THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 2, pp. 962–967, January 13, 2006

Leu^{309} Plays a Critical Role in the Encapsulation of Substrate Protein into the Internal Cavity of GroEL*

Received for publication, June 9, 2005, and in revised form, August 25, 2005 Published, JBC Papers in Press, October 20, 2005, DOI 10.1074/jbc.M506298200

Ayumi Koike-Takeshita[‡], Tatsuro Shimamura[§¶], Ken Yokoyama[‖], Masasuke Yoshida[‡‖1], and Hideki Taguchi[‡‡‡]**

*From the [‡]Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan, the [§]Department of Biological Sciences, Imperial College, London SW7 2AZ, United Kingdom, the [‖]ATP System Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Corporation, 5800-3 Nagatsuta, Midori-ku, Yokohama 226-0026, Japan, the **Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, the [‡‡]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562, Japan, and the [¶]Structural Biophysics Laboratory, RIKEN Harima Institute, Spring-8, 1-1-1 Kouto, Mikazuki, Sayo-gun, Hyogo 679-5148, Japan*

In the crystal structure of the native GroEL·GroES·substrate protein complex from *Thermus thermophilus*, one GroEL subunit makes contact with two GroES subunits. One contact is through the H-I helices, and the other is through a novel G*XX*LE region. The side chain of Leu, in the G*XX*LE region, forms a hydrophobic cluster with residues of the H helix (Shimamura, T., Koike-Takeshita, A., Yokoyama, K., Masui, R., Murai, N., Yoshida, M., Taguchi, H., and Iwata, S. (2004) *Structure* (*Camb.*) 12, 1471–1480). Here, we investigated the functional role of Leu in the G*XX*LE region, using *Escherichia coli* GroEL. The results are as follows: (i) cross-linking between introduced cysteines confirmed that the G*XX*LE region in the *E. coli* GroEL·GroES complex is also in contact with GroES; (ii) when Leu was replaced by Lys (GroEL(L309K)) or other charged residues, chaperone activity was largely lost; (iii) the GroEL(L309K)·substrate complex failed to bind GroES to produce a stable GroEL(L309K)·GroES·substrate complex, whereas free GroEL(L309K) bound GroES normally; (iv) the GroEL(L309K)·GroES·substrate complex was stabilized with BeF_x, but the substrate protein in the complex was readily digested by protease, indicating that it was not properly encapsulated into the internal cavity of the complex. Thus, conformational communication between the two GroES contact sites, the H helix and the G*XX*LE region (through Leu^{309}), appears to play a critical role in encapsulation of the substrate.

Chaperonins are a subclass of molecular chaperones capable of mediating ATP-dependent folding of polypeptides to their native states (1–4). GroEL is the best characterized chaperonin; it is found in the cytoplasm of *Escherichia coli* and is essential for cell viability and growth at all temperatures (5). The complete functional cycle of GroEL is dependent on the presence of ATP and the co-chaperonin GroES (6–11). GroEL is a large cylindrical protein complex comprising two heptamer rings of identical 57-kDa subunits stacked back to back (12). GroES is a dome-shaped, single heptamer ring of 10-kDa subunits (13). GroEL binds a wide variety of substrate proteins in non-native states and forms a binary complex (14–18), which then binds ATP and GroES to the same (*cis*) GroEL ring to form the *cis*-ternary complex (8, 9). The binding of GroES induces the encapsulation of the substrate protein into an enlarged cavity (the *cis*-cavity) inside the *cis*-ring, which is capped by GroES. In the *cis*-cavity, non-native protein initiates folding without the risk of aggregation (8, 9, 19). Based on studies of crystal structures and mutagenesis, it is thought that the residues of GroEL involved in binding of GroES are overlapped, to a large extent, with those for binding of the substrate protein (16). Therefore, it might appear that binding of GroES results in freeing of the unfolded protein into the *cis*-cavity through deprivation of its binding sites. However, simple competition between substrate protein and GroES for the same binding sites does not explain how the release of substrate protein always results in encapsulation into the *cis*-cavity rather than diffusion into the bulk solution. Analysis of an intermediate in the process of encapsulation may help clarify the mechanism by which GroEL operates at this critical stage.

We recently determined a crystal structure of the native GroEL·GroES complex purified from *Thermus thermophilus*, the *cis*-cavity of which is filled with cellular proteins (20). The structure shows several significant differences to the GroEL·GroES complex of *E. coli*, which was obtained by reconstitution of purified GroEL and GroES, in the presence of ADP (19). A new contact region between GroEL and GroES was identified in the *T. thermophilus* GroEL·GroES structure (Fig. 1*A*). In *E. coli* GroES, residues 24–27 are part of a mobile loop structure (comprising residues 24–30) that interacts with helices H and I at the apical domain of GroEL, located in the inner rim of the central cavity. In the GroEL·GroES of *T. thermophilus*, the same interactions are observed. The region ^{305}GFKLE^{309} of GroEL (corresponding to the *E. coli* GroEL sequence ^{306}GMELE^{310}) makes contact with the sequence ^{33}PDT^{35}, in the mobile loop of the adjacent GroES (^{28}TGS^{30} in *E. coli* GroES). The residues Gly^{305}, Leu^{308}, and Glu^{309} of *T. thermophilus* GroEL are well conserved across species, and hereafter, we refer this region as the G*XX*LE region. This region also has an intrasubunit interaction with the H helix; the side chain of Leu^{308} (Leu^{309} in *E. coli* GroEL) points at the N terminus of the H helix to form a hydrophobic cluster with other residues (Fig. 1, *A*, *circle*, and *B*). Fenton *et al.* (16) reported that a Leu^{309} mutant of *E. coli* GroEL (GroEL(L309K)) was unable to assist folding. The aim of this study was to examine the contribution of the G*XX*LE region to chaperonin function, using *E. coli* GroEL and GroES. We investigated the G*XX*LE region in the *E. coli* GroEL·GroES complex to determine whether it is also in contact with GroES and whether or not Leu^{309} in *E. coli* GroEL plays a role for the efficient encapsulation of substrate protein into the *cis*-cavity.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] To whom correspondence should be addressed. Fax: 81-45-924-5277; E-mail: myoshida@res.titech.ac.jp.

