A Chaperonin from a Thermophilic Bacterium, *Thermus thermophilus*, That Controls Refoldings of Several Thermophilic Enzymes*

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The chaperonin has been purified from a thermophilic bacterium, *Thermus thermophilus*. It consists of two kinds of proteins with approximate *M*, 58,000 and 10,000 and shows a 7-fold rotational symmetry from the top view and a “football”-like shape from the side view under the electron microscopic view. Its weak ATPase activity is inhibited by sulfite and activated by bicarbonate. ATP causes change of its mobility in nonnondenatured polyacrylamide gel electrophoresis. The *T. thermophilus* chaperonin can promote in vitro refolding of several guanidine HCl-denatured enzymes from thermophilic bacteria. At high temperatures above 60 °C, where the native enzymes are stable but their spontaneous refoldings upon dilution of guanidine HCl fail, the chaperonin induces productive refolding in an ATP-dependent manner. No or very poor refolding is induced when the chaperonin is added to the solution aged after dilution. An excess amount of the chaperonin is inhibitory for refolding. At middle temperatures (30–50 °C), where spontaneous refoldings of the enzymes occur, the chaperonin arrests refolding in the absence of ATP and refolding is induced when ATP is supplemented. At temperatures below 20 °C, where spontaneous refoldings also occur, the chaperonin arrests the refolding but ATP does not induce refolding.

The chaperonins are a ubiquitous class of proteins implicated in the folding of other proteins. They include two kinds of proteins, chaperonin-60 (cpn60), and chaperonin-10 (cpn10). The cpn60s have been found in prokaryotes (GroEL), mitochondria (hsp60), and chloroplasts (ribulose-1,5-bisphosphate carboxylase subunit-binding protein) (1, 2). The amino acid sequences of cpn60s, as well as cpn10s, from various organisms are highly conserved. The cpn60 forms a tetradecameric oligomer with an approximate *M*, 840,000 (3), which shows a heptagonal shape from a top view and a rectangular shape with four clear stripes from a side view (3, 4). The cpn60 oligomers have a weak ATPase activity (5). The cpn10 has been found in prokaryotes (GroES) (6, 7), and a functional equivalent was recently identified in mitochondria (8). Purified *Escherichia coli* chaperonins, cpn60 and cpn10, promote the reconstitution of active dimeric ribulose-1,5-bisphosphate carboxylase from an unfolded state. This reconstitution is dependent on the presence of both chaperonins, MgATP and potassium ion (1, 9). It has been proposed that the binding of cpn10 to cpn60 is required to couple the hydrolysis of ATP to the release of the folded monomers from cpn60 (1, 9). Similar chaperonin activity on protein folding has been observed in the folding of a monomeric protein, β-lactamase precursor (10), and a dimeric protein, citrate synthase (11). Since elucidation of the molecular mechanism of chaperonin activity on the refolding may contribute a great deal to our understanding about the general pathway of protein folding and since the in vitro chaperonin-dependent refolding assay system has been so far restricted in *E. coli* chaperonins, we have developed another system using a thermophilic bacterium, *Thermus thermophilus*. In the present paper, we describe the purification of a chaperonin composed of cpn60 and cpn10 from this bacterium and the demonstration of its activity to promote refolding of several thermophilic proteins from an unfolded state.

**Experimental Procedures**

*Materials*

Isopropylmalate dehydrogenase (IPMDH) (2R,3S)-3-isopropylmalate dehydrogenase (EC 1.1.1.85) and NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42) from *T. thermophilus* strain HB8 were kindly gifts from Dr. T. Oshima and his colleagues. Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase from *Bacillus stearothermophilus* were given by Unitika Corp. (2R*,3S*)-3-Isopropylmalic acid, a substrate of IPMDH, was obtained from Wako Pure Chemical Corp.

