Protein Science (1999), *8*:1899–1903. Cambridge University Press. Printed in the USA.

Nucleotide-dependent oligomerization of ClpB from *Escherichia coli*

MICHAL ZOLKIEWSKI,[1] MARTIN KESSEL,[2] ANN GINSBURG,[3] AND MICHAEL R. MAURIZI[4]

[1]Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506
[2]Laboratory of Structural Biology Research, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892
[3]Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892
[4]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

(RECEIVED March 3, 1999; ACCEPTED May 27, 1999)

Abstract

Self-association of ClpB (a mixture of 95- and 80-kDa subunits) has been studied with gel filtration chromatography, analytical ultracentrifugation, and electron microscopy. Monomeric ClpB predominates at low protein concentration (0.07 mg/mL), while an oligomeric form is highly populated at >4 mg/mL. The oligomer formation is enhanced in the presence of 2 mM ATP or adenosine 5′-*O*-thiotriphosphate (ATPγS). In contrast, 2 mM ADP inhibits full oligomerization of ClpB. The apparent size of the ATP- or ATPγS-induced oligomer, as determined by gel filtration, sedimentation velocity and electron microscopy image averaging, and the molecular weight, as determined by sedimentation equilibrium, are consistent with those of a ClpB hexamer. These results indicate that the oligomerization reactions of ClpB are similar to those of other Hsp100 proteins.

Keywords: electron microscopy; heat-shock proteins; nucleotide binding; protein association; sedimentation equilibrium; sedimentation velocity

Clp ATPases (also referred to as Hsp100 proteins) are involved in protein degradation and disaggregation in both prokaryotic and eukaryotic cells (Schirmer et al., 1996; Gottesman et al., 1997). Clp ATPases have one or two nucleotide-binding domains and express both basal and protein-stimulated ATPase activity. The first studied, ClpA, from *Escherichia coli* has two nucleotide-binding domains. It functions in a complex with the genetically unrelated ClpP protease to promote degradation of protein substrates and displays chaperone-like activity by promoting structural rearrangements of specific proteins (Wickner et al., 1994; Gottesman et al., 1997). ClpX from *E. coli* has a single nucleotide-binding domain but likewise promotes protein degradation by ClpP and displays ATP-dependent chaperone activity (Wawrzynow et al., 1995; Gottesman et al., 1997). ClpX promotes disassembly of tight protein complexes and prevents aggregation of specific protein substrates (Wawrzynow et al., 1995). A yeast Clp homolog, Hsp104, promotes the resolubilization of proteins from heat-induced aggregates (Parsell et al., 1994b). This activity requires cooperation between Hsp104 and yeast Hsp70, and Hsp40 (Glover & Lindquist, 1998).

ClpA (Kessel et al., 1995; Maurizi et al., 1998) and Hsp104 (Parsell et al., 1994a) were found as monomers and dimers in the absence of nucleotides and formed single hexameric rings in the presence of ATP or its nonhydrolyzable analogs. ClpY, a ClpX homolog, also forms hexamers in the presence of ATP (Kessel et al., 1996).

ClpB is an *E. coli* homolog of yeast Hsp104 (~40% identity between ClpB (Gottesman et al., 1990) and Hsp104 (Parsell et al., 1991) amino acid sequences) but substrates for ClpB in vivo have not been identified. The ClpB gene contains an internal translation initiation site and is expressed in vivo as two gene products: a 95- and an 80-kDa polypeptide chain (ClpB95, ClpB80) (Squires et al., 1991; Woo et al., 1992). Gel filtration-based size determination suggested that purified ClpB is a tetramer (~350,000 M_r) (Woo et al., 1992; Park et al., 1993) and its apparent oligomerization is not affected by nucleotides (Kim et al., 1998). This would indicate a significant difference in structural organization between ClpB and ClpA, or Hsp104.

In this paper, we used gel filtration chromatography, analytical ultracentrifugation, and electron microscopy to study the oligomerization of ClpB. In contrast with previous reports (Woo et al., 1992; Park et al., 1993; Kim et al., 1998), we found that at low concentrations in the absence of nucleotides, ClpB is monomeric but in the presence of ATP or ATPγS, or at high protein concen-

Reprint requests to: Michal Zolkiewski, Department of Biochemistry, 104 Willard Hall, Kansas State University, Manhattan, Kansas 66506; e-mail: michalz@ksu.edu.