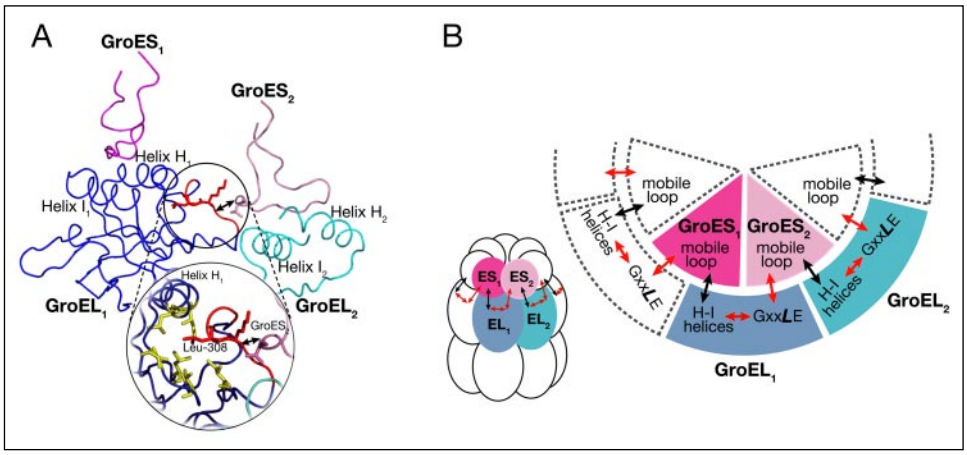

FIGURE 1. **Contacts between GroEL and GroES in the *cis*-ring of the GroEL·GroES complex.** *A*, the structure around the GroEL-GroES contact region of the *T. thermophilus* GroEL·GroES complex (Protein Data Bank entry 1WF4) (20). In the complex, one GroEL subunit ($GroEL_1$) makes contact with two GroES subunits, $GroES_1$ (*magenta*) in the known contact region (helices H and I) and $GroES_2$ (*pink*) in the undescribed contact region (*arrows*). The novel contact region in GroEL (the sequence ^{305}GFKLE^{309} in *T. thermophilus*; shown in *red*) is located immediately behind helices H and I. The GroES sequences that are interacting with the conventional and novel contact regions are in the same stretch of the loop region of GroES. Side chains of the *T. thermophilus* GroEL residues (Lys^{307} and Leu^{308}) and GroES (T35) are shown as stick models. A magnified view around the highly conserved Leu^{308} is shown by a *circle*. Hydrophobic residues Leu^{220}, Val^{222}, Val^{226}, Leu^{232}, Ile^{235}, and Ile^{300} are drawn as stick models in *yellow*. *B*, schematic drawing of the adjacent GroEL-GroES contacts. One GroEL subunit in the heptameric *cis*-ring interacts with two adjacent GroES subunits, at the H-I helices (*black arrows*) and at the G*XX*LE region (*red arrows*). The two GroES contact sites can communicate with each other through a hydrophobic cluster formed by Leu in the G*XX*LE region and residues at the entrance formed by the H helix (*red arrows*).

EXPERIMENTAL PROCEDURES

Reagent and Proteins—$BeCl_2$ was from Aldrich. NaF was obtained from Wako (Osaka, Japan). Chymotrypsin and hexokinase were from Sigma. Porcine malate dehydrogenase (MDH),[2] ATP, and ADP were obtained from Roche. The trace amount of contaminating ATP in the ADP solution was eliminated by hexokinase/glucose treatment (21). Cy3-NHS (Fluorolink Cy3 monofunctional dye) was from Amersham Biosciences. The following proteins were purified and prepared as previously described: green fluorescent protein (GFP) (22); GroEL, GroES, and bovine mitochondrial rhodanese (23); and Cy3-labeled GroES ($GroES_{Cy3}$) and Cy3-labeled MDH (MDH_{Cy3}) (24).

Strains and Plasmids—E. coli XL2-Blue (Stratagene) was used for site-directed mutagenesis and cloning. *E. coli* GroEL mutants were generated using QuikChange site-directed mutagenesis (Stratagene). The mutated *groEL* gene fragment was amplified using PCR, and the mutation containing pET-EL plasmid was used as a template. PCR products were digested with NcoI and HindIII and ligated into the NcoI/HindIII site of pTV118N (Takara), forming pTV-EL. The wild type *groES* gene fragment was amplified using PCR, digested with SalI and EcoRI, and ligated into the SalI/EcoRI site of pSTV29, forming pSTV-ES. *E. coli* MM100 (supplied by Dr. M. Masters) was used for complementation experiments (25). Mutated GroEL and wild type GroES were co-expressed (from expression plasmids pTV-EL and pSTV-ES, respectively) in *E. coli* MM100.

Formation of the GroEL Cross-linked Product—The mixtures (40 μl) containing HKM buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, and 5 mM $MgCl_2$), 0.25 μM GroEL, 0.5 μM GroES, 1 mM dithiothreitol (DTT) and, when indicated, 1 mM ATP, were loaded onto centrifugal ultrafiltration units (Microcon YM-100) to remove DTT gradually. After treatment for 60 min, 200 μM iodoacetamide was added to a final concentration of 10 μM to prevent excessive cross-linking. The intramolecular cross-linked product was formed as follows: 40 μl of mixture containing HKM buffer, 0.25 μM GroEL(E232C/L309C), 20 μM $CuCl_2$ and, when indicated, 1 mM ATP, 1 mM ADP, 1 mM DTT, and 0.5 μM GroES was incubated at 25 °C. After 30 min, iodoacetamide was added to a final concentration of 10 μM. Cross-linked products (~10 μg) were analyzed by polyacrylamide gel electrophoresis using 0.1% SDS (SDS-PAGE) in the absence of reducing agent.

