ATP Binding to Nucleotide Binding Domain (NBD1) of the ClpB Chaperone Induces Motion of the Long Coiled-coil, Stabilizes the Hexamer, and Activates NBD2

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The molecular chaperone ClpB can rescue the heat-damaged proteins from an aggregated state in cooperation with other chaperones. It has two nucleotide binding domains (NBD1 and NBD2) and forms a hexamer in a manner dependent on ATP binding to NBD1. In the crystal structure of ClpB with both NBDs filled by nucleotides, the linker between two NBDs forms an 85-Å-long coiled-coil that extends on the outside of the hexamer and leans to NBD1. To probe the possible motion of the coiled-coil, we tested the accessibility of a labeling reagent, fluorescence change of a labeled dye, and cross-linking between the coiled-coil and NBD1 by using the mutants with defective NBD1 or NBD2. The results suggest that the coiled-coil is more or less parallel to the main body of ClpB in the absence of nucleotide and that ATP binding to NBD1 brings it to the leaning position as seen in the crystal structure. This motion results in stabilization of the hexamer form of ClpB and promotion of ATP hydrolysis at NBD2.

A molecular chaperone ClpB is unique in its activity to rescue heat-damaged proteins from an aggregated state in cooperation with a trio DnaK chaperone set, DnaK/DnaJ/GrpE. To date, such disaggregation activities have been demonstrated for ClpB and its homologues of yeast, Thermus thermophilus, Escherichia coli, and mitochondria (1–8). ClpB is a member of AAA+ protein superfamily, and, similar to other members, it assembles into a hexamer in an ATP-dependent manner (9–15). It has two nucleotide binding domains (NBD(s)) in a single polypeptide, NBD1 and NBD2, each containing a set of Walker A and B motifs and having a RecA-like fold in which the nucleotide binding cleft exists between the P-loop subdomain and helical subdomain. In addition, ClpB has a unique inserted 120-amino-acid sequence between two NBDs, and this insertion has been inferred to contribute to the disaggregation activity of ClpB (15, 16). The crystal structure of ClpB from T. thermophilus revealed an unusual structure of the inserted region, an 85-Å-long, straight coiled-coil extending on the outside of the hexamer (17). The coiled-coil is tethered to the main body of ClpB at its middle point, resembling in structure the shape of a two-wing propeller, one wing (wing-1) leaning to NBD1 and another wing (wing-2) away from any other domains (see Fig. 1). This “leaning position” of the coiled-coil seems to be stabilized by the hydrophobic interactions between the wing-1 and the α-helical subdomain of NBD1. The coiled-coil structure by itself is stabilized by the leucine zipper-like interaction between two helices. The importance of the position and movement of the coiled-coil on the chaperone activity was demonstrated previously (17). To know further the movement of the coiled-coil in the chaperone function, we examined reactivity and fluorescence change of a fluorescent label and cross-linking of NBD1 and the wing-1 of T. thermophilus ClpB. The contribution of nucleotide occupancy and hydrolysis of the two NBDs to the movement of the coiled-coil was assessed from the results of defective mutants of NBDs. The results suggest that the coiled-coil is in the more parallel position than in the crystal structure relative to the main body of ClpB when NBD1 is empty, but it takes the leaning position when NBD1 is filled with ATP. This arrangement of the coiled-coil would stabilize the hexamer and promote ATP hydrolysis in NBD2.

EXPERIMENTAL PROCEDURES

Proteins—The recombinant plasmid pMCB1 (2) containing a ClpB gene of T. thermophilus was used for a mutagenesis template. Site-directed mutagenesis was performed by overlap extension PCR method (18, 19) using Ex-taq DNA polymerase (Takara). The mutations were confirmed by DNA sequence analysis. ClpB and its mutants were expressed in E. coli BL21(DE3) and purified as described (2) except that a 30-min heat treatment at 80 °C was added after cell disruption.

