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 (bisANS) 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.

INTRODUCTION

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 1, 2). Like its homologs, CCT[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 has 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 inter-subunit 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). Since 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 (16, 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 dependant 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.

1 The abbreviations used are: CCT, chaperonin containing TCP-1; bisANS, bis-(5,5')-

8-anilino-1-naphthalene sulfonic acid; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; GFP, green fluorescent protein; MOPS, 3-(N-morpholino) propanesulfonic acid; NEM, N-ethylmaleimide; P_i, inorganic phosphate.

EXPERIMENTAL PROCEDURES

Proteins —GroEL, and GroES, were expressed in and purified from *E. coli* as previously described (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 from 325 to 355 nm). Protein concentrations were determined spectrophotometrically based on the procedure outlined by Gill and von Hippel (35), using the following extinction coefficients: ε_{276} = 8700 $M^{-1}cm^{-1}$ for GroEL; ε_{276} = 1450 $M^{-1}cm^{-1}$ for GroES. Recombinant green fluorescent protein GFP was purified as described (36).

GroEL ATPase activity GroEL (125 nM) was equilibrated for 10 min at a given temperature in 60 µL of buffer A (25 mM MOPS-NaOH pH 7.5, 5 mM $MgCl_2$, 2 mM DTT) supplemented with various salts as described in the figure legends. Ionic strength of the salts was calculated according to $I = 1/2\Sigma cz^2$ where c is the concentration and z is the charge of each ionic species generated by a given salt in solution. Where indicated, GroES was present at a four-fold molar excess over GroEL, and α_{s1}-casein (Sigma) was present at a five-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 CDTA to 10 mM and 50 µL were withdrawn for quantification of

liberated inorganic phosphate by the malachite green assay as previously described (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 7.6, 5 mM $MgCl_2$, and increasing concentrations of $(NH_4)_2SO_4$ 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 7.6, 5 mM $MgCl_2$, and supplemented with 50 mM KCl or increasing concentrations of $(NH_4)_2SO_4$. GroEL samples were incubated for 20 min at 75°C and centrifuged for 5 min at 10,000 g to remove aggregates. The supernatants were desalted over a NICK column (Amersham Pharmacia) into 25 mM MOPS-NaOH pH 7.2, 100 mM NaCl, and were analyzed by non-denaturing 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 μM) was incubated for 1 hour in 8 mM Tris-Cl pH 7.5, 12.5 mM HCl, 1 mM DTT, 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 DTT) supplemented with either 50 mM KCl or 1.2 M $(NH_4)_2SO_4$. For chaperonin-mediated refolding, denatured GFP was diluted as above into buffer B containing a two-fold molar excess of GroEL. As indicated in the figure legends, folding was initiated by the addition of nucleotide (2 mM) or nucleotide plus GroES (at a four-

fold molar excess over GroEL). Where indicated, α_{S1}-casein was added at a five-fold molar excess over GroEL. Intrinsic GFP fluorescence was measured at 508 nm in 10 sec intervals using a FluoroMax-2 spectrofluorometer (Instruments S.A.) equipped with a thermostatted cuvette cell-holder. Samples were excited at 398 nm. The excitation and emission slit-widths were set at 5 and 3 nm of bandpass respectively. The refolding solution was mixed continuously with a built-in magnetic stirrer.

Thermal Aggregation of Citrate Synthase —Porcine citrate synthase (173 nM; Roche Molecular Biochemicals) was added to buffer C (25 mM MOPS-NaOH pH 7.6, 5 mM $MgCl_2$, 1.2 M $(NH_4)_2SO_4$), pre-equilibrated to 70°C, in the absence or presence of various amounts of GroEL as indicated in the figure legends. Aggregation was followed by monitoring light scattering at 320 nm in an Ultrospec300 spectrophotometer (Amersham-Pharmacia Biotech) equipped with a thermostatted cuvette cell-holder. Where indicated, release of GroEL-bound citrate synthase was accomplished by the addition of ATP to 2 mM.

α-*Glucosidase Folding* α-Glucosidase (270 nM) from *Bacillus stearothermophilus* (Sigma) was heat-inactivated in buffer C, pre-equilibrated to 70°C, in the absence or presence of chaperonins and ATP as indicated in the figure legends. Aliquots (50 µL) were removed at the indicated times and assayed for α-glucosidase activity. For reactivation experiments, α-glucosidase was first heat-inactivated for 60 min at 70°C in the presence of a three-fold molar excess of GroEL. Refolding was initiated by the addition of ATP (2 mM) and GroES (at a four-fold molar excess over GroEL).

Immediately upon addition, the sample was split in half. One half was shifted to 50°C, while the other remained at 70°C. At the indicated time points, 50 µL aliquots were mixed with 550 µL of assay buffer (20 mM MOPS-NaOH pH 7.2, 100 mM NaCl, 2 mM EDTA, 3 mM *p*-nitrophenyl-α-glucoside) and incubated for 20 min at room temperature. α-Glucosidase enzymatic activity was measured as increase in absorbance at 400 nm.

GroEL Binding of bisANS A dilution series of bis-(5,5)-8-anilino-1-naphthalene sulfonic acid dipotassium salt (bisANS; ICN) at various concentrations was prepared in water. 3 µL of each dilution was added to 300 µL of buffer (250 nM GroEL, 25 mM MOPS-NaOH pH 7.5, 5 mM $MgCl_2$, and 50 mM KCl or 1.2 M $(NH_4)_2SO_4$) and the fluorescence emission spectrum recorded. Intensity maxima were plotted as a function of bisANS concentration. Scatchard analysis was performed to determine the amount of bisANS bound and the approximate affinity. To determine the maximum fluorescence intensity of a given amount of bisANS that is totally bound by protein, 250 nM of bisANS in KCl- or $(NH_4)_2SO_4$-containing buffer was titrated with increasing amounts of 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 397 nm. The excitation and emission slit-widths were set at 5 and 3 nm of bandpass 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 find 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 $(NH_4)_2SO_4$ (lane 2) and 1.2 M NH_4Cl (lane 4) was found to be approximately three-fold higher than that in 50 mM KCl (lane 1). The effect was not solely dependent upon ionic strength, as 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, as 1.2 M Na_2SO_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 $(NH_4)_2SO_4$ and 1.2 M NH_4Cl even though the latter salt supplies only half as

