High Salt-induced Conversion of *Escherichia coli* GroEL into a Fully Functional Thermophilic Chaperonin*

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The GroE chaperonin system can adapt to and function at various environmental folding conditions. To examine chaperonin-assisted protein folding at high salt concentrations, we characterized *Escherichia coli* GroE chaperonin activity in 1.2 M ammonium sulfate. Our data are consistent with GroEL undergoing a conformational change at this salt concentration, characterized by elevated ATPase activity and increased exposure of hydrophobic surface, as indicated by increased binding of the fluorophore bis-(5,5’)-8-anilino-1-naphthalene sulfonic acid to the chaperonin. The presence of the salt results in increased substrate stringency and dependence on the full GroE system for release and productive folding of substrate proteins. Surprisingly, GroEL is fully functional as a thermophilic chaperonin in high concentrations of ammonium sulfate and is stable at temperatures up to 75 °C. At these extreme conditions, GroEL can suppress aggregation and mediate refolding of non-native proteins.

The chaperonin GroEL from *Escherichia coli* belongs to a class of proteins, termed molecular chaperones, whose collective function is to assist in the folding of newly synthesized proteins and in the refolding of non-native polypeptides generated under conditions of stress (reviewed in Refs. 1 and 2). Like its homologs, CCT (chaperonin containing TCP-1) in eukaryotes and the thermosome in archaea, GroEL forms a multi-subunit assembly arranged into twin rings stacked end-to-end (3–5). The resultant homotetradecamer of 57-kDa subunits provides a deep cavity where non-native protein species may bind and undergo productive folding (6). GroEL is assisted in its chaperoning function by GroES, a heptamer composed of identical 10-kDa subunits arranged into a single ring (7, 8). One of the functions of GroES is to act as a lid on the GroEL cylinder, thereby providing an enclosed environment for the folding polypeptide. However, its binding to GroEL plays other important roles too, such as modulating the low intrinsic ATPase activity of GroEL by coordinating the actions of nucleotide binding and hydrolysis (7–9). Crystallographic data have enabled the visualization of the GroEL complex and individual subunit architecture (10–12). It has revealed the regions implicated in both nucleotide and substrate binding. It is now known that each subunit of GroEL is arranged into three domains. An equatorial domain forms the majority of intersubunit interactions and is the site of nucleotide binding. The apical domain contains the substrate binding and GroES binding residues (13). The two domains are connected by a hinge region, which transmits information on the status of GroES, polypeptide, and nucleotide binding (14). Because GroEL/ GroES has been the most widely studied chaperonin system, its mechanism of protein folding is known in some detail. Briefly, GroEL binds substrate protein in one of its two ring cavities. The bound substrate protein is in a molten-globule state characterized by the presence of secondary structure but lacking well defined tertiary structure (8, 15). Cooperative binding of seven molecules of ATP to the same (cis) ring as the bound polypeptide is immediately followed by the binding of GroES, also to the cis ring, which causes the polypeptide to be displaced into the cavity (4, 17). The released polypeptide now folds in the protective environment of the enclosed chaperonin complex. Hydrolysis of the seven ATP molecules primes the cis complex for disassembly, and the binding of seven ATP molecules to the opposite (trans) ring of GroEL causes the release of both GroES and the folded substrate (18). The system is now reset and ready to either accept a new substrate or rebind the just-released but not yet native polypeptide for another round of folding.

Folding by GroEL involves a complex interplay of three ligands (i.e. substrate, GroES, and nucleotide), and all three are capable of inducing allosteric conformational changes within GroEL, either individually or in concert (19–21). Attempts to perturb the GroEL system by mutagenesis (13, 22–24), chemical modification (14, 25), substrate modification (26, 27), or solvent manipulation (28–30) have often resulted in chaperonins with altered functional properties. These can provide a wealth of information on the inner workings of the system as a whole. Here we report on the functional properties of one such altered state, induced by the presence of high concentrations of ammonium sulfate.

Our work was prompted by structural data on both GroEL and the thermosome from the archaeon *Thermoplasma acidophilum*, which have been obtained from the analysis of crystals grown in high concentrations of ammonium sulfate (10, 31). Furthermore, the *in vitro* assembly of functional chaperonins from certain species of archaea has required the presence of ammonium sulfate (32), and the ATPase activity of at least one archaeal chaperonin has been shown to be dependent on a relatively high concentration of ammonium ions (33). We find that high ammonium sulfate concentrations alter the functional properties of GroEL, resulting in, among other things, an increased hydrophobic surface area and increased stringency for protein folding. Most surprisingly, these conditions allow the extension of *E. coli* chaperonin action to thermophilic conditions.

