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A Chaperonin from a Thermophilic Bacterium, Thermus thermophilus [and Discussion]

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A chaperonin from a thermophilic bacterium, *Thermus thermophilus*

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SUMMARY

Unlike *Escherichia coli* chaperonins, a chaperonin (cpn) from a thermophilic bacterium, *Thermus thermophilus*, consisting of homologues to GroEL (cpn 60) and GroES (cpn 10) is co-purified as a large complex. *Thermus* chaperonin shows a bullet-like shape in the side view seen by electron microscopy, and antibody against cpn 10 binds only to the round side of the bullet. We conclude that a single cpn 60-heptamer ring with two stripes stacks into two layers and a cpn 10 oligomer binds to one side of the layers. The purified *Thermus* chaperonin contains endogenously bound ADP, and incubation with ATP causes a partial dissociation of chaperonin into cpn 60 monomers and a cpn 10 heptamer. The effect of *Thermus* chaperonin on protein refolding upon dilution from guanidine HCl is different at three temperature ranges. At high temperatures above 55°C, where the native proteins are stable but their spontaneous foldings fail, the chaperonin induces productive folding in an ATP-dependent manner. At middle temperatures (25–55°C) where spontaneous foldings of the enzymes occur, the chaperonin slows down the rate of folding without changing the final yield of productive folding. At lower temperatures below 25°C where spontaneous foldings also occur, the chaperonin arrests the folding even in the presence of ATP. When a solution of relatively heat labile protein is incubated at high temperatures, and then residual activity of the protein is measured at its optimal temperature after incubation with ATP, the temperature that causes irreversible heat denaturation of the protein is elevated about 10°C by inclusion of *Thermus* chaperonin in the solution. Furthermore, once the folding intermediate of a protein is captured by *Thermus* chaperonin, it retains the ability to resume productive folding even after exposure to the otherwise denaturing high temperature. These results indicate that during heat denaturation proteins assume the common structure which is recognizable by the chaperonin. Finally, a 'folding intermediate reservoir' model to explain the effect of chaperonin is proposed, and is compared with a 'marsupium' model.

1. INTRODUCTION

The chaperonins (cpns) are a ubiquitous subclass of molecular chaperones or proteins implicated in the folding of other proteins (Ellis & van der Vies 1991; Gething & Sambrook 1992; Lorimer 1992). They include two kinds of proteins, chaperonin 60 (cpn 60, GroEL of *Escherichia coli*), and chaperonin 10 (cpn 10, GroES of *E. coli*) (Hemmingsen *et al.* 1988). The cpn 60 forms a tetradecameric or heptameric oligomer (Hendrix 1979; Hohn *et al.* 1979; Ishii *et al.* 1992; Miller *et al.* 1990; Viitanen *et al.* 1992). It has been proposed that the binding of cpn 10 to cpn 60 is required to couple the hydrolysis of ATP to the release of the folding intermediate from cpn 60 (Bochkareva *et al.* 1992; Goloubinoff *et al.* 1989; Viitanen *et al.* 1990). Because elucidation of the molecular mechanism of chaperonin activity on protein folding may contribute a great deal to our understanding of the general pathway of protein folding, and the *in vitro* chaperonin-dependent folding assay system has been so far mainly restricted to *E. coli* chaperonins, we have developed another system using a thermophilic bacterium, *Thermus thermophilus* (Taguchi *et al.* 1991). In the present communication, we describe the effect of temperatures on folding, the spatial location of cpn 10 in the *Thermus* chaperonin molecule, ATP-induced partial dissociation of the *Thermus* chaperonin into cpn 60 and cpn 10, protection from heat denaturation of heat-labile proteins by the *Thermus* chaperonin, and a simple model of chaperonin function.

2. EXPERIMENTAL PROCEDURES

Isopropylmalate dehydrogenase (IPMDH; (2*R*,3*S*)-3-isopropylmalate dehydrogenase) from *Thermus thermophilus* strain HB8 and from *Escherichia coli* were kind gifts from Dr T. Oshima and his colleagues. Lactate dehydrogenase (LDH) from *Bacillus stearothermophilus* was kindly given by Unitika Corp. (2*R**, 3*S**)-3-isopropylmalic acid, a substrate of IPMDH, was obtained from Wako Pure chemical Corp. Rhodanese from bovine liver was purchased from Sigma. The

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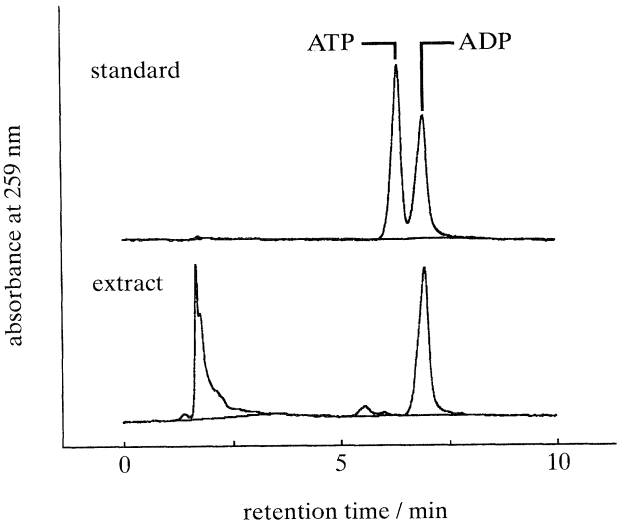

Figure 1. Analysis of the endogenously bound nucleotide of *Thermus* chaperonin by HPLC. Upper trace, ATP and ADP. A mixture of 50 pmol of ADP and ATP were injected into a Supelcosil LC-18T column equilibrated and eluted with 100 mM sodium phosphate, pH 6.0. Lower trace, supernatant fraction of acid-precipitated *Thermus* chaperonin. *Thermus* chaperonin (110 μg in 50 μl) was precipitated by addition of 2.5 μl of 20% of perchloric acid on ice. The supernatant was neutralized with 5 μl of 2.5 M potassium bicarbonate solution. The neutral supernatant fraction (10 μl) was injected to the column.

chaperonin from *Thermus thermophilus* strain HB8 (ATCC 27634) was purified as described in Taguchi *et al.* (1991).

In vitro folding assays were performed as described in Taguchi *et al.* (1991). Rhodanese activity was measured according to Sörbo (1953).