many ammonium ions on a per mole basis. The increased concentration of NH_4^+ provided by the ammonium sulfate may be necessary to counteract the inhibitory effect of the SO_4^{2-} 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 M $(NH_4)_2SO_4$ plus 0.6 M Na_2SO_4 is only 80% of that in 1.2 M $(NH_4)_2SO_4$ (data not shown) even though the two buffers have the same ionic strength and same SO_4^{2-} 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 M $(NH_4)_2SO_4$ 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. 2 A, upper panel). When GroEL was present in the renaturation buffer at a two-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, Fig. 2 A). The full GroEL/GroES system in the presence of ATP or ADP also supported folding in 50 mM KCl. In 1.2 M ammonium sulfate, GFP folded spontaneously upon dilution into renaturation buffer and the presence of GroEL in this buffer also suppressed folding (Fig. 2 A, lower panel). However, unlike in 50 mM KCl, ATP alone was not sufficient to mediate the folding of GFP by GroEL in 1.2 M $(NH4)2SO4$. Instead, the full GroE system was required to reactivate 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 re-bound 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. α_{s1}-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 α_{s1}-casein to a preformed GroEL-GFP complex resulted in release of the substrate in the presence of ATP and its subsequent productive refolding (Fig. 2 B). 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 (C138) in the intermediate domain of GroEL with N-ethylmaleimide 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 if 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 M $(NH_4)_2SO_4$ in the presence of GroES or substrate proteins. As expected, in 50 mM KCl, a four-fold molar excess of GroES over GroEL reduced the ATPase activity to 56%, whereas a five-fold molar excess of α_{s1}-casein over GroEL stimulated the ATPase activity by 74% (Fig. 3). When α_{s1}-casein was added to GroEL/GroES, stimulation of 84% over GroEL/GroES alone was observed. In ammonium sulfate, a different picture emerged. While the addition of GroES inhibited the ATPase activity three-fold, the addition of α_{s1}-casein to GroEL alone was without effect (Fig. 3). However, when α_{s1}-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 and equatorial domains is not compromised in 1.2 M $(NH_4)_2SO_4$ because substrate can still elicit the expected increase in ATPase activity when GroES is present.

Increased Binding of bisANS to GroEL in 1.2 M $(NH_4)_2SO_4$ — To determine in more detail the nature of the altered state of GroEL in the presence of 1.2 M $(NH_4)_2SO_4$, 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 bisANS binds readily to GroEL, presumably to this very region (45). We titrated a fixed amount of GroEL in both 50 mM KCl and 1.2 M

$(NH_4)_2SO_4$ with increasing concentrations of bisANS. At each concentration tested, the bisANS fluorescence was markedly higher in 1.2 M $(NH_4)_2SO_4$ than in 50 mM KCl (Fig. 4; representative spectrum). Also, the emission λ_{max} was at the same wavelength in both 50 mM KCl and 1.2 M $(NH_4)_2SO_4$. Since λ_{max} of fluorescence emission is an indicator of the degree of hydrophobicity of the fluorophore environment (46), these findings taken together suggest that the bisANS binding sites were not more hydrophobic in character in 1.2 M $(NH_4)_2SO_4$ than in low salt. Rather, more bisANS molecules were bound per GroEL complex in 1.2 M $(NH_4)_2SO_4$. To control for the possibility that ammonium sulfate was enhancing bisANS fluorescence or that potassium chloride was acting as a quencher, we titrated a fixed amount of bisANS with increasing amounts of GroEL in both 50 mM KCl and in 1.2 M $(NH_4)_2SO_4$. When bisANS is completely bound by GroEL, its fluorescence should be the same in both salts provided that the local binding environment is equally hydrophobic. Since λ_{max} is the same in both 50 mM KCl and 1.2 M $(NH_4)_2SO_4$ (Fig. 4), we are confident that the binding environment offered by GroEL is equally hydrophobic in both salts, and therefore any difference observed should be due to buffer composition alone. Having titrated 250 nM bisANS with increasing amounts of GroEL in both 50 mM KCl and 1.2 M $(NH_4)_2SO_4$, we graphically extrapolated the resultant fluorescence intensity maxima to infinite protein

concentration, as described by Bohnert et al (39). The values for all-bound bisANS obtained in this way in KCl and $(NH_4)_2SO_4$-containing buffers were found to differ by no more than 5% (data not shown). We conclude that buffer composition has no effect on the observed bisANS fluorescence. Using the fluorescence values obtained for the all-bound bisANS, we applied Scatchard analysis to the titration data and found the number of bisANS binding sites per 14-mer GroEL to be 21.6 in 50 mM KCl and 29.6 in 1.2 M $(NH_4)_2SO_4$, with apparent dissociation constants of 13.7 and 10.9 μM respectively. These values are comparable to, although somewhat higher, than those obtained in previous studies (28, 30). Thus, the increased bisANS fluorescence observed in 1.2 M $(NH_4)_2SO_4$ genuinely reflects an increase in the hydrophobic area available for bisANS binding. The increased exposure of solvent-accessible hydrophobic sites may at least in part be responsible for the increased substrate stringency observed with GFP.

Heat-stability and ATPase Activity of GroEL at 70°C in Ammonium Sulfate — It had previously been reported that GroEL ATPase activity reached a maximum near 60°C and that at higher temperatures, the protein rapidly denatured and became non-functional (47, 48). We describe here conditions under which the operational range of GroEL can be extended well beyond the previously reported maximum. While investigating the functional properties of GroEL in 1.2 M $(NH_4)_2SO_4$, we noticed the chaperonin to be stable and active at elevated temperatures. Figure 5 A shows that GroEL possesses significant ATPase activity at 70°C in 1.2 M $(NH_4)_2SO_4$ that was unequaled in other

buffers. This activity could be inhibited by GroES (data not shown). We were able to demonstrate similar ATPase activity even at a temperature of 75°C (data not shown). Notably, 1.2 M NH_4Cl (lane 4) was virtually unable to support ATPase activity at 70°C even though it was as effective as 1.2 M $(NH_4)_2SO_4$ at 30°C (refer to Fig. 1). Likewise, the ability of 3.6 M NH_4Cl (Fig. 5 A, lane 5), which has the same ionic strength as 1.2 M $(NH_4)_2SO_4$, 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. 5 A, lane 1), GroEL at 70°C in 50 mM KCl plus 1.2 M Na_2SO_4 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 $(NH_4)_2SO_4$ (0.6 - 0.8 M), GroEL aggregated rapidly at this high temperature (Fig. 5 B). Increasing the salt concentration had a protective effect such that at 1.2 M $(NH_4)_2SO_4$, GroEL remained fully soluble and active over the course of the experiment. Non-denaturing polyacrylamide gel electrophoresis confirmed this result (Fig. 5 C). When GroEL was incubated for 20 min at 75°C in buffer supplemented with 1.0 or 1.2 M $(NH_4)_2SO_4$, all of it was soluble and

fully assembled as judged by its migration on a native gel (Fig. 5 C, lanes 4 and 5). In contrast, only a small fraction of GroEL remained soluble and assembled in 0.6 M $(NH_4)_2SO_4$. 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).