Folding Assays—For measurement of GFP folding, an acid-denatured GFP solution (12.6 μM) was diluted 252-fold in HKM buffer (1.3 ml) containing 0.15 μM GroEL, 5 mM DTT, 200 mM glucose, and 0.3 μM GroES. Where indicated as "+trap", this was followed by the addition of 0.04 unit/μl hexokinase and 0.3 μM "trap-GroEL" (GroEL(N265A) (8)); trap-GroEL binds free unfolded proteins irreversibly even in the presence of ATP. Then 0.8 mM ATP was added to initiate folding. The intensity of GFP fluorescence was monitored continuously with a fluorometer (excitation at 485 nm, emission at 510 nm; FP-6500, Jasco). MDH was denatured in 6 M urea and 1 mM DTT for 1 h and diluted in HKM buffer containing 0.1 μM GroEL, 0.3 μM GroES, 5 mM DTT, and 2 mM ATP. The final MDH subunit concentration was 0.2 μM. At the times indicated, a 25-μl aliquot was injected into 1.2 ml of the assay solution containing 0.5 mM oxalacetic acid, 0.2 mM NADH, 1 mM DTT, and 0.1 mg/ml bovine serum albumin. The rate of oxidation of NADH at 25 °C was monitored at 340 nm. Rhodanese was denatured in 6 M guanidine HCl (20 μM) and 1 mM DTT for 1 h and diluted 40-fold into HKM buffer containing 1 μM GroEL, 2 μM GroES, 20 mM $Na_2S_2O_3$, and 1 mM DTT. ATP was then added to a final concentration of 4 mM. At the times indicated, 5-μl aliquots were added to 750 μl of a solution containing 100 mM KH_2PO_4, 150 mM $Na_2S_2O_3$, and 1 mM EDTA. Recovery of rhodanese activity was measured colorimetrically by absorbance at 460 nm, indicating formation of a complex between ferric ions and the thiocyanate reaction product (26).

Binding Assays Using Gel Filtration—The GroEL·Cy3-labeled MDH (MDH_{Cy3}) complex was formed in the presence or absence of GroES, and the GroEL·Cy3-labeled GroES ($GroES_{Cy3}$) complex was formed in the presence or absence of denatured MDH. Denatured MDH (or MDH_{Cy3}) was diluted in HKM buffer containing GroEL and incubated for 2 min at 25 °C. The solution containing ATP, with (or without) GroES, was added and incubated for 2 min at 25 °C. Final concentrations of the components were 0.125 μM MDH (or MDH_{Cy3}), 0.25 μM GroEL, 0.125 μM GroES, and 0.5 mM ATP. Aliquots (100 μl) were loaded onto a gel filtration HPLC column (G3000SW_{XL}; Tosoh, Japan) equilibrated with HKM buffer containing 50 mM Na_2SO_4 and 0.2 mM

[2] The abbreviations used are: MDH, malate dehydrogenase; GFP, green fluorescent protein; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

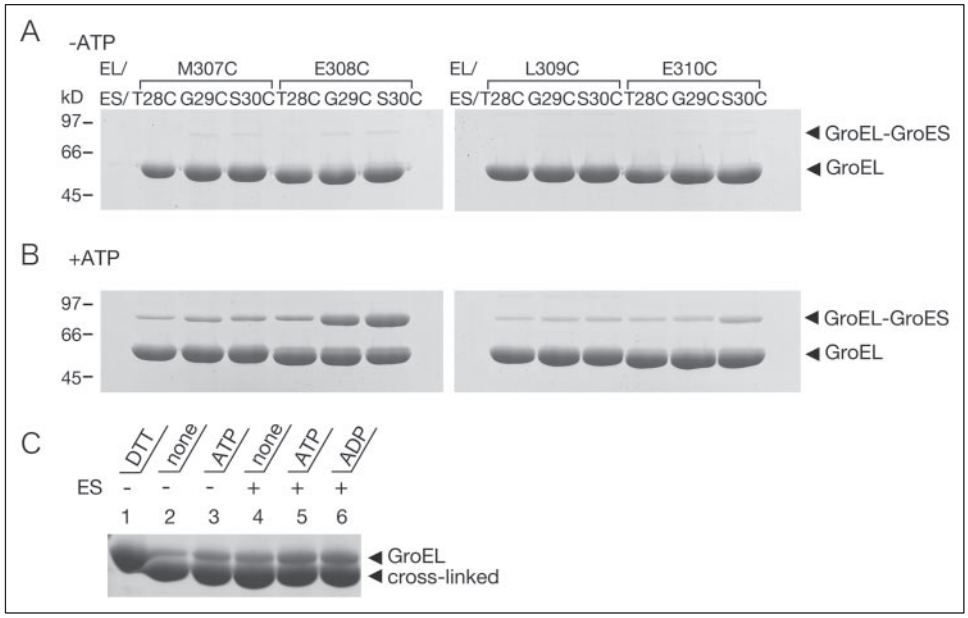

FIGURE 2. **Cross-linking between cysteine-incorporated *E. coli* mutants of GroEL and GroES.** *A* and *B*, intermolecular cross-linking between mutants of GroEL and GroES. The mixture containing purified *E. coli* GroEL and GroES mutants with 1 mM DTT was subjected to ultrafiltration to remove DTT gradually and in the absence (*A*) or presence (*B*) of 1 mM ATP to induce cross-linking. *C*, intrasubunit cross-linking between the G*XX*LE region and the H helix in GroEL. The GroEL(E232C/L309C) double cysteine mutant was incubated with 20 μM $CuCl_2$ in the absence or presence of GroES and 1 mM nucleotides. DTT was included in the leftmost sample (*lane 1*). Products were analyzed by SDS-PAGE in the absence of a reducing agent, and gels were stained with Coomassie Blue.

ATP. The flow rate was 0.5 ml/min, and elution was monitored by an in-line fluorometer (excitation at 550 nm, emission at 570 nm).

