FEBS Letters 362 (1995) 121–125 FEBS 15294

Equatorial split of holo-chaperonin from *Thermus thermophilus* by ATP and K^+

Noriyuki Ishii[a], Hideki Taguchi[b], Hiroyuki Sasabe[a], Masasuke Yoshida[b,*]

[a]*Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan*
[b]*Laboratory of Resources Utilization R-1, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan*

Received 24 January 1995; revised version received 22 February 1995

Abstract Holo-chaperonin molecule from *Thermus thermophilus* is a bullet-shaped particle whose cylinder part and round top are composed of two stacked rings of the cpn60 heptamer and a single ring of the cpn10 heptamer, respectively. We found that it splits at the plane between two cpn60 rings into two halves under physiological conditions, that is, in the presence of ATP (but not AMP-PNP, ADP) + K^+ (but not Na^+) at 60°C. This equatorial split could be functionally important although it has not been considered in any current mechanistic model of chaperonin functioning.

Key words: Chaperonin; Chaperonin, split of; GroEL; *Thermus thermophilus*

1. Introduction

Chaperonin has been reported to commit the folding of many different proteins both in vivo and in vitro [1–4]. Several models for the mechanism of chaperonin function have been proposed [5–7]. The two components of chaperonin, cpn60 (GroEL) and cpn10 (GroES), are purified separately from *Escherichia coli* [8–10], and the crystal structure of GroEL was recently reported [11]. From *Thermus thermophilus*, on the contrary, the two components of chaperonin are co-purified as a large complex called holo-chaperonin (holo-cpn) [12]. The gross structure of holo-cpn is bullet-like [13–15]. It is composed of two stacked heptamer rings of cpn60 ($[cpn60]_{14}$), which correspond to *E. coli* GroEL, and a heptamer ring of cpn10 ($[cpn10]_7$) which is associated at one axial end of $[cpn60]_{14}$. Our previous observation by immuno-electron microscopy using an antibody against the substrate protein showed that the folding intermediate binds to the bottom end of bullet-shaped holo-cpn, which is opposite to the end where $[cpn10]_7$ resides [16]. Here, we report the finding that, in the presence of Mg-ATP and K^+, the holo-cpn of *T. thermophilus* splits at its equator plane of the bullet into two components. This kind of equatorial split has not been reported previously.

2. Materials and methods

2.1. Preparation of Thermus holo-cpn

The preparation of holo-cpn from *Thermus thermophilus* strain HB8, isolated according to the method in a previous report [12], was further purified using a gel filtration HPLC column (Beckman UltraSpherogel SEC3000) which was eluted with 50 mM Tris-HCl (pH: 7.51 at 22°C, 7.93 at 0°C, 6.94 at 60°C) containing 200 mM NaCl (buffer A) in order to remove the dimeric form of holo-cpn. The purity was tested with the same gel filtration HPLC and the concentration was determined by the reported method [17].

2.2. Sample treatments and examination with HPLC

Thermus holo-cpn (58 μg) was dissolved in 110 μl of buffer A containing the indicated components (90 mM KCl, 1.5 mM Mg-ADP, 1.5 mM Mg-ATP, 1.5 mM Mg-ADP + 90 mM KCl, 1.5 mM Mg-AMP-PNP + 90 mM KCl, or 1.5 mM Mg-ATP + 90 mM KCl) and the solutions were incubated for 20 min or 60 min at three temperatures (0°C, 22°C, or 60°C). Each nucleotide solution contained the same concentration of $MgCl_2$. After the incubation, each sample solution was analyzed by gel filtration HPLC (Beckman UltraSpherogel SEC3000) at room temperature (22°C). For samples which included 90 mM KCl as a component, 50 mM Tris-HCl (pH 7.51 at 22°C) containing 200 mM KCl (buffer B) was used as an elution buffer. Buffer A was used for the rest of the samples. When adenine nucleotide was included in the sample solution, the elution buffer also contained 0.5 mM of the same adenine nucleotide, except for the case of AMP-PNP which was not added to the elution buffer.