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**EXPERIMENTAL PROCEDURES**

*Proteins—* GroEL and GroES were expressed in and purified from *E. coli* as described previously (8, 17). In a final additional step in purification of GroEL, contaminating peptides were removed on a Reactive Red 120 column (34). Removal of tryptophan-containing impurities was confirmed by measuring fluorescence emission spectra of eluted fractions (excitation, 295 nm; emission, 325–355 nm). Protein concentrations were determined spectrophotometrically based on the procedure outlined by Gill and von Hippel (35), using the following extinction coefficients:

\[ \varepsilon_{280} = 8700 \text{ M}^{-1} \text{cm}^{-1} \text{ for GroEL}; \varepsilon_{280} = 1450 \text{ M}^{-1} \text{cm}^{-1} \text{ for GroES}. \]

Recombinant green fluorescent protein GPP was purified as described (36).

**GroEL ATPase Activity**—GroEL (125 nm) was equilibrated for 10 min at a given temperature in 60 \( \mu \)l of buffer A (25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), 2 mM dithiothreitol) supplemented with various salts as described in the figure legends. Ionic strength of the salts was calculated according to \( I = 1/2 \Sigma c_i z_i^2 \) where \( c_i \) is the concentration and \( z_i \) is the charge of each ionic species generated by a given salt in solution. Where indicated, GroES was present at a 4-fold molar excess over GroEL, and \( \alpha_1\)-casein (Sigma) was present at a 5-fold molar excess over GroEL. ATPase activity was initiated by the addition of ATP to 2 mM and allowed to proceed for 10 min. The reaction was stopped by the addition of DTT to 10 mM, and 50 \( \mu \)l were withdrawn for quantification of liberated inorganic phosphate by the malachite green assay as described previously (37). The absorbance was measured at 640 nm.

**Analysis of GroEL Stability—**GroEL (530 nm) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), and increasing concentrations of (NH\(_4\))\(_2\)SO\(_4\) as indicated in the figure legends. Protein denaturation was followed as aggregation by measuring light scattering at 320 nm. In a parallel experiment, GroEL (300 nm) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), and supplemented with 50 mM KCl or increasing concentrations of (NH\(_4\))\(_2\)SO\(_4\) as indicated in the figure legends. Protein denaturation was followed as aggregation by measuring light scattering at 320 nm. In a parallel experiment, GroEL (300 nm) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), and supplemented with 50 mM KCl or increasing concentrations of (NH\(_4\))\(_2\)SO\(_4\). GroEL samples were incubated for 20 min at 75 °C and centrifuged for 5 min at 10,000 \( \times g \) to remove aggregates.

The supernatants were desalted over a NICK column (Amersham Pharmacia Biotech) into 25 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, and were analyzed by nondenaturing polyacrylamide gel electrophoresis as described (4).

**Analysis of GFP Folding**—All GFP experiments were carried out either at room temperature or at 70 °C. Acid-denatured GFP was prepared according to the method of Makino et al. (38). Briefly, GFP (22 \( \mu \)g/ml) was incubated for 2 h in 2 ml of Tris-CI, pH 7.5, 12.5 mM HCl, 1 mM dithiothreitol, 0.3 mM EDTA. For spontaneous refolding, denatured GFP was diluted 200-fold into 1 ml of buffer B (25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), 5 mM dithiothreitol) and supplemented with various salts as indicated in the figure legends. Protein denaturation was followed as aggregation by measuring light scattering at 320 nm. In a parallel experiment, GroEL (300 nm) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), to 50 °C, whereas the other remained at 70 °C. At the indicated time points, 50-\( \mu \)l aliquots were mixed with 550 \( \mu \)l of assay buffer (20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 2 mM EDTA, 3 mM p-nitrophenyl-\( \beta \)-glucoside) and incubated for 20 min at room temperature. \( \beta \)-Glucosidase enzymatic activity was measured as increase in absorbance at 400 nm.