*Purification of Chaperonin*

*T. thermophilus* strain HB8 (ATCC 27634) was cultured at 75 °C under strong aeration in a medium containing 10 g of yeast extract, 10 g of polypeptone, and 2 g of NaCl/liter (12). Cells were harvested at the late log phase and stored at −20 °C until use. The frozen cells were thawed, suspended in a 2-fold volume of Buffer A (25 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 μM dithiothreitol), and sonicated for 5 min at 0 °C. The disrupted cells were centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant (crude extract) was diluted with Buffer A to a final protein concentration at 10 mg ml⁻¹ and was fractionated with ammonium sulfate precipitation (30-40% saturation). The precipitate was dissolved in, dialyzed against Buffer A, and applied to a DE52-cellulose column equilibrated with Buffer A. The column was washed with Buffer A containing 50 mM NaCl and was subsequently eluted with Buffer A containing 100, 250, and then 500 mM NaCl. Fractions eluted at 256 mM NaCl were pooled and concentrated by ultrafiltration (Amicon, ultrafiltration cell 8200). The concentrated solution was applied on a Sepharose CL6B column equilibrated with Buffer A containing 100 mM Na₂SO₄. Fractions eluted at void volume were pooled and stored in 50% ammonium sulfate suspension at 4 °C.
**Thermophilic Chaperonin**

The purified protein was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), and protein bands were blotted to a polyvinylidene difluoride membrane (Millipore Corp.). The blotted membrane was stained with Coomassie Brilliant Blue and the 58- and 10-kDa bands were cut out. Pieces of membranes thus obtained were analyzed with a gas phase peptide sequenator (Shimadzu FSP-1). The NH2-terminal amino acid sequences of the native chaperonin were also determined to estimate the relative stoichiometry of 58- and 10-kDa polypeptides in the chaperonin. The native chaperonin (50 μg) dissolved in 25 mM Tris-HCl (pH 7.5) and 3 mM MgCl2, was blotted directly onto a polyvinylidene difluoride membrane. Blotted membrane was dried, washed with distilled water, and stained with Coomassie Brilliant Blue R-250. A protein spot was cut out and analyzed with a gas phase peptide sequenator.

**Electron Microscopy**

An aliquot of sample solution was applied on an electron microscope specimen grid covered with a carbon support film that had been hydrophilized by ion bombardment. The excess of the solution was blotted with a filter paper. The specimen was immediately stained with 1% uranyl acetate for 2 min and placed on the stage of the electron microscope (JEOL JEM-1200EXII). Images were recorded onto Mitsubishi electron microscope film at an accelerating voltage of 100 kV at a magnification of 60,000.

**Other Methods**

The circular dichroism spectrum was measured with a Jasco J-500C spectropolarimeter equipped with a thermometer regulating elevated temperature at a constant rate. Proteins were analyzed by polyacrylamide gel electrophoresis either on 15% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS), or on 7.5% polyacrylamide gels without SDS. A molecular weight standard kit of Coomassie Brilliant Blue R-250. Protein concentrations were measured by the method of Bradford (14) with bovine serum albumin as the standard. ATPase activities were assayed in 500 μl of a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM ATP, and 10 mM KCl. The reaction was initiated by the addition of ATP and was terminated after a 15-min incubation at 60°C by the addition of 250 μl of 2% perchloric acid. The amount of P1 produced was measured as described previously (15). One unit of activity is defined as the activity liberating 1 μmol of P1/min.

**In Vitro Refolding Assay**

The experiments are made up of three steps: denaturation by guanidine HCl, refolding by dilution of guanidine HCl, and assays of recovered enzyme activities. Unless otherwise mentioned, the experiments were carried out as follows.

**Denaturation**—B. stearothermophilus LDH and T. thermophilus isocitrate dehydrogenase were dissolved separately in 50 mM dithiothreitol, 6.4 mM guanidine HCl, and 40 mM potassium phosphate, pH 7.8. For B. stearothermophilus glucose-6-phosphate dehydrogenase, 50 mM Tris-HCl buffer, pH 7.8, was used instead of phosphate buffer.

**T. thermophilus IPMDH,** which lacks cysteine and cystine residues, was dissolved in 7.2 mM guanidine HCl and 15 mM potassium phosphate, pH 7.8. The protein concentrations of LDH, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and IPMDH in the denatured enzyme solutions were 18, 8, 50, and 150 μg/ml, respectively. The denatured enzyme solutions were incubated for 30 min at room temperature.