GroEL Chaperone Activity at 70°C in Ammonium Sulfate — The finding that *E. coli* GroEL was stable and possessed ATPase activity under thermophilic conditions opened the exciting possibility that it could function as a chaperone under these conditions as well. First we asked if GroEL can bind unfolded polypeptides and prevent them from thermal aggregation. As a model substrate, we used porcine citrate synthase, a homodimer of 48 kDa subunits. Citrate synthase is a thermolabile protein with a mid-point transition temperature for thermal denaturation of only 43°C and as such aggregates rapidly and irreversibly (49). When citrate synthase was diluted into buffer containing 1.2 M $(NH_4)_2SO_4$ at 70°C, it aggregated immediately (Fig. 6 A). Aggregation was inhibited by GroEL in a concentration-dependent manner, and was completely suppressed at a three-fold molar excess of GroEL. When ATP was added, citrate synthase was released from GroEL and aggregated rapidly. Interestingly, under these conditions ATP alone appears sufficient to affect release of substrate from GroEL, in contrast to the results obtained with GFP at room temperature (Fig. 2). At 70°C the thermal energy is expected to be sufficiently high to allow aggregation to occur on a faster scale than rebinding to GroEL. Unlike citrate synthase, GFP does not aggregate when released from GroEL and there is no side reaction to compete with rebinding to the chaperonin. We reasoned that

we should be able to reestablish increased substrate stringency in 1.2 M $(NH_4)_2SO_4$ at 70°C by using a more thermotolerant substrate for our experiments. The results described below show that this is indeed the case. α-Glucosidase from *Bacillus stearothermophilus*, a moderate thermophile with a growth optimum of 55°C (50), exhibited a gradual loss of activity upon incubation at 70°C in 1.2 M $(NH_4)_2SO_4$ (Fig. 6B). In the presence of GroEL, GroES, and ATP, only a marginal stabilization was observed. In the presence of a three-fold molar excess of GroEL alone, the loss of activity was accelerated. The faster loss of α-glucosidase activity in the presence of GroEL can be attributed to the binding of heat-inactivated intermediates of α-glucosidase to the chaperonin. As α-glucosidase slowly inactivates, an equilibrium condition likely exists between the native form and the non-native intermediate(s). GroEL binding to these intermediates would shift the equilibrium away from the native form, thereby causing a faster decline in α-glucosidase activity. After 60 min of heat-inactivation, residual α-glucosidase activity was no more than 5% of control (Fig. 6 C). At this point (t = 0), we attempted refolding of the protein by addition of ATP and GroES. ATP alone was unable to affect any recovery of α-glucosidase activity (data not shown). Only the full chaperonin system was capable of restoring α-glucosidase activity (Fig. 6 C). Enzymatic activity began to decline again after 10 min, presumably because the protein is thermodynamically unstable under these conditions. When refolding in a 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 $(NH_4)_2SO_4$.

Finally, we studied the folding of GFP at 70°C in 1.2 M $(NH_4)_2SO_4$. This protein appeared to be the most robust of the substrates tested in this study, as the fluorescence of native GFP under these conditions was nearly identical to that at room temperature and declined by no more than 5 to 10% over the course of the experiment (data not shown).

Acid-denatured GFP refolded spontaneously upon dilution into 1.2 M $(NH_4)_2SO_4$-containing buffer at 70°C with a recovery of about 30% (Fig. 6 D, trace 1). Dilution of GFP into buffer containing a two-fold molar excess of GroEL prevented spontaneous refolding caused by binding of the non-native protein to the chaperonin. Like at room temperature, addition of ATP alone was unable to release GFP from GroEL (trace 4). In the presence of GroES, together with ATP or ADP, folding resumed. We conclude that the presence of 1.2 M $(NH_4)_2SO_4$ extends 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 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 $(NH_4)_2SO_4$. 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-dependant 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, NH_4Cl and $(NH_4)_2SO_4$ 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, Na_2SO_4 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 SO_4^{2-}, 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 substrate 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 $(NH_4)_2SO_4$, 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 α_{s1}-casein stimulates the ATPase activity of GroEL, no such increase is observed in $(NH_4)_2SO_4$. 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 sub-maximal ATP hydrolysis. Consequently, in the presence of GroES, substrate should show more pronounced effects in ammonium sulfate by trying to shift the T/R equilibrium back toward the TT state. This is exactly what we have observed with α_{s1}-

casein which stimulates ATP hydrolysis in 1.2 M $(NH_4)_2SO_4$ 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 M $(NH_4)_2SO_4$ 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 which 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 M

$(NH_4)_2SO_4$ than in low salt. Moreover, the hydrophobic nature of the binding sites is comparable, as the emission λ_{max} is the same in both salts. There may well be additional reasons for the inability of ATP alone to mediate GFP release in $(NH_4)_2SO_4$. 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 M $(NH_4)_2SO_4$, 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 M $(NH_4)_2SO_4$.

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 M $(NH_4)_2SO_4$ in a "closed" conformation said to represent the Mg-ATP bound form (31). However,

Gutsche et al have recently demonstrated by small angle neutron scattering that in solution, the Mg-ATP bound thermosome favors the “open” conformation in low salt buffer (55). The closed conformation occurs only after ATP hydrolysis, but before release of P_i. Surprisingly, Gutsche et al also showed that the crystallization buffer can induce the closed conformation in solution (55). The crystals for the unliganded structure of GroEL were grown in similarly high $(NH_4)_2SO_4$ 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, as 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 in order 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 to and study of homologs from naturally occurring thermophiles. Some methanogenic archaea use increased intracellular ion concentrations to stabilize their proteins *in vivo* (56). 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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FIGURE LEGENDS

FIG. 1. **ATPase activity of GroEL under various salt conditions.** GroEL (125 nM) in buffer A was supplemented with various salts as indicated below. ATPase activity was initiated at 30°C by addition of ATP (2 mM). The ATPase activity of GroEL in 50 mM KCl (lane 1) is set as 100%. *Lane 1*: 50 mM KCl; *Lane 2*: 1.2 M $(NH_4)_2SO_4$; *Lane 3*: 1.2 M KCl; *Lane 4*: 1.2 M NH_4Cl; *Lane 5*: 1.2 M Na_2SO_4, 50 mM KCl; *Lane 6*: 3.6 M NH_4Cl.