Abbreviations: ATPγS, adenosine 5′′-*O*-thiotriphosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; Hepes, 4-(2-hydroxyethyl-)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

tration, ClpB forms oligomers (522,000 M_r) that are most likely hexamers.

Results

Figure 1 shows gel filtration elution profiles of ClpB in the absence and presence of nucleotides. Without nucleotides, ClpB eluted as a broad band with shoulders indicating the presence of molecules with different Stokes radii. With 2 mM ADP the amount of larger ClpB species apparently increased. When 2 mM ATP is present in the running buffer, the majority of ClpB eluted with an apparent size of ~600 kDa, with a smaller band corresponding to monomeric ClpB. When a nonhydrolyzable ATP analog, ATPγS, was used instead of ATP, only the 600 kDa band was observed (data not shown). This indicates that the oligomer formation does not require ATP hydrolysis and suggests that the fraction of ClpB eluting as monomers in the presence of ATP could be due to the ATP hydrolysis by ClpB on the column and a formation of a ClpB-ADP complex or a nucleotide-free ClpB. The SDS-PAGE analysis of fractions collected during the gel filtration experiment with ATP showed both 95- and 80-kDa ClpB bands present in all protein-containing fractions for the elution times from 50 to 68 min, indicating that both the longer and the shorter form of ClpB participate in the oligomer formation.

Figure 2 shows apparent distributions of the sedimentation coefficient ($g(s^*)$) for ClpB at different protein concentrations obtained from the time-derivative analysis (Stafford, 1992) of the sedimentation velocity data. In the absence of nucleotides, the concentration dependence of $g(s^*)$ profiles indicated self-association of ClpB (Stafford, 1994). Only at the lowest ClpB concentration (0.07 mg/mL) (Fig. 2A) and the highest (4.0 mg/mL) (Fig. 2D) could the $g(s^*)$ profiles be approximated by single gaussians. In Figures 2A and 2D the s^* values corresponding to maxima of $g(s^*)$ approximate the observed sedimentation coefficients for molecular species predominantly populated at a given protein concentration. For a low-molecular weight ClpB species, which dominates at 0.07 mg/mL: s^*_{max} = 2.8 S and $s_{20,w}$ = 4.4 S, and for the associated species (at 4 mg/mL): s^*_{max} = 10.6 S, $s_{20,w}$ = 17.1 S.

At intermediate ClpB concentrations (Fig. 2B,C), the $g(s^*)$ profiles were complex. It has been shown that for mixtures of oligomers participating in an association/dissociation equilibrium, the $g(s^*)$ profiles have multiple maxima, which do not necessarily correspond to sedimentation coefficients of any of the associating species (Stafford, 1994). An increasing area under the $g(s^*)$ profile for increasing s^* represents the progress of the association reaction (Stafford, 1994). Thus, the data in Figures 2B and 2C illustrate the sedimentation properties of mixtures of the low- and high-molecular weight forms of ClpB at different molar ratios and the progress of the ClpB association for increasing protein concentration.

As shown in Figures 2B and 2C, in the presence of 2 mM ADP, the $g(s^*)$ profiles were centered at the s^* values lower than those found in the absence of nucleotides for the same protein concentration. This indicates that ADP inhibits full association of ClpB.

Figure 3 shows the sedimentation equilibrium data for ClpB with 2 mM ATPγS. The protein concentration gradient could be described accurately (with small and predominantly random residuals) by a single-species model of 522,000 M_r.