**Refolding**—Chaperonin-dependent refolding was measured by diluting 5 μl (LDH, glucose-6-phosphate dehydrogenase, and isocitrate dehydrogenase) or 2.5 μl (IPMDH) of the denatured enzyme solutions into 95 or 97.5 μl, respectively, of a dilution buffer containing 50 mM potassium phosphate, pH 7.8, and 40 mM MgCl2. The B. stearothermophilus chaperonin was used with or without 500 μM MgATP. For B. stearothermophilus glucose-6-phosphate dehydrogenase, 50 mM Tris-HCl buffer, pH 7.8, was used instead of phosphate buffer. The dilution solution was preincubated at temperatures indicated in the figure legends. The final concentrations of guanidine HCl in the diluted solutions were 360 mM (LDH, glucose-6-phosphate dehydrogenase), and 180 mM (IPMDH), and those of proteins were 0.9 μg ml⁻¹ (LDH), 0.4 μg ml⁻¹ (glucose-6-phosphate dehydrogenase), 2.5 μg ml⁻¹ (isocitrate dehydrogenase), and 3.8 μg ml⁻¹ (IPMDH). For the assay of spontaneous refolding, *Thermus* chaperonin was omitted from the dilution buffer. The diluted solution was incubated for an appropriate period at desired temperatures, the solution was subjected to a cuvette containing 1 ml of an assay mixture, and the recovered enzyme activity was measured.

**Assay of Enzyme Activities**—Assay mixtures to measure activities of 1) LDH, 2) glucose-6-phosphate dehydrogenase, 3) isocitrate dehydrogenase, and 4) IPMDH contained the following components (described as final concentrations): 1) 50 mM potassium phosphate, pH 6.0, 0.25 mM NADH, and 10 mM sodium pyruvate, 2) 50 mM Tris-HCl, pH 8.8, 1 mM NADP⁺, 40 mM MgCl2, and 2.5 mM glucose 6-phosphate, 3) 50 mM potassium phosphate, pH 7.8, 0.45 mM NADP⁺, 0.9 mM di-isocitrate, and 1.8 mM MgCl2 (16), and 4) 100 mM potassium phosphate, pH 7.8, 1.0 mM KC1, 10 mM MgCl2, 0.8 mM NAD⁺, and 0.4 mM (2R*,3S*)-3-isopropylmalic acid (17). The reaction was started by the addition of the native or the refolded enzyme prepared as above, and the initial rate of increase (or decrease) of the absorbance at 340 nm was measured. Temperatures of enzyme assays were 60°C (LDH and glucose-6-phosphate dehydrogenase) or 68°C (isocitrate dehydrogenase and IPMDH). One unit of activity is defined as the activity producing 1 μmol of product/min.

**Continuous Refolding Assay**

IPMDH denatured as above (13 μg ml⁻¹) was diluted into a 19-fold volume of the dilution buffer containing both *Thermus* chaperonin and components for assays of recovered enzyme activity. Otherwise stated, denatured solution contained final concentrations of 100 mM potassium phosphate, pH 7.8, 60 μg ml⁻¹ of IPMDH, 0.5 mM MgATP, 360 mM guanidine HCl, 1.0 mM KC1, 1 mM MgCl2, 0.8 mM NAD⁺, and 0.4 mM (2R*,3S*)-3-isopropylmalic acid. Refolding of IPMDH was continually monitored by the rate of increasing absorbance at 340 nm at indicated temperatures.