Protease Sensitivity of Substrate Protein in the GroEL·GroES Complex—GroEL that had been saturated with denatured rhodanese was prepared as described previously (21, 27). The reaction mixtures contained 1 mM nucleotide, 10 mM NaF, 2 mM $BeCl_2$, 20 mM $Na_2S_2O_3$, 1 μM rhodanese-saturated GroEL, 2.0 μM GroES, and 1 mM DTT in HKM buffer. Unbound GroES and substrate proteins were removed by ultrafiltration (Microcon YM-100) at 90 min after initiation of the reaction. Chymotrypsin (final concentration, 1 μg/ml) and glycerol (final concentration, 10% v/v) were added to 25 μl of the mixture containing 1 mM DTT and 15 μg of protein in HKM buffer. Following incubation for 20 min at 25 °C, components with a molecular mass of <100 kDa were removed by ultrafiltration (Microcon YM-100). An aliquot of the resulting solution was analyzed by 13% SDS-PAGE. The intensity of band staining was quantified using the NIH Image program and calibrated using known protein concentrations.

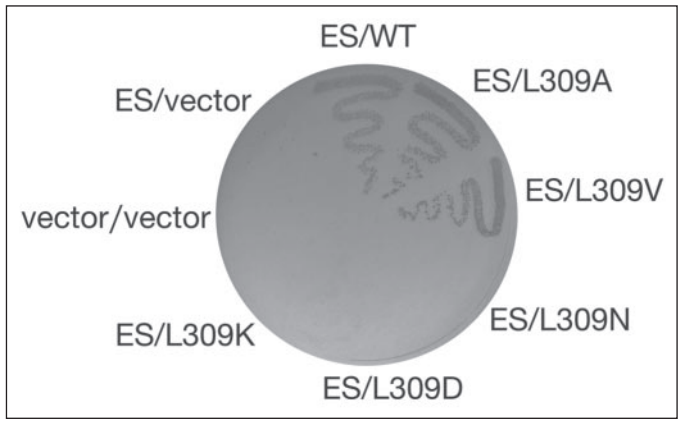

FIGURE 3. **Complementation of *E. coli* MM100 by expression of mutant GroEL proteins.** The viability of *E. coli* MM100 cells co-expressing GroES and either wild type (*WT*) or mutant GroEL when plated on LB in the absence of arabinose is shown.

RESULTS

Conservation of Novel Contacts in the E. coli GroEL·GroES Complex—To investigate whether or not the G*XX*LE region is in contact with GroES in the *E. coli* GroEL·GroES complex, we conducted a series of cross-linking experiments using mutants of *E. coli* GroEL and GroES. We replaced Met^{307}, Glu^{308}, Leu^{309}, and Glu^{310} in GroEL, and Thr^{28}, Gly^{29}, and Ser^{30} in GroES with Cys. The removal of DTT from the reaction mixture containing GroEL and GroES led to the generation of a single high molecular mass band, corresponding to cross-linked GroEL-GroES (Fig. 2, *A* and *B*). Binding of GroES to GroEL is known to be ATP (or ADP)-dependent, and the cross-linking was only successful when ATP was present. Among the mutants, the combination GroEL(E308C)/GroES(S30C) was most efficiently cross-linked, followed by the combination of GroEL(E308C)/GroES(G29C). We found efficient intrasubunit cross-linking between L309C and E232C at the N terminus of the H helix (Fig. 2*C*), and cross-link formation was not affected by nucleotides or GroES. These results confirm that the topological arrangement of the G*XX*LE region in the GroEL·GroES complex of *E. coli* is similar to that of the *T. thermophilus* complex, where the mobile loop of GroES and the H helix interact with the G*XX*LE region.

Effect of Mutation of Leu^{309} on Growth of E. coli—The results above indicate that Glu^{308} of GroEL and Gly^{29}/Ser^{30} of GroES are in close proximity. Next we investigated a role for the highly conserved Leu^{309} in chaperonin function. We replaced Leu^{309} of GroEL with Val, Ala, Asn, Asp, and Lys. *E. coli* MM100 (25), a strain in which expression of chromosomal GroEL-GroES is under control of the P_{BAD} promoter (arabinose induction), was co-transformed with the expression plasmids encoding the GroEL Leu^{309} mutants and wild type GroES. Transformants were cultured on LB plates, in the absence of arabinose (Fig. 3). Cells with expression plasmids encoding the mutants GroEL(L309A) and GroEL(L309V) grew normally, as did cells expressing wild type GroEL. In contrast, as previously described by Fenton *et al.* (16), cells expressing the mutant GroEL(L309K) could not grow in the absence of arabinose, Similarly, cells expressing the mutants GroEL(L309N) or GroEL(L309D) were unable to rescue GroEL-deficient *E. coli* MM100. Thus, the GroEL mutants, in which Leu^{309} was replaced by polar residues, could not support growth of GroEL-deficient *E. coli* MM100, indicating a critical role for Leu^{309} in chaperonin function *in vivo*.

Chaperone Activity of the Leu^{309} Mutants—The GroEL Leu^{309} mutants were purified, and their properties were examined. ATPase