**GroEL Binding of bisANS—**A dilution series of bisANS (ICN) at various concentrations was prepared in water. 3 \( \mu \)l of each dilution was added to 300 \( \mu \)l of buffer (250 mM GroEL, 25 mM MOPS-NaOH, pH 7.5, 5 mM MgCl\(_2\), and 50 mM KCl or 1.2 M (NH\(_4\))\(_2\)SO\(_4\)). GroEL and graphical analysis of the resultant fluorescence emission spectra was carried out according to Bohnert et al. (39). Fluorescence emission spectra were recorded at room temperature from 450 to 580 nm. Samples were excited at 97 nm. The excitation and emission slit widths were set at 5 and 3 nm of bandwidth, respectively.

**RESULTS**

*Increased GroEL ATPase Activity at High Concentrations of Ammonium Ions—* A key feature of the GroEL mechanism of action is its ATPase activity, which is modulated by interaction of GroES and substrate protein with the chaperonin (4, 7, 8, 40, 41). The rate of ATP hydrolysis responds to conformational changes in the chaperonin and can therefore serve as a sensitive indicator of possible structural alterations in GroEL. We investigated the effect of high salt concentrations on GroEL ATPase activity. It has been demonstrated previously that ammonium ions can support the ATPase activity of GroEL, although less efficiently than potassium ions (40). In contrast, we found that at high concentrations, ammonium ions have a stimulatory effect exceeding that of potassium ions considerably (Fig. 1). The ATPase activity of GroEL in 1.2 M (NH\(_4\))\(_2\)SO\(_4\) (lane 2) and 1.2 M NH\(_4\)Cl (lane 4) was found to be approximately 3-fold higher than that in 50 mM KCl (lane 1). The effect was not solely dependent upon ionic strength, because 1.2 M KCl (lane 3; same ionic strength as lane 4) failed to stimulate ATP hydrolysis above control. However, the stimulatory effect was ammonium-specific, because 1.2 M Na\(_2\)SO\(_4\) alone (data not shown) did not support ATPase activity. It actually had an inhibitory effect on the ATPase activity of GroEL in the presence of 50 mM KCl (lane 5). It is noteworthy that the level of stimulation of ATPase activity is roughly the same for both 1.2 M (NH\(_4\))\(_2\)SO\(_4\) and 1.2 M NH\(_4\)Cl even though the latter salt excess over GroEL. Immediately upon addition, the sample was split in half. One half was shifted to 50 °C, whereas the other remained at 70 °C. At the indicated time points, 50-\( \mu \)l aliquots were mixed with 550 \( \mu \)l of assay buffer (20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 2 mM EDTA, 3 mM p-nitrophenyl-\( \beta \)-glucoside) and incubated for 20 min at room temperature. \( \beta \)-Glucosidase enzymatic activity was measured as increase in absorbance at 400 nm.
Fig. 2. Chaperone activity of GroEL at high concentrations of ammonium sulfate. A, denatured GFP was diluted 200-fold into buffer B supplemented with 50 mM KCl (upper panel) or 1.2 mM (NH₄)₂SO₄ (lower panel) containing 0.22 μM GroEL. To initiate folding, the following additions were made at 60 s: 2 mM ATP and 0.88 mM GroES (trace 2); 2 mM ADP and 0.88 mM GroES (trace 3); and 2 mM ATP (trace 4). Spontaneous folding of GFP was observed upon dilution of denatured GFP into buffer without chaperonins (trace 1). Fluorescence of native GFP in buffer is set as 100%. B, a competing substrate protein can mediate release of GroEL-bound GFP in the presence of ATP at a high ammonium sulfate concentration. Denatured GFP was diluted 200-fold into buffer B supplemented with 1.2 mM (NH₄)₂SO₄ and 0.22 μM GroEL. ATP (2 mM) and s1-casein (1.1 μM) were added at 1 and 11 min, respectively. The refolding of GFP was followed by monitoring fluorescence at 508 nm.

The increased concentration of NH₄⁺ provided by the ammonium sulfate may be necessary to counteract the inhibitory effect of the SO₄²⁻ anions, with a strongly elevated ATPase activity as a net result. In agreement with this explanation, we find that the ATPase activity of GroEL in 0.6 mM (NH₄)₂SO₄ plus 0.6 mM Na₂SO₄ is only 80% of that in 1.2 mM (NH₄)₂SO₄ (data not shown) even though the two buffers have the same ionic strength and same SO₄²⁻ concentration. Despite sulfate having an inhibitory effect at lower temperatures, the stabilizing effect of this anion becomes important for the chaperone activity of GroEL at high temperatures, as we will demonstrate below. For this reason, in the remainder of this article we will focus on the effects of ammonium sulfate.