**RESULTS**

Purification of a Chaperonin from *T. thermophilus*—Since cpn60s from various sources have common features, such as their abundance in cells, their molecular weights (around 60,000), their existence as large tetradecameric complexes, and their weak ATPase activities, we have isolated a chaperonin from *T. thermophilus* by pursuing the protein complex with the above features. In fact, SDS-polyacrylamide gel electrophoresis analysis of crude extract from *T. thermophilus* showed the existence of an abundant protein with *M* = 58,000 in *T. thermophilus* (Fig. IA, lane 1). The ATPase activity staining of non-denaturing polyacrylamide gel electrophoresis and subsequent two-dimensional SDS-polyacrylamide gel electrophoresis showed that this protein existed as a large complex with weak ATPase activity (data not shown). This 58-kDa protein was purified by using modified procedures for *E. coli* cpn60 (GroEL) (4). Gel filtration column chromatography with a Sepharose CL6B column was the most effective step for the purification, and large protein complexes were eluted at almost void volume (data not shown). Analysis of this complex with SDS-polyacrylamide gel electrophoresis revealed that this fraction contained not only a polypeptide with an apparent *M* = 58,000 but also another polypeptide with an apparent *M* = 10,000 (Fig. IA, lane 2). This complex migrated as a single band with very low mobility in non-denaturing polyacrylamide gel electrophoresis (Fig. IB, lane 1), indicating that both polypeptides were indeed components of a single protein complex. This was further supported by the analysis of NH₂-terminal amino acid sequences of the complex, as described later, which gave two PTH-derivatives at each cycle. At least 200 mg of purified complex were obtained from 100 g of wet cells, reflecting its abundance in cells.

**NH₂-terminal Amino Acid Sequences**—In order to confirm that the purified complex was really the chaperonin, NH₂-terminal amino acid sequences of the 58- and 10-kDa poly-
Polyacrylamide gel electrophoresis of Thermus chaperonin. A, polyacrylamide gel electrophoresis in the presence of SDS. Lane 1, crude extract (20 μg of proteins) of T. thermophilus. Lane 2, purified Thermus chaperonin (10 μg). Molecular mass standards used are: phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carboxylic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). B, polyacrylamide gel electrophoresis in the absence of SDS. Purified Thermus chaperonin (12 μg) was incubated at 55°C for 20 min in 15 μl of 50 mM potassium phosphate buffer, pH 7.6, containing (Lane 1) no adenine nucleotide, (Lane 2) 500 μM MgATP, or (Lane 3) 500 μM MgAMP-PNP.

peptides were determined by Edman degradation and compared with those of the cpn60 and cpn10 from bacteria, plants, and mitochondria (Fig. 2) (2, 18). When Thermus 58-kDa polypeptide was compared with cpn60 from E. coli (GroEL), wheat ribulose-1,5-bisphosphate carboxylase (Rubisco) subunit-binding protein (Ref. 2), yeast mitochondrial hsp60 (Ref. 18), E. coli cpn10 (GroES) (Ref. 2), M. tuberculosis 10-kDa antigen (Ref. 6). Yeast mitochondrial hsp60 has a longer NH2-terminal amino acid sequence than the others, but the first 19 amino acid residues are not shown to save space. Amino acid residues identical with those of T. thermophilus are shown by white letters in black background.

Molecular Size and Shape of Thermus Chaperonin—Since the Thermus chaperonin was eluted at the void volume from a gel permeation HPLC column (G3000SWxL, Tosoh), its molecular mass should be larger than the exclusion limit of this HPLC column, 500 kDa. When Thermus chaperonin was analyzed with another gel permeation HPLC column (G4000SWxL, Tosoh), it was eluted slightly after blue dextran 2000 (data not shown). Since no appropriate molecular weight standard is available in this range, the molecular weight of Thermus chaperonin was only estimated to be larger than 1,000,000.

Fig. 4 shows an electron micrograph of purified Thermus chaperonin negatively stained with uranyl acetate. Similar to cpn60s from other sources, including E. coli cpn60 (GroEL), the particles have the characteristic 7-fold rotational symmetry when viewed from the top. The diameter of the particles is approximately 13 nm. The side view appears to be somewhat different from E. coli cpn60. Thermus chaperonin is longer than E. coli cpn60 in the direction perpendicular to the stripes, and the stripes themselves are less obvious. The edges of the particles are not sharp, and gross shapes of particles look more like a "football" or a "bullet" than the almost rectangular shapes of E. coli cpn60 (3, 4).

