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Catalysis, Commitment and Encapsulation during GroE-mediated Folding

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The *Escherichia coli* GroE chaperones assist protein folding under conditions where no spontaneous folding occurs. To achieve this, the cooperation of GroEL and GroES, the two protein components of the chaperone system, is an essential requirement. While in many cases GroE simply suppresses unspecific aggregation of non-native proteins by encapsulation, there are examples where folding is accelerated by GroE.

Using maltose-binding protein (MBP) as a substrate for GroE, it had been possible to define basic requirements for catalysis of folding. Here, we have analyzed key steps in the interaction of GroE and the MBP mutant Y283D during catalyzed folding. In addition to high temperature, high ionic strength was shown to be a restrictive condition for MBP Y283D folding. In both cases, the complete GroE system (GroEL, GroES and ATP) compensates the deceleration of MBP Y283D folding. Combining kinetic folding experiments and electron microscopy of GroE particles, we demonstrate that at elevated temperatures, symmetrical GroE particles with GroES bound to both ends of the GroEL cylinder play an important role in the efficient catalysis of MBP Y283D refolding. In principle, MBP Y283D folding can be catalyzed during one encapsulation cycle. However, because the commitment to reach the native state is low after only one cycle of ATP hydrolysis, several interaction cycles are required for catalyzed folding.

Keywords: catalysis of folding; chaperone mechanism; electrostatic interactions; ATP hydrolysis; apyrase

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Introduction

The *Escherichia coli* GroE system is a highly sophisticated chaperone machinery found in bacteria, mitochondria and chloroplasts. Great progress has been made in understanding the structure and mechanism of action of this chaperone family (Coyle *et al*., 1997; Ranson *et al*., 1998; Beißinger & Buchner, 1998; Sigler *et al*., 1998). Specifically, the ATP-dependent cooperation of the two constituent components, GroEL and GroES, allows proteins to fold under conditions otherwise non-permissive for folding (e.g. Peralta *et al*., 1994; Schmidt *et al*., 1994a; Smith & Fisher, 1995; Taguchi & Yoshida, 1995; Todd *et al*., 1996). During the functional cycle, the non-native protein is encapsulated in a cage inside the GroEL/GroES complex, imposing a size limit for proteins to be folded by GroE. *In vivo*, the size range for substrates seems to vary between 10 and 55 kDa for GroEL/ES (Ewalt *et al*., 1997) and between 15 and 90 kDa for the homologous hsp60/hsp10 system of mitochondria (Dubaquié *et al*., 1998). Almost all mechanistic studies were performed *in vitro* using artificial substrate proteins in the subunit range mentioned above. Much less is known about the *in vivo* substrates although GroE is an essential gene in *E. coli* (Georgopoulos *et al*., 1973; Fayet *et al*., 1989).

GroEL exhibits a complex structure composed of two heptameric rings stacking back to back and forming a 14-subunit hollow cylinder (Braig *et al*., 1994) with two identical binding sites for non-native proteins. The smaller co-chaperone GroES (10.3 kDa) forms a seven-membered, dome-shaped single ring (Hunt *et al*., 1996). Nucleotide binding to GroEL is responsible for the transition from a high-affinity to a low-affinity state of the chaperone for its substrates. Furthermore, it is a prerequi-

Abbreviations used: MBP, maltose-binding protein; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

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site for the interaction of GroEL with GroES. Binding of GroES to the ends of the GroEL cylinder in the presence of ATP leads to the formation of both asymmetric bullet-shaped 1:1 (EL_{14mer}:ES_{7mer}; Langer *et al*., 1992; Chen *et al*., 1994; Roseman *et al*., 1996) and symmetric, football-shaped 1:2 complexes (Azem *et al*., 1994; Llorca *et al*., 1994, 1997; Schmidt *et al*., 1994c). From cryo-electron microscopic studies (Roseman *et al*., 1996) as well as from the crystal structure of the asymmetric $GroES_7$:ADP_7:$GroEL_{14}$ complex (Xu *et al*., 1997), large en bloc movements about two hinge regions in GroEL became evident. The upward movement of the apical domains enlarges the substrate-accessible cavity under GroES. The rotation of the apical domains moves the hydrophobic substrate-binding patches away from the cavity into the intersubunit contacts. As a consequence, substrate is released into the shielded environment of the GroE cavity (*cis* complex).

The importance of the communication of the two GroEL rings is demonstrated by the fact that ATP binding to the *trans* ring is neccessary and sufficient to eject GroES as well as substrate from the opposite ring (Todd *et al*., 1994; Burston *et al*., 1995; Rye *et al*., 1997). Completion of one ATPase cycle takes about 15 seconds at room temperature. This implies that every 15 seconds the protein is ejected from the GroE complex, irrespective of its folding state (Todd *et al*., 1994; Burston *et al*., 1995).

In an environment restrictive for folding, the GroE system increases the refolding yield of substrate proteins by inhibition of aggregation (Buchner *et al*., 1991). In some cases, the GroE-system affects the folding kinetics in addition to the folding yield (Viitanen *et al*., 1991; Peralta *et al*., 1994; Todd *et al*., 1994, 1996; Fedorov & Baldwin; 1997; Ranson *et al*., 1997; Sparrer *et al*., 1997). Several models have been suggested for how GroE influences the folding process. These range from a passive effect by just decreasing the concentration of aggregation-prone intermediates, over providing a ''folding cage'' where the polypeptide can fold in quasi ''infinite dilution'', to a more active role of the GroE system where kinetically trapped species are unfolded by GroE thus giving them another chance to fold (Creighton, 1991; Agard, 1993; Ranson *et al*., 1995; Todd *et al*., 1996; Zahn *et al*., 1996; Frieden & Clark, 1997). With most GroE substrates used, a detailed analysis of folding kinetics is not accessible as they are either multimeric and require an association step after release from the chaperone or they do not fold spontaneously.

Recently, the maltose-binding protein (MBP) from *E. coli* has been introduced as a new substrate for GroE. A major advantage of the MBP system is that folding of the monomeric protein can be monitored directly in the presence of chaperones. This allowed a detailed quantitative analysis of the parameters determining the interaction with GroE in the presence and absence of nucleotides (Sparrer *et al*., 1996, 1997; Sparrer & Buchner, 1997). Even at temperatures similar to heat shock conditions *in vivo* (40 °C), MBP folds with high efficiency without aggregation.

MBP is a monomeric (40.6 kDa) periplasmic protein involved in sugar transport of *E. coli* as the primary acceptor for maltose and maltodextrin (Spurlino *et al*., 1991). Several folding mutants of MBP have been described that retard folding (Chun *et al*., 1993; Betton *et al*., 1996). In contrast to wild-type MBP, which exhibits a biphasic folding behaviour after formation of a burst phase intermediate in the dead time of mixing (Chun *et al*., 1993), the folding mutant MBP Y283D used in this study follows simple monophasic kinetics. A rapidly formed, partially folded intermediate of MBP Y283D binds to GroEL with very high affinity ($K_D \sim 10^{-11}$ M; Sparrer *et al*., 1996) leading to the complete suppression of refolding in the absence of nucleotide. In the presence of ATP, the dissociation constant increases by three orders of magnitude, which results in release and refolding of MBP (Sparrer *et al*., 1996).

Interestingly, in contrast to the systems where GroE increases the folding yield, the kinetics of MBP Y283D refolding are efficiently accelerated in the presence of GroEL/ES and ATP, especially at high temperature (Sparrer *et al*., 1997). Here, we set out to further define the requirements for catalyzed folding and especially the correlation between ATP hydrolysis, catalysis of folding, and commitment to fold to the native state.

Results

Folding of MBP Y283D is salt-dependent

High temperature is a parameter rendering the folding of MBP mutant Y283D non-permissive. This is demonstrated by the observation that the apparent folding rate deviates from the linear Arrhenius dependence observed for wild-type MBP at temperatures above 35 °C (Sparrer *et al*., 1997). Under these restrictive conditions, MBP Y283D refolding is efficiently catalyzed by GroEL/ES in the presence of ATP. In order to analyze the correlation of catalysis and ATP hydrolysis, we wanted to modulate the ATPase of GroEL by variation of the K^+ concentration (cf. Viitanen *et al*., 1990). However, the apparent folding rate for MBP Y283D is itself salt-dependent, i.e. folding is decelerated at high concentrations (Figure 1(a)). The observed salt-dependence is not linear, but is described by a single exponential behaviour and reaches a plateau at a salt concentration of around 200 mM ionic strength. The comparison of the monovalent salts KCl and NaCl showed that there is no specific dependence on the cations Na^+ *versus* K^+. Furthermore, MBP folding experiments in the presence of the divalent salt Na_2SO_4 showed that the folding rate of MBP Y283D depends on the ionic strength rather than the molarity of the salt added.