3. PURIFIED *THERMUS* CHAPERONIN CONTAINS ENDOGENOUS ADP

Usually cpn 60 and cpn 10 are separately purified, even though both of them are often required to refold protein. However, in the case of *Thermus* chaperonin they are co-purified as a functional complex (Taguchi *et al.* 1991). It has been shown that formation of the complex between GroEL and GroES from *Escherichia coli* is dependent on ATP (Viitanen *et al.* 1990). Bochkareva *et al.* (1992) reported that purified GroEL contains no endogenous adenine nucleotide and has no ability to bind adenine nucleotide in a stable manner; these authors suggested that ADP, as well as ATP, is effective for the formation of a complex between GroEL and GroES, and that, *vice versa*, dissociation of GroES from GroEL could occur when bound adenine nucleotides are released. Therefore, the stability of *Thermus* chaperonin may be attributable to a strong binding affinity for adenine nucleotide, so we examined the purified *Thermus* chaperonin to see if it contained endogenously bound adenine nucleotide. When *Thermus* chaperonin was precipitated by perchloric acid, and the supernatant fraction was analysed by reverse phase HPLC, a peak appeared at the same retention time as ADP (figure 1); no peak was detected at the position of ATP. Finally, a compound contained in a supernatant fraction was identified as ADP by an enzymic method, i.e. it can be a substrate for pyruvate kinase (data not shown). The stoichiometry of bound ADP differed from preparation to preparation, ranging from 0.3 to 2.8 mol ADP per mol of the *Thermus* chaperonin. The reason for this fluctuation is not known. More evidence may be required to exclude other possibilities, but it is most likely at this moment that the association of cpn 60 and cpn 10 of the *Thermus* chaperonin is stabilized by this tightly bound ADP.

4. LOCATION OF CPN 10 IN *THERMUS* CHAPERONIN

Purified *Thermus* chaperonin was observed by electron micrography (Ishii *et al.* 1992, Ishii *et al.* 1991). Although a heptagonal 'top view' of the *Thermus* chaperonin molecule looks similar to that of cpn 60s from other sources, a 'side view' is not a simple rectangle, but a bullet-like shape which has a round-cap on one side of the rectangle. It should also be noted that the stripes are perpendicular to the axis of the bullet. As a rectangle portion corresponds to $[\text{cpn } 60]_{14}$, the cap portion is probably occupied by cpn10. This conclusion was directly proved by immuno-electron micrography using a specific antibody to *Thermus* cpn 10 (Ishii *et al.* 1992). Two chaperonin molecules are connected by antibodies only through the round tops of the bullet-shaped molecules (figure 2). In addition, there are no chain-like linear aggregates of chaperonins connected by antibodies which should be observed if cpn 10s exist at both ends of the chaperonin. We previously suggested a 1:1 stoichiometry for cpn 60 and cpn 10 (Taguchi *et al.* 1991), but this might be an overestimation of cpn 10. Our current model of chaperonin structure is shown in figure 3. To account for the four stripes, the cpn 60 monomer is assumed to consist of two domains each of which is observed as a stripe when assembled into the $[\text{cpn } 60]_{14}$ structure.

5. ATP INDUCES PARTIAL DISSOCIATION OF *THERMUS* CHAPERONIN INTO CPN 60 MONOMERS AND A CPN 10 HEPTAMER

As reported for other cpn 60s, preincubation of *Thermus* chaperonin with MgATP caused the appearance of a new fast-running band in non-denaturing polyacrylamide gel electrophoresis (figure 4*a*). In contrast to results observed with GroEL–GroES which showed a new band only detectable by immunostaining (Goloubinoff *et al.* 1989), a significant population of the *Thermus* chaperonin shifted to a new band (Taguchi *et al.* 1991). Preincubation with ADP also showed the same effect but AMP and AMPPNP had no effect. This new protein band contained cpn 60 but not cpn 10, and its apparent molecular mass was estimated to be between 57–86 kDa from Ferguson plots of two series of non-denaturing polyacrylamide gel electrophoreses with different gel concentrations

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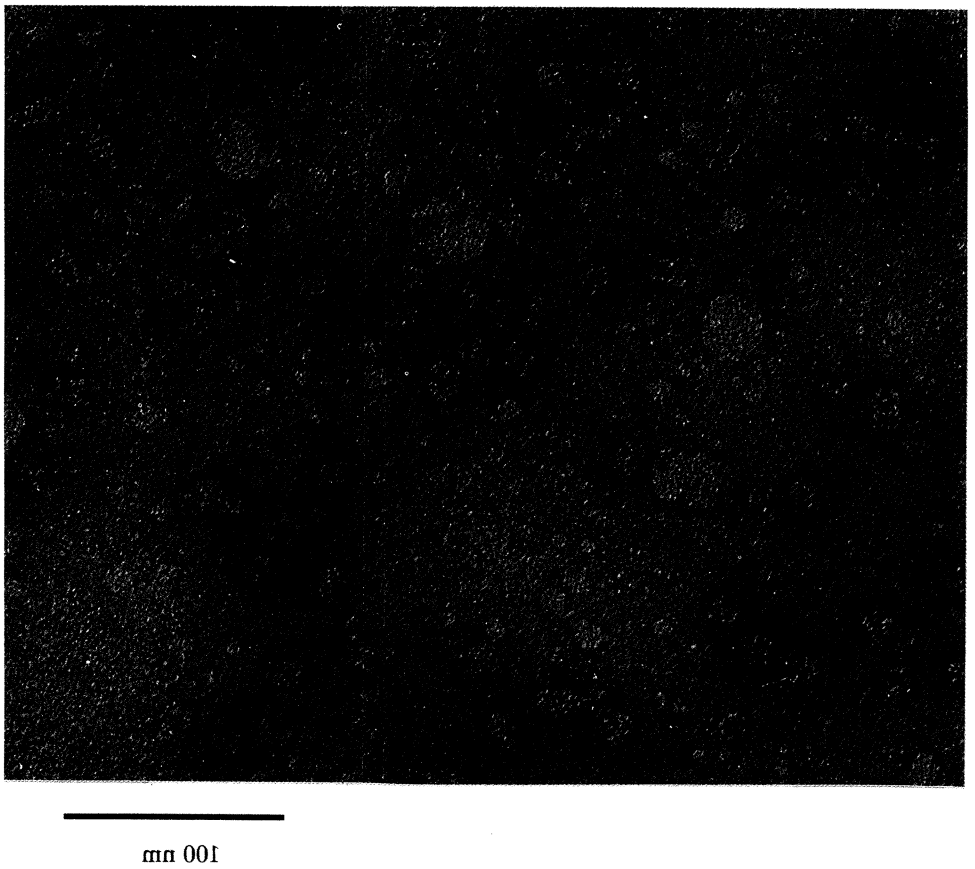

Figure 2. Electron microscopic view of *Thermus* chaperonin-anti-cpn 10 antibody complex. *Thermus* chaperonin and anti-cpn 10 antibody were mixed at a molar ratio of about 1:20 and the mixture was incubated for 24 h at 4°C. The mixture was subjected to gel filtration HPLC on a TSK G3000SW × 1 column with an elution buffer containing 20 mM Tris-SO_4 and 200 mM Na_2SO_4, pH 6.8. A fraction containing both free chaperonins and the chaperonin–antibody complexes was negatively stained with 1% uranyl acetate, and examined by electron microscopy as described previously (Ishii *et al.* 1992).