2.3. Electron microscopy

The sample solution was applied onto a specimen grid covered with a carbon support film which was previously made hydrophilic by ion bombardment. The staining solution (1% uranyl acetate) was applied to the grid immediately after the excess sample solution was adsorbed from the side of the grid using pre-water soaked filter paper without insertion of rinsing with water. We omitted the water rinsing step to avoid possible artifacts which might be brought on with a drastic change of ionic strength, chemical constituents and so on. When the sample solution contained inorganic phosphate and nucleotides, it was stained with 2.5% methylamine tungstic acid instead of uranyl acetate which easily formed some insoluble materials. The transmission electron microscope (JEOL JEM2000EX) was operated with an anode voltage of 160 kV. Images were recorded at a magnification of 43,500 on Kodak electron image films (SO-163).

3. Results and discussion

3.1. HPLC analysis of holo-cpn

Fig. 1 shows the elution profiles from the gel filtration HPLC column (Beckman SEC3000) of the *Thermus* holo-cpn after incubation with reagents indicated at each trace. A peak at 6.1 ml of elution volume in Fig. 1 corresponds to *Thermus* holo-cpn. A small shoulder at 5.6 ml of this peak contains the dimeric form of holo-cpn which is formed bottom-to-bottom binding of two *Thermus* holo-cpns (Ishii and Yoshida, unpublished observation). Peaks eluted after 8.0 ml are monomeric cpn60, and $[cpn10]_7$. Elution profiles of the samples incubated with Mg-ADP ± KCl or Mg-ATP were all similar to the elution profile of holo-cpn alone at three temperatures (0°C, 22°C and 60°C). Longer incubation (60 min) did not affect elution profiles (data not shown). However, only when holo-cpn was incu-

*Corresponding author. Fax: (81) (45) 924 5277.

Abbreviations: cpn60, chaperonin-60; cpn10, chaperonin-10; holo-cpn, holo-chaperonin; HPLC, high pressure liquid chromatography; AMP-PNP, adenylylimidodiphosphate.

SSDI 0014-5793(95)00222-7

bated for 20 min with Mg-ATP + KCl, the peak at 6.1 ml decreased and a new peak at 6.9 ml emerged (*trace g, o, w*), indicating that dissociation of the holo-cpn molecule occurred. The temperature of the gel filtration HPLC column used for *trace w* was 22°C but similar dissociation was observed when the column was heated at 60°C (data not shown). The percentages of the dissociated fractions estimated from the elution profiles were 59% at 0°C, 60% at 22°C, and 46% at 60°C. These figures were not changed significantly after prolonged incubation (60 min, *trace h, p*) except for *trace x* where the shoulder at 6.9 ml decreased. A relatively low yield of the dissociated fraction at 60°C and a further decrease after prolonged incubation might be the reflection of the ATPase activity at 60°C which is more than ten times higher than that at 22°C and 0°C [12]. During 20 min and 60 min incubations, produced ADP is accumulated which probably stimulates the re-association of