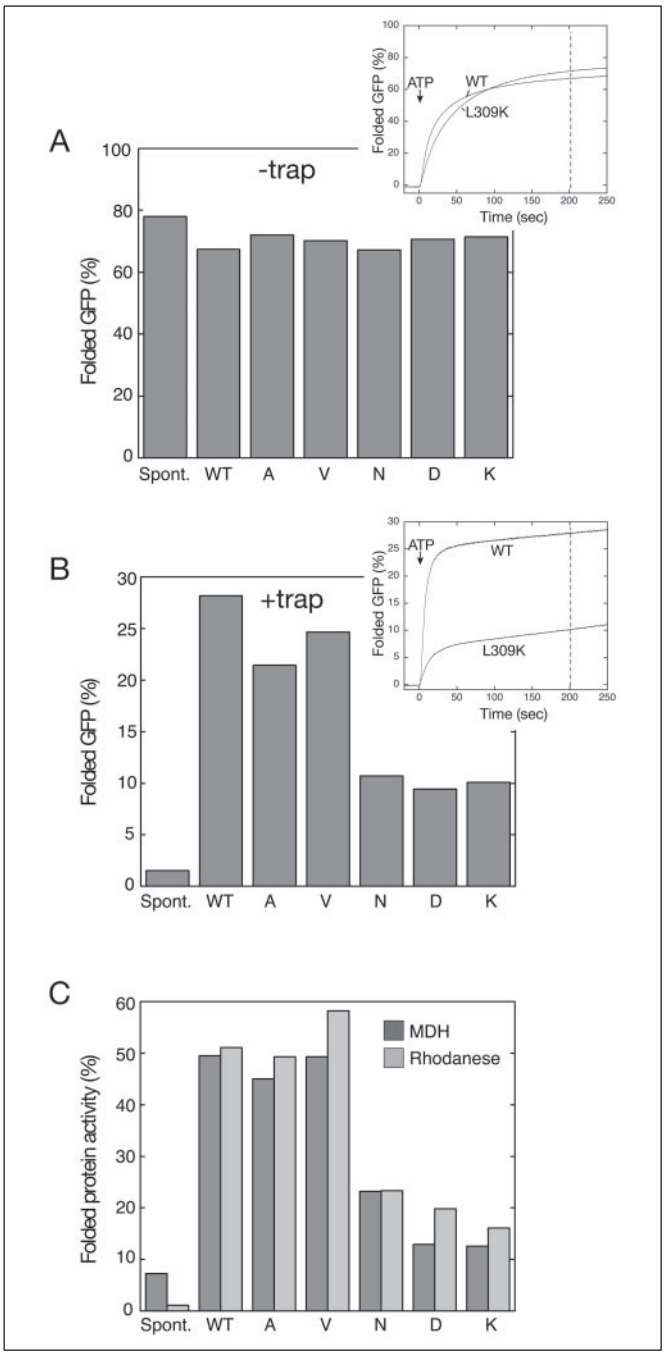

FIGURE 4. **Chaperone activities of GroEL Leu^{309} mutants.** Chaperone activity of GroEL (*WT*) and the mutants, in which Leu^{309} of GroEL was replaced by the alternative residues (*A*, *V*, *N*, *D*, and *K*). *A*, GroEL-assisted GFP folding for multiple reaction cycles. Denatured GFP was diluted into buffer containing GroEL, GroES, and 200 mM glucose. ATP (0.8 mM) was added to initiate folding. Recovery of GFP fluorescence was monitored with a fluorometer; GFP fluorescence measurements at 200 s, following the addition of ATP, are shown. Folding in the presence of bovine serum albumin instead of GroEL and GroES is shown as a spontaneous folding (*Spont.*). *Inset*, a representative example of the folding assay. *B*, GroEL-assisted GFP folding for a single reaction cycle. Denatured GFP was diluted as above (*A*), with 0.04 unit/μl hexokinase, and after incubating for 5 min, a GroEL trap mutant (GroEL(N265A)) was added. A single round of folding was initiated by the addition of ATP and monitored as above (*A*). Other experimental procedures are the same as those in *A*, except that spontaneous folding was done in the buffer containing the trap-GroEL. *Inset*, a representative example of the folding assay under the single cycle reaction conditions. *C*, GroEL-assisted folding of MDH and rhodanese. Denatured MDH or rhodanese were diluted in buffer containing GroEL and GroES. The recovery of activity was measured at 42 min (MDH) and at 40 min (rhodanese), following the initiation of folding, by the addition of ATP. Spontaneous folding (*Spont.*) levels were determined by measurement of folding in the presence of bovine serum albumin, instead of GroEL and GroES. For the purposes of comparison, the intensity of fluorescence (GFP) or activity (rhodanese and MDH) of the same amounts of native protein was considered to be 100% folding.

activities of the mutants were similar to those of the wild type (data not shown). Protein folding activity was tested using GFP. Denatured GFP was diluted into a solution of GroEL and GroES. Upon dilution, denatured GFP was bound efficiently to the GroEL mutant, because no spontaneous GFP folding occurred (Fig. 4*A*, *inset*). GFP started folding upon the addition of ATP, and regardless of mutations, a similar yield of folded protein (~70%) was achieved after 200 s (Fig. 4*A*). In the parallel experiments, hexokinase was included in the mixtures to eliminate ATP and to prevent the secondary turnover of the GroEL reaction cycle. Excess trap-GroEL (GroEL(N265A)) (8) was added prior to ATP addition, to capture unfolded proteins in the bulk solution. Under these conditions, only folding of proteins encapsulated in the *cis*-cavity during the first round of the GroEL reaction cycle would be observed. Under the single cycle reaction conditions, the yield of folded GFP differed among the mutants (Fig. 4*B*). Two mutants, GroEL(L309A) and GroEL(L309V), retained wild type-like folding activity, whereas GroEL(L309N), GroEL(L309D), and GroEL(L309K) gave significantly reduced yields of folded protein. These results indicate that the GroEL mutants in which Leu^{309} was replaced by polar residues tend to fail in encapsulating unfolded proteins into the *cis*-cavity. In addition, we tested the effect of these mutations on the folding of stringent substrate proteins, such as MDH and rhodanese, the folding of which depends on the GroEL/GroES system. GroEL(L309V) and GroEL(L309A) mediated efficient folding of both proteins, with the former giving a better yield of folded protein than wild type GroEL (Fig. 4*C*). As expected, substitution of Leu^{309} by polar residues, particularly Lys and Asp substitutions, resulted in a significant decrease in the yields of reactivated proteins (MDH and rhodanese).

Binding of Substrate Protein and GroES to GroEL(L309K)—In the light of previous investigations of GroEL(L309K) by Fenton *et al.* (16), we sought to analyze further the properties of GroEL(L309K). The mutant protein was mixed with denatured MDH_{Cy3} and analyzed using gel filtration HPLC with elution in a buffer containing ATP (Fig. 5*A*). Like the wild type protein, GroEL(L309K) bound and retained MDH_{Cy3}. The binding of MDH_{Cy3} to GroEL(L309K) was not affected by the presence of GroES in the mixture (Fig. 5*B*). Next we investigated the binding of $GroES_{Cy3}$. In the absence of denatured MDH, GroEL(L309K) formed a complex with $GroES_{Cy3}$ (Fig. 5*C*). However, in the presence of denatured MDH, $GroES_{Cy3}$ was primarily eluted as the free GroES heptamer (Fig. 5*D*). Under the same conditions, the wild type GroEL formed a complex with both MDH_{Cy3} (Fig. 5*B*) and $GroES_{Cy3}$ (Fig. 5*D*). These results demonstrate that GroEL(L309K) can bind denatured substrate protein or GroES individually but cannot form a GroEL·GroES·MDH *cis*-ternary complex that has sufficient stability to survive gel filtration.