(figure 4*b*,*c*). This result indicates that the new band corresponds to the monomer of cpn 60. In a separate experiment using pure cpn 10, it was shown that cpn 10 runs slightly slower than cpn 60 monomer in non-denaturing polyacrylamide gels (data not shown). The effect of ATP on the molecular structure of the chaperonin was also studied by gel permeation HPLC (figure 5). When *Thermus* chaperonin was preincubated with MgATP and loaded on a column equilibrated and eluted with a buffer containing MgATP, some protein eluted at 8–11 min as a broad shoulder. This observation indicates that part of the

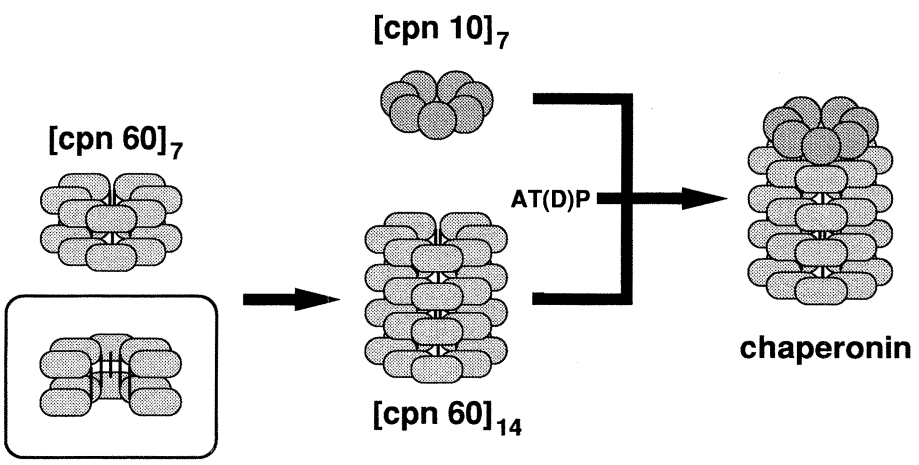

Figure 3. Suggested structure of *Thermus* chaperonin. A cpn 60 monomer has two domains and a single cpn 60-heptamer ring, denoted as $[cpn\ 60]_7$, shows two stripes in the side view. Two $[cpn\ 60]_7$ rings stack into two layers to form $[cpn\ 60]_{14}$ and cpn 10, which may also exist as a heptamer ring, binds only to one end of cylindrical $[cpn\ 60]_{14}$.

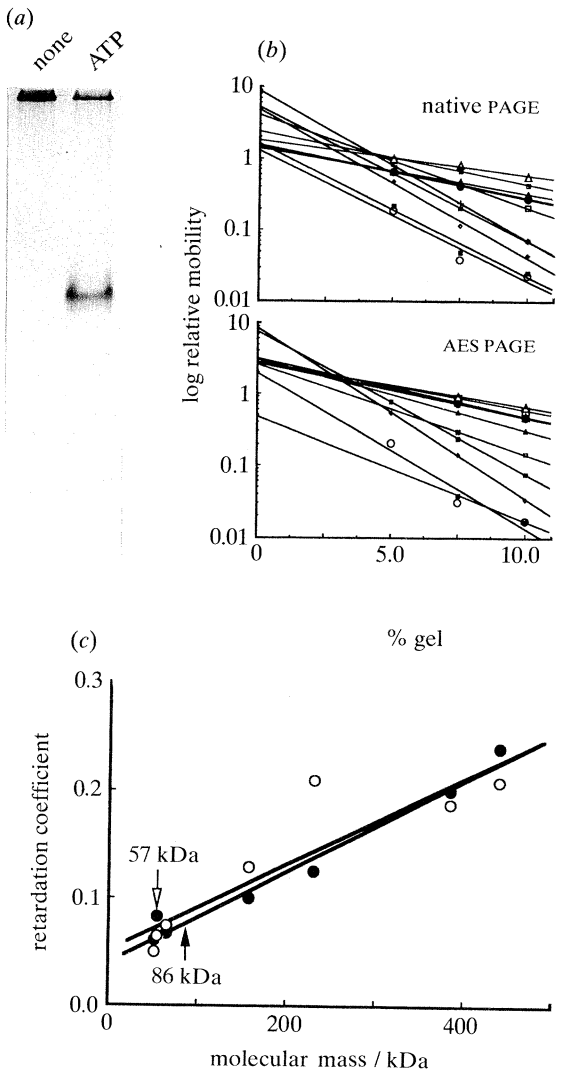

Figure 4. (*a*) Non-denaturing polyacrylamide gel electrophoresis of *Thermus* chaperonin. Purified *Thermus* chaperonin (12 μg) was incubated at 55°C for 20 min in 15 μl of 50 mM potassium phosphate buffer, pH 7.6, without (left lane) or with (right lane) 500 μM MgATP. The gel was stained by Coomassie Brilliant Blue R-250. (*b*) Ferguson plots of the band newly produced in the presence of MgATP. Upper panel non-denaturing polyacrylamide gel electrophoresis; lower panel, polyacrylamide gel electrophoresis in the presence of 0.1% alkyl ethoxy sulphate (AES PAGE). Alkyl ethoxy sulphate is a mild detergent and is expected not to destroy the structure of multisubunit proteins. Molecular mass standards used are thyroglobulin (669 kDa), ferritin (440 kDa), F_1-ATPase from a thermophilic *Bacillus* strain PS3 (385 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), the α subunit of F_1-ATPase (55 kDa), and the β subunit of F_1-ATPase (52 kDa). (*c*) A calibration plot of molecular mass versus retardation coefficient calculated from above Ferguson plots. Open circles, native PAGE; filled circles, AES PAGE. Retardation coefficient was defined as $(\log u - \log u_0)/T$ (u, mobility; u_0, free mobility; T, gel concentration). Arrows indicate the values of the newly produced band of the chaperonin when the *Thermus* chaperonin was preincubated and electrophoresed in the presence of MgATP. Open arrow and circles are results from the non-denaturing gel system and closed ones are those from the alkyl ethoxy sulphate gel system.