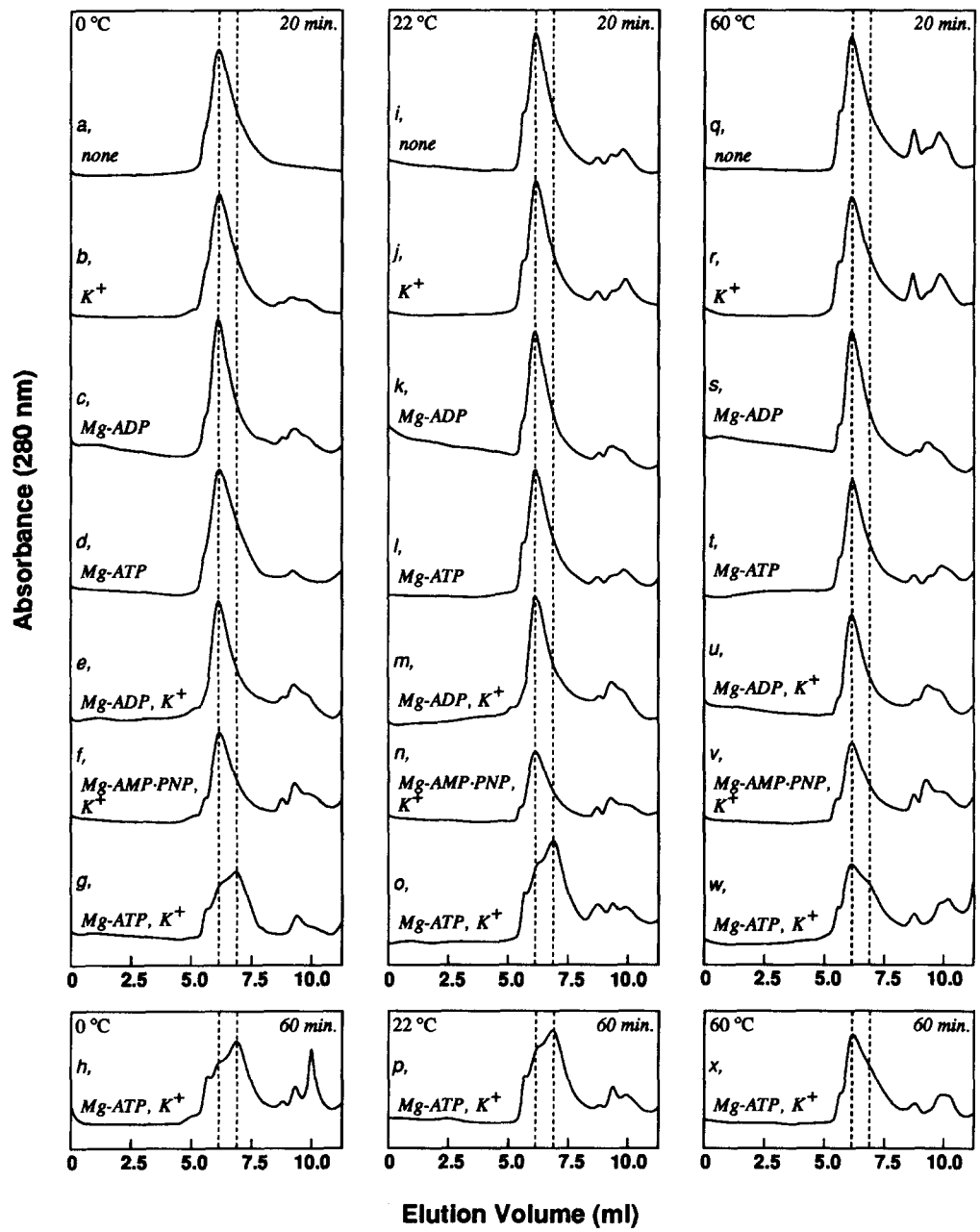

Fig. 1. Gel filtration HPLC chromatograms of *Thermus* holo-cpn after the incubation for 20 min at three temperatures (0°C, 22°C or 60°C). Constituents of solutions used for incubations and elutions are follows. *(a, i, q)* incubation, 50 mM Tris-HCl (pH: 7.51) and 200 mM NaCl (buffer A); elution, buffer A. *(b, j, r)* incubation, buffer A + 90 mM KCl; elution, 50 mM Tris-HCl (pH:7.51) and 200 mM KCl (buffer B). *(c, k, s)* incubation, buffer A + 1.5 mM Mg-ADP; elution, buffer A + 0.5 mM Mg-ADP. *(d, l, t)* incubation, buffer A + 1.5 mM Mg-ATP; elution, buffer A + 0.5 mM Mg-ATP. *(e, m, u)* incubation, buffer A + 1.5 mM Mg-ADP + 90 mM KCl; elution, buffer B + 0.5 mM Mg-ADP. *(f, n, v)* incubation, buffer A + 1.5 mM Mg-AMP-PNP + 90 mM KCl; elution, buffer B. *(g, o, w)* incubation, buffer A + 1.5 mM Mg-ATP + 90 mM KCl; elution, buffer B + 0.5 mM Mg-ATP. *(h, p, x)* are chromatograms of *Thermus* holo-cpn after the incubation for 60 min at three temperatures (0°C, 22°C or 60°C). Constituents of solutions are; incubation, buffer A + 1.5 mM Mg-ATP + 90 mM KCl; elution, buffer B + 0.5 mM Mg-ATP. All chromatographies were carried out at 22°C. The positions of holo-chaperonin (6.1 ml) and split particles (6.9 ml) are indicated by dashed lines. Detailed experimental conditions are described in section 2.

