The Chaperonin GroEL Is Destabilized by Binding of ADP*

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The urea-induced dissociation and subsequent conformational transitions of the nucleotide-bound form of GroEL were studied by light scattering, 4,4'-bis(1-anilino-8-naphthalenesulfonic acid) binding, and intrinsic tyrosine fluorescence. Magnesium ion alone (10 mM) stabilizes GroEL and leads to coordination of the structural transitions monitored by the different parameters. The midpoint of the light-scattering transition that monitored dissociation of the 14-mer with bound magnesium was raised to ~3 M, which is considerably higher than the ligand-free form of the protein, which exhibits a transition with a midpoint at ~2 M urea. Binding of ADP results in destabilization of the GroEL oligomeric structure, and complete dissociation of the 14-mer in the presence of 5 mM ADP occurs at about 2 M urea with the midpoint of the transition at ~1 M urea. The same destabilization by ADP and stabilization by Mg2+ were seen when the conformation was followed by the intrinsic fluorescence. Complexation with the nonhydrolyzable ATP analog, 5'-adenylylimidodiphosphate gave an apparent stability of the quaternary structure that was between that observed with Mg2+ and that with ADP. The ADP-bound form of the protein demonstrated increased hydrophobic exposure at lower urea concentrations than the uncomplexed GroEL. In addition, the GroEL-ADP complex is more accessible for proteolytic digestion by chymotrypsin than the uncomplexed protein, consistent with a more open, flexible form of the protein. The implication of the conformational changes to the mechanism of the GroEL function is discussed.

Molecular chaperones are proteins that can assist in the folding of other proteins (Hartl et al., 1994). One well studied example is GroEL that is found in Escherichia coli and that is homologous to Hsp60 found in the mitochondrial matrix. GroEL is a large, multisubunit, oligomeric protein consisting of two stacked rings. Each 7-fold rotationally symmetric ring contains seven identical 60-kDa subunits. Each subunit is folded into three domains: 1) an equatorial domain holding the two rings together, 2) an apical domain forming the ends of the cylinder, and 3) a small intermediate domain that connects the equatorial and apical domains (Braig et al., 1994).

GroEL participates in protein folding by first forming a tight, noncovalent complex with the partially folded target protein, which is thereby prevented from misfolding or precipitating, two activities that commonly compete with successful folding. During its function, GroEL requires the presence of adenine nucleotides and the cations, K+ and Mg2+. Release of a bound target protein is accomplished by GroEL-mediated ATP hydrolysis (Hartl et al., 1994), and some proteins require a second protein, the co-chaperonin GroES, which is a seven-subunit homooligomeric protein. For example, the most efficient folding of proteins such as Rubisco (Goloubinoff et al., 1989), rhodanese (Mendoza et al., 1991), 6-hydroxy-o-nicotine oxidase (Brandsch et al., 1992), and ornithine transcarbamylase (Zheng et al., 1993) require the complete chaperonin system, including GroES and ATP. On the other hand, ornithine transcarbamylase monomers that cannot assemble can be released from the GroEL-ornithine transcarbamylase complex by the sole addition of ATP or a nonhydrolyzable ATP analog (Zheng et al., 1993). Refolding of some other target proteins from their GroEL-polypeptide complexes can be induced just by addition of nucleotides. Proteins of this type include dihydrofolate reductase (Viitanen et al., 1991), tryptophanase (Mizobata et al., 1992), and the Fab fragment of a monoclonal antibody (Schmidt et al., 1992). GroEL-protein complexes of various proteins have been shown to be capable of dissociation just by the addition of ATP, ADP, ATPyS, or AMP-PNP. In some more complicated cases, the addition of nucleotide can initiate renaturation of a protein, but the rate of release is increased in the presence of GroES (Fisher, 1992, 1994; Höll-Neugebauer et al., 1991).

It has been suggested that during the GroEL-GroES refolding cycle, the GroEL molecule undergoes several conformational changes (Todd et al., 1993, 1994; Staniforth et al., 1994a). For example, the affinity of GroEL for the unfolded polypeptide is decreased upon binding of GroES and MgATP (Todd et al., 1994; Staniforth et al., 1994b). The binding of ATP leads to conformational changes in the GroEL-ATP complex such that the ATP initially forms a weak collision complex with GroEL (Kd = 4 mM), which isomerizes to a strongly binding state at a rate of 180 s−1 (J. Jackson et al., 1993). The GroEL conformation formed in the presence of ATP was proposed to be distinct from the conformation formed in the presence of ADP (Mizobata et al., 1992). Other investigators proposed the existence of two interconvertible forms of GroEL, one, stabilized by MgATP, which associates weakly with unfolded polypeptide, and a second destabilized by MgATP, which associates strongly with the target protein (Badooe et al., 1991).