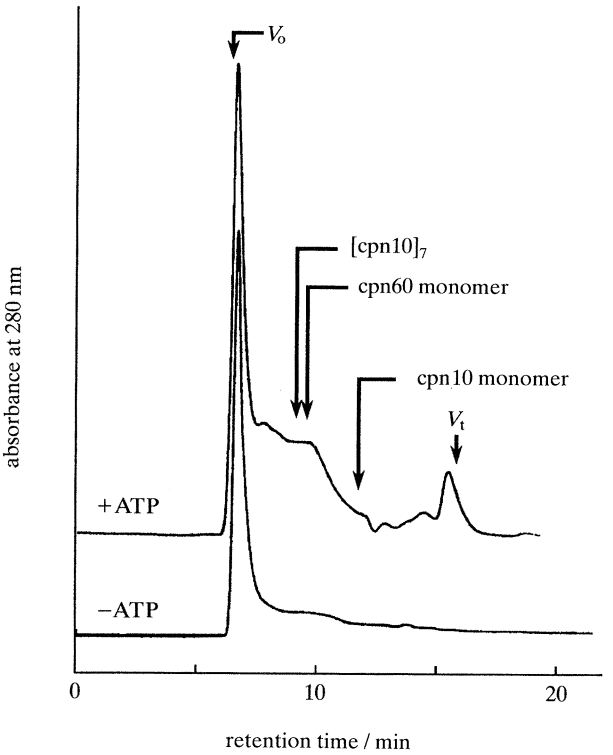

Figure 5. Gel permeation HPLC of *Thermus* chaperonin in the presence (upper trace) or in the absence (lower trace) of MgATP. The purified *Thermus* chaperonin was subjected to gel filtration on a TSK G3000SW × 1 column (flow rate 0.8 ml min^{-1}) with the elution buffer containing 50 mM Tris HCl, pH 6.8, and 100 mM Na_2SO_4. In the upper trace, the *Thermus* chaperonin was preincubated with 50 μM MgATP and was loaded on the column equilibrated with buffer containing 50 μM MgATP. Retention times of cpn 10 heptamer and cpn 60 monomer were obtained from the results using isolated *Thermus* cpn 60 and cpn 10. The retention time of cpn 10 monomer is calculated from a calibration curve of the column. V_o and V_t mean void volume and total volume of the column, respectively.

chaperonin dissociates into low molecular mass species. Analysis of the eluted fractions with polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate shows that the shoulder fractions contained both cpn 10 and cpn 60. If cpn 10 dissociates into the monomer, it should be eluted at about 11.7 min. Therefore, we speculate that cpn 10 exists as a heptamer even after dissociation from cpn 60. In the previous section, we speculate that ADP stabilizes the association of cpn 10 to cpn 60, but here, ATP, as well as ADP, destabilizes the association. This apparent discrepancy could be due to the possible presence of complexes formed between the chaperonin and folding intermediates of some other proteins in our chaperonin preparation, which dissociate into cpn 60 and cpn 10 with concomitant release of intermediate in the presence of AT(D)P. This model can also explain why only part of the chaperonin is dissociated by AT(D)P. However, the effect of adenine nucleotides on the chaperonin is not fully understood in general, and more unambiguous experiments are obviously needed.

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6. *THERMUS* CHAPERONIN ACTS DIFFERENTLY IN THREE TEMPERATURE RANGES

Judging from the temperature-dependency of ATPase activity and the decay of circular dichroism spectrum, the *Thermus* chaperonin is stable up to 80°C (Taguchi *et al.* 1991). When the spontaneous and chaperonin-dependent folding of several chemically denatured enzymes are examined at less than 80°C, three temperature ranges are generally recognized according to the mode of chaperonin action (figure 6). At temperature range I (below 25°C), *Thermus* chaperonin inhibits spontaneous folding and MgATP does not relieve the inhibition. The ATPase activity of *Thermus* chaperonin is almost zero at this temperature range, whereas its ability to bind folding intermediate remains intact. For productive folding the chaperonin should be omitted from the solution. At temperature range II (25–55°C), spontaneous folding is inhibited by *Thermus* chaperonin but this inhibition is relieved by MgATP. The chaperonin is dispensable for folding in this temperature range since spontaneous folding proceeds efficiently. At temperature range III (above 55°C), spontaneous folding no longer occurs and chaperonin plus MgATP is required for productive folding. The chaperonin is indispensable for folding as only chaperonin-dependent folding occurs in this temperature range. The border between temperature range II and III varies from one enzyme to another, and is dependent on experimental conditions. For example, for *Thermus* IPMDH, the temperature range III covers 55–75°C (figure 6*a*) while *B. stearothermophilus* LDH has an only narrow temperature range III, 58–65°C, because of the limited thermal stability of the native LDH (figure 6*b*). Which activities at different temperature ranges reflect the physiological function of the chaperonin? It is noteworthy that the spontaneous folding of proteins appears to involve an intermediate form which is generally more heat labile than the native protein, and is easily irreversibly denatured even at the optimal growth temperature of the cell. Therefore, it is reasonable to conclude that the chaperonin activity in the temperature range III, that is, the protection of folding intermediates from heat denaturation, might correspond to the physiological function. This conclusion also explains why the chaperonin is expressed abundantly at normal physiological temperatures for the *Thermus* cell in the absence of heat shock.