ATPase Activity and Effect of ATP—The Thermus chaperonin has a weak ATPase activity, 0.1 unit mg⁻¹ at 80°C, where it is most active (Fig. 5). Above 80°C, the chaperonin is not stable and its secondary structure starts to denature, as shown by the sharp decrease of the CD spectrum at 220 nm (Fig. 5). The ATPase activity was strongly temperature-dependent, and there was no or very little activity at temperatures lower than 40°C. The effects of various salts and

![Fig. 3. Yield of PTH-derivatives from Thermus cpn60 (©) and cpn10 (●) at each cycle of Edman degradation. Since the NH2-terminal amino acids of cpn60 and cpn10 are the same alanine, the amount of PTH-alanine at the first cycle was approximately double that of other cycles.](image-url)
The scale bar represents 20 nm. The detailed protocol is described under "Experimental Procedures.

The effect of various reagents on the ATP hydrolyzing activity of Thermus chaperonin

A, the effect of salts on the ATPase activity of Thermus chaperonin. Conditions of assays were the same as described under “Experimental Procedures,” except for inclusion of indicated salts in the assay mixtures. B, the effect of preincubation with some chemical modification reagents on the ATPase activity of Thermus chaperonin. The Thermus chaperonin (80 μg ml⁻¹) was dissolved in 0.5 ml of the solution containing 50 mM Tris-HCl, pH 8.0, 10 mM KCl, 5 mM MgCl₂, and the indicated compound. NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; DCCD, dicyclohexylcarbodiimide; NEM, N-ethylmaleimide. After the solution was incubated at 37 °C for 30 min, the assay of ATPase activity was started by adding 5 μl of 100 mM ATP into the solution. The solution was then incubated at 60 °C for 15 min, and the amount of released Pi was measured as described under “Experimental Procedures.

| Reagents | Concentration (mM) | Residual activity (%) |
|----------|--------------------|-----------------------|
| A. None  | —                  | 100                   |
| NaSO₄    | 50                 | 87                    |
| 200      | 14                 |
| Na₂SO₄  | 200                | 84                    |
| NaHCO₃  | 200                | 215                   |
| KF       | 1                  | 71                    |
| NaN₃     | 1                  | 89                    |
| NH₄VO₃  | 0.2                | 72                    |
| KNO₃     | 10                 | 105                   |
| B. None  | —                  | 100                   |
| NBD-Cl   | 0.1                | 69                    |
| NEM      | 0.1                | 68                    |
| DCCD     | 0.1                | 57                    |

As reported for other cpn60s, such as E. coli cpn60 (1), and ribulose-1,5-bisphosphate carboxylase subunit-binding protein (19), preincubation of Thermus chaperonin with ATP caused the appearance of a new fast running band in nondenaturing polyacrylamide gel electrophoresis (Fig. 1B, lane 2). In contrast to E. coli cpn60, which showed a new band only detectable by immunostaining, a significant population of the Thermus chaperonin shifted to a new band. Preincubation with AMP-PNP had no effect on electrophoretic mobility of Thermus chaperonin (Fig. 1B, lane 3).

Chaperonin-dependent Refolding of Several Thermophilic Proteins—We have examined the effect of Thermus chaperonin on the refolding of several enzymes isolated from thermophilic bacteria: IPMDH (a dimer of 37-kDa subunits) and isocitrate dehydrogenase (a dimer of 57.5-kDa subunits) from T. thermophilus and LDH (a tetramer of 35-kDa subunits) from B. stearothermophilus. They are denatured with a high concentration of guanidine HCl, and then guanidine HCl was diluted by injecting the denatured enzyme solution into a large excess of the dilution buffer containing indicated components (Fig. 6). Although they differed from each other in the efficiency of spontaneous refolding under the conditions examined, the effects of ATP on chaperonin-dependent refolding were all similar. In the absence of ATP, recoveries of the enzyme activities were null or significantly lower than spontaneous ones, indicating that Thermus chaperonin prevents spontaneous refolding. In the presence of ATP, maximal refolding was observed for all enzymes. The possibility of direct stimulation of refolded enzymes by Thermus chaperonin was eliminated, since treatment of the native enzymes with the same procedures, except omitting guanidine HCl from the enzyme solutions, caused neither stimulation nor inhibition of the enzyme activities (data not shown). Similar results were obtained when the enzymes were denatured by urea instead of guanidine HCl (data not shown). Thus, Thermus chaperonin can promote refolding of various proteins from thermophilic bacteria in an ATP-dependent manner. Refoldings of IPMDH and LDH were investigated in more detail.
Effect of Temperature on Refolding of IPMDH—Thermus. IPMDH is extremely thermostable (17). No detectable loss of the enzyme activity was observed after incubation at 68°C for 10 min under the same conditions as the refolding assay (Fig. 7A). However, spontaneous refolding of denatured *Thermus* IPMDH appeared to include a more heat-labile process and no or very little spontaneous refolding, as assessed by the recovery of the enzyme activity, was observed above 60°C. In contrast, chaperonin-dependent refolding in the presence of ATP was observed even at 75°C. At temperatures lower than 25°C, chaperonin-dependent refolding in the presence of ATP was very poor, even though spontaneous refolding occurred very efficiently. Since the rates of ATP hydrolysis by chaperonin at these temperatures were very low (Fig. 5), the added ATP could not be used to drive productive refolding processes, although the chaperonin retained the ability to bind refolding intermediates and interfered with the spontaneous refolding. Consistent with this postulation, when the dilution buffer contained *Thermus* chaperonin alone without ATP, only very poor enzyme activity was recovered at low temperatures, as well as at high temperatures.