Fluorescence Measurement—7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) was purchased from Molecular Probes. Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Sigma. ClpB mutants (2–6 mg/ml) were incubated with 1 mM ABD-F in 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, and 5 mM TCEP at 55 °C for 1–2 h. In the case of triple mutant (G167C/R475C/A419C), TCEP was omitted. Unreacted probes and TCEP were removed by the HPLC G3000SWXL gel-filtration column. The amount of Cys-ABD was determined spectrophotometrically using the extinction coefficients, ɛ394 nm = 7800 M⁻¹ cm⁻¹ (20). ABD-labeled ClpB mutants (0.1 mg/ml) in 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, in the presence or the absence of nucleotide were preincubated for 3 min in a sealed cuvette at 55 °C, and fluorescence was measured with a Hitachi F4500 fluorometer (excitation 390 nm, emission spectra 400–650 nm). In the case of the reducing condition, the fluorescence spectrum was measured in the presence of 20 mM TCEP, and the preincubation time was 10 min. The emission spectra were normalized by the amount of Cys-ABD. In the experiment of time-course monitoring of the ABD labeling, ClpB labeling, ClpB mutants (0.15 mg/ml) were preincubated with 5 mM TCEP in 50 mM...
MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl₂, in the presence or the absence of nucleotide at 55 °C for 2 min, and the reaction was started by adding ABD-F (final concentration of 0.5 mM). The fluorescence spectra (400–650 nm) were measured at appropriate time intervals. The percentages of the labeled Cys residues against total Cys residues at each nucleotide condition and mutants were independently calculated from the fluorescence intensity at 500 nm and absorption of Cys-ABD.

Gel-filtration Analysis—Purified wild-type or mutant ClpB (1 mg/ml) in 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl₂ with or without 2 mM ATP, 20 mM TCEP was preincubated for 1 min at 55 °C. Then the mixture was centrifuged for 1 min (no obvious precipitation). An aliquot (100 μl) of the solution was loaded on a HPLC gel-filtration column, TSK G-3000SWXL (Tosoh). The column was pre-equilibrated and eluted at 55 °C at a flow rate 0.5 ml/min with the same buffer used for preincubation. The elution of proteins was monitored by absorbance at 290 nm. The molecular size standards used were thyroglobulin (669 kDa), ferritin (440 kDa), T-DnaKJ complex from T. thermophilus (330 kDa), catalase (232 kDa), glucose-6-phosphate dehydrogenase from Bacillus stearothermophilus (212 kDa), and aldolase (158 kDa). Elution profiles of the heat stable standard proteins (T-DnaKJ complex, glucose-6-phosphate dehydrogenase) at 55 °C were the same as those at 20 °C, ensuring the column properties were not changed at high temperature.

ATPase Activity—ATPase activities of ClpB were measured spectrophotometrically with an ATP-regenerating system at 50 °C (21). The assay mixture contained 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl₂, 0.2 mM NADH, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, 3 mM ATP and 0.1 mg/ml casein when indicated. The reaction was initiated by the addition of ClpB, and the changes in the absorbance at 340 nm were monitored.

RESULTS

ATP-induced Fluorescence Change of ABD-labeled ClpB—We introduced a fluorescence probe ABD to the Cys residue of ClpB (A419C) mutant of T. thermophilus that is located at the edge of the wing-2 (Fig. 1A) with labeling yield 92%. Labeling specificity was ensured by the fact that authentic T. thermophilus ClpB does not contain a cysteine residue. At 55 °C, where T. thermophilus ClpB is fully active, fluorescence intensity of ABD-labeled A419C (hereafter, the term ClpB is omitted from the mutant names) was drastically decreased to 35% by the addition of 3 mM ATP (Fig. 1B). A small red shift of the fluorescence peak was also observed. Almost the same changes were observed by the addition of ADP or ATP/γS (Fig. 1E). It has been known that the fluorescence of Cys-ABD is very sensitive to the environment, and a decrease in intensity and red shift occur in the hydrophilic environment (22). Therefore, the observation implies that nucleotide binding to ClpB induces the movement of the wing-2 to the direction by which the wing-2 loses hydrophobic interactions with other domains and becomes more isolated. Next, we tested which NBD would be responsible for this nucleotide binding-induced change. Mutants were generated by substitution of Lys-Thr sequence in the Walker A sequence with Ala-Ala for each NBD. These mutants, termed as 1KT/AA for NBD1 and 2KT/AA for NBD2,