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 M $(NH_4)_2SO_4$ (*lower panel)* containing 0.22 μM GroEL. To initiate folding, the following additions were made at 60 sec: 2 mM ATP and 0.88 μM GroES (*trace 2*); 2 mM ADP and 0.88 μM GroES (*trace 3*); 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 M $(NH_4)_2SO_4$ and 0.22 μM GroEL. ATP (2 mM) and α_{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.

FIG. 3. **Effects of GroES and substrate protein on GroEL ATPase activity.** The ATPase of GroEL (125 nM) was assayed at 25°C in the presence or absence of α_{s1}-casein (0,6 µM). The reaction was carried out in buffer A plus 50 mM KCl or 1.2 M $(NH_4)_2SO_4$. Where indicated, GroES was added at 0.5 µM. ATPase activity was initiated by the addition of ATP (2 mM). The ATPase activity of GroEL in 50 mM KCl is set as 100%.

FIG. 4. **Increased binding of bisANS to GroEL in elevated concentrations of ammonium sulfate.** BisANS (10 µM) was added to GroEL (250 nm) in 25 mM MOPS-NaOH pH 7.5, 5 mM $MgCl_2$, supplemented with either 50 mM KCl (*bottom trace*) or 1.2 M $(NH_4)_2SO_4$ (*top trace*). Fluorescence emission spectra were recorded from 450 to 580 nm. Samples were excited at 397 nm.

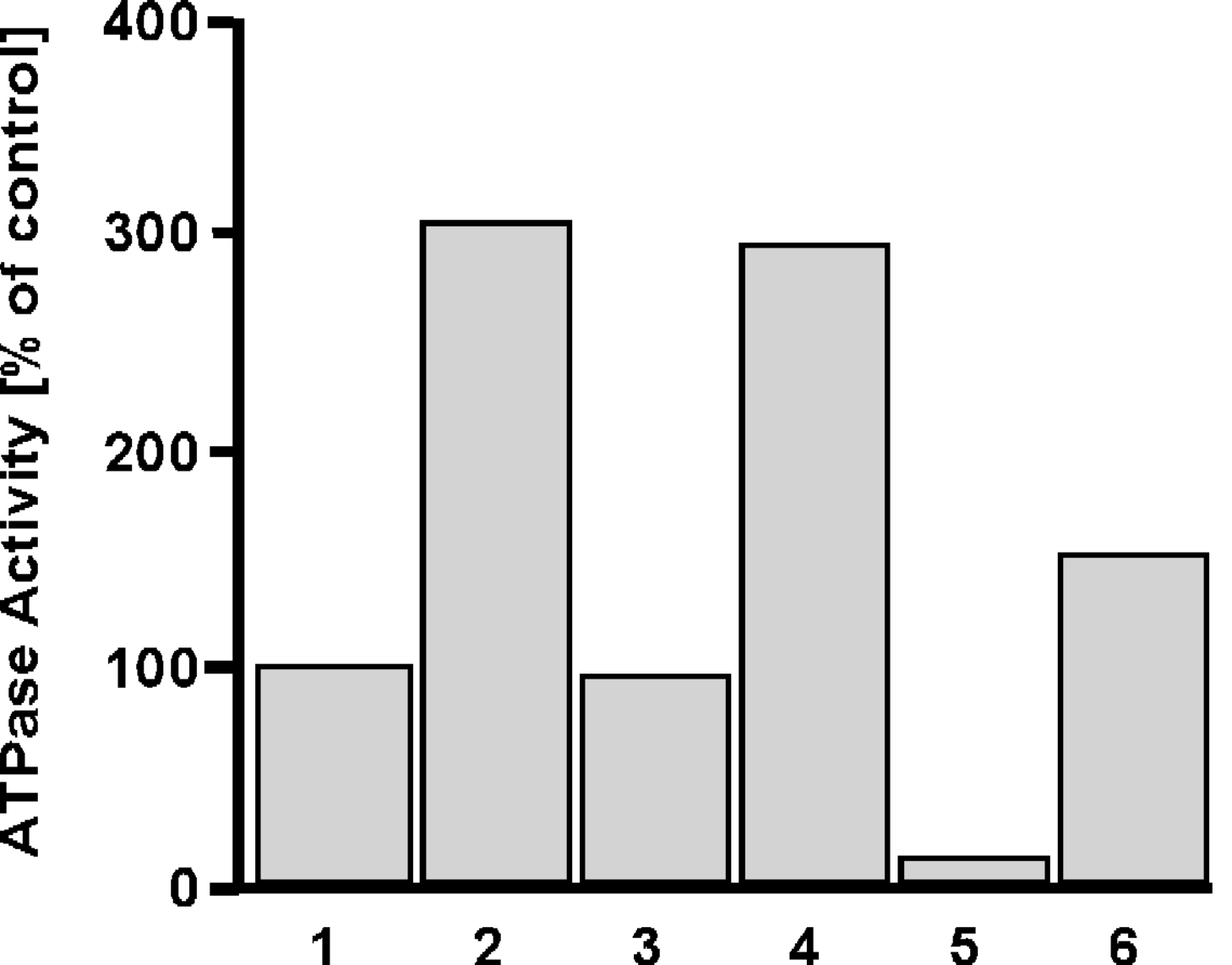
FIG. 5. **GroEL stability and ATPase activity at high temperatures in elevated concentrations of ammonium sulfate.** A) GroEL (125 nM) was added at 70°C to buffer A supplemented with various salts as indicated below. ATPase activity was initiated by addition of ATP (2 mM). The ATPase activity of GroEL 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 $(NH_4)_2SO_4$; *Lane 3*: 1.2 M KCl; *Lane 4*: 1.2 M NH_4Cl; *Lane 5*: 1.2 M Na_2SO_4, 50 mM KCl; *Lane*