In the present work, we have directly monitored conformational consequences of the binding of Mg2+ and the Mg2+-nucleotides of ADP and AMP-PNP. We show that the stability of the protein is greatly decreased in the GroEL-ADP complex, and it is significantly less affected upon binding of AMP-PNP. In contrast, the addition of Mg2+ stabilizes the GroEL oligomer.
It has been shown that GroEL can form monomers at moderate urea concentrations (Mendoza et al., 1994). Due to the large size of the GroEL oligomer, this transition can be easily monitored by light scattering (Price et al., 1993; Mizobata and Kawara, 1994).

The dissociation of the 14-mer, monitored by the decrease in the intensity of the scattered light, is shown for GroEL as a function of urea concentration in Fig. 1 (open circles). The protein was incubated in the standard Buffer A (see "Materials and Methods"). The dissociation transition of GroEL monitored by light scattering. Separate samples were prepared with 380 μg/ml of GroEL in the corresponding standard buffer (see "Materials and Methods"). Samples were preincubated for 90–120 min at the indicated concentration of urea and then measured at 323 nm. Signals were corrected for the corresponding blank values. The lines correspond to 1) GroEL-Mg complex in standard Buffer B (see "Materials and Methods") (closed circles), 2) GroEL in standard Buffer A (see "Materials and Methods") (open circles), 3) GroEL-ADP complex in standard Buffer B (see "Materials and Methods") (open squares). The lines are plotted according to nonlinear least squares fit to the data as described under "Materials and Methods." The fit for the GroEL-Mg complex uses two transitions, and the major one was used for calculation and comparison. All other transitions were fit to single transitions.

### RESULTS

**Analysis of the GroEL Dissociation Transition by Light Scattering**—It has been shown that GroEL can form monomers at moderate urea concentrations (Mendoza et al., 1994). Due to the large size of the GroEL oligomer, this transition can be easily monitored by light scattering (Price et al., 1993; Mizobata and Kawara, 1994).

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GroEL, analyzed by gel electrophoresis, increases upon addition of divalent cations such as Mg²⁺, Mn²⁺, Ca²⁺, or Zn²⁺ (Azem et al., 1994). The present results extend those previously reported, since the nondenaturing gel electrophoresis used to detect intact oligomers, as normally performed, removes denaturant, and, therefore, it detects only irreversible dissociation (Mendoza, et al., 1995).

Binding of MgADP Destabilizes Oligomeric GroEL—It was previously shown that ATP-ADP exchange plays important role in the mechanism of GroEL function (Todd et al., 1994). GroEL with bound nucleotide has been suggested to be more labile to dissociation than the unliganded form of the protein (Lissin, 1995; Jackson, et al. 1993; Gorovits et al., 1995). In the present study, light scattering was utilized to monitor the stability of the GroEL-ADP complex. Fig. 1 (open squares) shows that the GroEL oligomer was more easily dissociated in the GroEL-ADP complex (1 μM GroEL and 5 mM ADP). The transition begins at very low urea concentration (less than 0.5 M) and is practically complete by 1.5–1.7 M urea. The midpoint of the transition is estimated as 1.0 M urea.

Several distinct species were detected for the GroEL-MgADP complex at 0.8 and 0.896 M urea by native polyacrylamide gel electrophoresis, where the gel contained MgCl₂ (10 mM), ADP (10 mM), and the corresponding amount of urea (data not shown). This can reflect the fact that 7-mers and other forms of GroEL, produced as a result of the protein dissociation, have increased relative stability in the presence of MgADP. We cannot exclude the possibility that GroEL molecules with different mobilities could reflect different numbers of bound ADP molecules.