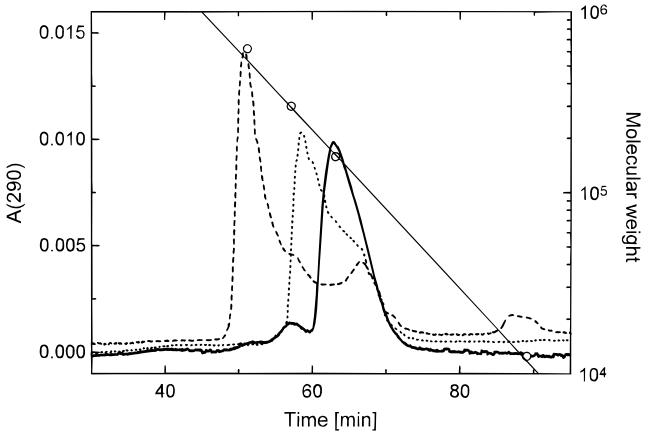

Fig. 1. Gel filtration analysis of ClpB. Aliquots of purified ClpB (50 μL of ~3 mg/mL) were injected onto a Superdex 200 column with a 0.2 mL/min flow rate (running buffer: 50 mM Tris, 0.2 M KCl, 10% glycerol, 20 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, pH 7.5). Protein elution profiles (monitored at 290 nm) are shown for ClpB without nucleotides (solid line), with 2 mM ADP in the running buffer (dotted line), and with 2 mM ATP in the running buffer (dashed line). Open circles correspond to elution times for glutamine synthetase (M_r 622,000), glutamate dehydrogenase (M_r 300,000), aldolase (M_r 158,000), and cytochrome *c* (M_r 12,500).

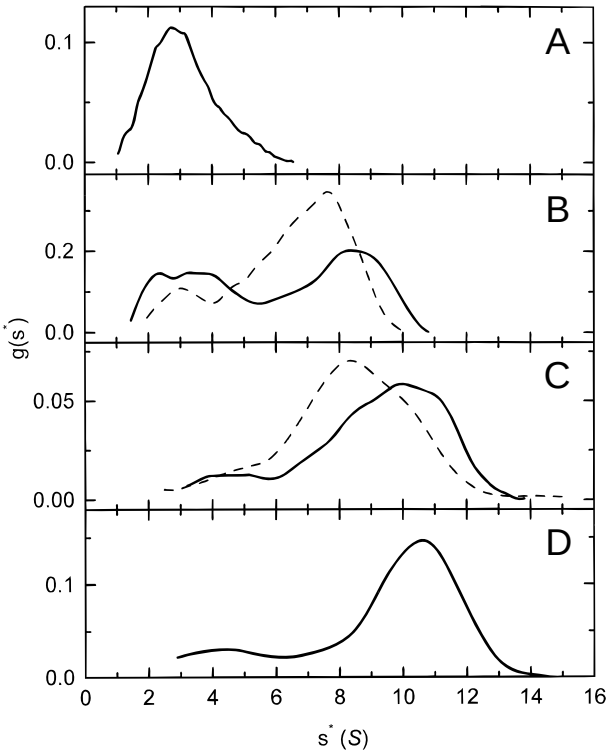

Fig. 2. Sedimentation velocity experiments for ClpB at 20 °C. Shown are the results from time-derivative analysis (Stafford, 1992) of four to six late radial protein concentration profiles. Solid lines show apparent distribution functions $g(s^*)$ vs. the sedimentation coefficient s^* in Svedberg units (S) for (**A**) 0.07 mg/mL, (**B**) 0.5 mg/mL, (**C**) 2.3 mg/mL, and (**D**) 4.0 mg/mL ClpB without nucleotides. Dashed lines show the data obtained in the presence of 2 mM ADP (**B**, **C**). Centrifugation speed was: (**A**) 48,000 rpm, (**B**) 39,000 rpm, (**C**) 38,000 rpm, and (**D**) 52,000 rpm. Protein concentration profiles were obtained using absorbance at (**A**) 229 nm, (**C**) 291 nm, (**D**) 290 nm, or (**B**) the interference detection system.