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Nucleotide-induced fluorescence changes of ABD-labeled ClpB. A, structure of ClpB from T. thermophilus. N-terminal domain (green), NBD1 (blue), NBD2 (red), and coiled-coil (yellow) are shown. The coiled-coil is divided into two motifs, wing-1 and wing-2. NBD1 and NBD2 consist of the P-loop subdomain and helical subdomain. The residue Ala-419 (A419), which was replaced by Cys to introduce ABD-F labeling, is shown in pink. B–D, fluorescence spectra of ABD-labeled ClpB (B), 1KT/AA (C), and 2KT/AA (D) in the absence (black) or the presence of 3 mM ATP (gray). The excitation wavelength was 390 nm. E, relative fluorescence intensities at 500 nm of ABD-labeled ClpB in the presence of no nucleotide, 3 mM ATP, 3 mM ADP, or 3 mM ATP/γS as indicated. The intensities in the absence of nucleotide were taken as 100%. Experimental details are described under “Experimental Procedures.” a.u., arbitrary units.
lost the ability of nucleotide binding to NBD1 and NBD2, respectively (14). The A419C mutation was further introduced into these mutants. Labeling yields of these mutants, 1KT/AA-A419C and 2KT/AA-A419C, by ABD-F were 79 and 77%, respectively. The fluorescence intensities of ABD-labeled 1KT/AA-A419C and 2KT/AA-A419C, normalized by labeling yields, were 127 and 118%, respectively, of that of ABD-labeled A419C, and the emission spectra of these two mutants were almost the same as ABD-labeled A419C. The fluorescence intensity of ABD-labeled 1KT/AA-A419C was decreased by the addition of ATP (Fig. 1C), but the extent of decrease (to 73%) was much less significant as compared with the case of ABD-labeled A419C. In contrast, a large decrease of fluorescence down to 35% by ATP addition was observed for of ABD-labeled 2KT/AA-A419C (Fig. 1D). A similar large decrease in fluorescence was observed when ATPγS and ADP were added to ABD-labeled 2KT/AA-A419C (Fig. 1E). Because the 2KT/AA (and 2KT/AA-A419C) mutant retains nucleotide binding ability to NBD1 but not to NBD2, these results indicated that the shift of the wing-2 to a relatively more hydrophilic environment is induced by nucleotide binding mainly to NBD1.

**ATP-induced Change of Accessibility of the Wing-1/NBD1 Interface**—The G167C/R475C mutant of ClpB was designed to make a disulfide cross-link between the wing-1 (R475C) and NBD1 (G167C) (Fig. 2A) (17). Indeed, when the mutant was loaded on SDS-PAGE in the non-reducing condition, the band was shifted to the upper position (Fig. 2B). The mutant lost its chaperone activity almost completely under the non-reducing condition. In the presence of a reducing reagent, TCEP, the mutant retained 70% of the chaperone activity of that of the wild-type, and Cys residues were labeled by ABD-F with an efficiency dependent on the nucleotide. Because free ABD-F has no fluorescence by itself, we could monitor the progress of the labeling reaction as the increase of fluorescence (Fig. 2C). From the fluorescence intensity at 500 nm and absorption spectrum of Cys-ABD, yields of the labeled Cys residues against total Cys at each time point were obtained and plotted (Fig. 2D). In the absence of nucleotides, 42% of total Cys in G167C/R475C was calculated from fluorescence intensities at 500 nm. E, the yield (%) of ABD-labeled Cys residues after a 39-min incubation of ClpB mutants with ABD-F in the presence of no nucleotide, 3 mM ATP, 3 mM ADP, or 3 mM ATPγS as indicated. Experimental details are described under “Experimental Procedures.” a.u., arbitrary units.
beled triple mutant in the absence (black) or the presence of 20 mM TCEP (gray). The excitation wavelength was 390 nm. B, fluorescence intensities at 500 nm of the ABD-labeled triple mutant, in the presence of no nucleotide, 3 mM ADP, or 3 mM ATP as indicated. Experimental details are described under “Experimental Procedures.” a.u., arbitrary units.

Stabilization of Hexamer Structure of ClpB by Fixing the Wing-1 to NBD1—The oligomer states of ClpB mutants were analyzed by gel-filtration chromatography. As reported previously, the wild-type ClpB can form a stable hexamer in the presence of 2 mM ATP at 55 °C. For the stable hexamer formation, ATP binding to NBD1, but not ATP hydrolysis, is required. Therefore, the 1KT/AA mutant did not form a stable hexamer even in the presence of ATP and was eluted at positions corresponding to a molecular weight lower than the hexamer with a long tailing (Fig. 4A). On the contrary, 1KT/AA-G167C/R475C was eluted at the position of the hexamer under a non-reducing condition (Fig. 4A). However, in the presence of a reducing reagent in the elution buffer, the hexamer of 1KT/AA-G167C/R475C was destabilized and eluted in a similar chromatographic pattern to that of 1KT/AA (Fig. 4B). 2KT/AA-G167C/R475C was eluted as a hexamer either in non-reducing or reducing conditions (Fig. 4A and B). These results suggest that oxidized G167C/R475C with the wing-1 being fixed to NBD1 mimics the structure in which NBD1 is filled by ATP and stabilizes the hexamer form. Indeed, even in the absence of ATP, the majority of G167C/R475C was eluted at the position of the hexamer with a significant peak, whereas wild-type was eluted as a broad peak (Fig. 4C).