Structural Transitions in GroEL Can Be Monitored by Intrinsic Tyrosine Fluorescence—The GroEL monomer contains seven tyrosines (Tyr-199, Tyr-203, Tyr-360, Tyr-476, Tyr-478, Tyr-485, and Tyr-506) and no tryptophans. Fig. 4 demonstrates that structural transitions in GroEL can be monitored by the tyrosine fluorescence and shows (closed squares) the transition for GroEL in standard Buffer B (see “Materials and Methods”) measured at 310 nm (samples were excited at 280 nm). The solid line represents a fit of this transition to a two-state model using a nonlinear least squares fit to the equation described by Pace (1990). The transition extends from approximately 2.7 M urea to 3.3 M urea. The addition of MgADP resulted in a significant decrease of the GroEL stability, which is reflected by large shift in the transition with the midpoint moving to 1.44 M urea (Fig. 4, open squares). This transition in the presence of ADP monitored by tyrosine fluorescence reflects the dissociation and not unfolding since the bis-ANS fluorescence remains high (Fig. 6) at urea concentrations producing nearly the minimum observed intensity of the intrinsic fluorescence. The binding to GroEL of the nonhydrolyzable ATP analog, AMP-PNP, resulted in a considerably smaller decrease of the protein stability, compared with ADP (Table I).

FIG. 4. The urea concentration dependence of the tyrosine-intrinsic fluorescence of GroEL. Separate samples for each point were made with 1.0–1.7 μM GroEL in the corresponding standard buffer (see “Materials and Methods”). Samples were incubated for 90–120 min. prior to measurements. Fluorescence was excited at 280 nm, and it was detected at 300–340 nm. The lines correspond to 1) GroEL-Mg complex in Buffer B (closed squares), and 2) GroEL-ADP complex in Buffer B with 5 mM ADP (open squares). The lines are plotted according to nonlinear least square fits to the data as described under “Materials and Methods.”
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| TABLE I |
| --- |
| Changes in free energy and $U_{14}$ of GroEL upon binding Mg$^{2+}$, ADP, and AMP-PNP |
| | Free energy ($\Delta G_{th}^{2}$) | $U_{14}^{b}$ |
| --- | --- | --- |
| GroEL only | 13.05 ± 1.53 (TIF)$^c$ | 3.02 |
| GroEL-Mg complex | 3.17 ± 0.3 (LSA)$^a$ | 2.06 |
| GroEL-MgAMP-PNP complex | 18.15 ± 3.3 (TIF) | 3.05 |
| GroEL-MgADP complex | 15.16 ± 1.52 (LSA) | 3.12 |
| GroEL-MgAMP-PNP | 7.65 ± 1.17 (TIF) | 2.85 |
| GroEL-MgADP complex | 4.03 ± 0.60 (TIF) | 1.44 |
| GroEL-MgAMP-PNP | 2.55 ± 0.22 (LSA) | 1.01 |

$^{a} \Delta G_{th}$ is the free energy of the transition in the absence of denaturant determined as the extrapolated y intercept from the equation $\Delta G = \Delta G_{th} - m [\text{urea}]$, where $\Delta G$ is the free energy determined from the equilibrium constant in the transition region.

$^{b} U_{14}$ urea concentration at the midpoint of transition. $U_{14} = \Delta G_{th}/m$, where $m$ is defined above.

$^{c}$ TIF, calculated from transition monitored by intrinsic tyrosine fluorescence.

$^{d}$ LSA, calculated from transition monitored by light scattering.


duction of several distinct polypeptides (Horowitz et al., 1995).

Fig. 5 shows the results of an experiment in which GroEL was treated with chymotrypsin (1%, w/w) in the absence of urea at 37°C in the absence (lanes 4 and 5) or the presence of 10 mM ADP (lanes 2 and 3). Proteolysis was stopped after either 60 (lanes 2 and 4) or 120 (lanes 3 and 5) min. The appearance of a distinct ~26-kDa band in the case of the sample that contained nucleotide is consistent with the idea that the protein is more open in the GroEL-Md complex (Fig. 5, arrow). Similar species have been observed before (Horowitz et al., 1995; Seale et al., 1995) for the GroEL digest, and it was shown that this band represents the N-terminal portion of the protein (approximately up to 250 amino acids) and contains parts of the equatorial and apical domains.

GroEL-ADP Complex Exhibits Increased Hydrophobic Exposure at Low Urea Concentrations—bis-ANS is a reporter of exposed hydrophobic surfaces on proteins. It has been shown that GroEL displays restricted hydrophobic surfaces and binds tightly 1–2 molecules of bis-ANS/14-mer (Horowitz et al., 1995). The two phases, dissociation of the GroEL-14-mer and the unfolding of the resulting monomers with increasing urea concentration, are reflected in an increase followed by a decrease in fluorescence phases shown in Fig. 6 with bis-ANS fluorescence as organized hydrophobic surfaces are first exposed and then disrupted (Horowitz et al., 1995) in a process that, in the absence of ADP, occurs at urea concentrations that have been shown to disrupt GroEL secondary structure (Mendoza et al., 1994).