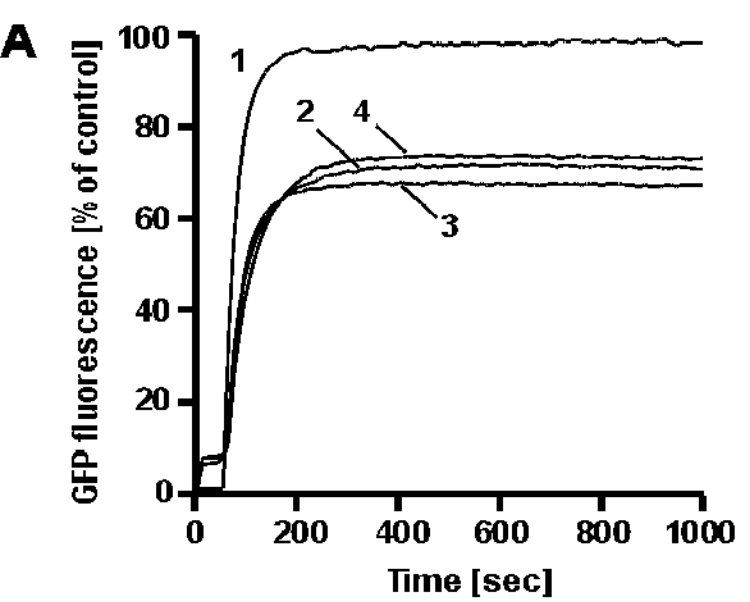
6: 3.6 M NH_4Cl. B) GroEL (530 nM) was diluted at 75°C into buffer containing 25 mM MOPS-NaOH pH 7.6, 5 mM $MgCl_2$, and increasing concentrations of $(NH_4)_2SO_4$: 0.6 M (*grey squares*); 0.8 M (*black triangles*); 1.0 M (*black squares*); 1.2 M (*open circles*). Denaturation of GroEL was followed 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 $MgCl_2$, 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 non-denaturing polyacrylamide gel. *Lane 1:* 1.2 M $(NH_4)_2SO_4$ (control); *Lane 2:* 0.6 M $(NH_4)_2SO_4$; *Lane 3:* 0.8 M $(NH_4)_2SO_4$; *Lane 4:* 1.0 M $(NH_4)_2SO_4$; *Lane 5:* 1.2 M $(NH_4)_2SO_4$; *Lane 6:* 50 mM KCl; *Lane 7:* 50 mM KCl (control).

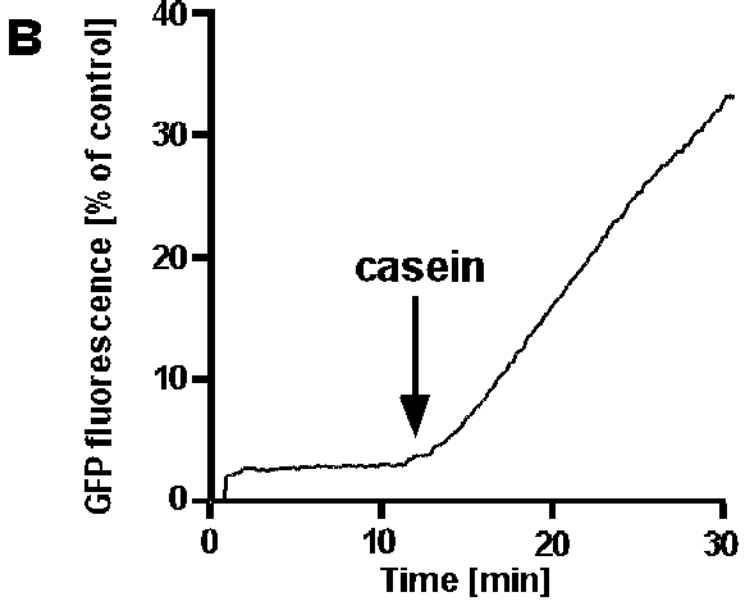
FIG. 6. **Chaperone activity of GroEL at high temperatures.** A) Suppression of citrate synthase aggregation. Citrate synthase (176 nM) was diluted at 70°C into buffer C, in the absence or presence of various amounts of GroEL. Where indicated (arrow), ATP was added to 2 mM. Protein aggregation was followed by measuring light scattering at 320 nm. Citrate synthase (*grey squares*); GroEL : Citrate synthase = 1 : 1 (*open circles)*; GroEL : Citrate synthase = 3 : 1 (*black triangles*). B) 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

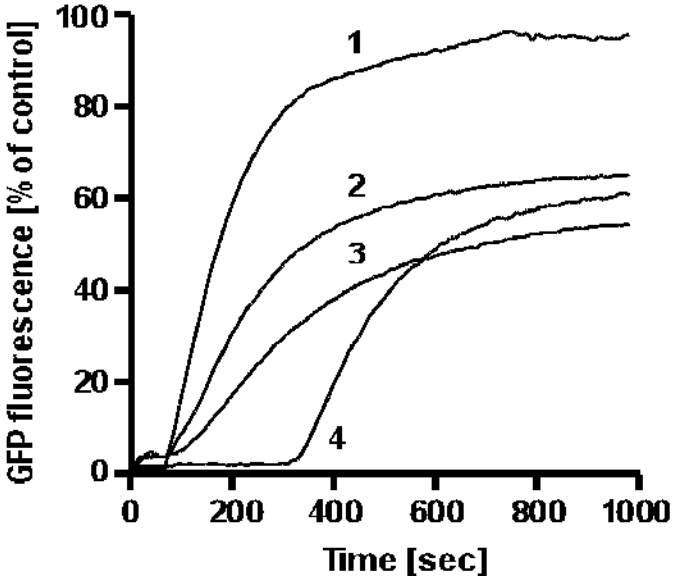
indicated time points, aliquots were withdrawn and assayed for α-glucosidase activity. α-Glucosidase (*open diamonds*); α-glucosidase plus GroEL (*black triangles*); α-glucosidase plus GroEL/GroES and ATP (*black squares*). C) GroEL-mediated refolding of heat-inactivated α-glucosidase. α-Glucosidase (270 nM) was heat-inactivated for 60 min at 70°C in the presence of a three-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*) while the other remained at 70°C (*open squares*). At the indicated time points, aliquots were withdrawn and analyzed 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

