ATP Hydrolysis Is Critical for Induction of Conformational Changes in GroEL That Expose Hydrophobic Surfaces*

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The degree of hydrophobic exposure in the molecular chaperone GroEL during its cycle of ATP hydrolysis was analyzed using 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonic acid (bisANS), a hydrophobic probe, whose fluorescence is highly sensitive to the environment. In the presence of 10 mM MgCl₂ and 10 mM KCl the addition of ATP, but not ADP or AMP-PNP, resulted in a time-dependent, linear increase in the bisANS fluorescence. The rate of the increase in the bisANS fluorescence depended on the concentrations of both GroEL and the probe. The effect could be substantially inhibited by addition of excess ADP or by converting ATP to ADP using hexokinase, showing that the increase in the bisANS fluorescence was correlated with ATP hydrolysis. The rate of ATP hydrolysis catalyzed by GroEL was uncompetitively inhibited in the presence of bisANS. This uncompetitive inhibition suggests that the probe can interact with the GroEL-ATP complex. The inability of the nonhydrolyzable ATP analog, AMP-PNP, to cause a similar effect is explained by the interaction of bisANS with a transient conformational state of GroEL formed consequent to ATP hydrolysis. It is suggested that this short lived hydrophobic exposure reflects a conformational shift in GroEL that results from electrostatic repulsion between the bound products of ATP hydrolysis, and it plays an important role in the mechanism of the chaperonin cycle.

The molecular chaperones have been shown to facilitate protein folding and transport of nascent polypeptides in vivo (1). The chaperonin system, involving Escherichia coli proteins GroEL and GroES, has been demonstrated to promote in vitro refolding of a wide variety of proteins (4), including rubisco (2) and rhodanese (3). This refolding activity is associated with ATP binding and hydrolysis. Recently, a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL has been suggested (5, 6). This model implies that there is a thermodynamic partitioning between folding of the protein to internalize its hydrophobic residues and the hydrophobic interaction of the protein with hydrophobic surfaces on GroEL. This proposal emphasizes the importance of investigating the role of hydrophobic interactions in the mechanism of GroEL-assisted protein folding. In addition, all models for interactions involving GroEL during the folding cycle recognize the importance of nucleotide binding and hydrolysis in driving the chaperonin cycle that alternately binds interactive intermediates and releases species that are capable of folding. No models to date have identified explicitly how the act of hydrolysis is coupled to the alteration of hydrophobic interactions. Does the hydrolysis serve simply to exchange ATP and ADP or does the hydrolytic event provide the motive force for the requisite conformational changes? If it is the latter, how could the hydrolysis be coupled to the structural events?

In the present study, we have investigated the level of hydrophobic exposure in the molecular chaperone GroEL during its cycle of ATP hydrolysis. The hydrophobic probe, 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonic acid (bisANS), has been used. The influence of different nucleotides has been studied. In the presence of MgCl₂ and KCl, addition of ATP, but not ADP or AMP-PNP, resulted in a linear time-dependent increase in the bisANS fluorescence. It is shown that the increase in the bisANS fluorescence is correlated with ATP hydrolysis itself, rather than with the binding of nucleotides. The importance of the hydrophobic exposure, occurring during the ATP hydrolysis, is discussed.

MATERIALS AND METHODS

Reagents and Proteins—1,1'-bis(4-Anilino)naphthalene-5,5'-disulfonic acid was purchased from Molecular Probes (Eugene, OR). ATP, ADP, and AMP-PNP were purchased from Sigma. All reagents were analytical grade.

Protein Purification—The chaperonin GroEL was expressed in E. coli and purified as described previously (7). After purification, GroEL was dialyzed against 50 mM Tris-HCl, pH 7.6, containing 1 mM dithiothreitol, then made 10% (v/v) in glycerol, rapidly frozen, and stored at −70 °C. The GroEL protomer concentration was measured using the bicinchoninic acid protein assay (Pierce) according to the procedure recommended by the manufacturer and assuming a molecular mass of 60 kDa.

Standard Buffer—The following solution was used as a standard buffer: 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM KC1.

Fluorescence Detection of the bisANS Interaction with GroEL—Fluorescence measurements were made on a SLM 500e spectrofluorometer (SLM Instruments, Urbana, IL). Samples contained 0.1–0.4 μM GroEL and 0.2–40 μM bisANS in the corresponding Standard Buffer. Samples were excited at 399 nm, and fluorescence was monitored at 500 nm.

Catalysis of the ATP Hydrolysis by GroEL—The ATPase activity of GroEL was measured by the method of Viitanen et al. (8) with the modifications of Horovitz et al. (9). The concentration of GroEL oligomer was 10 μM in Standard Buffer, containing 0–40 μM [γ-³²P]ATP (72–235 Ci/mmol) and 0–40 μM bisANS. The reaction was carried out for 0–16 min.