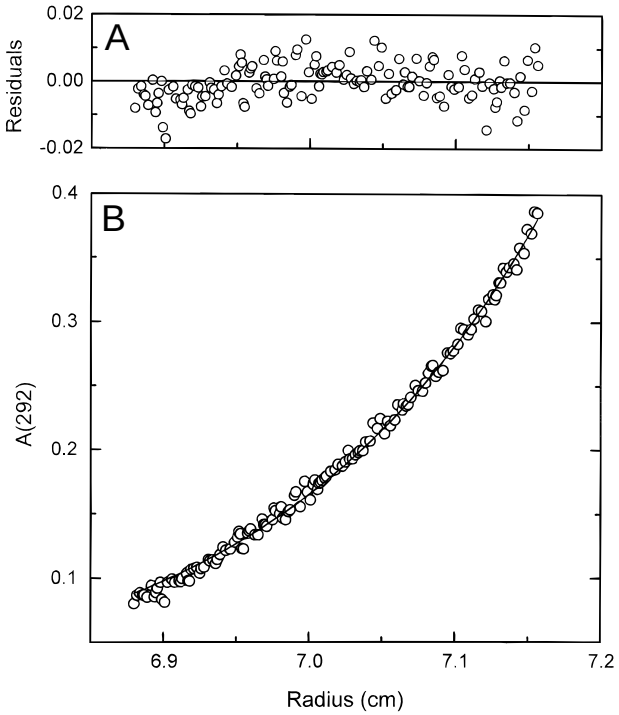

Fig. 3. Sedimentation equilibrium experiment for ClpB (~2 mg/mL) with 2 mM ATPγS at 4°C and 3,500 rpm. The absorption data at 292 nm (**B**, open circles) are shown together with a model fit assuming a single molecular component of 522,000 molecular weight (solid line). **A:** The residuals ($A_{exp} - A_{model}$) are shown. **B:** The 0.146 base line absorption has been subtracted from the data.

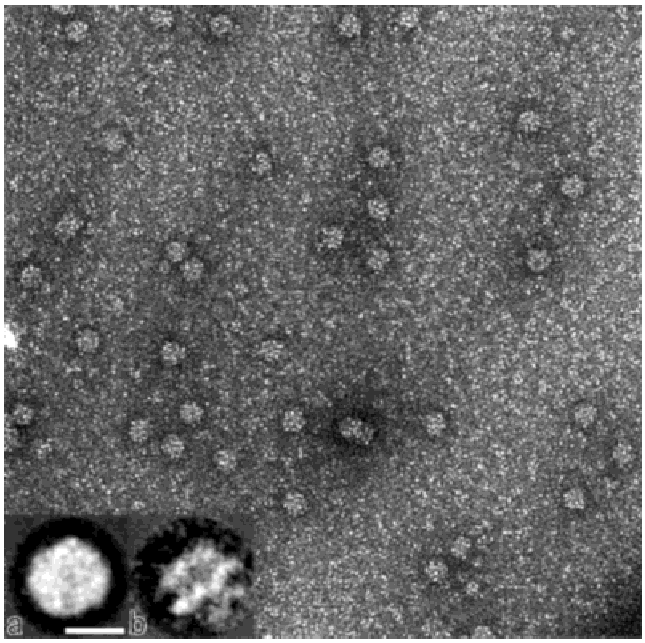

Fig. 4. Electron micrograph of negatively stained ClpB. ClpB was prepared in solution containing 2 mM ATPγS and micrographs were obtained after negative staining with uranyl acetate. The bar is 10 nm. Insets, average images of top views (a) of 120 particles and side views (b) of 35 particles.

Figure 4 shows an electron micrograph of ClpB in the presence of ATPγS. The uniform distribution of particles indicates the size homogeneity of ClpB under these conditions. The insets show top views and side views of ClpB obtained by translational and rotational alignment and averaging (Kessel et al., 1995). The particle has a diameter (average ~13.5 nm) similar to that of ClpA (Kessel et al., 1995). The disk-like appearance is reminiscent of the hexagonal rings seen with ClpA (Kessel et al., 1995) and Hsp104 (Parsell et al., 1994a), but no clear symmetry could be calculated for ClpB, possibly because the presence of both large (95 kDa) and small (80 kDa) subunits in the oligomer introduce an intrinsic heterogeneity. The side view suggests that, as with ClpA (Kessel et al., 1995), the N- and C-terminal nucleotide-binding domains form structurally distinct rings.

Discussion

Previous investigators had suggested that ClpB forms a stable, nucleotide-independent tetrameric complex (Woo et al., 1992; Park et al., 1993; Kim et al., 1998). However, our results show a protein concentration-dependent association of ClpB (Fig. 2). The ClpB association reactions are coupled to the nucleotide-binding (Figs. 1–3). The electron micrographs (Fig. 4) indicate that ClpB in the presence of ATPγS has a structure that is analogous to that of ClpA, a result that was expected given the high degree of homology (42% identity and 64% similarity) between the proteins and similarities in their functions.