This result was surprising, because no salt-dependence of folding had been observed before for wild-type MBP (Sparrer *et al*., 1996). As these

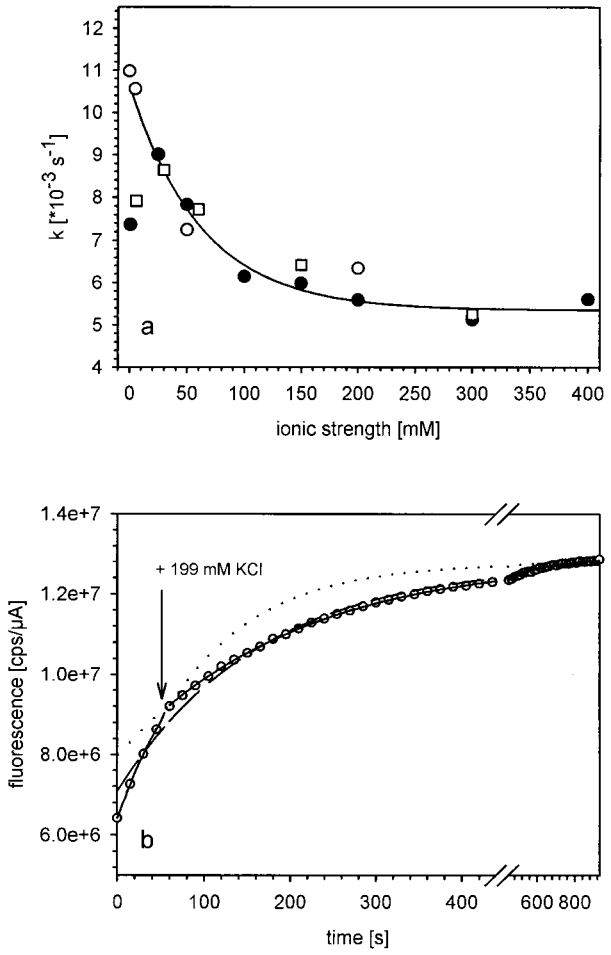

Figure 1. Ionic strength-dependence of the apparent rate constants of MBP Y283D folding. MBP Y283D (50 nM) was refolded spontaneously in 20 mM Tris-HCl (pH 7.2 at 40 °C), 5 mM $MgCl_2$, 200 µM ATP. (a) KCl (●), NaCl (○) or Na_2SO_4 (□) were added at the ionic strength indicated on the abscissa. The symbols represent the apparent first-order rate constants derived from respective fits to the refolding traces observed by fluorescence emission. The line through the data shows a single exponential fit for the ionic strength-dependence of the folding rate constants. (b) Refolding of MBP Y283D was started in the above-mentioned buffer supplied with 1 mM KCl. After 60 seconds, 199 mM KCl was added and further refolding was monitored. The broken line represents a single exponential fit through the complete kinetic trace of the data (○), the continuous lines represent separate single exponential fits through the data for the first 60 seconds (rate constant 11.0 (±1.3) × 10^{-3} s^{-1}) and the period from 60 seconds on (rate constant 4.6 (±0.35) × 10^{-3} s^{-1}). The dotted lines mark extrapolations of the separate fits.

measurements were performed under permissive conditions (25 °C), we repeated the wild-type experiments under the non-permissive temperature used for mutant MBP refolding in this study (40 °C). Wild-type MBP exhibited a more complicated folding behaviour, including two folding phases observed after manual mixing as described before (Chun *et al.*, 1993; Sparrer *et al.*, 1996). Both phases were faster than MBP Y283D folding by at least one order of magnitude (rate constants of 0.10 (±0.01) s^{-1} and 0.9 (±0.3) s^{-1} for the slow and fast refolding phases, respectively) and essentially salt-independent in the range tested (data not shown).

In order to test whether high ionic strength affects only the early folding intermediates of MBP Y283D, we increased the salt concentration 60 seconds after starting spontaneous refolding (Figure 1(b)). The folding reaction cannot be described by a single exponential fit to the data. Individual fits for the two folding phases observed under the different salt concentrations gave rate constants of 11.0 × 10^{-3} s^{-1} and 4.6 × 10^{-3} s^{-1}. These correspond nicely to the respective values observed for folding under low and high salt conditions described above (Figure 1(a)). Obviously, a partially folded intermediate along the folding pathway of MBP Y283D is negatively influenced by high ionic strength.

GroE compensates the salt-dependence of MBP Y283D folding

The complete GroE system (GroEL, GroES and ATP) was shown to be required for effectively catalyzing refolding of the slow-folding mutant MBP Y283D at restrictive temperatures (Sparrer *et al.*, 1997). These experiments were done under conditions where the data presented above showed that folding of mutant MBP was decelerated by high ionic strength (200 mM KCl). To analyze how catalysis of folding and salt-dependence correlate, we repeated the above-mentioned experiment in the presence of the complete GroE system. Figure 2(a) shows that GroE is able to render MBP Y283D folding salt-independent over the whole range of ionic strength tested. In Figure 2(b), two representative MBP Y283D refolding kinetics for spontaneous (lower curve) and GroE-assisted refolding (upper curve) under high salt conditions are depicted (the respective rate constants were 3.10 × 10^{-3} s^{-1} and 11.64 × 10^{-3} s^{-1}). Both kinetic traces are perfectly described by single exponential fits to the data (thicker continuous lines). As shown before (Sparrer *et al.*, 1997), the catalysis of MBPY283D folding is strictly dependent on the ratio of GroES to GroEL. Using higher GroES to GroEL ratios, the acceleration by the GroE system exceeded by far the increase in the apparent folding rate that can be achieved by lowering the salt concentration in spontaneous MBP Y283D folding (Figure 2(c)). As fluorescence measurements use relatively low protein concentrations, the experiments do not strictly monitor the stoichiometry of the components used, but are influenced by affinity. Therefore, maximum acceleration is not achieved at a 2 to 1 ratio of GroES to GroEL, but at a ratio of around 8 to 1 (Figure 2(c); compare also Figures 2 and 4 of Sparrer *et al.*, 1997).

The experiments show that under conditions restrictive for MBP Y283D folding, the complete GroE system is able to compensate the salt effect

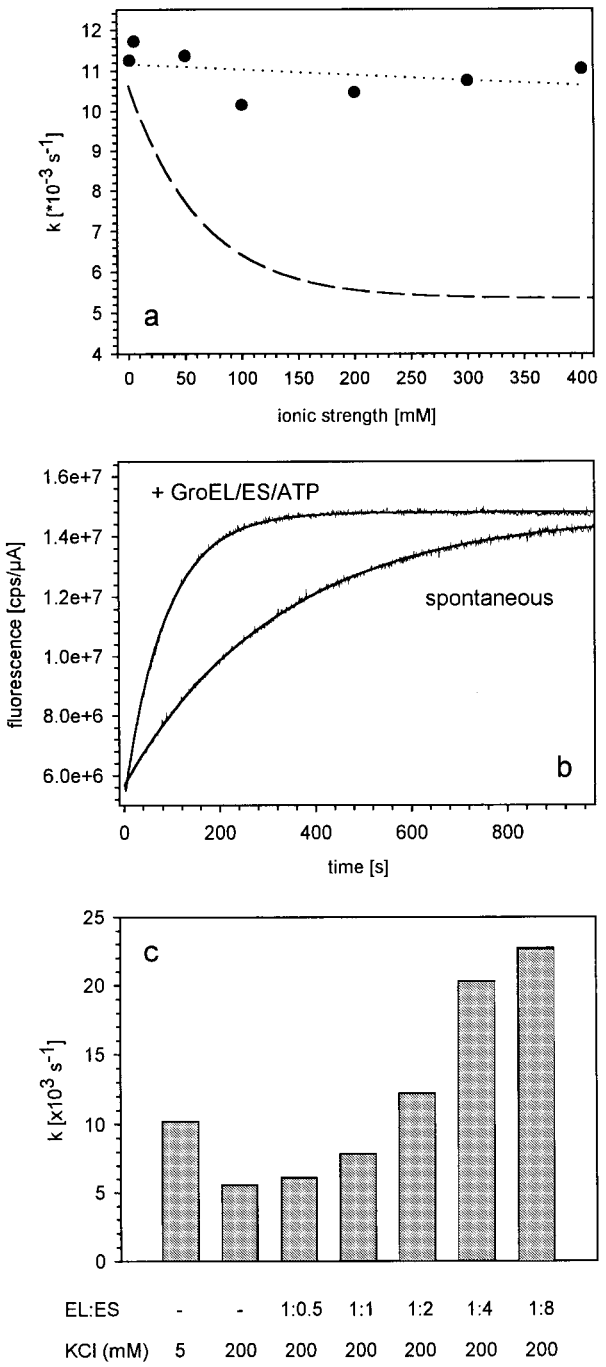

Figure 2. Ionic strength-dependence of the apparent rate constants of MBP Y283D folding in the presence of GroEL and GroES. MBP Y283D (50 nM) was refolded in 20 mM Tris-HCl (pH 7.2 at 40 °C), 5 mM $MgCl_2$, 200 µM ATP in the presence of GroEL (50 nM) and GroES (150 nM). (a) KCl was added at the ionic strength indicated. The symbols (●) represent the apparent first-order rate constants derived from respective fits of the refolding traces observed by fluorescence emission. The dotted line through the data is a linear fit through the data. The broken line represents the ionic strength-dependence observed in the absence of the GroE system (see Figure 1(a)). (b) Representative kinetic traces for spontaneous (rate constant for single exponential fit 3.10×10^{-3} s^{-1}) and GroE-assisted refolding (rate constant for single exponential fit 11.64×10^{-3} s^{-1}) of MBP Y283D in standard buffer (200 mM KCl) observed by fluorescence spectroscopy. The continuous lines represent single exponential fits through the data for slow (spontaneous) and catalyzed GroE-assisted folding. (c) Acceleration of MBP Y283D folding at low ionic strength and in the presence of increasing GroES to GroEL ratios.

and alleviates the folding defect caused by high temperature. Thus, GroE renders MBP Y283D folding largely independent of the environmental conditions.

MBP is able to fold in a sequestered position under GroES in the presence of ATP

Having established conditions to monitor catalyzed folding, we wanted to know whether MBP is able to fold *in cis*, i.e. inside GroES/GroEL complexes after one round of ATP hydrolysis, or, whether several cycles of binding and release are required for catalyzed folding. Using size-exclusion HPLC and the tryptophan fluorescence of MBP, we first quantified MBP-GroE complexes formed during refolding under conditions exactly matching those of the kinetic experiments measured ''on-line''.

The elution profiles depicted in Figure 3(a) show that in the absence of ATP, MBP was completely bound to GroEL over a period of time comparable to that of spontaneous MBP folding. The fluorescence intensity of the bound MBP folding intermediate did not change and exhibited a value between those of completely denatured and native MBP. After addition of ATP, the MBP fluorescence at the position of the complex peak decreased and fluorescence at the position of free MBP began to appear. Twenty minutes after addition of ATP, all fluorescence was observed at the position of free MBP, reaching more than 90 % of the fluorescence signal of the native control, confirming that refolding of MBP Y283D and interaction with GroE is fully reversible. The HPLC data with wild-type GroEL exactly match the results for the kinetic experiments (cf. Figures 2(b) and 5), including the increase of the fluorescence signal with time. However, these data do not allow us to determine whether MBP folds in GroE, as the complexes are highly dynamic under folding conditions.