RESULTS

The Binding of the Hydrophobic Probe bisANS Changes in the Presence of Nucleotides—One of the methods for monitoring changes in the exposure of hydrophobic surfaces in a protein involves use of noncovalent fluorescent probes. One such probe, bisANS, has been widely used to study conformational changes in proteins (10, 11). bisANS fluorescence is increased in the presence of GroEL, showing that some of the hydrophobic sites of the protein are solvent-exposed under normal conditions.
The fluorescence intensity reaches a plateau after prolonged incubation (data not shown). For example, under the conditions shown in Fig. 1 (0.4 μM GroEL, 1 mM ATP, 10 μM bisANS) the fluorescence plateaus at a value of ~10 units after a 10-min incubation. The rate of the increase of the bisANS fluorescence at a given initial ATP concentration depends on the concentrations of both the protein and the bisANS. For example, at 5 μM bisANS, increasing GroEL concentrations result in higher rates of bisANS fluorescence increase, which reach an apparent maximum rate at [GroEL] = 0.3–0.4 μM (data not shown). It should be noted that under these conditions there are 5.6 μM GroEL monomers so there is no large excess of bisANS. The dependence of the rates on the bisANS concentration is somewhat more complex. The rates of the fluorescence change increase up to about a 5 μM concentration of the probe, and then the rates decrease as the bisANS concentration is raised further. The latter effect can be explained by the inhibition of the ATPase activity of GroEL at high concentrations of the probe, >5 μM (see below). For example, the rate of GroEL-catalyzed ATP hydrolysis is inhibited 46.6% at [bisANS] = 40 μM.

To elucidate the type of inhibition of the GroEL-catalyzed ATP hydrolysis by bisANS, the reaction was performed at different concentrations of bisANS (Fig. 2A). The initial ATPase velocities were fit to the Hill equation. The dependences of the maximal initial ATPase velocities (V$_{max}$), apparent dissociation constants (K), and Hill coefficients (n) were analyzed as a function of [bisANS]. Both V$_{max}$ and K were reduced at high concentrations of the probe (Fig. 2B), indicating that bisANS is an uncompetitive inhibitor of the ATPase activity of GroEL. The value of the Hill coefficient obtained in the absence of bisANS (n = 2.41) corresponds to the previously reported value (9). It is interesting that n does not change considerably up to 40 μM bisANS, although the rate of the reaction is reduced to 46.6% of the initial value (Fig. 2B). ATPase activities were constant after preincubation of GroEL for at least 10 min at the concentrations of bisANS used. This indicates that bisANS does not cause any progressive changes in GroEL under the present experimental conditions.

bisANS was observed to dissociate from GroEL when samples that had been incubated with ATP and bisANS were diluted 1:10 into buffer without the probe to give a final bisANS concentration of 1 μM. The decrease of the fluorescence signal could be fit with two exponentials having approximately equal amplitudes and rate constants of 47.5 × 10⁻³ s⁻¹ and 5.3 × 10⁻³ s⁻¹ without ATP in the dilution buffer and 82.2 × 10⁻³ s⁻¹ and 12.1 × 10⁻³ s⁻¹ in the presence of 0.2 mM ATP. These results demonstrate reversibility of the bisANS-GroEL interaction, and they show that ATP can accelerate both binding and dissociation of the probe.

The Increase in the bisANS Fluorescence Is Related to the GroEL-catalyzed ATP Hydrolysis—Several possibilities were examined as contributing to the increasing bisANS fluorescence in the presence of GroEL and ATP. No covalent phospho-
Hexokinase (5 units) was added to the solution containing 0.4 mM ADP using hexokinase and glucose (Fig. 4). In this experiment GroEL and ATP could also be quenched by converting ATP to ADP. Excessive amounts of ADP (≥2.0 M) caused a slight decrease in the bisANS fluorescence in the presence of ATP and GroEL. ATP and GroEL were pre-mixed, and ATP hydrolysis was allowed to proceed in the absence of bisANS. When bisANS was then added to replicate samples after various intervals, the initial fluorescence increment and the subsequent rates of increase of the bisANS signal were close to those observed if the bisANS had been added at t = 0. Finally, under conditions where GroEL ATPase activity was largely suppressed (low concentration of K+), no significant increase in the bisANS fluorescence was observed (data not shown).

Addition of increasing amounts of ADP to the mixture containing 0.1 μM GroEL, 0.2 mM ATP, and 5 μM bisANS, successfully inhibited the fluorescence increase (Fig. 3). The effect was completely quenched when the ADP concentration reached 1 mM. Excessive amounts of ADP (>2 mM) caused a slight decrease in the fluorescence signal (data not shown).

The increase in the bisANS fluorescence in the presence of GroEL and ATP could also be quenched by converting ATP to ADP using hexokinase and glucose (Fig. 4). In this experiment hexokinase (5 units) was added to the solution containing 0.4 μM GroEL, 0.2 mM ATP, 10 μM bisANS, and 0.1 mM glucose. ATP is quickly hydrolyzed to ADP in the enzymatic reaction catalyzed by hexokinase. As a result, the rate of increase in the bisANS fluorescence was largely inhibited (Fig. 4, lower trace).