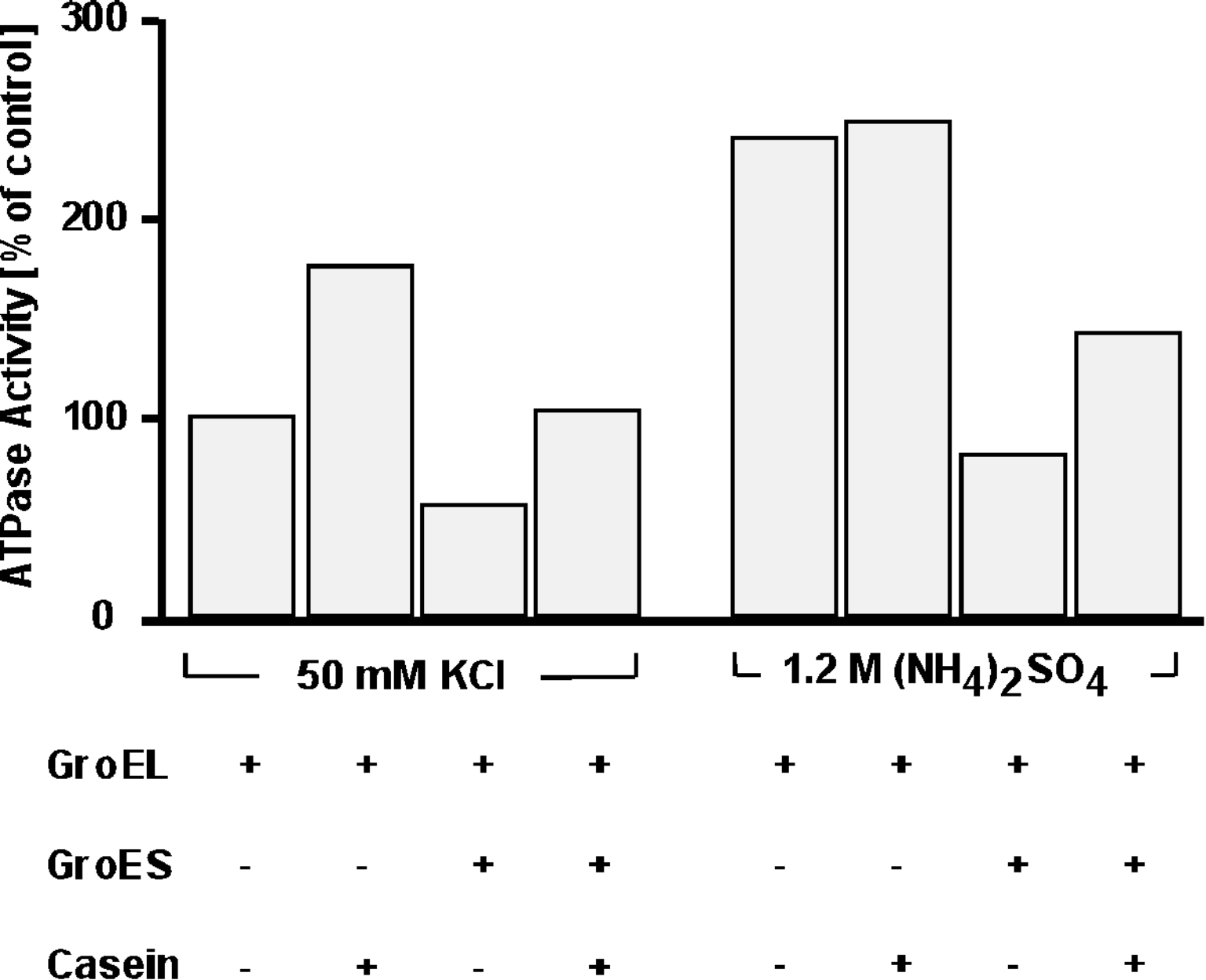
$(NH4)2SO4$ and 0.22 µM GroEL. To initiate folding,the following additions were made at 60 sec: 2 mM ATP and 0.88 µM GroES (*trace 2*); 2 mM ADP and 0.88 µM GroES (*trace 3*); 2 mM ATP (*trace 4*). Spontaneous folding of GFP was observed upon dilution of denatured GFP into buffer B (*trace 1*). The amount recovered was set as 100%, representing approximately 30% of the fluorescence of native GFP at 70°C. GFP fluorescence was monitored at 508 nm.