We therefore wanted to address the question of whether MBP Y283D is able to fold to the native state inside GroE using SR1 instead of GroEL as a simplified experimental system (Figure 3(b)). SR1 is a single ring mutant of GroEL, which had been described to bind GroES and perform only one round of ATP hydrolysis (Weissman *et al.*, 1995, 1996; Hayer-Hartl *et al.*, 1996). In the absence of ATP, MBP Y283D remained completely bound to SR1, indicating that the affinity of SR1 for MBP Y283D is comparable to that of GroEL. However, 20 minutes after addition of ATP, comparable to wild-type GroEL, most of the fluorescence was

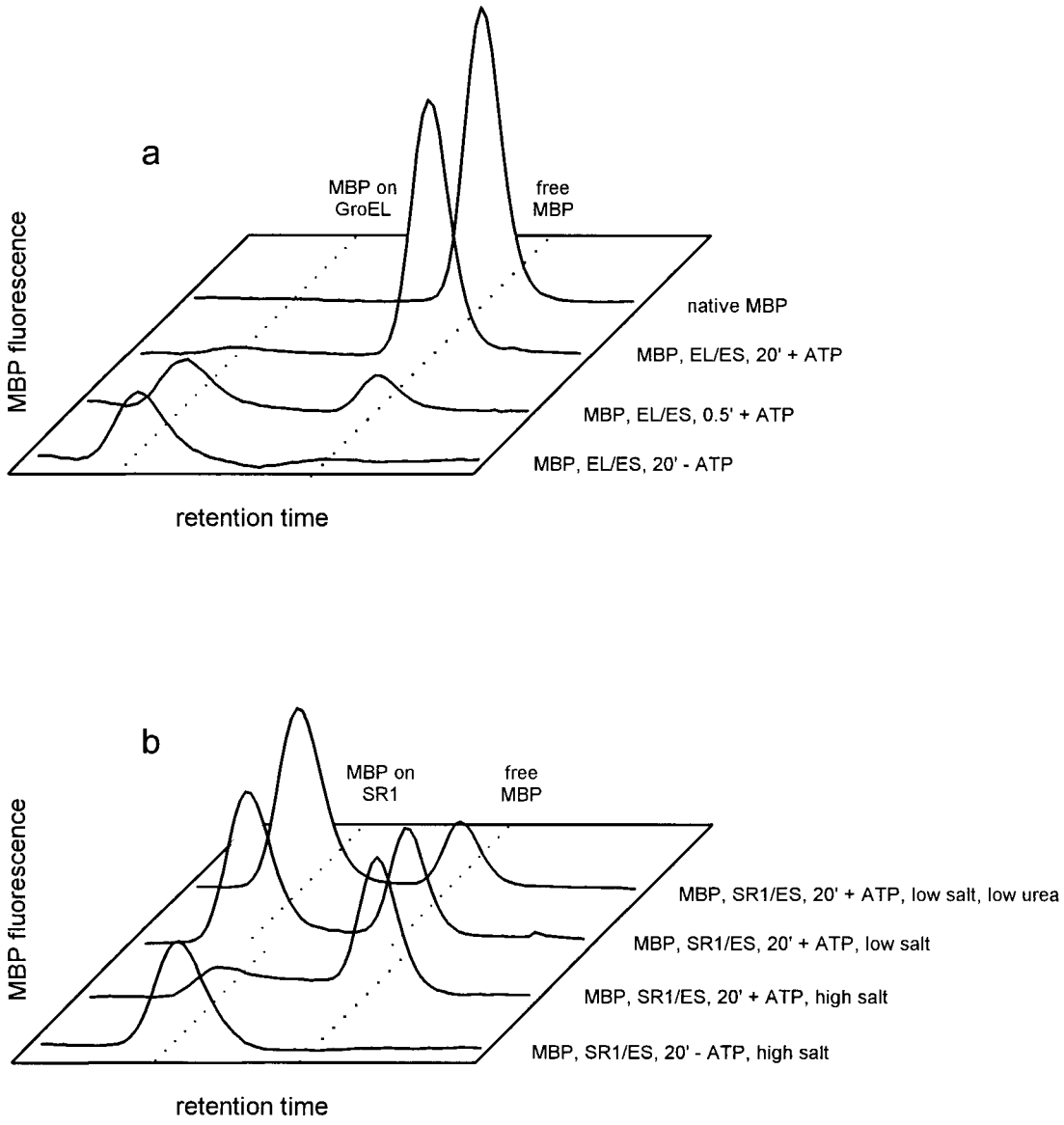

Figure 3. Size-exclusion HPLC chromatography of GroE-MBP complexes. Protein was detected by the tryptophan fluorescence of MBP. (a) MBP Y283D (50 nM) was bound to GroEL (50 nM), GroES (200 nM), in the absence of ATP in 20 mM Tris-HCl (pH 7.2 at 40 °C), 5 mM $MgCl_2$, 1 mM KCl, 199 mM NaCl. At time zero, ATP (200 μM) was added and the samples were applied to a TSK 4000 PW column after the time indicated. Retention times of MBP were 10.7 minutes in complex with GroEL/ES and 13.5 minutes in the free form. (b) MBP Y283D (50 nM) was bound to SR1 (100 nM), GroES (200 nM), in the absence of ATP at 40 °C in 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$ supplemented with either 1 mM KCl (low salt) or 1 mM KCl, 199 mM NaCl (high salt). At time zero, ATP (200 μM) was added and the samples were applied to a TSK 4000 PW column after the time indicated. The retention time for MBP in the SR1/ES complex was 11.2 minutes under high salt conditions, as SR1 is smaller compared to GroEL. Under low salt conditions, retention times of complex and free form are somewhat smaller (10.8 and 13.1 minutes, respectively) due to slightly different binding properties of the column. In the upper trace, the MBP:SR1:ES ratio was increased to 1:3:6 and the residual urea concentration was decreased to 3 mM (compared to 30 mM).

observed at the position of free MBP. ATP hydrolysis experiments showed that ATP is hydrolyzed by SR1 in a GroES-dependent manner at a rate similar to that of GroEL under high salt conditions and at a low, but still measurable rate under low salt conditions in the presence of ES (data not shown). This indicates that the SR1 complexes are not completely stable, although the trigger from the second EL ring to eject ES is absent. As there is some indication in the literature that the SR1/ES complex is salt-labile (Hayer-Hartl *et al.*, 1996; Weissman *et al.*, 1996), we repeated the experiments under low salt conditions (1 mM KCl). Here, under standard conditions (SR1/MBP/ES = 2:1:4, 30 mM residual urea), after completion of refolding 64% MBP remained bound to SR1, while 36% of the fluorescence was observed at the position of free MBP. It was possible to slightly increase the amount of

MBP bound to SR1 after refolding to 77 % by increasing the SR1 to MBP ratio (SR1/MBP/ES = 3:1:6) and reducing the residual urea concentration to 3 mM. Although some MBP was still released, a large percentage of MBP remained in the complex with SR1 under these conditions after refolding was completed. Interestingly, the fluorescence signal of the sample increased to about 90 % of the native signal, indicating refolding of MBP in the SR1-GroES complex.

Taken together, the experiments show that MBP Y283D folding is productive both in GroEL and SR1 complexes. Furthermore, when release of MBP from GroE complexes is artificially blocked (as in the case of SR1) the protein can, in principle, fold inside the chaperone.

GroE-bound MBP reaches the native state only in the presence of GroES and ATP

In addition to the increase in fluorescence amplitude upon refolding, we used maltose binding as an assay for the native state of MBP. Maltose-binding to wild-type MBP is accompanied by a red-shift of the fluorescence emission of the protein and a slight decrease in the fluorescence signal (Miller *et al.*, 1983).

Upon addition of maltose (5 mM), native MBP Y283D exhibited a shift in the fluorescence emission maximum from 345 nm to 349 nm and a concomitant 10 % decrease in the fluorescence signal. In contrast, completely unfolded MBP exhibits 25 % of the native MBP fluorescence signal and an emission maximum of 354 nm (data not shown), MBP stably bound to GroEL in the absence of ATP showed an emission maximum at 343 nm and about half the signal amplitude of native MBP (Figure 4(a)), indicating that the GroEL-bound intermediate did not bind maltose. MBP Y283D released from GroEL and refolded in the presence of GroES and ATP, however, bound maltose like native MBP Y283D (Figure 4(b)). Addition of maltose to MBP Y283D trapped in the SR1/ES/ADP complex under low salt conditions exhibited the characteristic red-shift in the fluorescence emission maximum, but not the small decrease in fluorescence signal (Figure 4(c)). As native MBP assayed under these buffer conditions shows only the change in the emission maximum without the decrease in signal amplitude (data not shown), we assume that MBP Y283D becomes functional when sequestered in the GroE cavity. Interestingly, MBP Y283D complexed to GroEL in the presence of GroES does not bind maltose after addition of the non-hydrolyzable ATP analogon AMP-PNP, suggesting that ATP hydrolysis is required to regain maltose-binding (Figure 4(d)).

Quenching the ATPase of GroEL during MBP refolding

The importance of ATP hydrolysis for acceleration of MBP folding was reinforced by the observation that the non-hydrolyzable ATP analogon AMP-PNP arrested the refolding of MBP at the state of a folding intermediate (Figure 5(a)).

To address the question of how MBP Y283D folding is connected to the ATPase activity of GroEL under non-permissive salt conditions, we used different methods to quench the ATP hydrolysis of wild-type GroEL during MBP folding. To start the GroE cycle under well-defined conditions, MBP Y283D was stably bound to GroEL in the absence of nucleotide at the beginning of each experiment. Refolding was then initiated by the addition of nucleotide (Figure 5).