Activation of ATPase Activity of NBD2 by Fixing the Wing-1 to NBD1—Because 1KT/AA is unable to bind nucleotide at NBD1, ATPase activity of this mutant is solely attributed to that of the intact NBD2. Similarly, ATPase activity of 2KT/AA is solely attributed to the intact NBD1. The sum of the ATPase activities of the two mutants is far smaller than the activity of the wild type. Thus, each of NBD1 and NBD2 has a basic ATPase activity, represented by the activities of 2KT/AA and 1KT/AA, respectively, and cooperative stimulation is assumed...
between two NBDs. Then we examined the effect of cross-linking between G167C and R475C on the ATPase activity of ClpB (Fig. 5). Under the reducing condition, ATPase activity and its casein-stimulation of G167C/R475C were almost the same as those of the wild-type ClpB (columns 1 and 2). The cross-linking of G167C/R475C resulted in enhancement of the ATPase activity 2-fold, whereas the casein-stimulation was marginal (columns 3). Under the reducing condition, the ATPase activities of 1KT/AA-G167C/R475C and 2KT/AA-G167C/R475C were almost the same as those of 1KT/AA and 2KT/AA, respectively (columns 4 and 5, 7 and 8). When oxidized, the ATPase activity of 2KT/AA-G167C/R475C was not changed (columns 8 and 9) but that of 1KT/AA-G167C/R475C was remarkably activated, nearly 30 times as compared with that in the reducing condition (columns 5 and 6). Marginal casein-stimulation of column 6 was the same as observed for column 3. Thus, when the wing-1 is fixed to NBD1, ATPase activity of NBD2 is greatly activated.

**DISCUSSION**

Based on the crystal structure of ClpB, the results of fluorescence change reported here are explained by the motion of the coiled-coil induced by the ATP binding to NBD1 (Fig. 6). The fluorescence of the dye attached at the edge of the wing-2 changed by the addition of ATP in such a way that indicated the shift of the wing-2 into a more hydrophilic environment, that is, a more isolated position from other domains (Fig. 1B). The wing-1 appears to move to the opposite direction with the addition of ATP; it moves and leans to NBD1, and the interface between the wing-1 and NBD1 becomes less accessible for a water-soluble labeling reagent (Fig. 2D). In addition, the edge of wing-2 shifted to the hydrophilic environment when the disulfide bridge between wing-1 and NBD1 was formed (Fig. 3A). These results suggest that the changes of fluorescence and dye accessibility reflect the same structural change. The changes of fluorescence and dye accessibility were obtained for ClpB with the intact NBDs. The mutant ClpB in which only NBD1 is intact also showed the similar response on addition of ATP (Figs. 1D and 2E), whereas the mutant that has defective NBD1 (and intact NBD2) did not exhibit such typical response (Figs. 1C and 2E). Therefore, it appears that ATP-binding to NBD1 induces the partial rotary motion of the coiled-coil around the center of the coiled-coil from a position relatively parallel to the main body of ClpB to a position leaning to NBD1 as seen in the crystal structure. Because the disulfide bridge between G167C and R475C is formed even in the absence of a nucleotide, the parallel conformation might not be rigid and might allow some thermal random movement in which an occasional leaching conformation is trapped slowly but irreversibly by a covalent linkage. Referring to the close examination of the crystal structure with two NBDs filled by AMPPNP, we can assume the sequence of events as follows. (i) ATP comes into the nucleotide binding cleft of NBD1, (ii) several residues in the helical subdomain of NBD1 are recruited to buttress the bound ATP, (iii) this accompanies closure of the cleft, and (iv) the cleft-closure movement brings the coiled-coil from a relatively parallel position into the leaning position. To be cautious, two reservations should be made. Although ATP hydrolysis is needed for chaperone function of ClpB (14), differences between ADP and ATP or ATP·P·S are not clear in this study. ATP hydrolysis might be needed for turning over the cycle of the structural changes induced by the binding and release of nucleotide. The leaning conformation is seen in a crystal structure, but the parallel conformation should be demonstrated by a more direct method.

Another finding is that ClpB can make a stable hexamer without ATP when the leaning position of the coiled-coil is fixed by cross-linking between the wing-1 and NBD1 (Fig. 4). In the native ClpB, ATP binding to NBD1 is necessary to stabilize the hexamer (14). The conformation fixed by the cross-linking likely mimics that of ClpB with NBD1 being filled by ATP (Fig. 6). The crystal structure of ClpB most likely represents the conformation that we mentioned here, although the ClpB promoters in the crystal are packed in helical arrangement rather than the ring hexamer (17).