cis-Ternary Complexes of GroEL(L309K) Formed in BeFx and Their Sensitivity to Protease Digestion—In general, BeF_x can mimic the phosphate of enzyme-bound nucleotides and stabilize transient complexes in ATP- and GTP-metabolizing proteins (28). In the case of GroEL, BeF_x stabilizes the *cis*-ternary complex of GroEL; a 1:2:2 GroEL·GroES·substrate protein complex with double *cis*-cavities and a 1:1:2 GroEL·GroES·substrate protein complex with a single *cis*-cavity are formed with ATP and ADP, respectively (27). Each cavity contains a substrate protein that is able to fold and that is protected from the attack by protease (27). In anticipation that BeF_x would also stabilize the *cis*-ternary complex of GroEL(L309K), we included BeF_x in the reaction mixtures that contained rhodanese as a substrate protein. The complex was isolated using ultrafiltration and analyzed with SDS-PAGE (Fig. 6, *lanes 1–6*). In a control experiment, 1 mol of free wild type GroEL or GroEL(L309K) (without GroES, nucleotide and BeF_x) bound 2 mol of rhodanese (*lanes 1* and *2*). The GroEL(L309K) complex that formed in

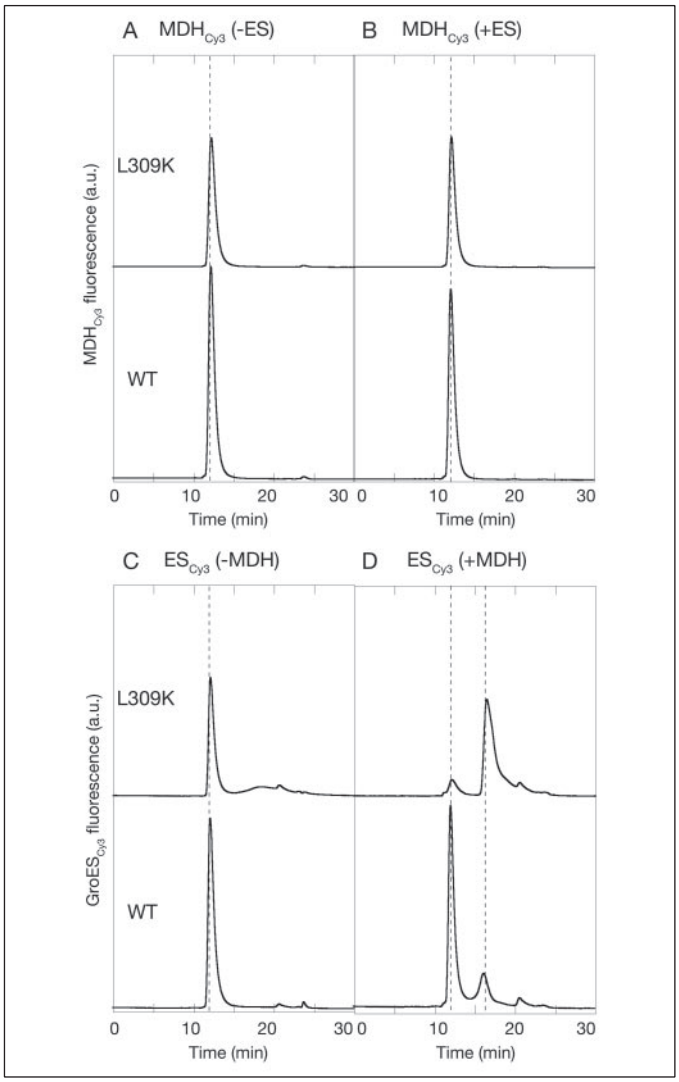

FIGURE 5. **Binding of substrate protein and GroES to GroEL(L309K) and wild type GroEL.** *A* and *B*, Binding of MDH$_{Cy3}$ to wild type (*WT*) GroEL or GroEL(L309K) in the absence (*A*) or presence (*B*) of GroES. Denatured MDH$_{Cy3}$ was diluted in the buffer containing GroEL and incubated for 2 min. GroES was added to *B*, and ATP was added to both reactions, which were incubated at 25 °C for 2 min. The mixtures were applied to a gel filtration HPLC column and eluted with a buffer containing 0.2 mM ATP. Fluorescence (excitation at 550 nm, emission at 570 nm) was monitored in-line. GroEL and complexes with GroES with the substrate or with both GroES and substrate were eluted at approximately 12 min (indicated by a *dotted line*). *C* and *D*, binding of GroES$_{Cy3}$ to GroEL in the absence (*C*) or presence (*D*) of denatured MDH. The reactions were prepared as above, and then GroEL and free GroES $_{Cy3}$ were eluted at 12 and 16 min, respectively (indicated by *dotted lines*).

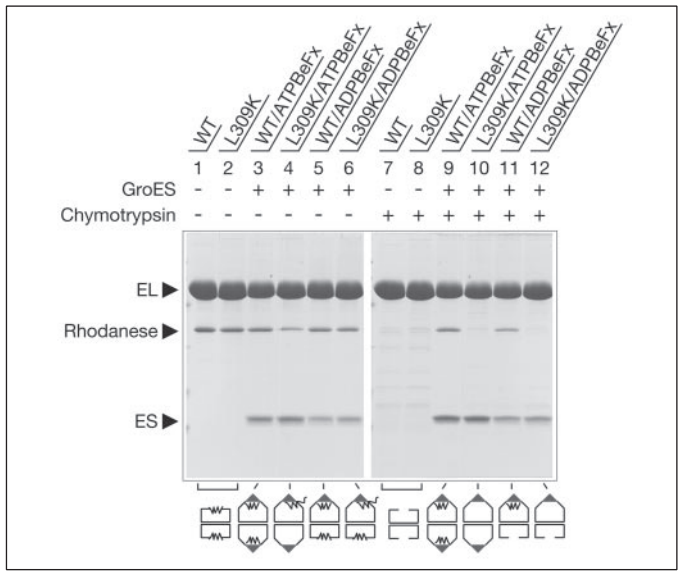

FIGURE 6. **Sensitivity of substrate protein trapped in the GroEL complexes to protease digestion.** GroES, ATP, and BeF$_x$ or GroES, ADP, and BeF$_x$ were added to GroEL (for both wild type (*WT*) and the L309K mutant) that had been saturated with denatured rhodanese. After 90 min, the aliquots underwent one of the two following treatments: ultrafiltration (100-kDa cut) and SDS-PAGE (*lanes 1–6*); or ultrafiltration (100-kDa cut), chymotrypsin treatment, a second ultrafiltration (100-kDa cut), and SDS-PAGE (*lanes 7–12*). The gels were stained with Coomassie Blue.