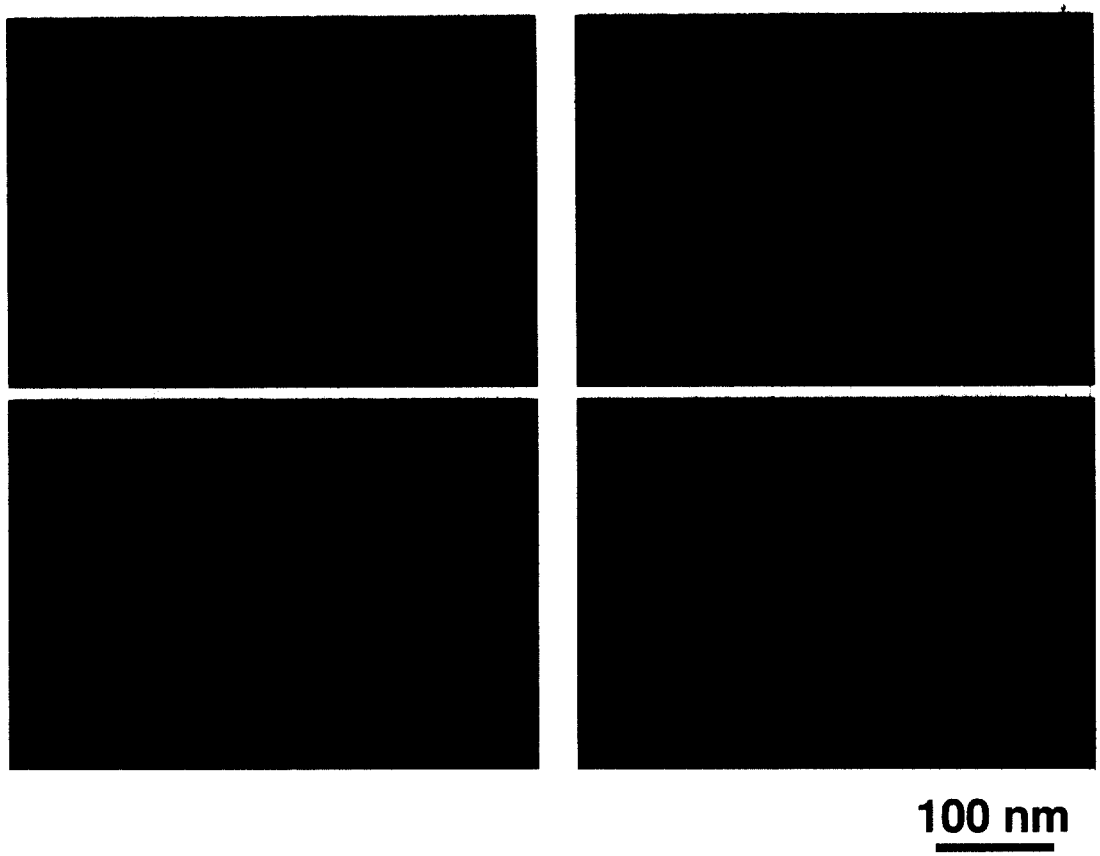

Fig. 2. Electron micrographs of (A) native *Thermus* holo-cpn stained with methylamine tungstic acid, (B) native *Thermus* holo-cpn stained with uranyl acetate for reference, (C) and (D) the fractions at 6.9 ml from gel filtration HPLC (trace o in Fig. 1) stained with methylamine tungstic acid, different fields of the same specimen grid. Detailed experimental conditions are described in section 2. Typical bullet-shaped *Thermus* holo-cpn molecules in side-on and in end-on views are seen in both (A) and (B). Cone-like particles are seen in (C) and round particles with clear central cavity are seen in (C). Round particles with obscured central cavity are seen both in (C) and (D).

the dissociated forms and thus reversibility of the dissociation is suggested. Buffers used for experiments of *trace d, l, t* contained 200 mM NaCl and it was evident that NaCl could not replace KCl to induce the dissociation. Since the dissociation was observed at 60°C where *T.thermophilus* can grow and *Thermus* holo-cpn has a good chaperone activity, the dissociation may have functional significance. Because Mg-AMP-PNP cannot replace Mg-ATP to induce dissociation (*trace f, n, v*), ATP hydrolysis is necessary for this dissociation. Although the rate is slow [12], *Thermus* holo-cpn can still hydrolyze ATP at low temperature; for example, one holo-cpn hydrolyzed three ATP molecules per min at 18°C. A small number of (or even a single) catalytic turnover of ATP hydrolysis seems to be sufficient to induce the dissociation of holo-cpn.

3.2. Electron microscopic observation of split holo-cpn