The third message of this report concerns the origin of cooperative stimulation of ATPase activity between two NBDs. Cooperative kinetics of ATP hydrolysis have been reported for several members of the AAA+ superfamily with two NBDs, but the molecular bases are yet unclear (12, 23–25). We found that the ATPase activity of NBD2 is activated when the coiled-coil is fixed to the leaning position by a cross-link between the wing-1 and NBD1 (Fig. 5). The stimulation is large enough to explain most fractions of the cooperative stimulation between NBD1 and NBD2 of the native ClpB. It is obvious now that ATP binding to NBD1 brings about activation of ATPase activity of NBD2 through the motion of the coiled-coil.

Recently, a strong support for the threading model of ClpB was reported and the importance of the central pore of the ClpB hexamer was proposed (26–28). We speculate that the coiled-coil might play in the stage before threading, such as recognition of the aggregate, disassembling large aggregates into small aggregates, and/or organization of other partner chaperones.

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REFERENCES
1. Glover, J. R., and Lindquist, S. (1998) Cell 94, 73–82
2. Motohashi, K., Watanabe, Y., Yoshida, M., and Yoshida, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7184–7189
3. Zolkiewski, M. (1999) J. Biol. Chem. 274, 28083–28086
4. Goloubinoff, P., Mogk, A., Zvi, A. P., Tomoyasu, T., and Bukau, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15732–15737
5. Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H., and Bukau, B. (1999) EMBO J. 18, 6934–6940
6. Watanabe, Y. H., Motohashi, K., Taguchi, H., and Yoshida, M. (2000) J. Biol. Chem. 275, 12388–12392
7. Krzewska, J., Langer, T., and Liberek, K. (2001) FERS Lett. 489, 92–96
8. Watanabe, Y. H., and Yoshida, M. (2004) J. Biol. Chem. 279, 15723–15727
9. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) Genome Res. 9, 27–43
10. Parsell, D. A., Sanchez, Y., Stitzel, J. D., and Lindquist, S. (1991) Nature 353, 270–273
11. Parsell, D. A., Kowal, A. S., and Lindquist, S. (1994) J. Biol. Chem. 269, 4480–4487
12. Schlee, S., Groemping, Y., Herde, P., Seidel, R., and Reinstein, J. (2001) J. Mol. Biol. 306, 889–899
13. Krzewska, J., Konopa, G., and Liberek, K. (2001) J. Mol. Biol. 314, 901–910
14. Watanabe, Y. H., Motohashi, K., and Yoshida, M. (2002) J. Biol. Chem. 277, 5804–5809
15. Mogk, A., Schlieker, C., Strub, C., Rist, W., Weibezahn, J., and Bukau, B. (2003) J. Biol. Chem. 278, 17615–17624
16. Schirmer, E. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996) Trends Biochem. Sci. 21, 289–296
17. Lee, S., Sowa, M. E., Watanabe, Y. H., Sigler, P. B., Chiu, W., Yoshida, M., and Tsai, F. T. (2003) Cell 115, 229–240
18. Higuchi, R., Krummel, E., and Saiki, R. K. (1988) Nucleic Acids Res. 16, 7351–7367
19. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
20. Toyooka, T., and Imai, K. (1985) Anal. Chem. 57, 1931–1937
21. Stiggall, D. L., Galante, Y. M., and Hatefi, Y. (1979) Methods Enzymol. 55, 308–315
22. Hiratsuka, T. (1993) J. Biol. Chem. 268, 24742–24750
23. Hattendorf, D. A., and Lindquist, S. L. (2002) EMBO J. 21, 12–21
24. Hattendorf, D. A., and Lindquist, S. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2732–2737
25. Cashikar, A. G., Schirmer, E. C., Hattendorf, D. A., Glover, J. R., Ramakrishnan, M. S., Ware, D. M., and Lindquist, S. L. (2002) Mol. Cell. 9, 751–760
26. Schlieker, C., Weibezahn, J., Patzelt, H., Tessarz, P., Strub, C., Zeth, K., Erbse, A., Schneider-Mergener, J., Chin, J. W., Schultz, P. G., Bukau, B., and Mogk, A. (2004) Nat. Struct. Mol. Biol. 11, 607–615
27. Lum, R., Tkach, J. M., Vierling, E., and Glover, J. R. (2004) J. Biol. Chem. 279, 29139–29146
28. Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E. U., Dougan, D. A., Tsai, F. T., Mogk, A., and Bukau, B. (2004) Cell 119, 653–665
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