An effective method to quench the ATPase of GroEL is the removal of magnesium by the addition of EDTA. The Mg-chelator blocked refolding instantaneously when added after the start of the refolding reaction (Figure 5(b)). The reaction was specific for magnesium as the structurally similar Ca-chelator EGTA had no effect (upper curve). These results showed that, upon removal of MgATP-complexes by EDTA, all non-native MBP molecules released from GroE were immediately rebound to nucleotide-free GroEL. If MBP released into solution were committed to reach the native state, it should fold to the native state spontaneously as EDTA does not influence this reaction (lower curve).

Another method to quench the ATPase is the addition of apyrase, an enzyme that hydrolyzes ATP to ADP and finally to AMP. As depicted in Figure 5(c), apyrase treatment did not result in an immediate stop of MBP refolding. Instead, a folding reaction with a smaller amplitude was observed, suggesting that apyrase is not able to remove ATP from GroEL/ES complexes that are committed to hydrolysis (Todd *et al.*, 1994). This result opened the possibility to study *cis*-folding of a substrate protein in wild-type GroE ''on-line''.

To further analyze catalysis of MBP Y283D folding under ''single turnover conditions'', we next asked how many cycles of ATP hydrolysis were required for MBP Y283D refolding. The time for one round of ATP hydrolysis by GroEL was reported to be around 15 seconds at 25 °C (Todd *et al.*, 1994; Weissman *et al.*, 1994; Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995). As ATP hydrolysis is accelerated at higher temperatures, one turnover under our experimental conditions occured roughly every ten seconds (data not shown). Nucleotide analysis showed that ATP was degraded within less than three seconds after addition of apyrase, and after ten seconds, the ADP content was 14 % and most of the nucleotide was already hydrolyzed to AMP. Thus, addition of apyrase allows us to perform single turnover experiments.

To analyze whether one or more cycles of ATP hydrolysis are required for catalysis of folding, MBP Y283D was bound to GroEL, refolding was started with ATP and after 10, 30, 60 or 180 seconds apyrase was added. The kinetic traces showed that at each time-point the folding amplitude observed after the quench (line with

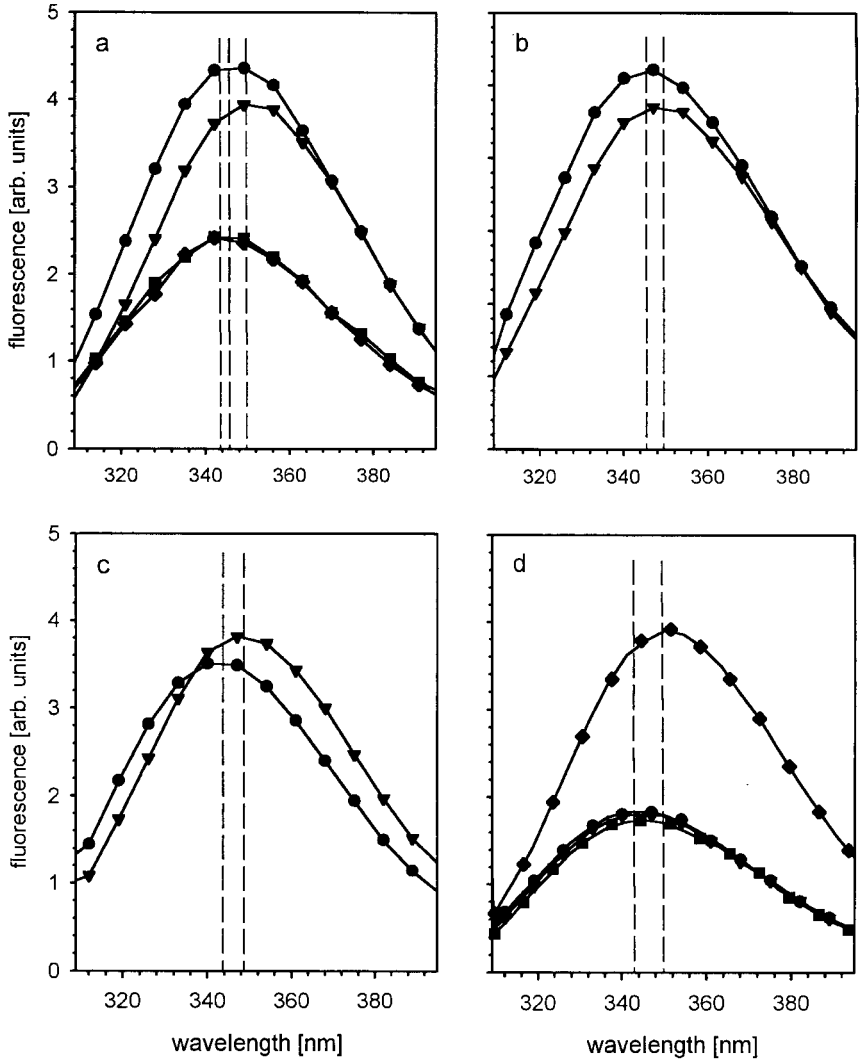

Figure 4. Maltose-binding to MBP Y283D. Maltose (5 mM) was added to native or refolded MBP Y283D (50 nM). Binding was indicated by signal changes in the fluorescence emission spectra (excitation at 295 nm). (a) Native MBP Y283D (●, emission maximum 345 nm) and after addition of maltose (▼, emission maximum 350 nm). MBP Y283D bound to GroEL (50 nM; ■, emission maximum 343 nm), and after addition of maltose (◆, emission maximum 343 nm). (b) MBP Y283D after refolding in the presence of GroEL (50 nM), GroES (200 nM) and ATP (200 μM) (●, emission maximum 345 nm), and after addition of maltose (▼, emission maximum 350 nm). (c) Standard buffer with 1 mM KCl. MBP Y283D after refolding in the presence of SR1 (100 nM), GroES (200 nM) and ATP (200 μM; ●, emission maximum 344 nm), and after addition of maltose (▼, emission maximum 349 nm). (d) MBP Y283D bound to GroEL (50 nM) in the absence (●, emission maximum 343 nm) and presence of 200 μM AMP-PNP (▼, emission maximum 343 nm), and after addition of maltose (■, emission maximum 343 nm). MBP Y283D after refolding initiated by addition of 200 μM ATP in the presence of GroES (200 nM; ◆, emission maximum 350 nm).

symbols) represents a fraction of the missing amplitude (Figure 6). Thus, there is no commitment for MBP Y283D to fold to the native state after one or a few cycles of ATP hydrolysis. Furthermore, folding is accelerated under these conditions with respect to spontaneous folding. If the folding amplitude observed after addition of apyrase would represent *cis*-folding species, it should be dependent on the occupancy of GroEL with GroES. Therefore, we next determined the GroES to GroEL ratio required for optimal catalysis of folding.

MBP folding after an apyrase quench is dependent on the GroES to GroEL ratio

We knew from earlier experiments (Figure 2(c) and Sparrer *et al.*, 1997) that catalyzed MBP Y283D folding correlates with the formation of symmetrical GroE particles with GroES bound to both ends of the GroEL cylinder. Another consideration was that GroEL loaded with substrate in both cavities of the double ring should lead to a productive *cis* interaction with GroES in every case, whereas at a 1 to 1 stoichiometry of substrate and GroEL, GroES

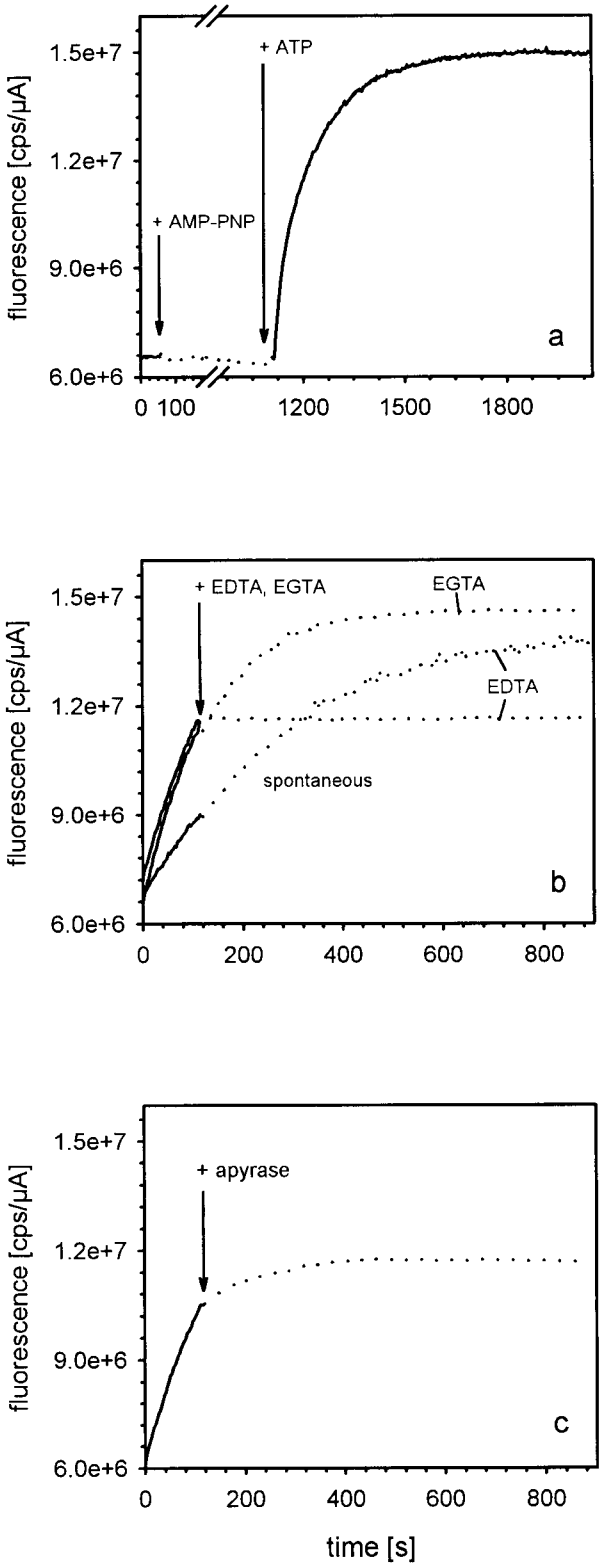

Figure 5. Effect of an ATPase quench on GroE-mediated MBP Y283D folding. MBP Y283D (50 nM) was bound to GroEL (50 nM) in standard buffer in the presence of 100 nM GroES. (a) After 60 seconds, AMP-PNP was added (200 μM, dotted line), after 1100 seconds catalyzed refolding was initiated by addition of ATP (200 μM, continuous line) rate constant for single exponential fit: 8.46×10^{-3} s^{-1}. (b) At time zero, ATP (200 μM) was added to initialize folding from GroEL-MBP complexes in the presence of GroES (upper curves) or spontaneous MBP Y283D refolding was started in the presence of 200 μM ATP (lower curve; rate constant for single exponential fit 3.34×10^{-3} s^{-1}). After 60 seconds, EDTA or EGTA (10 mM) were added as indicated by the arrow (rate constant for single exponential fit 7.85×10^{-3} s^{-1}). The kinetic traces after the quench are presented as dotted lines. (c) Folding was initiated as in (b), and after 60 seconds five units of apyrase were added to quench the ATPase of GroEL (rate constant for single exponential fit after apyrase addition 9.80×10^{-3} s^{-1}).