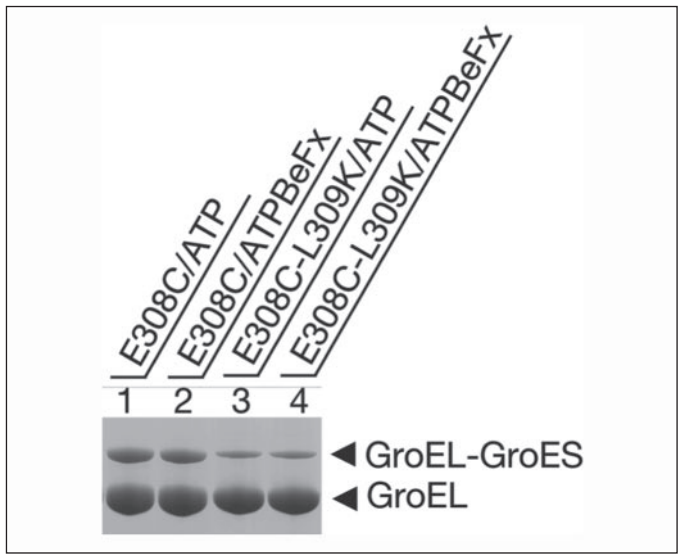

FIGURE 7. **Cross-linking between GroES(S30C) and GroEL(E308C) in the GroEL(L309K) mutant.** Cross-linking was performed between GroES(S30C) and GroEL(E308C) (*lanes 1* and *2*) or GroES(S30C) and GroEL(E308C/E309K), in which a second mutation, GroEL(E308C), was introduced into GroEL(L309K) (*lanes 3* and *4*). The mixture containing 1 mM DTT and the two mutants was subjected to ultrafiltration as described in the legend to Fig. 2 in the presence of either ATP (*lanes 1* and *3*) or ATP and BeF$_x$ (*lanes 2* and *4*).

the presence of ATP and BeF$_x$, GroEL(L309K)·GroES·rhodanese, had an apparent composition of 1:2:1 (*lane 4*). This indicated that, for the GroEL(L309K) mutant, one of the two rhodanese molecules in the 1:2:2 GroEL·GroES·rhodanese complex (*lane 3*) had dissociated. The GroEL(L309K) complex, formed in the presence of ADP and BeF$_x$, had an apparent composition of 1:1:2 (GroEL(L309K)·GroES·rhodanese) (*lane 6*). This composition is the same as found for wild type (*lane 5*). In this complex, one substrate occupies the *cis*-cavity, whereas the other is positioned on the opposite (*trans*) GroEL ring, without GroES. In parallel experiments, the isolated complexes were treated with chymotrypsin, reisolated using ultrafiltration, and analyzed with SDS-PAGE (Fig. 6, *lanes 7–12*). The rhodanese molecules that had bound to free GroEL (wild type or GroEL(L309K)) were digested completely (*lanes 7* and *8*). Rhodanese molecules in the 1:2:2 GroEL·GroES·rhodanese complex were fully protected from digestion (*lane 9*). Rhodanese molecules in the 1:2:1 GroEL(L309K)·GroES·rhodanese complex were digested completely (*lane 10*). For the complexes formed in the presence of ADP and BeF$_x$, only one rhodanese molecule in the wild type 1:1:2 GroEL·GroES·rhodanese complex was digested (*lane 11*). Both rhodanese molecules in the 1:1:2 GroEL(L309K)·GroES·rhodanese complex were digested (*lane 12*). Thus, the substrate proteins in the GroEL(L309K)·GroES complexes are all accessible to attack by chymotrypsin. Similar results were obtained when MDH was used as a substrate protein (data not shown). It appears, therefore, that GroEL(L309K) can form a relatively stable ternary complex with GroES and substrate proteins in the presence of BeF$_x$; however, it cannot prop-

erly shield the substrate proteins from the bulk medium. Thus, the substrate polypeptide is exposed, at least partially, to the protease-containing medium in this experiment. These results indicate that GroEL(L309K) is impaired in its ability to encapsulate substrate protein efficiently into the *cis*-cavity.

Decreased Cross-linking between GroEL(L309K) and GroES—We tested whether or not the GroEL(L309K) mutation affects the contact between the G*XX*LE region and a loop region of GroES. The level of cross-linking between E308C of GroEL and S30C of GroES was lower in the GroEL(L309K) mutant that in wild type, in the presence of ATP or ATP and BeF_x (Fig. 7). These results suggest an aberrant contact between the G*XX*LE region of GroEL(L309K) and GroES.