Molecules contained in the peak fraction at 6.9 ml of *trace o* in Fig. 1 were observed by electron microscopy. Native *Thermus* holo-cpn negatively stained with methylamine tungstic acid is shown in Fig. 2A. When inorganic phosphate was included in the specimen, we used methylamine tungstic acid instead of uranyl acetate which produced insoluble material in the presence of nucleotide and inorganic phosphate. Native *Thermus* holo-cpn stained with uranyl acetate in the absence of inorganic phosphate is shown in Fig. 2B as a reference. Images of molecules in the fractions at 6.9 ml are shown in Fig. 2C and D. Both were derived from the same fractions, but from different fields of the specimen grid. Holo-cpn (bullet-like particle) was not seen in any fields. In Fig. 2C, it is easy to distinguish between a side-on view of 'half a football' (cone-like particles) and the end-on view looking down the seven-fold axis of symmetry whose central cavity is not seen. In Fig. 2D, there are two kinds of round particles; one is the same as round particles with obscured central cavity seen in Fig. 2C and the other has a clear central cavity (ring-like particle). We assume that the cone-like particle corresponds to the upper half of the bullet-shaped holo-cpn and the ring-like particle corresponds to the lower half. The population of images of cone-like particles, round particles with obscured central cavity, round particles with clear central cavity are 25%, 42%, and 32%, respectively. Particles with two stripes apparently corresponding to a side-on view of the lower half were seen only rarely (1%). Similar images were observed for the 6.9 ml fractions at 60°C (data not shown).

3.3. Immuno-electron micrograph of split holo-cpn

Holo-cpn molecules were treated with anti-cpn10 IgGs at first by the same procedure employed in our previous report [14]. Two holo-cpn molecules were connected by anti-cpn10 IgGs (Fig. 3A). Then the solution was incubated for 30 min