Effect of Temperature on Refolding of LDH—*B. stearothermophilus* LDH is less stable than *Thermus* IPMDH and is not stable above 60°C. Different from Thermus IPMDH, there was only a very narrow temperature range where *Thermus* chaperonin-dependent refolding occurred but a spontaneous one did not (Fig. 7B). Maximum temperature of productive refolding, irrespective of whether it was spontaneous or chaperonin-dependent, appeared to be limited by the stability of the refolded (native) enzyme. The optimum temperature of the chaperonin-dependent refolding in the presence of ATP was approximately 55°C. As observed in IPMDH refolding, the chaperonin alone in the absence of ATP interfered with the spontaneous refolding at all temperatures.

Chaperonin-dependent Refolding of IPMDH at 68 and 50°C—Chaperonin-dependent refolding of the denatured IPMDH was at first examined at 68°C, where spontaneous refolding did not proceed but once-refolded IPMDH was fully stable. The dilution buffer contained components for the assay of IPMDH activity so as to monitor the recovery of activity continually. When both chaperonin and ATP were included in the dilution buffer, IPMDH activity was recovered gradually after a lag time of about 2 min and then reached a constant rate, 40% of the native enzyme, in about 5 min (Fig. 8, trace A). When ATP was omitted from the dilution buffer, *Thermus* chaperonin alone did not promote the recovery of the activity of IPMDH, but the activity started to recover when ATP was supplemented to the mixture at 6 min after dilution (Fig. 8, trace B). The final activity thus recovered was 20% of the native enzyme. An ATP analogue, AMP-PNP, could not replace ATP to induce refolding (Fig. 8, trace C). When *Thermus* chaperonin and ATP were omitted from the dilution buffer and supplemented to the mixture at 1 or 5 min after dilution, only a very poor or no recovery of the activity was induced (Fig. 8, traces D and E). The addition of *Thermus* chaperonin at the moment of or immediately after the dilution appeared to be essential for efficient refolding and delayed addition of *Thermus* chaperonin to the solution aged after dilution were no longer effective to induce refolding. As pointed out by Goloubinoff et al. (1), an unstable refolding intermediate is formed upon dilution of the chaotrope and, unless chaperonin is present, it rapidly binds each other to form inactive aggregates or it decays to the next forms, which then undergo prodactively assembly. *Thermus* chaperonin appears to bind to this intermediate and to prevent it from aggregation and decay. Indeed, refolding experiments at 50°C, where 55% of *Thermus* IPMDH can refold spontaneously, justified the presence of such interaction between *Thermus* chaperonin and refolding intermediate (Fig. 8, trace G). When
the dilution buffer contained both Thermus chaperonin and ATP, IPMDH activity was recovered gradually and reached a constant rate, 48% of the native enzyme, in about 12 min (Fig. 8, trace B). However, when ATP was omitted from the dilution buffer, recovery of the activity did not occur (Fig. 8, trace I). Thus, Thermus chaperonin interfered with the spontaneous refolding in the absence of ATP. This interference was transient, that is refolding was just arrested, since recovery of the activity started when ATP was supplemented to the mixture at 8 min after dilution (Fig. 8, trace I). ATP alone had no effect on the spontaneous refolding (data not shown).