GroES Dependence of GroEL-mediated Refolding of Proteins in Ammonium Sulfate—How does the changed ATPase rate in ammonium sulfate affect the ability of GroEL to bind substrate protein and mediate folding? We determined the ability of GroEL to fold proteins in the presence of 1.2 mM (NH₄)₂SO₄ using GFP, a monomeric 29-kDa protein, which is an established GroEL substrate in vitro. Because GFP fluoresces only in the native state, folding can be monitored by following its intrinsic fluorescence (38, 42). Acid-denatured GFP folds spontaneously upon dilution into a renaturation buffer (38) containing 50 mM KCl (Fig. 2A, upper panel). When GroEL was present in the renaturation buffer at a 2-fold molar excess, folding was suppressed as the non-native GFP was bound by GroEL. The addition of ATP alone was sufficient to release GFP from GroEL for productive folding (Ref. 38 and Fig. 2A). The full GroEL/GroES system in the presence of ATP or ADP also supported folding in 50 mM KCl. In 1.2 mM ammonium sulfate, GFP folded spontaneously upon dilution into renaturation buffer, and the presence of GroEL in this buffer also suppressed folding (Fig. 2A, lower panel). However, unlike in 50 mM KCl, ATP alone was not sufficient to mediate the folding of GFP by GroEL in 1.2 mM (NH₄)₂SO₄. Instead, the full GroE system was required to reconvert GFP under these conditions (trace 4). Under high salt conditions, where ATPase rates are strongly increased, the time span in which the chaperonin is in a low affinity state for unfolded polypeptide is expected to be shortened. After the rate-limiting ATP hydrolysis step, GroEL would then regain the acceptor state for substrate protein before released GFP could internalize its hydrophobic structure elements. It would be rebound immediately, resulting in a steady-state association with the chaperonin. In that case it should be possible to prevent rebinding of GFP by adding a competitor molecule. αs1-casein is a relatively hydrophobic, yet soluble protein that binds readily to chaperonins and has been used as an effective substrate competitor under refolding conditions (8). We found that addition of αs1-casein to a preformed GroEL-GFP complex resulted in release of the substrate in the presence of ATP and its subsequent productive refolding (Fig. 2B). The inability of GroEL to release GFP in the presence of ATP alone can thus be explained by a cycle of release and rapid recapture of substrate. This situation is reminiscent of that observed with N-ethylmaleimide-modified GroEL (NEM-GroEL; Ref. 14). It was shown that covalent modification of a cysteine residue (Cys¹³⁸) in the intermediate domain of GroEL with NEM results in a chaperonin with increased basal ATPase activity and a more stringent requirement for the folding of substrate proteins (i.e. previously GroES-independent substrates like dihydrofolate reductase now were GroES-dependent). The altered properties of GroEL were attributed to a disruption in the communication between the apical and equatorial domains (14). Typically, binding of GroES to GroEL attenuates its ATPase activity by about 50%, whereas substrate binding enhances it (4, 7, 8, 40, 41). The latter effect is expected because if ATP binding and hydrolysis affect the affinity of GroEL for substrate protein, the reverse should apply as well. In contrast, in an uncoupled system the two ligands, ATP and substrate protein, should not affect each other. We were curious to see whether the ammonium-induced changes in the functional properties of GroEL were, as in NEM-GroEL, the result of an uncoupling of the chaperonin system, and investigated the ATPase activity of GroEL in 1.2 mM (NH₄)₂SO₄ in the presence of GroES or substrate proteins. As expected, in 50 mM KCl, a 4-fold molar excess of GroES over GroEL reduced the ATPase activity to 56%, whereas a 5-fold molar excess of αs1-casein over GroEL stimulated the ATPase activity by 74% (Fig. 3). When αs1-casein was added to GroEL/
GroES, stimulation of 84% over GroEL/GroES alone was observed. In ammonium sulfate, a different picture emerged. Although the addition of GroES inhibited the ATPase activity 3-fold, the addition of α1-casein to GroEL alone was without effect (Fig. 3). However, when α1-casein was added to GroEL/GroES, we again observed stimulation of ATPase activity by 78% over that of GroEL/GroES alone. We conclude that, in contrast to NEM-GroEL, communication between the apical domain implicated in substrate binding (11, 13) and that GroES, we again observed stimulation of ATPase activity by 3-fold, the addition of α1-casein to GroEL alone was without effect (Fig. 3). However, when α1-casein was added to GroEL/GroES, we again observed stimulation of ATPase activity by 78% over that of GroEL/GroES alone. We conclude that, in contrast to NEM-GroEL, communication between the apical domain implicated in substrate binding (11, 13) and that GroES, we again observed stimulation of ATPase activity by 3-fold, the addition of α1-casein to GroEL alone was without effect (Fig. 3). However, when α1-casein was added to GroEL/GroES, we again observed stimulation of ATPase activity by 78% over that of GroEL/GroES alone. We conclude that, in contrast to NEM-GroEL, communication between the apical domain implicated in substrate binding (11, 13) and that since substrate can still elicit the expected increase in ATPase activity when GroES is present.