The ClpB samples used in this study contained ~67% ClpB95 and ~33% ClpB80 (see Materials and methods). Such lack of homogeneity prevents an unequivocal characterization of the ClpB oligomerization reactions, as too many molecular species have to be accounted for in the data analysis. For example, Figure 2 shows complex superposition of the sedimentation coefficient distributions for different incompletely and completely associated species of ClpB containing both ClpB95 and ClpB80. In this discussion, we will focus, therefore, on the qualitative description of ClpB self-association. Full quantitative characterization of the ClpB association equilibria will require studying homogenous samples containing either ClpB95 or ClpB80 and combinations of these two protein forms at controlled molar ratios. Such studies will be possible by using mutated constructs of *clpB* that produce one or the other form of ClpB (Park et al., 1993).

The sedimentation coefficient for the ClpB species predominating at high protein concentration without nucleotides (Fig. 2D) is very close to $s_{20,w}$ = 17.2 S measured for ClpA with ATPγS (Maurizi et al., 1998), under which conditions ClpA (84 kDa monomer) forms hexamers (Kessel et al., 1995). The sedimentation coefficient of the low concentration ClpB species (Fig. 2A) is much lower than $s_{20,w}$ = 8.7 S for the mixed population of monomeric and dimeric ClpA (Maurizi et al., 1998). These data suggest that the smallest stable species of ClpB (Fig. 2A) is a monomer and the largest species (Fig. 2D) is a hexamer.

The results in Figure 2 illustrate the importance of studying the particle size for an associating system over a broad concentration range and raise the possibility that at the high intracellular concentrations expected, most Clp ATPases may exist predominantly in the oligomeric state regardless of nucleotide binding.

The effect of ADP binding on the size of ClpB oligomers appears to depend on ClpB concentration: apparent increase in the molecular weight with 2 mM ADP at <0.1 mg/mL ClpB (Fig. 1) and apparent decrease in the molecular weight for 0.5–2 mg/mL

ClpB (Fig. 2B,C). This can be interpreted as the ADP-induced stabilization of an intermediate, possibly dimeric, form of ClpB. Full assembly of ClpB hexamers does not occur in the presence of ADP.

In contrast to ADP, ATP and ATPγS promote ClpB association into hexamers (Figs. 1, 3). The molecular weight of the ClpB oligomer at equilibrium in the presence of ATPγS (Fig. 3) is consistent with that of a hexamer formed by a mixed population of ClpB95 and ClpB80. In spite of the heterogeneity among ClpB monomers, the population of ATPγS-induced ClpB hexamers is apparently homogenous, as shown by the single-species sedimentation equilibrium data (Fig. 3). At present, we do not know the distribution of the 95- and 80-kDa subunits in ClpB oligomers. However, the observed molecular weight (522,000) is close to that predicted for a hexamer formed by ClpB95 and ClpB80 in proportion to their relative concentrations (i.e., four ClpB95 monomers associating with two ClpB80).

In summary, although the nucleotide-dependent self-association has been found for all members of the Hsp100 family studied so far, the oligomerization of ClpB in vitro differs from that of Hsp104. Both ATP (ATPγS) and ADP stabilize hexameric form of Hsp104 (Parsell et al., 1994a), while only ATP (ATPγS), but not ADP, stabilizes the ClpB hexamer.