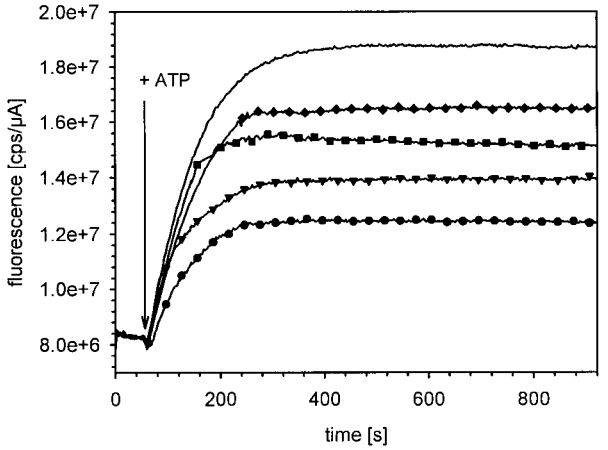

Figure 6. Effect of ATPase quench after several hydrolysis cycles. MBP Y283D (50 nM) was bound to GroEL (50 nM) under standard buffer conditions in the presence of 100 nM GroES. After 60 seconds, ATP (200 μM) was added to initiate refolding. Ten units of apyrase was added ten seconds (●), 30 seconds (▼), 60 seconds (■) or 180 seconds (◆) later. The continuous line represents the folding trace for the unquenched reaction (full amplitude reference), the lines with symbols represent the folding traces observed after the ATPase quench.

could bind either *cis* or *trans* relative to MBP. Therefore, we varied the GroES to GroEL ratio as well as the MBP to GroEL ratio in the apyrase quench experiments and looked for effects on the folding amplitude. MBP Y283D was bound to GroEL, ten seconds after folding was started by ATP, apyrase was added and MBP refolding was monitored until the final folding amplitude was reached. As a control, a large excess of ATP was added later on to release all MBP molecules still bound to GroEL (Figure 7).

Surprisingly, the refolding amplitudes were mainly dependent on the GroES to GroEL ratio and, to a much lower extent, on the occupancy of GroEL with substrate. With a MBP to GroEL ratio of 1 to 1 (Figure 7(a)), 62% folding was observed for a high GroES to GroEL ratio in contrast to 42% for a 1 to 1 ratio of GroES to GroEL. With a ratio of two MBP per GroEL (Figure 7(b)) the respective values were similar, 72% *versus* 50%. If we injected the samples of experiment 7 on a size-exclusion HPLC column,

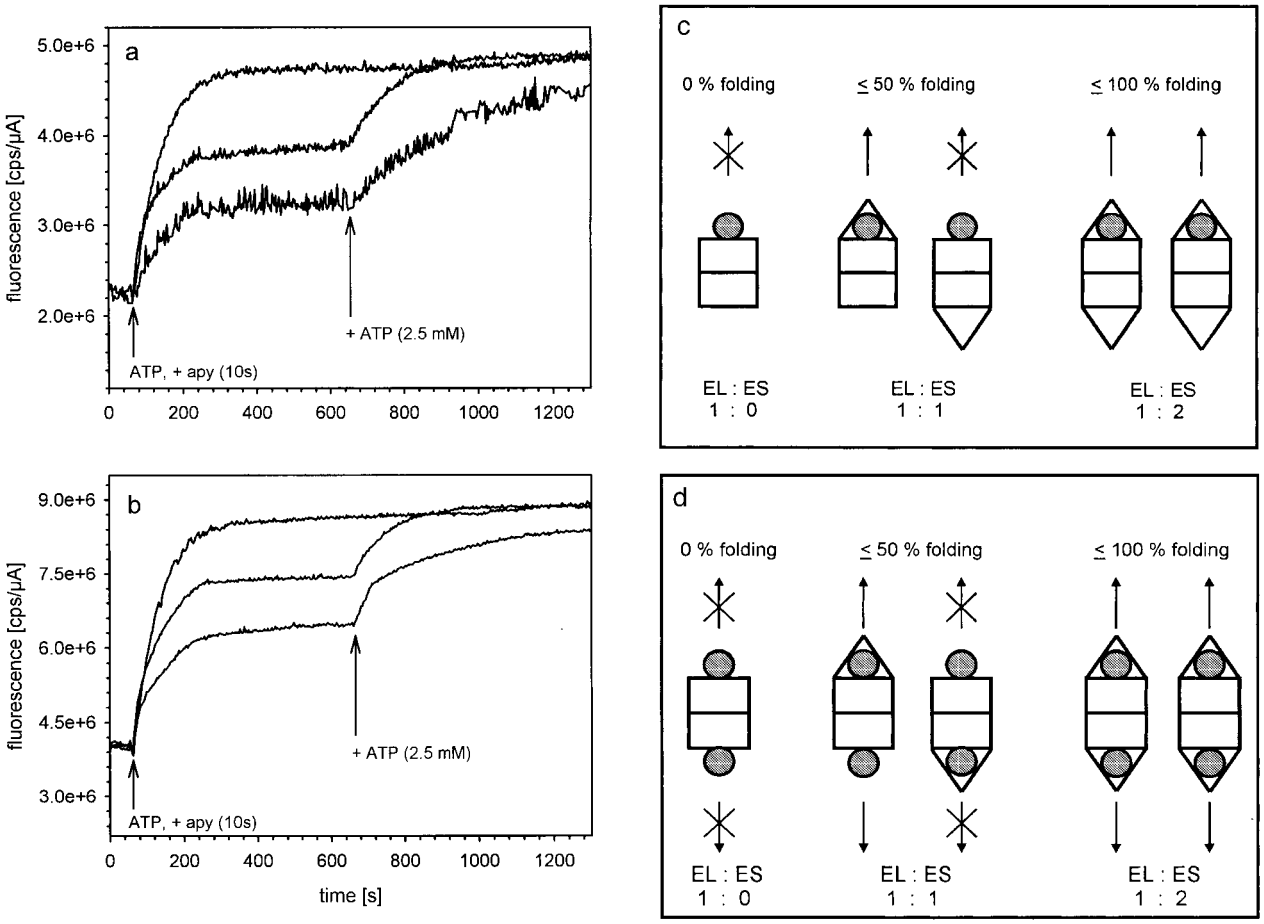

Figure 7. Variation of the MBP to GroEL and GroES to GroEL ratios in the apyrase quench experiment. MBP Y283D was bound to GroEL (25 nM) under standard buffer conditions in the presence of GroES. After 60 seconds, ATP (200 μM) was added to initiate refolding, and ten units of apyrase were added to quench the ATPase ten seconds later. After 650 seconds, ATP (2.5 mM) was added to release and initiate refolding of MBP stably bound to GroEL. The upper traces represent the controls without addition of apyrase, the middle traces a GroES:GroEL ratio of 4:1, and the lower traces a GroES:GroEL ratio of 1:1. (a) MBP to GroEL ratio 1:1 (rate constants for single exponential fits are 1.34×10^{-2} s^{-1}, 1.58×10^{-2} s^{-1} and 1.32×10^{-2} s^{-1} for the upper, middle and lower traces, respectively). (b) MBP to GroEL ratio 2:1 (rate constants for single exponential fits are 1.32×10^{-2} s^{-1}, 1.42×10^{-2} s^{-1} and 1.11×10^{-2} s^{-1} for the upper, middle and lower traces, respectively). (c) and (d) Simplified model for GroE-mediated folding after one round of ATP hydrolysis. GroEL is represented by two rectangles, GroES by a triangle, and MBP by a circle. (c) Results for a half occupancy of GroEL with MBP; (d) full occupancy. MBP is assumed to fold productively in a sequestered position under GroES, and to remain in the state of a folding intermediate when stably bound to GroEL.

we were able to exactly reproduce the refolding amplitudes of 70 % and 40 % for high and low GroES to GroEL stoichiometries, respectively (data not shown). It should be noted that at a high GroES to GroEL ratio, the observed amplitude is considerably more than 50 %. This cannot be explained by the existence of asymmetrical, bullet-shaped GroEL/ES particles only. Upon titration with GroES, the observed folding amplitude reaches a maximum of 80 % at a GroES to EL ratio of 8 to 1 (data not shown). This correlates with the GroES-dependence of the catalysis of the MBP Y283D folding rate under non-permissive conditions (Figure 2(c)) and most likely reflects both affinity and stoichiometry of GroES and GroEL under the conditions of the experiment.