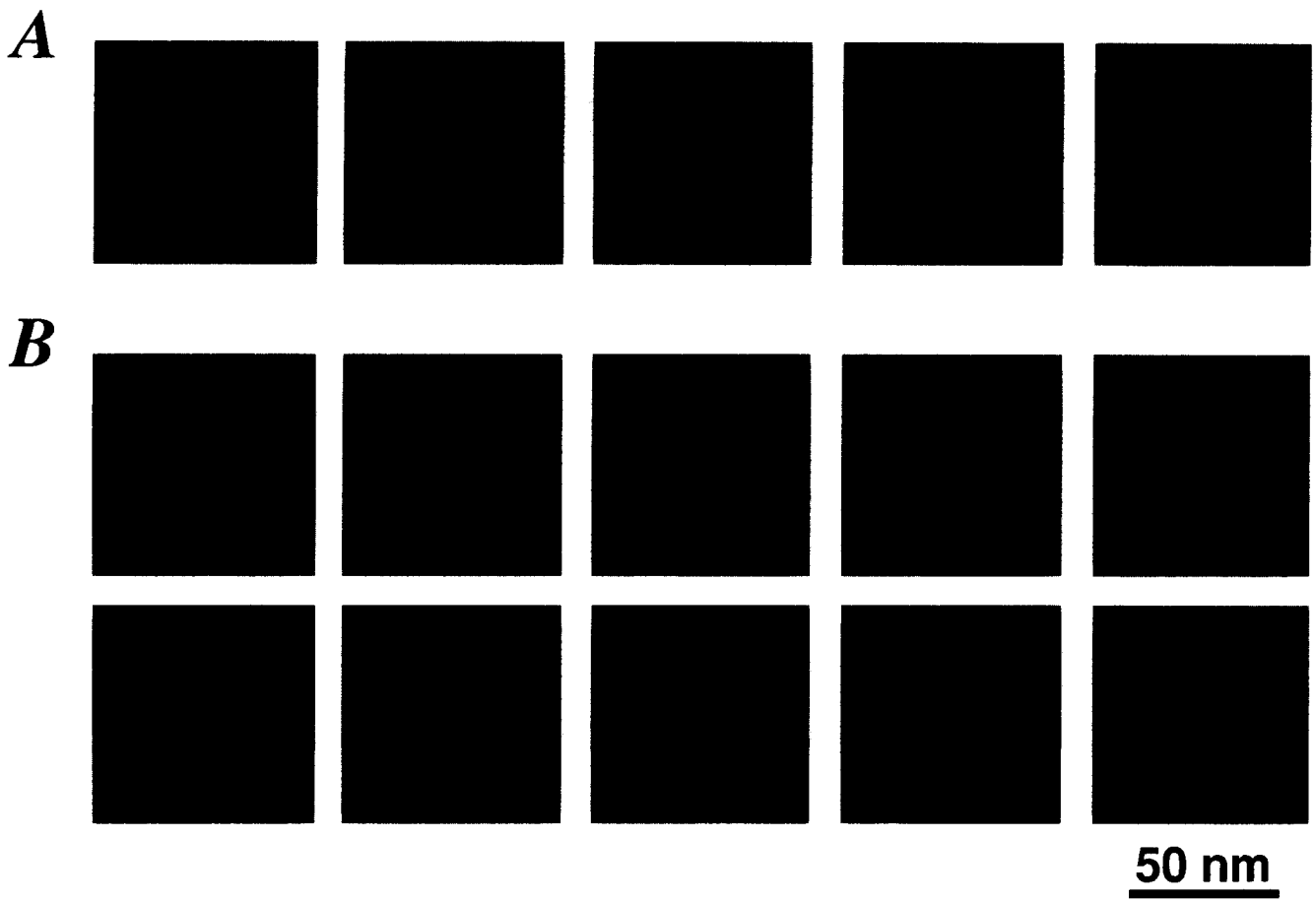

Fig. 3. Galleries of electron micrographs of (A) two native *Thermus* holo-cpn molecules connected by anti-cpn10 IgGs, and (B) those after the incubation for 30 min at 22°C with 50 mM Tris-HCl buffer (pH: 7.51) containing 200 mM NaCl, 90 mM KCl and 1.5 mM Mg-ATP. Detailed experimental conditions are described in section 2. Particles in (B) lost lower halves of connected holo-cpns in (A).