Materials and methods

Protein purification

ClpB was overexpressed in *E. coli* using a multicopy pClpB plasmid kindly provided by Dr. Catherine L. Squires (Squires et al., 1991). The purification procedure was analogous to that used to obtain ClpA (Maurizi et al., 1994). Approximately 80 mg of ClpB has been purified from 25 g of *E. coli* cells. Three chromatography steps: anion-exchange (Q Sepharose, Pharmacia Biotech, Piscataway, New Jersey), gel filtration (Bio-Rad BIO-SIL TSK-250), and another anion-exchange (Mono Q, Pharmacia Biotech), were used to purify ClpB. Fractions from the Mono Q column that contained ClpB showed two protein bands on SDS-PAGE (data not shown) corresponding to the 95- and 80-kDa polypeptides (ClpB95, ClpB80) as previously observed (Woo et al., 1992). The identities of the two bands were confirmed by amino terminal sequencing of each species by automated Edman degradation (data not shown). The relative amounts of ClpB95 and ClpB80 were determined by gel scanning and band intensity integration using Scion Image software (Scion Corp., Frederick, Maryland). For the studies presented here, the ClpB preparation contained ~67% ClpB95 and ~33% ClpB80 (data not shown). The ATPase activity (Hess & Derr, 1975; Lanzetta et al., 1979) of ClpB was 5.2 nm Pi released per hour by 1 μg ClpB and exhibited an ~10-fold activation in the presence of 0.1 mg/mL casein. For further studies, ClpB samples were concentrated by precipitation with $(NH_4)_2SO_4$ and dialyzed extensively against 50 mM Hepes (pH 7.5), 0.2 M KCl, 10% glycerol, 20 mM $MgCl_2$, 1 mM EDTA, and 2 mM β-mercaptoethanol. Protein concentration was measured using the calculated absorption coefficient $A_{280} = 0.38$ cm^2/mg (Pace et al., 1995).

Gel filtration chromatography

HPLC gel filtration experiments were performed at room temperature using Superdex 200 HR 10/30 and Superdex 200 PC 3.2/30 columns (Pharmacia Biotech) with Hewlett Packard 1090 and 1100 liquid chromatography systems.

Analytical ultracentrifugation

The Beckman Optima XL-A and XL-I analytical ultracentrifuges with four-position AN-Ti rotors were used for sedimentation velocity and sedimentation equilibrium experiments. The density of the dialysate buffer at 20 °C was determined to be 1.0427 g/mL with a Paar DMA 58 densitometer, and the relative viscosity (1.427 at 25 °C) was measured as described previously (Shapiro & Ginsburg, 1968). The partial specific volume of ClpB (0.724 mL/g) was calculated according to Zamyatnin (1984).

For sedimentation velocity experiments, ClpB samples (0.34 mL) and dialysate buffer (0.35 mL for absorbance and 0.34 mL for interference detection) were loaded into the right and left side, respectively, of the double-sector 1.2 cm analytical cell. After equilibration at 3,000 rpm at 20 °C, the rotor was accelerated to a desired speed (see Fig. 2) and radial scans of the cell were performed using either ultraviolet absorption or interference detection system. Apparent sedimentation coefficient distributions were calculated using the time-derivative analysis (Stafford, 1992) and the data analysis software from Beckman (Palo Alto, California) supplied with the instrument. Observed sedimentation coefficients were corrected to values corresponding to the viscosity and density of water ($s_{20,w} = 1.615\ s_{obs}$).

For sedimentation equilibrium experiments, 0.11 mL of ClpB solutions and 0.12 mL of buffer were placed in a two-channel cell. The samples were equilibrated at a desired speed at 4 °C for ~48 h (Fig. 3). To monitor the approach to equilibrium, the cells were scanned every 3 h after the initial ~30 h equilibration. After the final data collection, the rotor was accelerated to 40,000 rpm for 3–4 h and the protein sedimented to the bottom of the cell. Subsequently, the low centrifuge speed was restored and the cell was scanned immediately to obtain the base line absorption value. The data were analyzed using the Beckman software.

Electron microscopy of ClpB and image averaging was performed as previously described (Kessel et al., 1995).

Acknowledgments

M.Z. thanks the Kansas Agricultural Experiment Station for support(contribution 99–358-J).

References

Glover JR, Lindquist S. 1998. Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell 94*:73–82.

Gottesman S, Squires C, Pichersky E, Carrington M, Hobbs M, Mattick JS, Dalrymple B, Kuramitsu H, Shiroza T, et al. 1990. Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proc Natl Acad Sci USA 87*:3513–3517.

Gottesman S, Wickner S, Maurizi MR. 1997. Protein quality control: Triage by chaperones and proteases. *Genes Dev 11*:815–823.

Hess HH, Derr JE. 1975. Assay of inorganic and organic phosphorus in the 0.1–5 nm range. *Anal Biochem 63*:607–613.