The model depicted in Figure 7(c) and (d) explains the results observed in Figure 7(a) and (b). At a 1 to 1 ratio of MBP and GroEL, 50 % refolding is observed if GroES to EL is 1:1, as half of the MBP is bound stably to GroEL at high-affinity binding sites and therefore refolding is prevented. At higher GroES to EL ratios, maximally 100 % of MBP could fold if all binding sites were occupied by GroES. With fully occupied GroEL (i.e. MBP to GroEL 2:1) the situation in terms of percentage folding amplitude is the same, because at a 1 to 1 stoichiometry of GroES to GroEL there is not enough GroES available to productively fold all MBP in one cycle of ATP hydrolysis. However, the absolute amplitudes observed for MBP Y283D folding differ between full and half occupancy.

MBP folding observed in apyrase quench experiments results from folding *in cis* and free in solution

To answer the question of whether MBP Y283D folds in GroE complexes or free in solution, we performed two different experiments. In the first one, depicted in Figure 8(a), we added empty GroEL as a ''trap'' to an apyrase-treated MBP-GroE folding reaction. Wild-type GroEL acts as a trap in this experiment, as all ATP is hydrolyzed to AMP by apyrase, leaving the chaperone in the high-affinity state for substrate. Excess GroES cannot bind, due to the lack of nucleotide. Folding of MBP Y283D bound to GroEL was initiated by addition of ATP. After ten seconds, the ATPase cycle was stopped by the addition of apyrase. The dotted line in Figure 8(a) represents the folding amplitude observed under these conditions. When the MBP folding reaction was supplemented with excess GroEL after addition of apyrase but before the complete folding amplitude is achieved, MBP refolding was blocked, indicating binding of MBP intermediates to empty GroEL (continuous line). Thus, MBP Y283D is not committed to fold to the native state after release from GroEL.

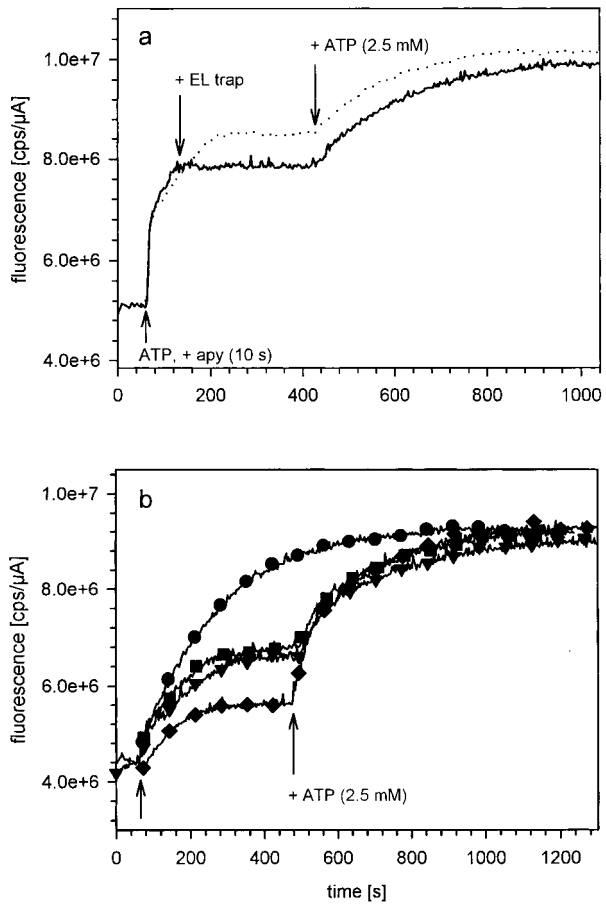

Figure 8. Analysis of the folding state of MBP released from GroEL. MBP Y283D (50 nM) was bound to GroEL (25 nM) under standard buffer conditions in the presence of GroES (200 nM). After 60 seconds, ATP (200 µM) was added to initiate refolding, and ten units of apyrase were added to quench the ATPase ten seconds later. After 450 seconds, ATP (2.5 mM) was added to release and initiate refolding of MBP stably bound to GroEL (second arrow). (a) No ''trap'' was added after apyrase quench (dotted line). At 100 seconds after initiation of refolding, GroEL (50 nM) was added as a trap for non-committed MBP free in solution (continuous line). (b) (●) GroE-catalyzed folding of MBP, addition of ATP is marked by the first arrow. (■) Apyrase was added ten seconds after addition of ATP (first arrow). (▼) Apyrase was added ten seconds before addition of ATP (first arrow). (◆) ATP was added at time zero, apyrase after ten seconds, and refolding was started by addition of MBP 50 seconds later (marked by the first arrow). This trace starts at the first arrow as no MBP is present before.

Figure 8(b) shows an ''order of addition'' experiment. In addition to trap experiments, another possibility to analyze folding under the conditions of the apyrase quench experiment is to block binding sites on GroEL by GroES. To this end, we added MBP to preformed GroEL/ES complexes treated with apyrase (Figure 8(b), line with diamond symbols), in contrast to the standard experiment, where we started with MBP bound to GroEL and afterwards added ATP and apyrase (Figure 8(b), line with rectangles). The circles in Figure 8(b) represent folding in the presence of GroEL, GroES and ATP without addition of apyrase. There is almost no difference between the orders of addition MBP - ATP - apyrase (rectangles) and MBP - apyrase - ATP (triangles). This shows that binding of ATP to GroEL is considerably faster than ATP hydrolysis by apyrase, which takes less than three seconds. However, there is a significant difference between the folding of MBP bound to GroEL before the addition of apyrase (giving the possibility to form *cis* complexes) and the folding of MBP added later on, where the observed folding amplitude correlates with the blocking of substrate binding sites by GroES. Taken together, the two experiments show that both folding in the GroE cavity and folding free in solution occur during catalyzed folding of MBP Y283D.

Electron microscopy and image processing reveal the presence of a large percentage of football particles under refolding conditions

The GroES dependence of catalysis of MBP Y283D refolding as well as the folding amplitudes observed after apyrase quench suggested that symmetrical particles with GroES located on both sides of the GroEL cylinder are involved in the catalysis of folding. Although the existence of football-shaped particles in complex with MBP had been shown before (Sparrer *et al.*, 1997), it was not clear which complexes might be populated under the specific conditions used here. Therefore, we performed electron microscopic studies under the standard conditions used in this study. Using saturating concentrations of GroES, a high percentage (58 %) of symmetrical, football-shaped GroE

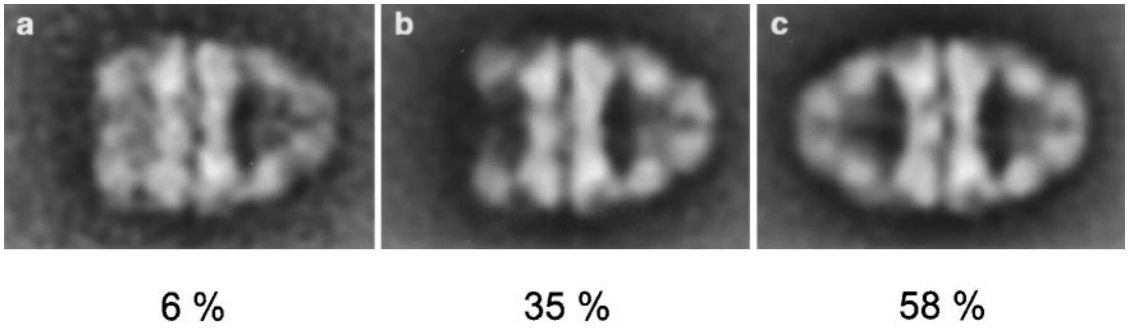

Figure 9. Side-views of GroEL-GroES complexes under standard MBP Y283D refolding conditions analyzed by electron microscopy and image analysis. In contrast to Sparrer *et al*. (1997), particles were generated under more physiological magnesium concentrations (5 mM compared to 40 mM) and using ATP instead of the ATP analogon AMP-PNP. GroEL and GroES concentrations were 25 nM and 200 nM, respectively. Three major structural classes were observed in the sample (810 particles). (a) Bullet with the *trans* ring in the closed state (no nucleotide bound, 6 %). (b) Bullet with the *trans* ring in the open state (nucleotide bound, 36 %). (c) Football with GroES and nucleotide bound on both sides of the GroEL cylinder (58 %).

particles was observed (Figure 9(c)). In contrast to the results presented by Sparrer *et al*. (1997), most of the asymmetic, bullet-shaped particles (35 %) exhibited an open conformation of GroEL on the ring opposed to GroES, indicating nucleotide binding in the second ring, which is a prerequisite for binding a second GroES molecule (Figure 9(b)). In all, 6 % of the particles appeared as bullets with a closed conformation in the *trans* ring of GroEL (Figure 9(c)). Only about 1 % of all particles observed had no GroES bound at all. This makes a model where GroE switches *via* empty GroEL particles between bullet-shaped complexes less likely than one with football-particles as an intermediate step (compare e.g. Sigler *et al*., 1998).

Discussion

A model for the GroE-mediated folding of MBP Y283D

The kinetic experiments together with electron microscopy allow us to present a detailed model for the interaction of GroEL with non-native proteins (Figure 10).