	I	II	III
native	○	○	○
spontaneous	○	○	×
cpn–dependent	×	○	○

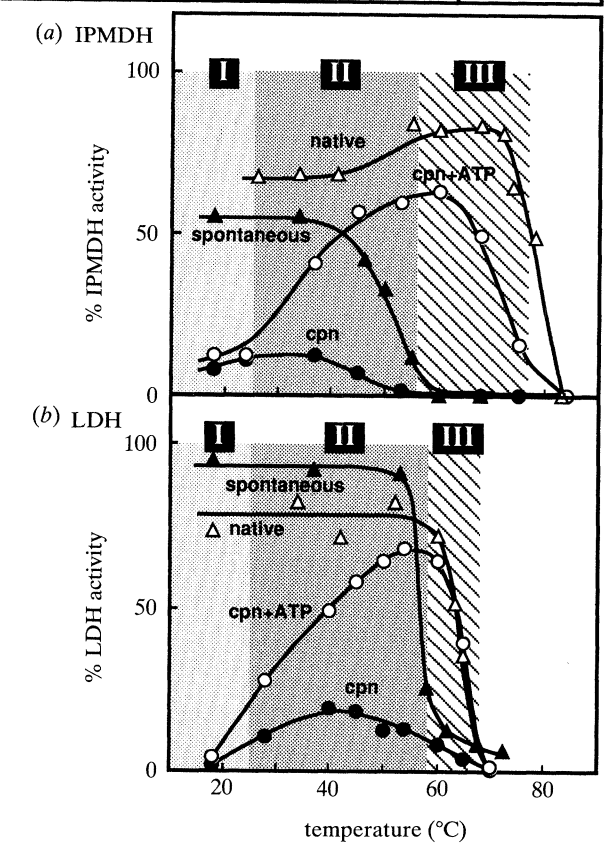

Figure 6. Temperature-dependency of spontaneous and chaperonin-dependent folding of (*a*) *T. thermophilus* IPMDH and (*b*) *B. stearothermophilus* LDH. The enzyme solution containing guanidine HCl-denatured IPMDH or LDH was diluted into the dilution buffer preincubated at the indicated temperature. Incubation was continued for another 10 min (IPMDH) or 30 min (LDH), and then the recovered activities were measured (Taguchi *et al.* 1991). The dilution buffer contained: *Thermus* chaperonin and MgATP (open circle); *Thermus* chaperonin (filled circles); or neither chaperonin nor MgATP (filled triangles). As a control (open triangles), native IPMDH or LDH was treated by the same procedures, except that guanidine HCl was contained not in the enzyme solution but in the dilution buffer at the concentration which gave the same final concentration of guanidine HCl as in the above experiments. Temperature ranges I, II, and III are shown by different shades. Characteristics of each temperature range are shown on top of the figures.

7. *THERMUS* CHAPERONIN INCREASES HEAT STABILITY OF OTHER PROTEINS

It has been demonstrated that the chaperonins from *Escherichia coli* promote folding of proteins that are denatured by high concentration of guanidine HCl or urea. Since chaperonins are heat shock proteins, it is also expected that they can protect native proteins from heat denaturation. Höll–Neugebauer *et al.* (1991) showed that GroEL suppresses the formation of aggregates by binding to thermally denatured α-glucosidase. *Thermus* chaperonin is stable and functional up to 80°C. Heterologous combination of *Thermus* chaperonin and relatively heat-labile enzymes provides a suitable assay system to study the effect of this chaperonin on heat denaturation *in vitro*. A solution of enzyme is incubated at various high temperatures for 30 min; it is then incubated at a fixed moderate temperature in the presence of MgATP, and the residual enzyme activity is assayed. The denaturing temperature of the enzyme thus obtained is elevated by about 10°C by inclusion of *Thermus*

chaperonin in the solution (table 1*a*). Addition of MgATP in the solution during the first incubation abolishes the effect of the chaperonin. The addition of *Thermus* chaperonin to the completely heat-denatured protein does not restore the activity of the protein. These results indicate that during heat denaturation the protein assumes a conformation which is recognizable by the chaperonin. Therefore, an unfolding intermediate produced by thermal denaturation and a refolding intermediate produced after chemical denaturation should have similar structural characteristics. As expected, the complex formed between *Thermus* chaperonin and the folding intermediate of chemically denatured proteins is highly thermostable (table 1*b*). It seems that thermostability of the chaperonin–intermediate complex is limited only by the thermostability of the *Thermus* chaperonin itself. During either refolding or unfolding, once the intermediate is captured by chaperonin, the intermediate retains the ability to resume productive folding even after exposure to high temperatures which otherwise cause rapid denaturation of the protein. Thus, the *Thermus* chaperonin can act as a shelter for labile proteins from transient exposure to high temperature or protein-destabilizing chemicals.

Table 1. *Temperature of 50% loss of activity (°C)*

	LDH (°C)	rhodanese (°C)	IPMDH (°C)
(*a*)			
+BSA	65	53	58
+BSA, then ATP	65	52	58
+cpn	65	52	58
+cpn, then ATP	73	67	67
(*b*)			
[cpn-I], then ATP	74	73	74

(*a*) Protection of relatively heat-labile proteins from irreversible heat denaturation by *Thermus* chaperonin. The experiment consisted of three steps. First step (exposure to high temperature); the enzyme solution (0.1 mg ml^{-1}) containing *B. stearothermophilus* LDH, bovine rhodanese, or *E. coli* IPMDH was diluted 50-fold (LDH) or 25-fold (rhodanese, IPMDH) into the dilution buffer preincubated at various high temperatures, and incubation was continued for 30 min. The dilution buffer contained 5 mM dithiothreitol, 50 mM potassium phosphate, pH 7.8, and, when indicated, bovine serum albumin or *Thermus* chaperonin (0.4 mg ml^{-1} for LDH, 0.2 mg ml^{-1} for the rhodanese, and 0.35 mg ml^{-1} for the IPMDH system). In the rhodanese system, the dilution buffer also contained 50 mM sodium thiosulphate in addition to the above components. Second step (refolding); the solution was transferred to a water bath thermostated at 55°C (LDH) or 37°C (rhodanese, IPMDH) with concomitant addition of 50 μM MgATP (final concentration) and incubated for 30 min (LDH) or 60 min (rhodanese, IPMDH). Third step (assay of residual enzyme activity); the enzyme activity in the solution was measured. This activity is the sum of the activity surviving the first incubation and activity recovered during the second incubation. Temperatures of the first incubation that cause 50% loss of enzyme activity are shown in the table. (*b*) Thermal stability of 'I' captured in the 'cpn–I' complex. The designation of 'I' in the 'cpn–I' is described in figure 7. The stability of 'I' was defined as the retention of the ability to resume refolding when MgATP is supplied. The experiments consisted of four steps. First step ('cpn–I' formation); the enzyme (*B. stearothermophilus* LDH, bovine rhodanese, or *E. coli* IPMDH) was denatured in 6.4 M guanidine HCl and was diluted into the buffer containing *Thermus* chaperonin. The diluted solution was incubated for 5 min at 37°C (rhodanese and IPMDH) or at 55°C (LDH). Second step (exposure to high temperature); the solution was shifted to various high temperatures and incubated for 30 min. Third step (refolding); the solution was shifted again to 37°C (rhodanese and IPMDH) or to 55°C (LDH) with concomitant addition of 50 μM MgATP. Incubation was continued for 60 min (rhodanese and IPMDH) or 30 min (LDH). Fourth step (assay of recovered enzyme activity); the enzyme activity in the solution was measured at 37°C (rhodanese and IPMDH) or at 55°C (LDH). Incubation temperatures of the second step that resulted in 50% loss of enzyme activity at the fourth step are shown in the table.