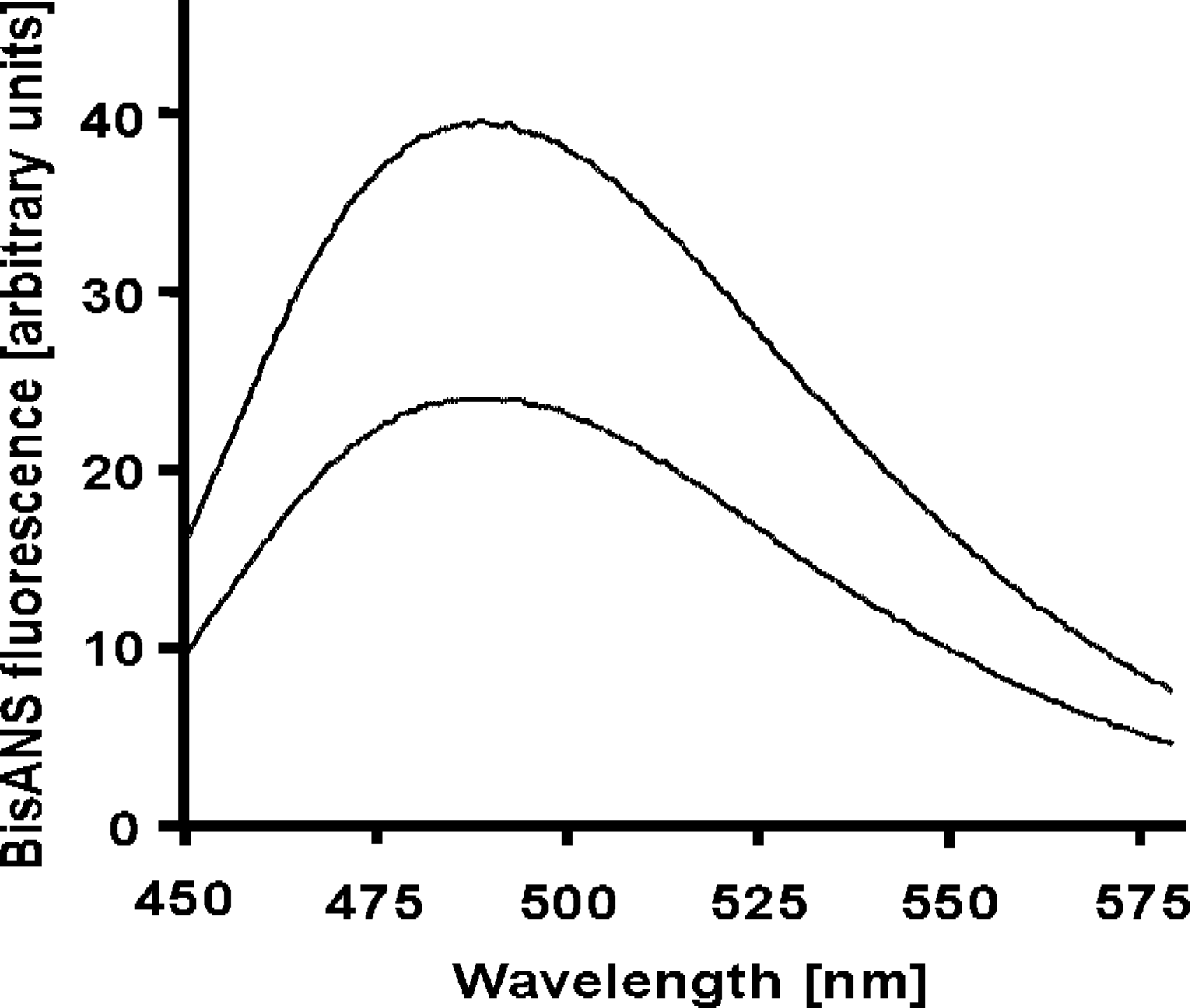

ATPase Activity [% of control]
400
300
200
100
0
1
2
3
4
5
6

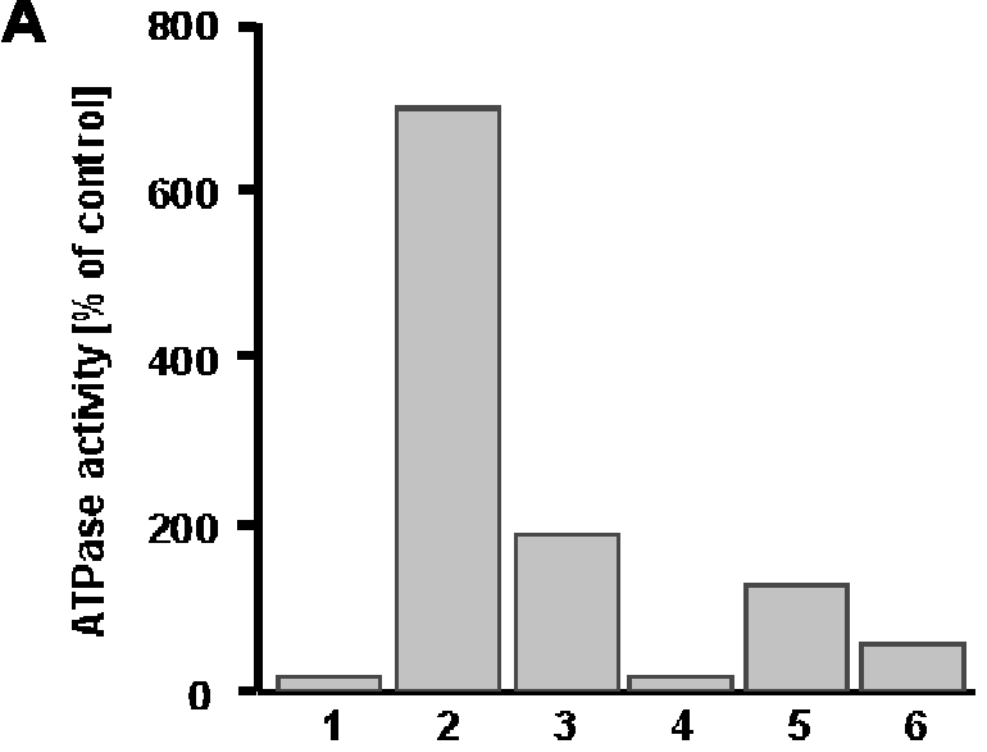

A
GFP fluorescence [% of control]
100
80
60
40
20
0
1
2
4
3
0
200
400
600
800
1000
Time [sec]

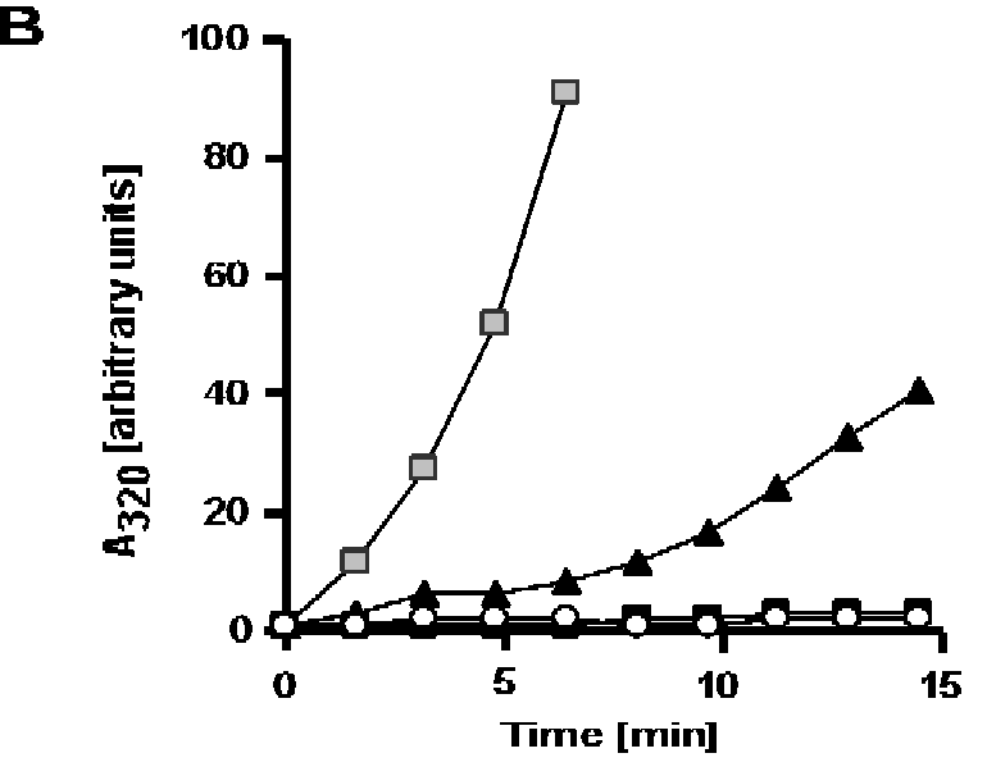

B
GFP fluorescence [% of control]
40
30
20
10
0
casein
0
10
20
30
Time [min]