For simplicity, only the situation for a 1:1 stoichiometry of substrate and protein is considered (compare Figure 7(c) and (d)). The starting point is a high-affinity GroEL particle with MBP stably bound in the absence of nucleotide. The upper part of Figure 10 represents the situation for a 1:1 stoichiometry of GroES to GroEL and the lower half shows the scenario for higher GroES to GroEL ratios. In cases (1) and (2), GroES is assumed to associate on the same side as the substrate forming productive *cis* complexes. (1) With one cycle of ATP binding and hydrolysis, all MBP will stay in the GroE cavity and a folding amplitude of up to 100 % will be observed. (2) If ATP bound also on the *trans* side, substrate, GroES and nucleotide would be ejected from the *cis* side in a second turnover (Todd *et al*., 1994) leaving half of the GroEL binding sites as high affinity sites for substrate. The folding amplitude in this case could vary between 0 and 100 % depending on the commitment for folding to the native state after one interaction with GroE. For MBP, our experiments show that the commitment for folding is very low, suggesting a small folding amplitude in case (2). For simplicity, GroEL is presented in the low-affinity state for substrate as soon as nucleotide has bound, although the conformational switch from high to low affinity is not instantaneous (Jackson *et al*., 1993; Sparrer & Buchner, 1997). If GroES binds on the side opposite to the substrate, one turnover does not result in productive folding, as all substrate remains bound to GroEL (3). A second round of nucleotide binding and hydrolysis (4) will exactly repeat the situation observed in case (2) with the difference that this time the ejected substrate had not been sequestered in the GroEL/ES cavity before. As binding of GroES to GroEL has the same probability independent of whether substrate is bound (Weissman *et al*., 1995), one has to combine cases (1) and (3) to achieve 50 % refolding with one ATP turnover, whereas in the combination of cases (2) and (4) for two turnovers the observed refolding amplitude depends very much on the folding state MBP has already reached when it is released into solution after one interaction with GroE. As the commitment for folding is low, an overall folding amplitude somewhat smaller than 50 % is expected for a GroES to GroEL ratio of 1:1 (cf. Figure 7).

In the lower part of Figure 10, the folding cycle is shown for a higher GroES to GroEL ratio where the formation of football-shaped GroE particles is possible (cf. Figure 9). For one turnover (5), a folding amplitude of 50 % is expected, as observed for a lower GroES to GroEL stoichiometry. If ATP was able to bind also to the second ring (6), which was shown to be the case under the conditions used (cf. Figure 9), a second GroES could associate, forming particles that have MBP enclosed in the cavity independent of whether the first GroES is associated *in cis* or *in trans* relative to the substrate. With

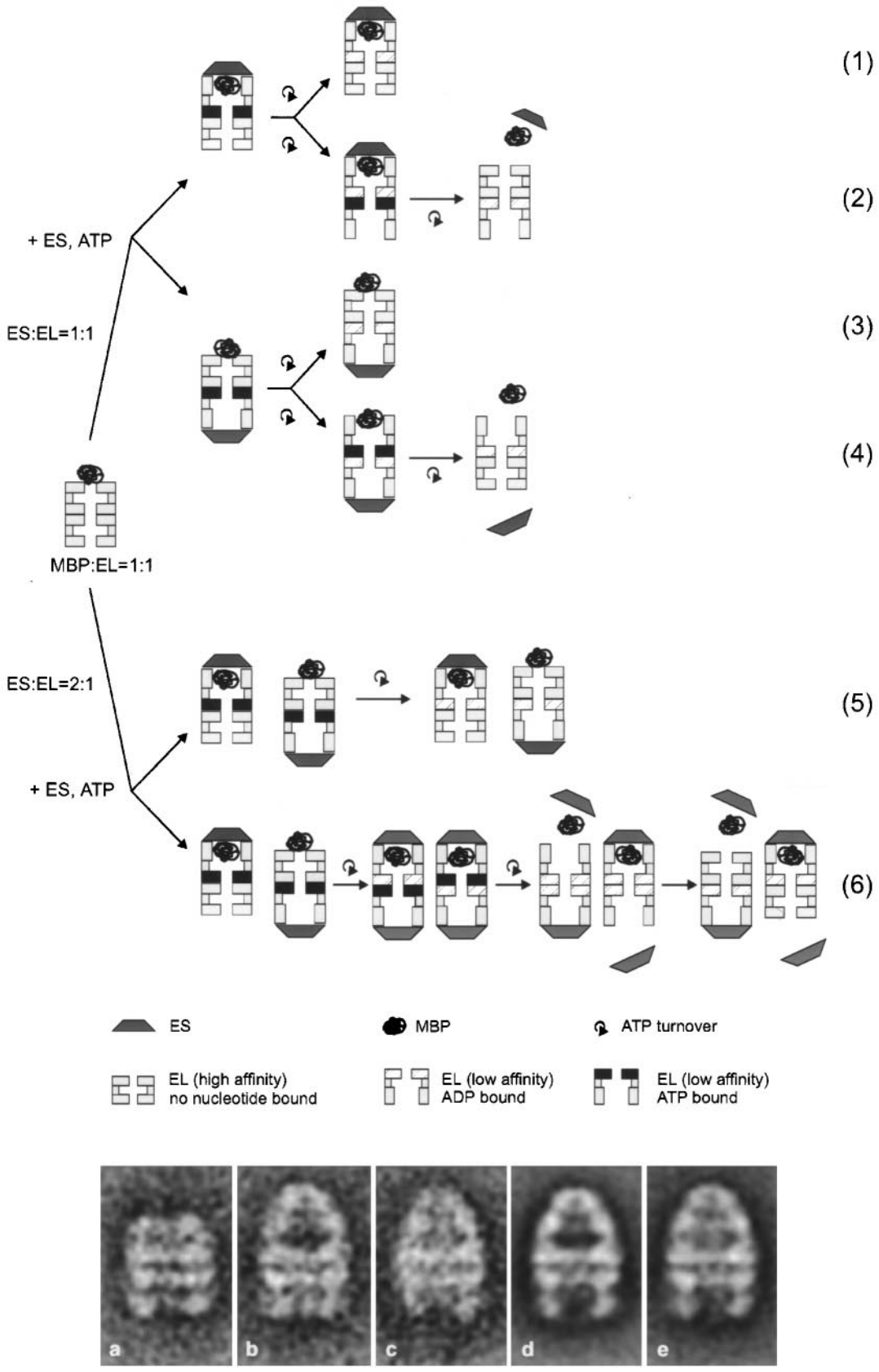

Figure 10. A schematic representation of GroE-mediated folding. MBP is bound to nucleotide-free, high-affinity GroEL. The upper branch depicts the situation for a 1:1 stoichiometry of GroES to GroEL, the lower branch for a higher GroES to GroEL ratio implicating the possibilty to form football-shaped GroE particles. After addition of GroES and ATP, asymmetric bullet-shaped particles with MBP either *in cis* or *in trans* to GroES are formed in all cases. After one ATP turnover (cases (1) and (3)), bullets persist, with 50 % MBP sequestered under GroES and 50 %

the second turnover, half of the substrate molecules will be released from the GroE cavity while the other half will remain bound to an asymmetric GroES/EL particle. Case (6) seems to be the only situation where folding amplitudes higher than 50 %, which were observed at high GroES to GroEL ratios (Figure 7(b)), are predicted. Here, in contrast to the other cases, MBP always encounters a productive *cis*-folding situation, and a large number of potential substrate-binding sites remain occupied by GroES, reducing the rebinding probability for non-native MBP folding intermediates.

Digitally processed electron micrographs of samples after the apyrase quench are in accordance with the predictions of our model (depicted at the bottom of Figure 10). As predicted for the final conformation to exist after the apyrase quench, the majority of particles were bullets, since footballs are only a transient form dependent on the presence of nucleotide. Free GroEL (Figure 10(a)) was observed with a very low frequency (<10 %). Particles showing MBP inside the cavity (Figure 10(c) and (e)) and empty bullets (Figure 10(b) and (d)) occurred with about the same frequency. The *trans* ring of GroEL was observed in closed (nucleotide free; Figure 10(b) and (c)) as well as in open (nucleotide bound; Figure 10(d) and (e)) conformations, with the open form being more populated. Both conformations were predicted by our model: the closed form representing a final conformation after two turnovers, the open form being a transient one that is capable of binding a second GroES ring or has just released one GroES ring (cf. Figure 9).

It should be noted that an important aspect in this model is not to have two rounds of ATP hydrolysis, but ''two turnovers'', which requires only that ATP binds to the second GroEL ring after hydrolysis in the first ring.

The functional significance of football-shaped symmetrical GroE particles

Although the existence of football-shaped particles with GroES bound to both sides of the GroEL cylinder was shown several times (Azem *et al*., 1994; Llorca *et al*., 1994, 1997; Schmidt *et al*., 1994c; Corrales & Fersht, 1996; Behlke *et al*., 1997; Sparrer *et al*., 1997), the functional significance of these chaperone complexes was questioned (Engel *et al*., 1995; Weissman *et al*., 1995, 1996; Mayhew *et al*., 1996). For MBP, efficient catalysis of folding by GroE was highly dependent on the ratio of GroES to GroEL (Sparrer *et al*., 1997; Figure 2(c) in this work). In addition, the GroES-dependence of the observed folding amplitude after apyrase quench strongly argues for the participation of football-shaped particles. The existence of only asymmetric particles would not allow folding amplitudes higher than 50 % (cf. Figures 7 and 10). Although football particles appear symmetrical with respect to GroES binding, they are predicted to exhibit an asymmetrical nucleotide composition, as only ATP hydrolysis in the *cis* ring of the bullet particle primes the *trans* GroEL ring to bind nucleotide, which is a prerequisite for binding a second GroES (Burston *et al*., 1995; Gorovits *et al*., 1997; Rye *et al*., 1997; Kad *et al*., 1998). The electron micrographs show a large population of bullet-shaped particles with the *trans* ring in an open conformation (Figures 9(b) and 10(c) and (e)). These species have nucleotide bound in both GroEL rings and most likely represent snapshots of species before binding a second GroES or directly after release of one GroES from a football particle. One argument against the significance of football particles was the observation of negative cooperativity of the two GroEL rings with respect to nucleotide binding (Burston *et al*., 1995; Yifrach & Horovitz, 1995, 1996). Higher temperatures, more closely resembling *in vivo* and especially stress conditions, however, seem to decrease the negative cooperativity between the two GroEL rings (Llorca *et al*., 1998) making symmetrical particles much more likely under the conditions used in this study. In addition, Sparrer & Buchner (1997) showed that the slow rate of substrate release from the *trans* position kinetically favors substrate encapsulation upon binding of a second GroES. Football-shaped GroE particles are not obligatory to perform the essential steps of the GroE folding cycle but they seem to be required under near-physiological conditions for efficient *trans* to *cis* conversion and the efficient use of ATP hydrolysis.

bound to the *trans* side of GroEL. Assuming two turnovers without the possibility for a second GroES to bind (cases (2) and (4)) all MBP is set free, and half of the binding sites on GroEL are left in a nucleotide-free form, immediately allowing rebinding of MBP not committed to fold to the native state. A higher GroES to GroEL ratio gives the same result as a 1:1 ratio if the binding of a second ATP is not allowed (case (5)). In the case of two ATP-binding cycles at high GroES to GroEL ratios (6), a second GroES can bind to GroEL, resulting in football particles as transient intermediates. After a second round of ATP hydrolysis, this scenario also produces bullet-shaped particles as the final conformation (first in the open, later on in the closed conformation) with the difference that all MBP that is set free into solution comes from a position formerly sequestered underneath GroES. This represents the scenario where the observed folding amplitude reaches values higher than 50 % (compare Figure 7). Lower part: main structural classes of particles observed in electron microscopic pictures of an apyrase quench experiment (high GroES to GroEL ratio, apyrase added after 30 seconds; sample corresponds to case (6)). (a) Free GroEL (closed form); (b) empty bullet (closed form); (c) bullet with MBP bound (closed form); (d) empty bullet (open form); (e) bullet with MBP bound (open form).