8. IS THE CHAPERONIN A "RESERVOIR" OR A "MARSUPIUM" FOR THE FOLDING INTERMEDIATE?

General agreement has been reached among researchers on the chaperonins that the main role of these proteins is the suppression of aggregate formation from unfolded or folding intermediates of target proteins (Buchner *et al.* 1991; Gatenby *et al.* 1990; Höll-Neugebauer *et al.* 1991; Kiefhaber *et al.* 1991). When completely unfolded proteins are put into the physiologically relevant solution, hydrophobic interactions at first drive the unfolded proteins to fold into compact forms which continue to fold into the native structures. However, exactly the same interaction also drives the aggregation of the unfolded or partially folded form of proteins. The factor which can affect the kinetic competition between these two processes is the concentration of a common molecular species of protein (a folding intermediate) which can successively undergo either productive folding or irreversible aggregation. The rate of the former process is independent of the concentration of the intermediate, while the latter process is accelerated very much when the concentration of the intermediate increases. Therefore, the simplest model to account for chaperonin function only needs to assume that the chaperonin acts as a "buffer" of folding intermediates (figure 7). According to this "reservoir" model, the role of the chaperonin is a passive one and it is not necessary to assume that protein folding occurs while the protein is bound to the chaperonin. This model assumes only that the chaperonin captures the intermediate rapidly, and releases it slowly with coupling to slow ATP hydrolysis, and thus keeps the concentration of free intermediate in the solution very low to facilitate productive folding. We simulated the kinetics of folding based on the 'reservoir' model by a computer and found that, if parameter were suitably chosen, a similar time course of folding to actual results is obtained (figure 8). Another model is also possible where the folding process of the captured intermediate goes forward while attached to the chaperonin to

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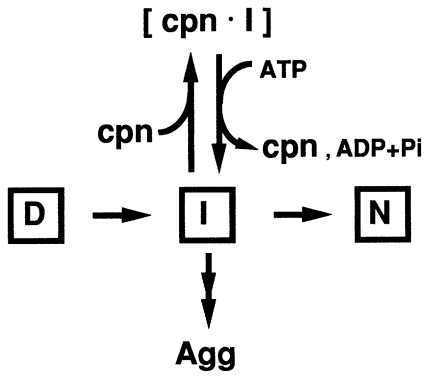

Figure 7. The 'reservoir' model to explain the effect of chaperonins on protein folding. 'D', 'I', 'N', and 'Agg' represent a protein with completely unfolded structure, a folding intermediate, a protein with native structure, and aggregated protein, respectively. 'cpn' indicates functional chaperonin and 'cpn–I' is a complex formed between chaperonin and a folding intermediate.

produce another, more native, intermediate which no longer has a tendency to aggregate in the solution (Figure 9*b*, inset). In this 'marsupium' model, an unstable intermediate is cherished in a safe room in the chaperonin until it grows relatively mature. The results reported by Martin et al favour this model (Martin *et al.* 1991). Is it possible to evaluate the validity of these two models by experiment? According to the 'reservoir' model, the final yield of native protein should increase as the ATP concentration decreases, although the rate of its appearance decreases (figure 9*a*). This latter effect occurs because as the ATP concentration decreases, the rate of ATP hydrolysis becomes more sluggish; thus the release of the intermediate from the chaperonin slows down, the concentration of the free intermediate in the solution decreases, and aggregation is suppressed. In contrast, if the step from I_1 to I_2 is irreversible, the final yield of native protein is independent of ATP concentration according to the "marsupium" model, even though the rate of appearance of native protein decreases as the ATP concentration decreases (figure 9*b*). Thus, the validity of the two models can be tested by experiment and we are attempting to make an appropriate assay system for this purpose.

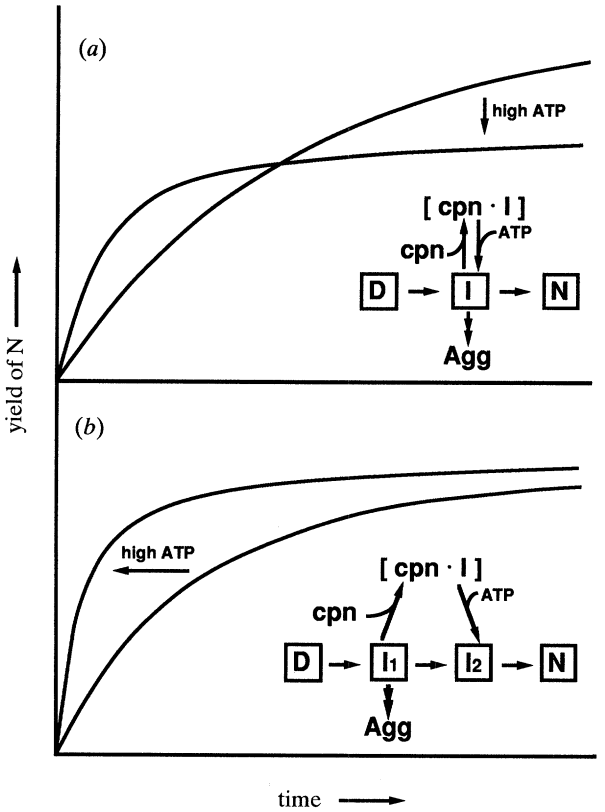

Figure 9. Schematic illustration of the timecourse of folding and the effect of ATP concentration predicted from two possible models. (*a*) Reservoir model; (*b*) marsupium model. The dependency of the yield of productive folding (N) on ATP concentration should be different between the two models. Symbols as in figure 7.