**DISCUSSION**

Hydrophobic interactions have been suggested to be the main driving force for the association of protein-substrates with GroEL (13). Therefore, the oligomer of the chaperonin should possess, or be able to express under certain conditions, hydrophobic surfaces, that are necessary for this interaction. By using the hydrophobic probe bisANS, only limited accessible hydrophobic regions are found in the intact chaperonin GroEL (3). Due to the high degree of the intramolecular flexibility, GroEL is able to expose hydrophobic sites under certain conditions (14, 15). For example, a noticeable increase in the number of bisANS binding sites in the unperturbed GroEL 14-mer can be induced upon addition of salt (12) or under conditions where GroEL is dissociated into folded monomers (e.g., ≥2.0 M urea) (16).

Although interaction of the GroEL oligomer with nucleotide under conditions where no hydrolysis is possible results in a conformational change (17), the present data show that there is no considerable change in the level of accessible hydrophobic surfaces (Fig. 1).

In the presence of K+, GroEL possesses weak ATPase activity (18). Under such conditions, addition of the hydrophobic probe results in a time-dependent increase in the bisANS fluorescence (Fig. 1), demonstrating the ability of the probe to bind to the hydrophobic surfaces that can be exposed on GroEL during the catalytic cycle of ATP hydrolysis. Since excess ADP or complete conversion of ATP to ADP by using the hexokinase-catalyzed reaction can completely inhibit the effect, the increase in the bisANS fluorescence can be directly correlated to the GroEL-catalyzed ATP hydrolysis. It should also be noted that the addition of ATP does not induce quickly a large fluorescence increment (Fig. 1, nucleotide added). This supports further the idea that the increase in bisANS fluorescence follows the hydrolysis and not the binding of ATP.

The results are consistent with the model presented in Scheme 1. In this model, the binding of ATP to GroEL is followed by the hydrolysis step where an intermediate state (GroEL-ADP-Pi), I, is produced. This GroEL conformation (shown in brackets) is not stable, and it transiently contains both bound ADP and Pi. In fact, because of the positive cooperativity among the ATPase sites within one ring of the GroEL oligomer, the products from the hydrolysis of 7 mol of ATP would be bound to that ring. The electrostatic repulsion between the bound ADP and Pi presumably contributes to this instability. The transient state, I, would have increased accessibility of hydrophobic surfaces, and, therefore, it would be able to bind bisANS with a consequent increase of the fluorescence signal. It has been shown that substrate and ligand interactions can modulate hydrophobic exposure in GroEL, so it cannot be excluded that the interactions of bisANS can contribute to the appearance of hydrophobic sites. Thus, the metastable state, I, or one that is accessible from it forms only after ATP hydrolysis and is not observed with any equilibrium states that can be produced by incubation with nucleotides. This potential...
Conformational Changes in GroEL Are Linked to ATP Hydrolysis

A large conformational change in the GroEL molecule has been detected by using electron microscopy (19). Interestingly, similar changes have been found when GroEL is incubated with ADP and P_i, but not with ADP alone (20). These changes were observed by electron microscopy of samples incubated in 30 mM ADP and P_i before fixation. In the present study, no large increases in hydrophobic exposure were observed at 3 mM ADP and P_i (data not shown). This presumably reflects the apparently large energy from concerted ATP hydrolysis. The conformational shift, occurring during ATP hydrolysis by GroEL, has been suggested to result in the formation of an open conformation in which the apical domain of each GroEL monomer moves upward and outward. This has the effect of exposing previously hidden areas of the GroEL molecule. A GroEL mutant has recently been prepared which was constrained by inserting a disulfide bridge in such a manner that movements of the apical domain of each GroEL monomer would be hindered. This mutant is locked in a closed conformation, and the hydrolysis of ATP is inhibited although ATP is bound (21). Those results are consistent with the picture presented here showing that ATP hydrolysis and structural changes are coupled, and hydrolysis per se and not only nucleotide binding have important conformational consequences. The same method was used to suggest that the release of substrate protein from GroEL is directly coupled with the ATP hydrolysis step (21).

The results presented here are consistent with the suggestion that GroEL can undergo a large conformational shift accompanied by large hydrophobic exposure during ATP hydrolysis. Since the unstable, transient GroEL conformation generated during ATP hydrolysis contains both ADP and P_i bound to it, the high energy of the electrostatic repulsion between these two species could produce the force necessary for GroEL to undergo the conformational shift. This molecular movement enables GroEL to unfold partially folded protein bound on the chaperonin. The subsequent release of the substrate protein, due to its tendency to fold, will allow the polypeptide to form a native-like conformation or correct a misfolded state. Further, it follows that the major exposure of hydrophobic surfaces is transient, thus explaining why no extensive hydrophobic surfaces are commonly detected on GroEL in complexes at equilibrium.

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