond to the last intermediate complex of cis-folding pathway (Fig. 6, species D) in which folding is already completed in the cavity of the GroEL ring underneath GroES. The result that 1.4 mol ATP/mol GroEL subunit promotes the production of the GroEL/ES-bound native GFP indicates that only one or two ATP mol/mol GroEL subunit is required to produce species D. To let native GFP escape from the cavity into the bulk solution, GroES should dissociate from the GroEL ring, and this process requires ATP hydrolysis (species D → E). When the amount of added ATP is small and exhausted by the steps prior to species D, species D will be accumulated. When the amount of ATP is enough to sustain multiple catalytic turnover of chaperonin, the GroEL/ES-bound native GFP are all released into the medium as free native GFP. Probably, the ATP binding, but not necessarily ATP hydrolysis, is required for the production of species D because a small amount of GroEL/ES-bound native GFP was produced by guest on July 25, 2018
when 1 mM AMP-PNP was added instead of ATP (Figs. 2B and 5A). Weissman et al. (19) observed also the GroEL/ES-bound native rhodanese in the presence of AMP-PNP. Mayhew et al. (18) observed the cis-folding of dehydrofolate reductase in the presence of ATP. In our experiments, the GroEL/ES-bound native GFP was not detected in the presence of hexokinase-treated ADP (Fig. 3). When ADP preparation without hexokinase treatment was used, a small amount of the GroEL/ES-bound native GFP was not detected in the presence of hexokinase.

**Folding in the Cavity of GroEL/ES Proceeds as Fast as Spontaneous Folding**—According to the model shown in Fig. 6, the ATP-triggered folding of GFP captured by GroEL/ES can proceed through three possible pathways to produce native GFP. One is *cis*-folding (A → B → C → D → E). The other two pathways contain spontaneous folding (u → n) of free unfolded GFP, which is released from either species A or B. The result of Fig. 4A indicates that the pathway (B → u → n) is slow (t½ = 250 s) and may not be operating when folding is initiated from species A. The folding pathway through *trans*-complex (A → u → n) can be operative, but the extent is not determined from our experiment. The presence of the GroEL/ES-bound native GFP (Fig. 2) provides evidence that at least a part of native GFP is folded through *cis*-folding. The overall rate of GFP folding from species A initiated by ATP (t½ = 24 s at 1 mM ATP) is the same as that of spontaneous folding (t½ = 24 s) (Fig. 1). This implies that any steps in A → B → C → D, as well as those in A → u → n, are faster than or the same as spontaneous folding. The step D → E cannot be discussed here because we observed fluorescence of the sum of species D and free GFP that was released from species D. Weissman et al. (19) observed the rate of fluorescence anisotropy decay of GFP and suggested that native GFP sequestered in the central cavity formed by GroEL and GroES is not freely tumbling. Our data indicate that folding of GFP in the central cavity proceeds as fast as spontaneous folding, despite the confinement and restriction of the movement of GFP. Based on this conclusion, one can argue that the microscale folding process is also similar between chaperonin-mediated and spontaneousfoldings.

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