Increased Binding of bisANS to GroEL in 1.2 M (NH₄)₂SO₄—To determine in more detail the nature of the altered state of GroEL in the presence of 1.2 M (NH₄)₂SO₄, we revisited the observation that ATP alone was not sufficient to release GFP from GroEL (Fig. 2B) and tested whether or not other factors, in addition to a shortened time window for folding, could be contributing to this phenomenon. Because substrate binding to GroEL has been demonstrated to involve numerous hydrophobic interactions (13, 43), we considered the possibility that the substrate has a higher affinity for GroEL. This could be because binding sites in GroEL are now more hydrophobic or because there are more binding sites for substrate available in GroEL. A method of choice for studying GroEL-mediated folding has been the use of chemical probes such as bisANS. BisANS is a fluorescent probe whose quantum yield increases with increasing hydrophobicity of its environment. It binds readily and noncovalently to exposed hydrophobic regions on proteins and has thus been used extensively to probe both conformational changes and folding in a number of protein systems (28, 30, 39, 44, 45). It has been demonstrated that GroEL exposes a region of hydrophobic residues in its apical domain implicated in substrate binding (11, 13) and that since substrate can still elicit the expected increase in ATPase activity when GroES is present.

Since GroES, stimulation of 84% over GroEL/GroES alone was observed. In ammonium sulfate, a different picture emerged. Although the addition of GroES inhibited the ATPase activity 3-fold, the addition of α1-casein to GroEL alone was without effect (Fig. 3). However, when α1-casein was added to GroEL/GroES, we again observed stimulation of ATPase activity by 78% over that of GroEL/GroES alone. We conclude that, in contrast to NEM-GroEL, communication between the apical domain implicated in substrate binding (11, 13) and that since substrate can still elicit the expected increase in ATPase activity when GroES is present.
ATPase activity was initiated by addition of ATP (2 mM). The ATPase activity of GroEL in 50 mM KCl at 30 °C (as shown in Fig. 1, lane 1) is set as 100%. Lane 1, 50 mM KCl; lane 2, 1.2 M (NH₄)₂SO₄; lane 3, 1.2 M KCl; lane 4, 1.2 M NH₄Cl; lane 5, 1.2 M Na₂SO₄; 50 mM KCl; lane 6, 3.6 M NH₄Cl. GroEL (530 nM) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.6, 5 mM MgCl₂, and increasing concentrations of (NH₄)₂SO₄: 0.6 M (NH₄)₂SO₄; 1.0 M (NH₄)₂SO₄; 1.2 M (NH₄)₂SO₄; 1.2 M NH₄Cl; 0.6 M (NH₄)₂SO₄; 1.2 M NH₄Cl; 1.2 M (NH₄)₂SO₄; 0.6 M Na₂SO₄ (lanes 4 and 5). In contrast, only a small fraction of GroEL remained soluble and assembled in 0.6 M (NH₄)₂SO₄. No soluble GroEL was detected in 50 mM KCl in agreement with previous reports that GroEL is unstable at temperatures above 60 °C in low salt buffers (47, 48).