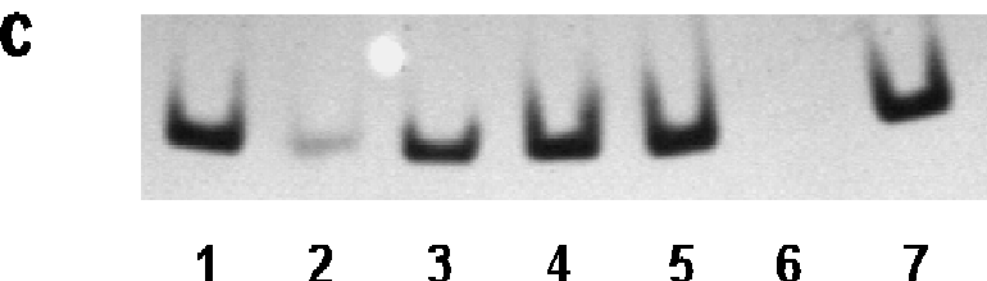

GFP fluorescence [% of control]
100
80
60
40
20
0
1
2
3
4
0
200
400
600
800
1000
Time [sec]

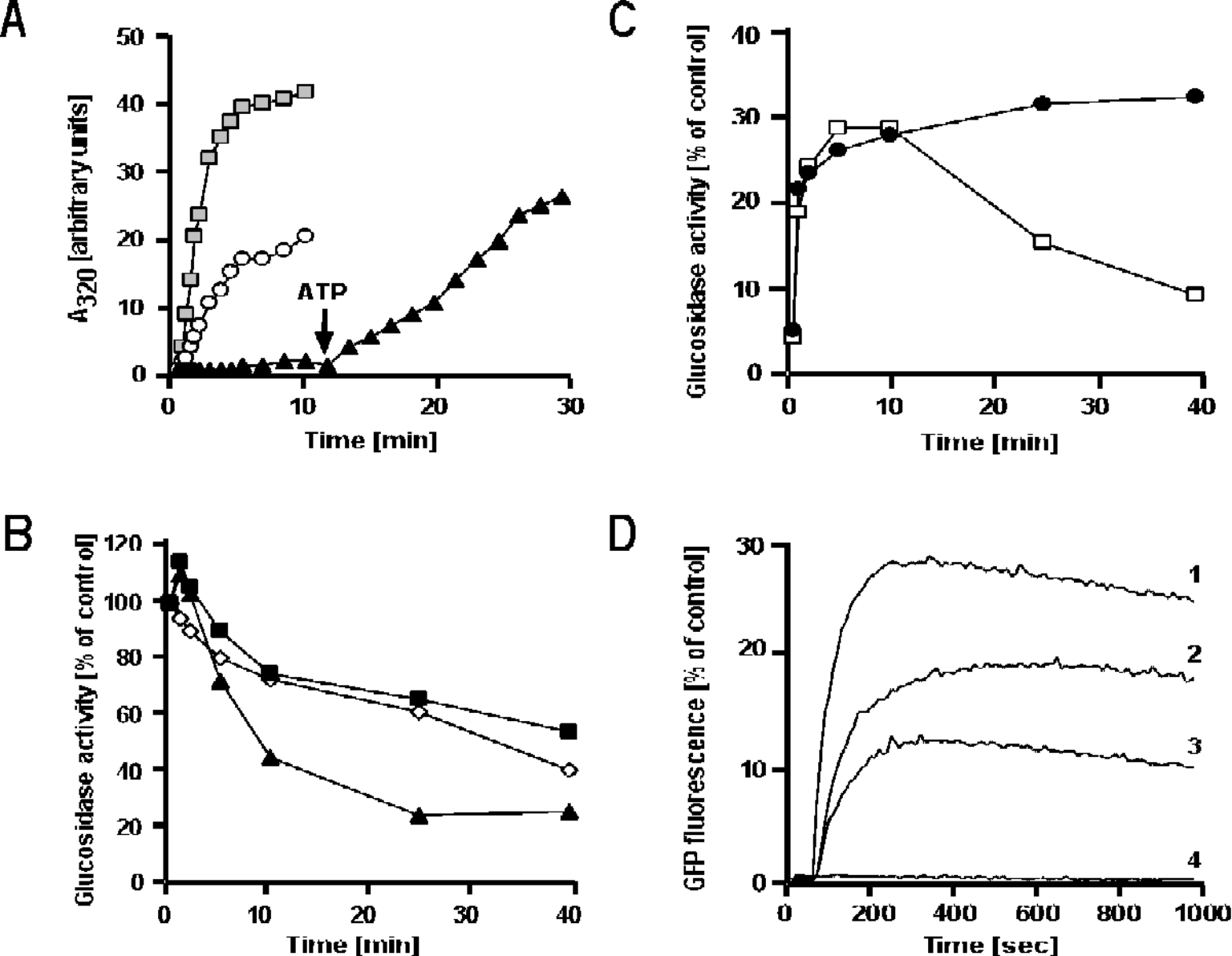

ATPase Activity [% of control]
300
200
100
0
50 mM KCl
1.2 M $(NH_4)_2SO_4$
GroEL + + + + + + + +
GroES - - + + - - + +
Casein - + - + - + - +


BisANS fluorescence [arbitrary units]
0
10
20
30
40
450
475
500
525
550
575
Wavelength [nm]


A
ATPase activity [% of control]
800
600
400
200
0
1
2
3
4
5
6


B
A320 [arbitrary units]
100
80
60
40
20
0
0
5
10
15
Time [min]


C
1
2
3
4
5
6
7


A
A_{320} [arbitrary units]
ATP
Time [min]
C
Glucosidase activity [% of control]
Time [min]
B
Glucosidase activity [% of control]
Time [min]
D
GFP fluorescence [% of control]
1
2
3
4
Time [sec]