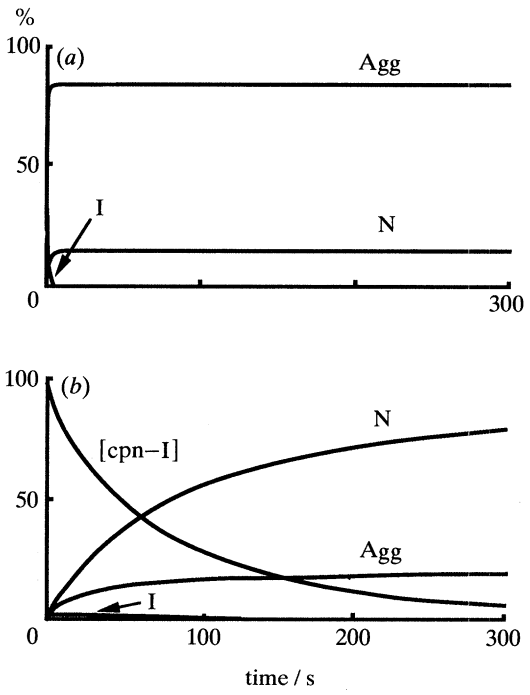

Figure 8. Simulation of the timecourse of the protein folding process based on the 'reservoir' model. (*a*) Without cpn (D: 1 μM); (*b*) with cpn (D: 1 μM; cpn: 3 μM). Assumed parameters are; k (cpn–I formation) = 10^8 M^{-1} s^{-1}; k (cpn–I decay) = 5 s^{-1}; k (aggregation) = 10^7 M^{-1} s^{-1}; k (formation of N) = 0.5 s^{-1}. The formation of dimers of I was regarded as aggregation. Symbols as in figure 7.

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Discussion

G. H. Lorimer (*Du Pont de Nemours, Wilmington, U.S.A.*). What chaperonin molecules have the authors produced in crystalline form, and do these diffract and to what resolution?

M. Yoshida. We have two types of crystal. One is of the chaperonin 60–chaperonin 10 complex and the other is of the chaperonin 60 alone; these crystals diffract to about 7 Å.

G. H. Lorimer. What is the melting temperature of the thermophilic chaperonin?

M. Yoshida. About 80°C.

R. Jaenicke (*Department of Biophysics and Physical Biochemistry, University of Regensburg, F.R.G.*). Have the authors tried to make hybrid molecules between their thermophilic chaperonin and the one from *E. coli*? One could for example dissociate the two types of chaperonin together in 8 M urea and then see if hybrids form when the urea is removed.

M. Yoshida. This has not yet been tried; the thermophilic chaperonin 60–chaperonin 10 complex is so stable that we have not been able to dissociate it into two parts and retain its function.

P. Viitanen (*Du Pont de Nemours, Wilmington, U.S.A.*). Have the authors tried to see whether the chaperonin 10 present in the stable thermophilic chaperonin complex will exchange with chaperonin 10 from *E. coli* in the presence of ATP?

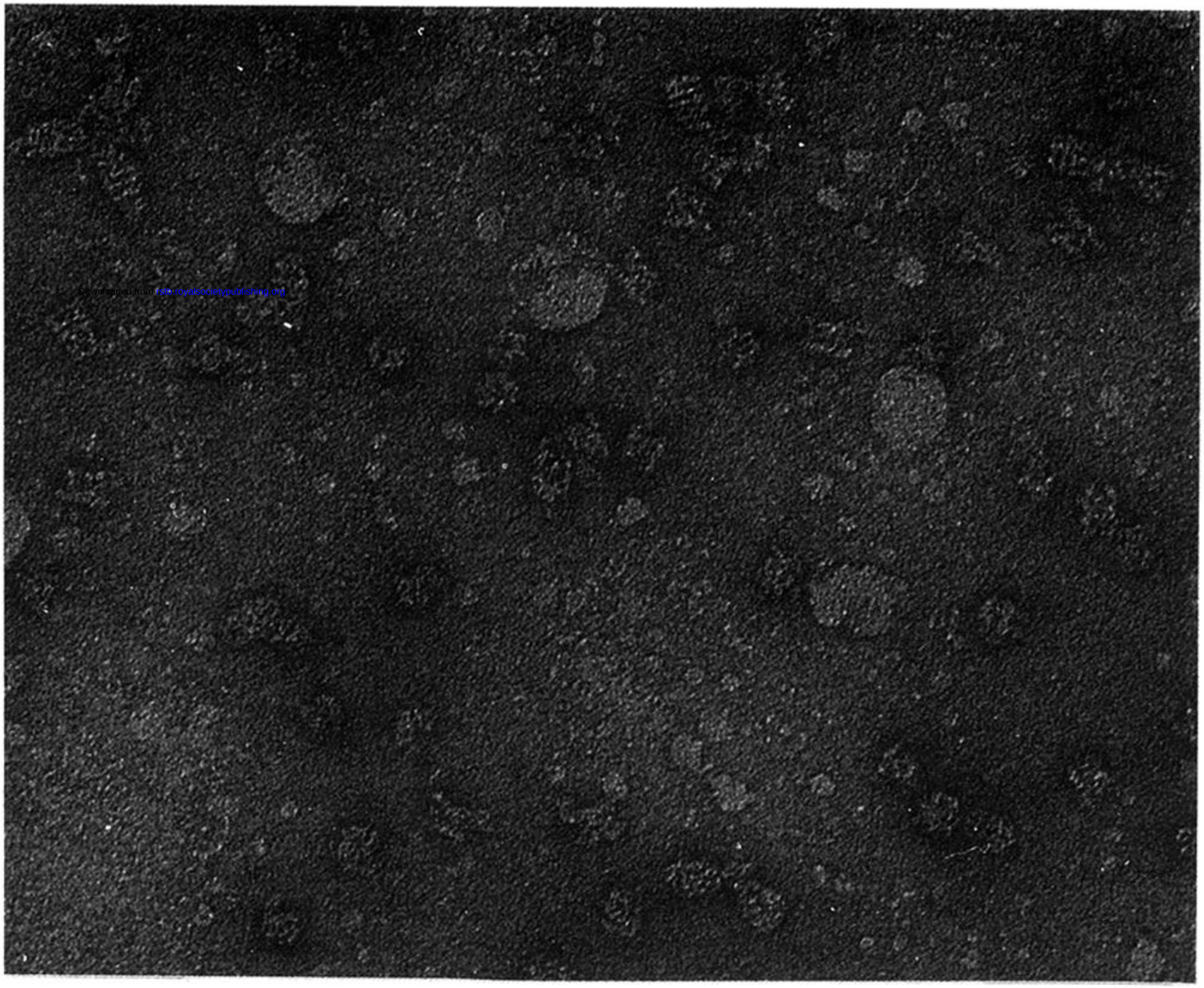
M. Yoshida. The effects of adenine nucleotides are not simple. The chaperonin complex contains bound ADP, but whether this is necessary for stability is not clear. Addition of either ADP or ATP produces a new band as seen on gel electrophoresis.

H. Saibil (*Birkbeck College, University of London, U.K.*). We have shown that the chaperonin 60–chaperonin 10 complex from *E. coli* also looks bullet-shaped in the electron microscope, so it would not be easy to distinguish by this method any hybrids that may be formed with the thermophilic chaperonin.