DISCUSSION

Leu^{309} Plays a Critical Role in the Encapsulation of Substrate—It was previously reported by Fenton *et al.* (16), that GroEL(L309K) was unable to assist with either folding *in vitro* or the rescue of GroEL-deficient *E. coli*. The results from this study indicate that GroEL(L309K) cannot properly encapsulate substrate protein into the *cis*-cavity. It appears that the GroEL(L309K)·substrate protein complex cannot bind GroES in a stable and productive manner. When the complex is stabilized by BeF_x, the substrate protein is unable to fold and is susceptible to protease digestion. This indicates that the substrate protein in the BeF_x-stabilized complex is still tethered to GroEL and is exposed to the outside medium because of incomplete capping by GroES. It is possible that GroES cannot completely sequester the common binding sites on all GroEL subunits, and so both GroES and the substrate protein remain bound to GroEL. Because BeF_x is thought to stabilize transient complexes in many ATP-metabolizing proteins, it is likely that the wild type GroEL also forms a transient intermediate like the GroEL(L309K)·GroES·substrate protein complex, before assuming the productive *cis*-ternary complex structure. If this suggestion is correct, then the next step of the wild type GroEL reaction cycle must be the discharge of substrate into the *cis*-cavity, accompanied by completion of GroES capping. The contention above is similar to the two-timer model of GroEL functioning (11). In that model, the GroEL·substrate complex binds GroES to generate a key intermediate whereby the substrate protein is tethered at some point to GroEL, and decay of this complex (~3 s) accompanies discharge of the substrate protein into the *cis*-cavity. In our experiments, the substrate protein was still in a non-native, protease-susceptible state in the BeF_x-stabilized GroEL(L309K)·GroES·substrate complex. Thus, this ternary complex may resemble the intermediate described in the two-timer model.

The Role of the GXXLE Region—GroEL(L309K) and the other mutants in the G*XX*LE region bind substrate protein normally (Figs. 4 and 5, *A* and *B*). In nucleotide-dependent GroES binding, GroEL(L309K) binds GroES normally in the absence of substrate (Fig. 5*C*). This demonstrates that the G*XX*LE region does not contribute to GroES binding in the absence of substrate. These results confirm previous studies showing that the H-I helices of GroEL are sufficient for GroES binding (16, 19). In contrast, the GroEL(L309K)·substrate protein complex binds GroES poorly (Fig. 5*D*), unless BeF_x is included in the reaction mix to stabilize the transient ATP-bound complex (Fig. 6). These findings indicate that the integrity of the G*XX*LE region is critical for the binding of GroES to the GroEL/substrate protein complex. The following model suggests a means by which Leu^{309} plays a pivotal role in subunit association and function (Fig. 1*B*). When associated with GroEL, one GroES subunit interacts with two adjacent GroEL subunits, using different stretches of the mobile loop (Fig. 1*B*). Similarly, one GroEL subunit is in contact with two adjacent GroES subunits at the H-I helices and the G*XX*LE region (Fig. 1*B*). The two contact sites in a GroEL subunit can communicate with each other through a hydrophobic cluster formed by the side chain of Leu in the G*XX*LE region and residues at the entrance formed by the H helix. Consequently, in the whole GroEL-GroES ring structure, these contacts form an interaction sequence connecting all of the GroES/substrate-binding sites in the apical domain of GroEL and the mobile loop of GroES. Therefore, one can assume that binding of one GroES subunit to the G*XX*LE region can induce conformational transitions in the H helix, which can stimulate release of the substrate and free the binding site for the next GroES subunit. If communication between the G*XX*LE region and the H helix is interrupted, as in the L309K mutation, the interaction sequence will halt and produce an unstable ternary complex. However, further study is required to confirm this hypothesis.

Acknowledgment—We thank Dr. M. Masters for kindly providing us with E. coli MM100.

REFERENCES

1. Bukau, B., and Horwich, A. L. (1998) *Cell* **92,** 351–366
2. Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A., and Horwich, A. L. (1998) *Annu. Rev. Biochem.* **67,** 581–608
3. Thirumalai, D., and Lorimer, G. H. (2001) *Annu. Rev. Biophys. Biomol. Struct.* **30,** 245–269
4. Saibil, H. R., and Ranson, N. A. (2002) *Trends Biochem. Sci* **27,** 627–632
5. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) *J. Bacteriol.* **171,** 1379–1385
6. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature* **342,** 884–889
7. Martin, J., Mayhew, M., Langer, T., and Hartl, F. U. (1993) *Nature* **366,** 228–233
8. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) *Cell* **83,** 577–587
9. Mayhew, M., da Silva, A. C. R., Martin, J., Erdjument-Bromage, H., Tempst, P., and Hartl, F. U. (1996) *Nature* **379,** 420–426
10. Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) *Nature* **388,** 792–798
11. Ueno, T., Taguchi, H., Tadakuma, H., Yoshida, M., and Funatsu, T. (2004) *Mol. Cell* **14,** 423–434
12. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) *Nature* **371,** 578–586
13. Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L., and Deisenhofer, J. (1996) *Nature* **379,** 37–45
14. Viitanen, P. V., Gatenby, A. A., and Lorimer, G. H. (1992) *Protein Sci.* **1,** 361–369
15. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N., and Furtak, K. (1993) *Cell* **74,** 909–917
16. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994) *Nature* **371,** 614–619
17. Ewalt, K. L., Hendrick, J. P., Houry, W. A., and Hartl, F. U. (1997) *Cell* **90,** 491–500
18. Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F., and Hartl, F. U. (1999) *Nature* **402,** 147–154
19. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) *Nature* **388,** 741–750
20. Shimamura, T., Koike-Takeshita, A., Yokoyama, K., Masui, R., Murai, N., Yoshida, M., Taguchi, H., and Iwata, S. (2004) *Structure* (*Camb.*) **12,** 1471–1480
21. Motojima, F., and Yoshida, M. (2003) *J. Biol. Chem.* **278,** 26648–26654
22. Makino, Y., Amada, K., Taguchi, H., and Yoshida, M. (1997) *J. Biol. Chem.* **272,** 12468–12474
23. Motojima, F., Makio, T., Aoki, K., Makino, Y., Kuwajima, K., and Yoshida, M. (2000) *Biochem. Biophys. Res. Commun.* **267,** 842–849
24. Taguchi, H., Ueno, T., Tadakuma, H., Yoshida, M., and Funatsu, T. (2001) *Nat. Biotechnol.* **19,** 861–865
25. McLennan, N., and Masters, M. (1998) *Nature* **392,** 139
26. Sörbo, B. H. (1953) *Acta Chem. Scand.* **7,** 1129–1136
27. Taguchi, H., Tsukuda, K., Motojima, F., Koike-Takeshita, A., and Yoshida, M. (2004) *J. Biol. Chem.* **279,** 45737–45743
28. Chabre, M. (1990) *Trends Biochem. Sci.* **15,** 6–10