**Fig. 5. GroEL stability and ATPase activity at high temperatures in elevated concentrations of ammonium sulfate.** A, GroEL (125 nM) was added at 70 °C to buffer A supplemented with various salts as indicated. ATPase activity was initiated by addition of ATP (2 mM). The ATPase activity of GroEL in 50 mM KCl at 30 °C (as shown in Fig. 1, lane 1) is set as 100%. Lane 1, 50 mM KCl; lane 2, 1.2 M (NH₄)₂SO₄; lane 3, 1.2 M KCl; lane 4, 1.2 M NH₄Cl; lane 5, 1.2 M Na₂SO₄; 50 mM KCl; lane 6, 3.6 M NH₄Cl. B, GroEL (530 nM) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.6, 5 mM MgCl₂, and increasing concentrations of (NH₄)₂SO₄: 0.6 M (white squares); 0.8 M (black triangles); 1.0 M (black squares); and 1.2 M (open circles). Denaturation of GroEL was followed as protein aggregation by measuring light scattering at 320 nm. C, GroEL (300 nM) was diluted at 75 °C into buffer containing 25 mM MOPS-NaOH, pH 7.6, 5 mM MgCl₂, and supplemented with the indicated salts. The samples were incubated for 20 min at 75 °C. Control samples were incubated at room temperature. After centrifugation to remove aggregates, samples were desalted, concentrated, and electrophoresed on a nondenaturing polyacrylamide gel. Lane 1, 1.2 M (NH₄)₂SO₄ (control); lane 2, 0.6 M (NH₄)₂SO₄; lane 3, 0.8 M (NH₄)₂SO₄; lane 4, 1.0 M (NH₄)₂SO₄; lane 5, 1.2 M (NH₄)₂SO₄; lane 6, 50 mM KCl; lane 7, 50 mM KCl (control).

ATPase activity even at a temperature of 75 °C (data not shown). Notably, 1.2 M NH₄Cl (lane 4) was virtually unable to support ATPase activity at 70 °C even though it was as effective as 1.2 M (NH₄)₂SO₄ at 30 °C (refer to Fig. 1). Likewise, the ability of 3.6 M NH₄Cl (Fig. 5A, lane 5), which has the same ionic strength as 1.2 M (NH₄)₂SO₄, to support ATPase activity was significantly lower by comparison. In accordance with earlier observations (48), GroEL in 50 mM KCl is virtually inactive at 70 °C (lane 1). We ascribe this novel feature of *E. coli* GroEL, functioning as a thermophilic protein, to stabilization provided by the sulfate ions. This is inferred from the observation that unlike in 50 mM KCl alone (Fig. 5A, lane 1), GroEL at 70 °C in 50 mM KCl plus 1.2 M Na₂SO₄ exhibited ATPase activity significantly higher than that of GroEL in 50 mM KCl at 30 °C (lane 5). The stabilization effect was also observed directly by following the time course of unfolding of GroEL at 75 °C. At 50 mM KCl (data not shown) and at medium concentrations of (NH₄)₂SO₄ (0.6–0.8 M), GroEL aggregated rapidly at this high temperature (Fig. 5B). Increasing the salt concentration had a protective effect such that at 1.2 M (NH₄)₂SO₄, GroEL remained fully soluble and active over the course of the experiment. Nondenaturing polyacrylamide gel electrophoresis confirmed this result (Fig. 5C). When GroEL was incubated for 20 min at 75 °C in buffer supplemented with 1.0 or 1.2 M (NH₄)₂SO₄, all of it was soluble and fully assembled as judged by its migration on a native gel (Fig. 5C, lanes 4 and 5). In contrast, only a small fraction of GroEL remained soluble and assembled in 0.6 M (NH₄)₂SO₄. No soluble GroEL was detected in 50 mM KCl in agreement with previous reports that GroEL is unstable at temperatures above 60 °C in low salt buffers (47, 48).
parallel sample was done at 50 °C, a temperature at which the protein is stable, activity did not decline. These results confirm that the GroEL/GroES chaperonin system is functional at thermophilic conditions in 1.2 M (NH₄)₂SO₄. Finally, we studied the folding of GFP at 70 °C in 1.2 M (NH₄)₂SO₄. This protein remained at 70 °C (Fig. 6D, black triangles). GroEL binding of heat-inactivated α-glucosidase, α-Glucosidase (270 nm) was diluted into buffer C at 70 °C in the absence or presence of chaperonins (810 nM GroEL; 3.2 μM GroES) and ATP (2 mM). At the indicated time points, aliquots were withdrawn and assayed for α-glucosidase activity. Open diamonds, α-glucosidase; black triangles, α-glucosidase plus GroEL; black squares, α-glucosidase plus GroEL/GroES and ATP. C, GroEL-mediated refolding of heat-inactivated α-glucosidase. α-Glucosidase (270 nm) was heat-inactivated for 60 min at 70 °C in the presence of a 3-fold molar excess of GroEL in buffer C. At 60 min (t = 0), refolding was initiated by the addition of 3.2 μM GroES and 2 mM ATP, and the sample was split in half. One of the two halves was shifted to 50 °C (black circles), whereas the other remained at 70 °C (open squares). At the indicated time points, aliquots were withdrawn and assayed for α-glucosidase activity. D, refolding of acid-denatured GFP by GroEL at 70 °C. Denatured GFP was diluted 200-fold into buffer B at 70 °C supplemented with 1.2 M (NH₄)₂SO₄ and 0.22 mM GroEL. To initiate folding, the following additions were made at 60 s: 2 mM ATP and 0.88 mM GroES, together with ATP or ADP, folding resumed. We conclude that the presence of 1.2 M (NH₄)₂SO₄ extents considerably the range of GroEL action, preserving its chaperone activity at high temperatures.