The profile shown here for GroEL in the absence of ADP (Fig. 6, closed squares) demonstrates a sharp increase in the bis-ANS fluorescence between 2 and 3 M urea. This considerable increase in the bis-ANS fluorescence demonstrates that a large amount of hydrophobic surface is hidden in the unperturbed conformation of GroEL. Addition of ADP to the GroEL leads to a large shift in the bis-ANS fluorescence profile to the lower concentrations of urea (Fig. 6, open squares). The high level of bis-ANS fluorescence at about 1.5 M urea for protein that is mainly monomeric (Fig. 1, open squares) supports the conclusion that the oligomeric form of GroEL is less stable in the presence of ADP.

Comparison of Urea-induced Transitions of GroEL in the Presence of Mg$^{2+}$ and Nucleotides Monitored by Tyrosine Intrinsic Fluorescence and Light Scattering—The urea-induced transitions for GroEL monitored by intrinsic fluorescence and light scattering can be compared, and the transitions fall into three groups: 1) a group at approximately 3 M urea includes all the transitions for the GroEL-Mg complex and the transition for the uncomplexed GroEL and the GroEL-MgAMP-PNP complex monitored by intrinsic fluorescence; 2) a group centered at approximately 2 M urea that includes the GroEL-MgAMP-PNP monitored by light scattering (data not shown) and uncomplexed GroEL; and 3) a group below 1.5 M urea that includes the transitions detected by both fluorescence and light scattering for the GroEL-MgADP complex. The increasing fluorescence phases shown in Fig. 6 with bis-ANS correspond closely to the transitions noted above for the GroEL-Mg complex or the GroEL-MgADP complex.

Based on these transitions, the values of free energy of stabilization of GroEL were derived from the parameters of the nonlinear, least squares fitting procedure described under "Materials and Methods" (Table I). The analysis shows that the structure of GroEL monitored by intrinsic fluorescence is slightly stabilized by binding of Mg$^{2+}$ ($\Delta \Delta G = -5.10$ kcal/mol), while the quaternary structure monitored by light scattering is stabilized to a greater extent ($\Delta \Delta G = -11.99$ kcal/mol). Binding ADP in the presence of Mg$^{2+}$ produces a large destabilization ($\Delta \Delta G = -14.12$ kcal/mol by intrinsic fluorescence) of the quaternary structure. Binding of AMP-PNP under these conditions produces a smaller change ($\Delta \Delta G = -10.5$ kcal/mol).
DISCUSSION

Two features of GroEL that have been suggested to be important for binding target proteins are 1) coordination of the multiple sites in the digomer, and 2) conformational changes that could lead to exposure of interactive surfaces. For example, studies with fluorescent probes suggested that conformational changes in GroEL are required for extensive hydrophobic exposure, so that important functional aspects of chaperonin-assisted refolding reside in the ease with which ligand interactions can trigger conformational changes (Horowitz et al., 1995). Previous studies have demonstrated that relevant conformational changes are related to changes in subunit interactions that can be monitored by the facility with which chaperonin-assisted refolding reside in the ease with which ligand interactions can trigger conformational changes (Horowitz et al., 1995). Previous studies have demonstrated that relevant conformational changes in GroEL are required for extensive hydrophobic exposure, so that important functional aspects of chaperonin-assisted refolding reside in the ease with which ligand interactions can trigger conformational changes (Horowitz et al., 1995).

The present work has demonstrated major changes in the stability of GroEL when it binds functionally relevant ligands such as MgADP or the MgADP complex of ATP at concentrations common to in vivo conditions (Diamant et al., 1995). In general, MgADP increased the stability of GroEL to produce a state in which conformational changes are correlated. Nucleotide binding destabilized this state, with ATP giving the greatest change. These results are consistent with previous observations that nucleotide binding can influence cooperativity among subunits (Diamant et al., 1995). The present results provide further support since they show that ligand binding can couple conformational changes in the monomer to quaternary structure and, therefore, increase cooperativity involved in binding folding intermediates.