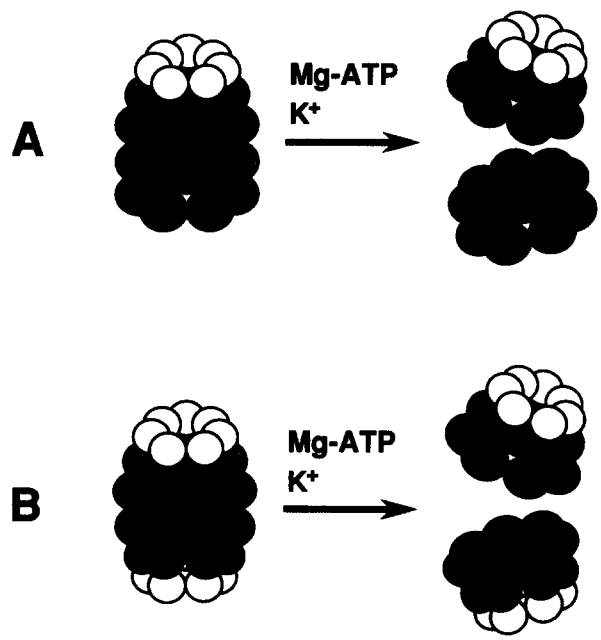

Fig. 4. Models of splitting *Thermus* holo-cpn into two halves at the equator plan. This dissociation occurs only in the presence of Mg-ATP + K^+. cpn60 and cpn10 are shown as shaded and white particles, respectively. (A) Asymmetric split. (B) Symmetric split.

with Mg-ATP + K^+ at 22°C, and the sample was observed with electron microscope. The bullet-shaped holo-cpn disappeared and two cone-like particles connected by anti-cpn10 IgGs through round tops were observed (Fig. 3B). Irregular aggregates which seemed to be connected by IgGs were also observed (not shown). Particles shown in Fig. 3B are not side-on view of $[cpn60]_{14}$ since four stripes are more obscure, the distance between the central two stripes is much larger than that of $[cpn60]_{14}$, and there are spike-like protrusions between central stripes which represent attached IgG molecules. Thus, cpn10s reside at the round top of the cone-like particle which is derived from upper half of the bullet-shaped holo-cpn. From these observations, it was concluded that holo-cpn splits into two particles in the presence Mg-ATP + K^+ and the split occurs at the boundary plane ('equatorial' split) between two rings of $[cpn60]_7$ in the bullet-shaped holo-cpn molecule as illustrated by Fig. 4A.

3.4. Implication of the equatorial split

The conditions to induce the equatorial split, 1.5 mM Mg-ATP and 90 mM KCl, are nearly physiological conditions [18] and are suitable for holo-cpn to mediate protein folding in vitro. ATP hydrolysis seems to be required for the split. K^+ is also required. The effect of K^+ is not due to simple ionic strength since Na^+ cannot replace K^+. Specific requirement for K^+ may be a consequence of its specific stimulating effect on

ATPase activity of chaperonin [19,20]. Thus, the equatorial split of holo-cpn reported here is not an artifact which is observed only under nonphysiological extreme conditions. Although the functional role of the split remains unknown, observations reported for mammalian mitochondrial cpn60 could be relevant to our finding. Mammalian mitochondrial cpn60 is purified as a single heptamer ring and it can facilitate the protein folding with the aid of mammalian mitochondrial homolog of cpn10 in the presence of Mg-ATP + K^+ [21]. No tetradecameric form of mitochondrial cpn60 has been found and there is a possibility that functional cycle of mitochondrial chaperonin does not include an intermediate with tetradecameric form of cpn60. If so, there should be a cone-like complex, made up from a heptamer of cpn60s and a heptamer of cpn10 homologs, as one of intermediates in the catalytic cycle of the mitochondrial chaperonin. Also the symmetrical complex of *E. coli* chaperonin [22–24] in which rings of $[GroES]_7$ are bound to both ends of $[GroEL]_{14}$ can be related to the split reported here. This symmetrical complex has been proposed to be one of the intermediates in the chaperonin catalytic cycle [7]. As illustrated in Fig. 4B, a possibility is arising that this symmetrical complex is a precursor form to split into two cone-like forms since our preliminary result shows that the addition of excess *Thermus* cpn10 into *Thermus* holo-cpn stimulates the split (Yamakoshi and Yoshida, unpublished observation).