Chaperonin-dependent Refolding of LDH at 55°C—The time course of Thermus chaperonin-dependent refolding of LDH was examined at 55°C, where denatured LDH refolded spontaneously and recovery of the activity reached the maximum level in 5 min (Fig. 9A). In the presence of chaperonin and ATP, 100% of the activity was recovered, but a much longer time, more than 12 min, was required to reach full recovery. In other words, rate-limiting steps on the refolding pathway are not accelerated by the chaperonin. The other difference between spontaneous and chaperonin-dependent refolding was that spontaneously refolded enzyme, but not the enzyme refolded in the presence of the chaperonin, started losing the activity after maximum refolding was attained (Fig. 9A). This was not due to the specific effect of chaperonin to protect denaturation, since bovine serum albumin, which itself did not have an effect on the time course of refolding, showed a similar protective effect from denaturation on the spontaneously refolded enzyme. When ATP was omitted from the dilution buffer, recovery of the activity was less than 20%, indicating that refolding was arrested by chaperonin (Fig. 9B). However, when ATP was supplemented to this mixture after a 20-min incubation, recovery of the activity was induced and 90% of the activity was finally recovered in 15 min. On the other hand, AMP-PNP was not effective to induce refolding, indicating that the hydrolysis of ATP is required for the release of LDH monomer polypeptides from the chaperonin complex in the form that can undergo productive refolding and subunit assembly.

Molar Ratio of Chaperonin to IPMDH—The chaperonin-dependent refolding of IPMDH was examined as a function of the Thermus chaperonin concentrations (Fig. 10). Optimal refolding occurred when the concentration of Thermus chaperonin was 400 μg ml⁻¹. Since the solution contained 100 nM of the denatured IPMDH (calculated as monomer, 38 kDa), the molar ratio of Thermus chaperonin to IPMDH monomer

**Fig. 9.** Time course of (A) spontaneous and Thermus chaperonin-dependent refolding of LDH and (B) release from arrested refolding by the addition of MgATP at 55°C. Fifteen microliters of the guanidine HCl-denatured LDH (100 μg ml⁻¹) were diluted into 735 μl of the dilution buffer. After the indicated time of incubation at 55°C, the LDH activity was measured at 55°C. (A), the dilution buffer contained (●) 408 μg ml⁻¹ of Thermus chaperonin and 500 μM MgATP, (○) 500 μg ml⁻¹ of bovine serum albumin, or (△) none. B, the dilution buffer contained 408 μg ml⁻¹ of Thermus chaperonin but not MgATP. MgATP at a final concentration of 500 μM (●), MgAMP-PNP at a final concentration of 500 μM (○), or none (△) was added to the solution at 20 min after dilution. Other detailed experimental conditions were described under “Experimental Procedures.”
was about 4:1 when the molecular mass of *Thermus* chaperonin was assumed to be 1,000 kDa. The arrested refolding of IPMDH by *Thermus* chaperonin in the absence of ATP at 40 °C was also measured as a function of the concentration of *Thermus* chaperonin. The molar ratio of *Thermus* chaperonin to IPMDH monomer to achieve nearly complete arrest of refolding was estimated to be about 2:1. These values imply that *Thermus* chaperonin reacts with IPMDH stoichiometrically but not catalytically under the conditions examined. An excess amount of *Thermus* chaperonin caused an inhibition of ATP-dependent refolding. The reason of this inhibition is not known.