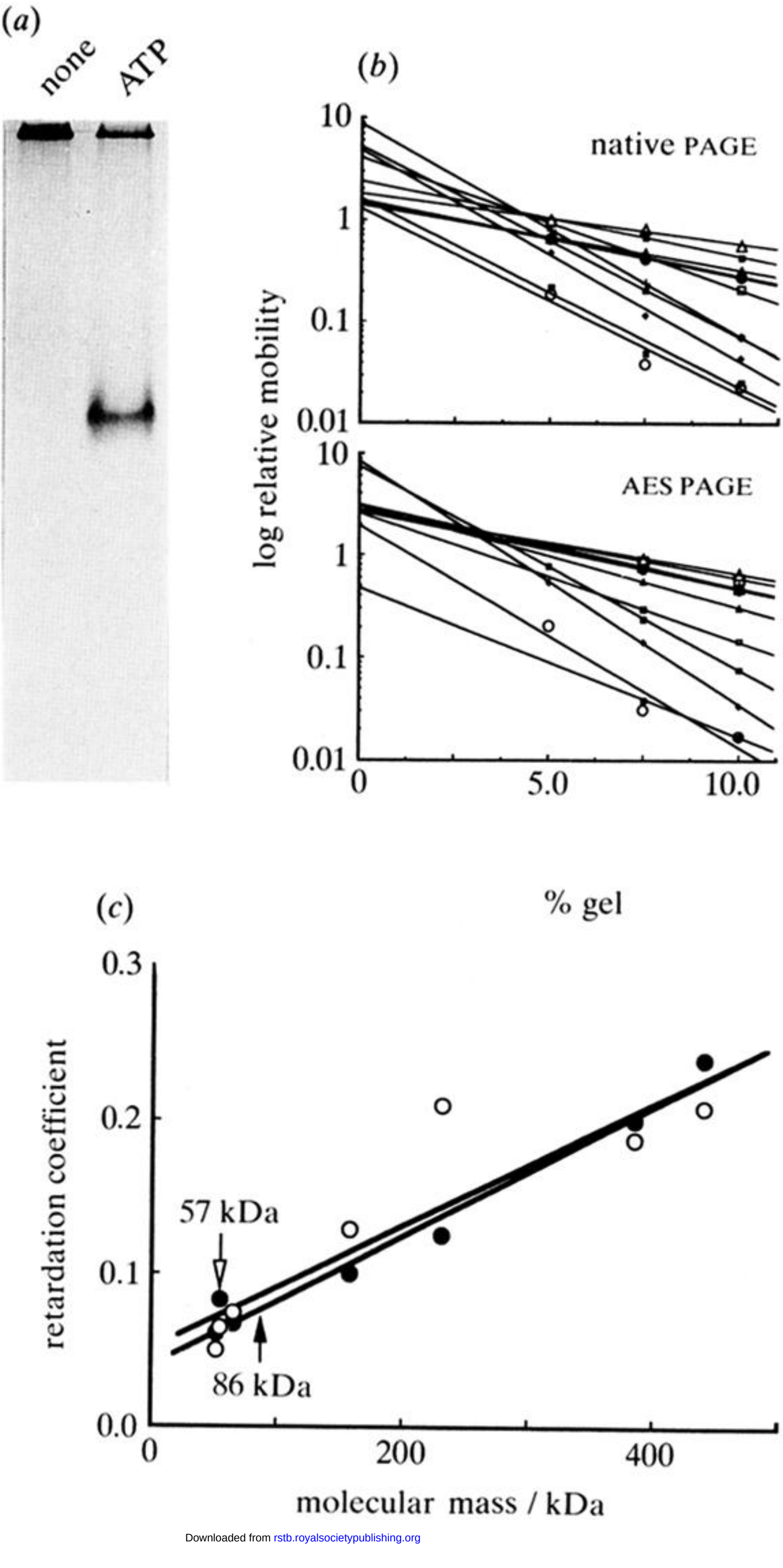
Has anyone examined in the electron microscope, complexes of the beef mitochondrial heptameric chaperonin 60 with chaperonin 10?

G. H. Lorimer. No.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 2. Electron microscopic view of *Thermus* chaperonin-anti-cpn 10 antibody complex. *Thermus* chaperonin and ti-cpn 10 antibody were mixed at a molar ratio of about 1:20 and the mixture was incubated for 24 h at 4°C. The ixture was subjected to gel filtration HPLC on a TSK G3000SW × l column with an elution buffer containing mM Tris-SO_4 and 200 mM Na_2SO_4, pH 6.8. A fraction containing both free chaperonins and the chaperonin–tibody complexes was negatively stained with 1% uranyl acetate, and examined by electron microscopy as scribed previously (Ishii *et al.* 1992).



gure 4. (*a*) Non-denaturing polyacrylamide gel electro-
oresis of *Thermus* chaperonin. Purified *Thermus* chapero-
n (12 µg) was incubated at 55°C for 20 min in 15 µl of
mM potassium phosphate buffer, pH 7.6, without (left
ne) or with (right lane) 500 µM MgATP. The gel was
ined by Coomassie Brilliant Blue R-250. (*b*) Ferguson
ots of the band newly produced in the presence of
gATP. Upper panel non-denaturing polyacrylamide gel
ctrophoresis; lower panel, polyacrylamide gel electro-
oresis in the presence of 0.1% alkyl ethoxy sulphate (AES
GE). Alkyl ethoxy sulphate is a mild detergent and is
pected not to destroy the structure of multisubunit
oteins. Molecular mass standards used are thyroglobulin
69 kDa), ferritin (440 kDa), F_1-ATPase from a thermophi-
Bacillus strain PS3 (385 kDa), catalase (232 kDa), aldo-
se (158 kDa), bovine serum albumin (66 kDa), the α
bunit of F_1-ATPase (55 kDa), and the β subunit of F_1-
TPase (52 kDa). (*c*) A calibration plot of molecular mass
rsus retardation coefficient calculated from above Fergu-
n plots. Open circles, native PAGE; filled circles, AES PAGE.
tardation coefficient was defined as $(\log u - \log u_0)/T$ (u,
obility; u_0, free mobility; T, gel concentration). Arrows
dicate the values of the newly produced band of the
aperonin when the *Thermus* chaperonin was preincubated
d electrophoresed in the presence of MgATP. Open arrow
d circles are results from the non-denaturing gel system
d closed ones are those from the alkyl ethoxy sulphate gel
stem.