Kessel M, Maurizi MR, Kim B, Kocsis E, Trus BL, Singh SK, Steven AC. 1995. Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteasome. *J Mol Biol 250*:587–594.

Kessel M, Wu WF, Gottesman S, Kocsis E, Steven AC, Maurizi MR. 1996. Sixfold rotational symmetry of ClpQ, the *Escherichia coli* homolog of the 20 S-proteasome, and its ATP-dependent activator, ClpY. *FEBS Lett 398*:274–278.

Kim KI, Woo KM, Seong IS, Lee ZW, Baek SH, Chung CH. 1998. Mutational analysis of the two ATP-binding sites in ClpB, a heat shock protein with protein-activated ATPase activity in *E. coli*. *Biochem J 333*:671–676.

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. 1979. An improved assay for nm amounts of inorganic phosphate. *Anal Biochem 100*:95–97.

Maurizi MR, Singh SK, Thompson MW, Kessel M, Ginsburg A. 1998. Molecular properties of ClpAP protease of *E. coli*: ATP-dependent association of ClpA and ClpP. *Biochemistry 37*:7778–7786.

Maurizi MR, Thompson MW, Singh SK, Kim SH. 1994. Endopeptidase Clp: ATP-dependent Clp protease from *E. coli*. *Methods Enzymol 244*:314–331.

Pace CN, Vajdos F, Fee L, Grimsley G, Gray Y. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci 4*:2411–2423.

Park SK, Kim KI, Woo KM, Seol JH, Tanaka K, Ichihara A, Ha DB, Chung CH. 1993. Site-directed mutagenesis of the dual translational initiation sites of the *clpB* gene of *E. coli* and characterization of its gene products. *J Biol Chem 268*:20170–20174.

Parsell DA, Kowal AS, Lindquist S. 1994a. *Saccharomyces cerevisiae* Hsp104 protein. Purification and characterization of ATP-induced structural changes. *J Biol Chem 269*:4480–4487.

Parsell DA, Kowal AS, Singer MA, Lindquist S. 1994b. Protein disaggregation mediated by heat-shock protein Hsp104. *Nature 372*:475–478.

Parsell DA, Sanchez Y, Stitzel JD, Lindquist S. 1991. Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. *Nature 353*:270–273.

Schirmer EC, Glover JR, Singer MA, Lindquist S. 1996. HSP100/Clp proteins: A common mechanism explains diverse functions. *Trends Biochem Sci 21*:289–296.

Shapiro BM, Ginsburg A. 1968. Effects of specific divalent cations on some physical and chemical properties of glutamine synthetase from *E. coli*. Taut and relaxed enzyme forms. *Biochemistry 7*:2153–2167.

Squires CL, Pedersen S, Ross BM, Squires C. 1991. ClpB is the *E. coli* heat shock protein F48.1. *J Bacteriol 173*:4254–4262.

Stafford WF III. 1992. Boundary analysis in sedimentation transport experiments: A procedure for obtaining sedimentation coefficient distributions using time derivative of the concentration profile. *Anal Biochem 203*:295–301.

Stafford WF III. 1994. Sedimentation boundary analysis of interacting systems: Use of the apparent sedimentation coefficient distribution function. In: Schuster TM, Laue TM, eds. *Modern analytical ultracentrifugation*. Boston: Birkhauser. pp 119–137.

Wawrzynow A, Wojtkowiak D, Marszalek J, Banecki B, Jonsen M, Graves B, Georgopoulos C, Zylicz M. 1995. The ClpX heat-shock protein of *E. coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. *EMBO J 14*:1867–1877.

Wickner S, Gottesman S, Skowyra D, Hoskins J, McKenney K, Maurizi MR. 1994. A molecular chaperone, ClpA, functions like DnaK and DnaJ. *Proc Natl Acad Sci USA 91*:12218–12222.

Woo KM, Kim KI, Goldberg AL, Ha DB, Chung CH. 1992. The heat-shock protein ClpB in *E. coli* is a protein-activated ATPase. *J Biol Chem 267*:20429–20434.

Zamyatnin AA. 1984. Amino acid, peptide, and protein volume in solution. *Annu Rev Biophys Bioeng 13*:145–165.