**DISCUSSION**

We have reported here the purification of the *Thermus* chaperonin, which is similar to other chaperonin families in subunit molecular weights, NH2-terminal amino acid sequences, electron microscopic images with a 7-fold rotational symmetry from the top view, and the weak ATPase activity. However, in the case of *T. thermophilus*, two protein components of chaperonin, cpn60 and cpn10, were co-purified as a large complex. It was suggested that an equal number of copies of cpn60 and cpn10 was present in *Thermus* chaperonin. This is in contrast to *E. coli* chaperonin, whose two protein components, cpn60 (GroEL) and cpn10 (GroES), have been purified separately (3, 4, 7). Two chaperonin proteins purified from *E. coli* bind with each other to form a binary complex in the presence of ATP (7, 9). Probably, stability of the binary complex of cpn60 and cpn10 of *T. thermophilus* is stronger than that of chaperonins from other sources and did not dissociate into cpn60 and cpn10 during purification procedures.

Possibly related to the above observations, molecular shape of *Thermus* chaperonin is also slightly different from other cpn60 families. Although electron microscopic images of *E. coli* cpn60 from the side view are a characteristic “square” with four clear stripes, those of *Thermus* chaperonins are a football-like structure with less obvious stripes. The “square” images of *E. coli* cpn60 have been interpreted as two layers of a seven-member ring (14-mer of cpn60) (3, 4). Since the *Thermus* chaperonin contains both cpn60 and cpn10, a speculation is possible that the round edges of the football-like structure are occupied by cpn10. However, more extensive electron microscopic studies are required before we can draw molecular architectures of chaperonins.

*In vitro* assays of chaperonin-dependent protein refolding have been first established in the *E. coli* chaperonin system (1). We have shown here that the *Thermus* chaperonin also promotes the refoldings of several thermophilic proteins from their chemically denatured states. The fact that *Thermus* chaperonin can promote refoldings of all of four different proteins examined confirms the concept that chaperonin can promote refolding of a wide range of proteins (20). This implies that the folding intermediates have some common structural features by which the chaperonin can distinguish the intermediates from the other structures and that these features are more or less independent from their amino acid sequences.

It has been postulated that chaperonin-dependent protein refolding proceeds through at least two steps. First (the association step), chaperonin binds to the refolding intermediate of the target protein, which otherwise decays rapidly. Second (the dissociation step), coupling with ATP hydrolysis, the intermediate is released from the complex as a form that preferably follows the productive refolding and/or assembly (1, 9). As were observed for the *E. coli* chaperonin system, two observations, that is, the arrest of refolding by the *Thermus* chaperonin in the absence of ATP and the requirement of a molar excess *Thermus* chaperonin over the target protein for optimal refolding, support the presence of the association step.

In the association step of the *E. coli* chaperonin system, the cpn60 can bind refolding intermediate to form the arrested complex to which the cpn10 and ATP bind subsequently (1, 21). It has been also proposed that *E. coli* cpn60 binds at first cpn10 in the presence of ATP and then binds refolding intermediate (9). In the *Thermus* chaperonin system, the cpn60 and cpn10 form a complex from the beginning that then binds refolding intermediate and ATP. These results mean that the order of binding to cpn60, refolding intermediate at first and cpn10 next or vice versa, is not obligatory.

Since *T. thermophilus* grows optimally at 75 °C and cannot grow under 50 °C, it is not surprising that *Thermus* chaperonin is most active as molecular chaperone at temperatures higher than 50 °C. The effect of the chaperonin on the refolding is varied, as it is dependent on the temperature range of experiments. 1) At high temperatures, above 60 °C, spontaneous refolding fails, but, if once-refolded (native) protein is sufficiently stable, the chaperonin induces productive refolding in an ATP-dependent manner. Delayed addition of ATP is effective to induce refolding, but that of *Thermus* chaperonin is not. 2) At middle temperatures, around 50 °C, spontaneous refolding of the proteins occurs, and the chaperonin arrests this spontaneous refolding in the absence of ATP. Refolding is initiated at the time when ATP is supplemented to the solution of arrested refolding. 3) At low temperatures, below 30 °C, spontaneous refolding also occurs, and the chaperonin arrests this refolding, but the addition of ATP, either at the time of or after dilution, does not induce refolding. These results are well interpreted by the postulation described above. It appears that the *Thermus* chaperonin is active with the association step at all temperatures up to 75 °C but is not so with the dissociation step below 20 °C.

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