DISCUSSION

In this study, we have characterized the functional properties of the GroEL/GroES chaperonin system in the presence of high salt concentrations. The most surprising result is the ability of GroEL to function as a chaperone under thermophilic conditions in 1.2 M (NH₄)₂SO₄. GroEL is able to suppress thermally induced aggregation of citrate synthase. It can bind to intermediates of heat-inactivated α-glucosidase and mediate their refolding in a GroES-dependent manner, and, as at room temperature, it can bind and refold acid-denatured GFP with the same increased substrate stringency. We find that high concentrations of ammonium ions have a stimulatory effect on the ATPase activity of GroEL. At 30 °C, NH₄Cl and (NH₄)₂SO₄ are equally effective, even though the former contributes only half the number of ammonium ions on a per mole basis. A higher concentration of ammonium ions may counteract negative effects of sulfate. In fact, Na₂SO₄ has an inhibitory effect on the ATPase activity of GroEL. At high concentrations, some salts are thought to make a protein more rigid (51) and are widely used as protein stabilizers. This is particularly true of SO₄²⁻, which has a high charge density and resides high on the Hofmeister series of anions. Sulfate ions, by virtue of making the protein more rigid, may hinder the ability of the chaperonin to hydrolyze ATP. Nevertheless, the stabilizing effect of the sulfate ions enables GroEL to function at thermophilic conditions by preventing its denaturation and keeping it soluble. This is evident in the fact that at temperatures of up to 75 °C, only ammonium sulfate is able to support a markedly enhanced chaperonin activity.

Several features of the GroEL/GroES chaperonin system can be explained in terms of a Monod-Wyman-Changeux representation (52). Each of the two rings can either be in a tense acceptor state (T), in which GroEL has high affinity for sub-
strate protein, low affinity for ATP, and high ATP hydrolysis rates, or in a relaxed state (R) with high affinity for ATP and low affinity for protein substrate. With increasing ATP concentrations, the equilibrium of conformations shifts first to the TR state, and when most GroEL subunits are occupied by ATP, the RR state dominates in which ATP hydrolysis rates are slightly decreased and substrate protein is released (19, 20, 24, 53, 54). Our data are consistent with the possibility that (NH$_4$)$_2$SO$_4$ rather than acting as an uncoupler, induces a conformational change in GroEL to a TT-like state in which the ATPase activity is at or near capacity. Unlike in low concentrations of KCl, where the substrate protein $\alpha_1$-casein stimulates the ATPase activity of GroEL, no such increase is observed in (NH$_4$)$_2$SO$_4$. Indeed, with GroEL already in a TT-like state, substrate protein should have no further effect. It has been established that GroES binding to GroEL regulates the ATPase activity of the chaperonin (4, 7, 8, 40, 41). When GroES binds to GroEL, it is able to shift the chaperonin conformation to TR and RR states of submaximal ATP hydrolysis. Consequently, in the absence of GroES, substrate should show more pronounced effects in ammonium sulfate by trying to shift the TR equilibrium back toward the TT state. This is exactly what we have observed with $\alpha_1$-casein, which stimulates ATP hydrolysis in 1.2 mM (NH$_4$)$_2$SO$_4$ in the presence of GroES. GroEL is fully able to fold proteins under these conditions at both ambient and thermophilic temperatures. Interestingly, the presence of the high concentration of ammonium sulfate increases substrate stringency such that GFP, capable of folding in a GroES-independent manner under low salt conditions, now becomes strictly GroES-dependent. A strongly favored TT-like state in 1.2 mM (NH$_4$)$_2$SO$_4$ would explain this inability of ATP hydrolysis alone to mediate GFP release from GroEL. In the absence of GroES, the nucleotide is not able to induce on its own the conformational shifts toward the TR and RR state that are necessary to dissociate the substrate. The predominantly present TT-like GroEL form can thus be seen as locked in a conformation with high substrate affinity. Moreover, in this conformation GroEL exposes more hydrophobic binding surface than in low salt, which may affect the interaction with substrate protein. GroEL-bound substrates are typically in a molten globule-like state (8, 15). This quasi-ordered condition, in which secondary structure is present but tertiary structure is undefined, is characterized by the exposure of hydrophobic residues that would normally be buried in a native protein. GroEL contains a number of hydrophobic residues in its apical domain that have been demonstrated to be necessary for binding of non-native substrate protein (13). It has been demonstrated that perturbation of the ionic strength of the solvent can increase exposure of hydrophobic residues on GroEL (28, 30). Ammonium sulfate seems to elicit a similar change in the chaperonin. Titration data presented here suggest that GroEL can bind more bisANS per tetradecamer in 1.2 mM (NH$_4$)$_2$SO$_4$ than in low salt. Moreover, the hydrophobic nature of the binding sites is comparable, because the emission $\lambda_{\text{max}}$ is the same in both salts. There may well be additional reasons for the inability of ATP alone to mediate GFP release in (NH$_4$)$_2$SO$_4$. For example, increased GFP stringency could be the result of some change within GFP itself induced by the high salt. The fluorescence of native GFP is virtually the same in both 50 mM KCl and 1.2 mM (NH$_4$)$_2$SO$_4$, and the spontaneous recovery of fluorescence of acid-denatured GFP is essentially complete in both buffers. This suggests that GFP behaves similarly in both buffers. Nevertheless, it is conceivable that non-native GFP intermediate(s) bound by GroEL upon dilution from denaturant are different in nature such that those in 50 mM KCl are more amenable to release from GroEL by ATP alone than those in 1.2 mM (NH$_4$)$_2$SO$_4$.