Acknowledgements: We thank Drs. T. Furuno, C. Toyoshima and W. Knoll for discussion. This work was supported by a grant for 'Special Researchers' Basic Science Program' from the Science and Technology Agency and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of the Japanese Government (to N.I.).

References

[1] Ellis, R.J. and van der Vies, S.M. (1991) Annu. Rev. Biochem. 60, 321–347.
[2] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[3] Lorimer, G.H. (1992) Curr. Opinion Struct. Biol. 2, 26–34.
[4] Hendrick, J.P. and Hartl, F.-U. (1993) Annu. Rev. Biochem. 62, 349–384.
[5] Agard, D.A. (1993) Science 260, 1903–1904.
[6] Martin, J., Mayhew, M., Langer, T. and Hartl, F.U. (1993) Nature 366, 228–233.
[7] Todd, M.J., Viitanen, P.V. and Lorimer, G.H. (1994) Science 265, 659–666.
[8] Hendrix, R.W. (1979) J. Mol. Biol. 129, 375–392.
[9] Hohn, T., Hohn, B., Engel, A., Wurtz, M. and Smith, P.R. (1979) J. Mol. Biol. 129, 359–373.
[10] Chandrasekhar, G.N., Tilly, K., Woolford, C., Hendrix, R. and Georgopoulos, C. (1986) J. Biol. Chem. 261, 12414–12419.
[11] Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimiak, A., Horwich, A.L. and Sigler, P.B. (1994) Nature 371, 578–586.
[12] Taguchi, H., Konishi, J., Ishii, N. and Yoshida, M. (1991) J. Biol. Chem. 266, 22411–22418.
[13] Ishii, N., Taguchi, H., Yoshida, M., Yoshimura, H. and Nagayama, K. (1991) J. Biochem. 110, 905–908.
[14] Ishii, N., Taguchi, H., Sumi, M. and Yoshida, M. (1992) FEBS Lett. 299, 169–174.
[15] Yoshida, M., Ishii, N., Muneyuki, E. and Taguchi, H. (1993) Phil. Trans. R. Soc. Lond. B 339, 305–312.
[16] Ishii, N., Taguchi, H., Sasabe, H. and Yoshida, M. (1994) J. Mol. Biol. 236, 691–696.
[17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[18] Christian, J.H.B. and Waltho, J.A. (1962) Biochim. Biophys. Acta 65, 506–508.
[19] Viitanen, P.V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P. and Lorimer, G.H. (1990) Biochemistry 29, 5665–5671.
[20] Todd, M.J., Viitanen, P.V. and Lorimer, G.H. (1993) Biochemistry 32, 8560–8567.
[21] Viitanen P.V., Lorimer, G.H., Seetharam, R., Gupta, R.S., Oppenheim, J., Thomas, J.O. and Cowan, N.J. (1992) J. Biol. Chem. 267, 695–698.
[22] Llorca, O., Marco, S., Carrascosa, J.L. and Valpuesta, J.M. (1994) FEBS Lett. 345, 181–186.
[23] Azem, A., Kessel, M. and Goloubinoff P. (1994) Science 265, 653–656.
[24] Schmidt, M., Rutkat, K., Rachel, R., Pfeifer, G., Jaenicke, R., Viitanen, P., Lorimer, G. and Buchner, J. (1994) Science 265, 656–659.