In the absence of added ligands, the structural changes in GroEL are uncorrelated. The light-scattering transition occurred at lower concentrations of urea than the transition monitored by the intrinsic fluorescence. This is in keeping with the previous results showing that monomers could be formed at lower concentrations of urea than those causing denaturation of the protein (Mendoza et al., 1994). The transition monitored by the bis-ANS fluorescence increase starts before significant changes in the intrinsic fluorescence, and the maximum of the hydrophobic exposure occurs at the end of the scattering transition and before the end of the changes in the intrinsic fluorescence. In short, the transitions followed by the three parameters are not coincident.

There are two major effects when MgADP is added to solutions of GroEL: 1) the protein becomes more stable relative to the unliganded state; and 2) the transitions become closely correlated, in that they occur over the same range of urea concentrations. The most obvious change is that the light-scattering transition is moved to higher urea concentrations. Thus, the MgADP binding stabilizes the quaternary structure of the protein (Table I). These results are compatible with previous studies using chemical cross-linking of GroEL, which showed that MgADP increased the interactions among the monomers of GroEL, particularly the contacts between monomers within the heptameric rings (Azem et al., 1994).

The most surprising result is that the quaternary structure of GroEL is very markedly destabilized by MgADP. The transitions become closer to being coincident, and it is much easier to expose hydrophobic surfaces in the presence of MgADP. The ∆G was 14.12 kcal/mol relative to the MgADP bound state for the transition monitored by intrinsic fluorescence.

When AMP-PNP was used, the structure of GroEL was destabilized relative to the magnesium-bound state as assessed by light scattering, although to a smaller extent than with MgADP. For the transition monitored by intrinsic fluorescence, the ∆G was 10.5 kcal/mol relative to the MgADP bound state. A comparison of these results with those reported previously in which ATP appeared to destabilize GroEL suggests that at least part of the reported effect might have been due to the spontaneous hydrolysis of ATP (Horowitz et al., 1995).

An interesting feature of GroEL is that many diverse proteins can interact with this chaperonin and there are different requirements for their release (Lorimer et al., 1993). ATP binding is sufficient for release of several target proteins, so there is no obligatory requirement for ATP hydrolysis or for GroES. Thus, nonhydrolyzable analogs of ATP can release glutamine synthase (Fisher, 1994), ornithine transcarbamylase (Zheng et al., 1993), barnase (Gray and Fersht, 1993), lactate dehydrogenase (Badcoe et al., 1991), and dehydrofolate reductase (Viitanen et al., 1991). In a number of cases, target proteins are bound more tightly in the presence of MgADP. Thus, lactate dehydrogenase and dehydrofolate reductase remain bound to GroEL in the presence of ADP under conditions that would lead to release if ATP were used (Badcoe et al., 1991; Staniforth et al., 1994a; Viitanen et al., 1991). Specifically, in the presence of GroES, the exchange of ADP for ATP at one of the sets of nucleotide binding sites on GroEL results in considerably tighter binding of lactate dehydrogenase with bound ADP (Kd = 34 nM) compared with bound ATP (Kd = 440 nM) (Staniforth et al., 1994a). Even GroES itself, whose binding sites on GroEL overlap the binding sites for bound polypeptides, binds tightly in the presence of MgADP (Todd et al., 1994, Schmidt et al., 1994). These results are reminiscent of nucleotide effects on the binding of proteins to the chaperonin, Hsp70. In that system, oscillation between a loose binding ATP state and a tight binding ADP state are involved in the alternate binding and release of target proteins during the cycle of this chaperonin (Pallerios et al., 1994).

It has been suggested that hydrophobic exposure on unper- turbated GroEL is less important than the ease with which hydrophobic surfaces can be induced when the chaperonin interacts with targets or ligands (Horowitz et al., 1995; Gibbons et al., 1995). Therefore, the effects of nucleotides on the ease of exposure of hydrophobic surfaces may have important consequences. If hydrophobic binding involving GroEL were too strong and uncoordinated, two possibilities are suggested: 1) GroEL might aggregate or the 14-mer might bury those surfaces internally so they would not be available for interactions with target polypeptides, and/or 2) Hydrophobic interactions with the target would be so strong that subsequent release would be difficult. Therefore, facile and reversible binding and release of polypeptide chains requires a balance between the exposure and coordination of hydrophobic surfaces on the chaperonin. Thus, nucleotide and ion binding to GroEL can provide a number of functions, and they can 1) shift the energy for exposure of hydrophobic surfaces into a biologically relevant range, 2) help coordinate the changes among the subunits in the 14-mer; and 3) permit structural differences induced by ATP and ADP to participate in the binding and release of peptide segments required for productive folding of proteins.

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