The presence of high concentrations of ammonium ions and ammonium sulfate pertaining to chaperonin structure and function has surfaced a few times in recent literature (10, 31–33). Notably, high ammonium sulfate concentrations were used to obtain crystals for the determination of the structures of both E. coli GroEL and the thermosome from T. acidophilum. Although the structures represent well the overall architecture of the chaperonins, questions have arisen as to the nature of the actual state, in terms of functional properties, that these structures represent. For instance, the unliganded thermosome from T. acidophilum was crystallized in 2 mM (NH$_4$)$_2$SO$_4$ in a “closed” conformation said to represent the Mg-ATP bound form (31). However, Gutsche et al. (55) have recently demonstrated by small angle neutron scattering that in solution, the Mg-ATP bound thermosome favors the “open” conformation in low salt buffer. The closed conformation occurs only after ATP hydrolysis, but before release of P$_i$. Surprisingly, Gutsche et al. (55) also showed that the crystallization buffer can induce the closed conformation in solution. The crystals for the unliganded structure of GroEL were grown in similarly high (NH$_4$)$_2$SO$_4$ concentrations as those employed in this study (10). Based on our results it is conceivable that the GroEL represented in that crystal structure has solution properties similar to the functional state observed here. Although these salt conditions are not physiologically relevant for E. coli in vivo, the changes in chaperonin function that they induce are nevertheless informative. For example, it was noted that high concentrations of sodium sulfate resulted in a stimulation of the ATPase activity of the archaeal chaperonin because of the aforementioned induction of the “closed” conformation, which occurs after ATP hydrolysis (55). The situation is different with bacterial chaperonins, because our findings show that sulfate inhibits the ATPase of GroEL; an effect that is in turn counterbalanced by high concentrations of ammonium ions. This difference serves to underscore the likelihood that despite having structurally conserved ATP-binding domains, the molecular basis of ATP hydrolysis in the two chaperonin systems may differ in some respects.

It appears that examination of the solution properties of chaperonins, under the solvent conditions used for crystallization, is a worthwhile endeavor to better assign the functional state that the respective structures represent. Moreover, the ability to convert a mesophilic chaperonin into a thermophilic chaperonin opens interesting possibilities for direct comparison with and study of homologs from naturally occurring thermophiles. Some methanogenic archaea use increased intracellular ion concentrations to stabilize their proteins in vivo (16). Whether or not a similar method of thermoadaptation is used by some extremophilic bacteria remains to be seen, but the results presented here suggest that this is a distinct possibility.

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