Folding in a sequestered position under GroES *versus* an iterative annealing mechanism

Whether folding occurs in the cavity of the GroEL/ES-complex or in solution after release from the chaperone is a controversial issue (Todd *et al*., 1996; Martin & Hartl, 1997). An important difference between the two models is whether the protein is released from GroE in a native form or given the chance to partition between folding to the native state and rebinding to GroEL as a non-native intermediate. For both models, shortly summarized as ''encapsulation'' *versus* ''iterative annealing'' mechanisms, respectively, examples can be found in the literature. Multiple cycles of binding and release were described for Rubisco, ornithine transcarbamylase, mitochondrial malate dehydrogenase and rhodanese (Todd *et al*., 1994, 1996; Weissman *et al*., 1994; Ranson *et al*., 1995; Smith & Fisher, 1995; Taguchi & Yoshida, 1995; Burston *et al*., 1996). On the other hand, productive folding to the native state after one *cis* interaction with GroEL/ES was also reported for ornithine transcarbamylase, mitochondrial malate dehydrogenase, rhodanese, dihydrofolate reductase and green fluorescent protein (Ranson *et al*., 1995; Weissman *et al*., 1995, 1996; Burston *et al*., 1996; Mayhew *et al*., 1996; Makino *et al*., 1997). Especially multimeric substrate proteins seem to need more than one interaction with GroE before they are able to associate to their native oligomeric state. MBP is an example where folding in *cis* is possible in principle, when the timer for release is artificially set to ''infinity'' either by using SR1 or by depleting nucleotide in the wild-type GroE system. Under normal conditions, however, where GroES and substrate are ejected several times per minute, the released MBP Y283D exhibits a high tendency to rebind to the chaperone and therefore needs several cycles of ATP hydrolysis before the native state is reached. This may result from the slow folding rate of MBP Y283D and the fact that the misfolding trap in the MBP folding pathway affects relatively late, already structured intermediates (see below).

Acceleration of folding under restrictive conditions

In most of the studies performed under conditions restrictive for protein folding, the presence of the complete GroE system increases the yield of native protein. In some cases, the apparent rate of refolding was also increased. This was partly attributed to the reversal of early steps on the aggregation pathway (Peralta *et al*., 1994; Todd *et al*., 1994; Ranson *et al*., 1995). For the slow-folding mutant MBP Y283D used in this study, GroEL/ES in the presence of ATP increases the apparent rate of refolding without altering the refolding yield, because spontaneous refolding is reversible to higher than 90 % under non-permissive conditions (Sparrer *et al*., 1997). In addition to high temperature as a restrictive condition, we found that high ionic strength decelerates MBP Y283D folding (Figure 1). In the native structure, residue 283 is located in the so-called N-domain of MBP, a region very sensitive for mutations affecting the folding and stability of the protein (Chun *et al*., 1993; Betton *et al*., 1996). These mutations are found in different elements of secondary structure and seem to be context-sensitive, as mutations in structurally similar positions in the C-domain have less dramatic effects (Raffy *et al*., 1998). The formation of a supersecondary structural element in the N-domain was considered a rate-limiting step in the folding pathway of MBP, as many mutations in this part of the molecule slow folding considerably (Chun *et al*., 1993). It is therefore reasonable to assume that an intermediate in the folding pathway for MBP is prone to misfolding. The examination of the environment of the tyrosine residue at position 283 in the structure of MBP (Sharff *et al*., 1992) suggests a plausible explanation for the salt dependence of the mutant. In wild-type MBP, the hydroxyl group of tyrosine 283 forms a hydrogen bond to aspartate 30. The interaction is missing when tyrosine 283 is exchanged for the smaller side-chain of aspartate. This may contribute to the destabilization of the mutant. Aspartate 283 could eventually form hydrogen bonds to asparagine 283 or threonine 286. An increase of the ionic strength weakens hydrogen bonds and thus destabilizes folding intermediates under high salt conditions. It is not clear how GroEL/ES is able to render MBP folding independent of the ionic strength, it should however be noted that in addition to hydrophobic interactions between GroE and substrate, electrostatic interactions have been reported to be important (Zahn & Plückthun, 1994; Hoshino *et al*., 1996; Lin & Eisenstein, 1996; Coyle *et al*., 1997; Perrett *et al*., 1997). Rubisco is the only example of GroE substrates the folding of which is known to be dependent of ionic strength, and GroE might play a role in lowering energy barriers on its folding pathway (Todd *et al*., 1996). In the case of MBP, the complete GroE system increased the efficiency of refolding more strongly than just changing the buffer conditions.

Acceleration of substrate refolding in the presence of chaperones was explained by a model of a ''rugged energy landscape'' the polypeptide chain faces on its refolding pathway (Todd *et al*., 1996). Folding is slowed when the protein gets stuck in local energy minima corresponding to ''folding traps''. To give the polypeptide chain the possibility to escape misfolding traps, folding *via* the iterative annealing mechanism seems optimal, as the protein has the chance to partition between productive folding and misfolding after every interaction with GroE (Todd *et al*., 1996; Frieden & Clark, 1997). Whether the GroE system assists refolding of the misfolded species *via* a kinetic mechanism (smoothing the energy landscape) or a thermodynamic control (changing the energy land-

scape) is not clear and hardly accessible experimentally. Some evidence for both mechanisms is available (Ranson *et al*., 1995, 1997; Walter *et al*., 1996; Persson *et al*., 1997; unpublished results).

Conclusions

Taken together, the experiments using MBP Y283D as a substrate for GroE improve our knowledge of the chaperone mechanism. Several steps in the reaction cycle of GroE (Sparrer *et al*., 1997) were confirmed. Furthermore, our data strengthen the importance of football-shaped particles for efficient catalysis of folding. In addition, we found out that under physiological conditions where the ''timer'' function of the GroE ATPase ejects substrate and GroES several times per minute, MBP Y283D requires several cycles of binding and release, as the commitment to fold to the native state is very low for MBP. Thus, even under catalyzed folding conditions, folding of MBP Y283D takes several times longer than the sequestration time within the GroE cavity. When the cycle time of GroE is artificially prolonged, MBP is able to fold and become native in a sequestered position in the GroE cavity. Although the degree of acceleration achieved in a single interaction with GroE cannot be determined precisely, in principle, accelerated folding can take place within GroE. In the case of MBP Y283D, we could show that GroE overcomes the restrictive effect of high ionic strength as well as high temperature on the apparent folding rate. As GroES and ATP are essential to catalyze folding under these conditions, MBP escapes misfolding traps in an ATP-dependent mechanism involving symmetric and asymmetric GroE particles.

Materials and Methods

Purification of proteins

GroEL and GroES were purified from the *E. coli* strain JM 109 TZ 136 bearing the multicopy plasmid DH(pOF 39 (Fayet *et al*., 1989) essentially as described (Schmidt *et al*., 1994b). SR1 was purified essentially like GroEL from the *E. coli* strain BL21(DE3) pLysS (Phillips *et al*., 1984) transformed with the plasmid pTrc99a (Amann *et al*., 1988). The protein concentrations were determined spectrophotometrically using the following extinction coefficients: ε_{276} 1456 M^{-1} cm^{-1}, ε_{276} 11,287 M^{-1} cm^{-1}, ε_{276} 11,032 M^{-1} cm^{-1} for GroES, GroEL and SR1 monomers, respectively. The extinction coefficients were determined individually for each preparation based on amino acid sequences and the results of the tryptophan titration. Fluorescence titrations were carried out to check for tryptophan-containing proteins in the GroE preparations (Pajot, 1976). On average 0.1 to 0.2 tryptophan residues per monomer could be detected. The absorption spectra for GroEL and SR1 were corrected for light-scattering of the solutions due to the large particle size of the chaperones.

Wild-type MBP and the mutant MBP Y283D were purified from the *E. coli* strains HB1045 and HB1204, respectively (Chun *et al*., 1993). The purification essentially followed the protocol described by Sparrer *et al*. (1996) with the exception that the Q-Sepharose column was substituted by a hydroxyapatite column (Bio-Scale CHT2-I, BioRad) as the first purification step. MBP was eluted with a linear gradient from 200 mM to 500 mM potassium phosphate buffer at pH 7.0. Protein concentrations were determined spectrophotometrically using molar extinction coefficients of ε_{280} 64,720 M^{-1} cm^{-1} and ε_{280} 63,440 M^{-1} cm^{-1} for wild-type and mutant MBP, respectively.

GroE proteins were stored in concentrated solutions at −70 °C in 50 mM Tris-HCl (pH 8.0) and MBP proteins in 20 mM Tris-HCl (pH 7.6).

Un/refolding

MBP was denatured in 6 M urea, 20 mM Tris-HCl (pH 7.6) for at least one hour at room temperature. Refolding was initiated by a rapid 1:200 dilution step into refolding buffer, leading to a residual urea concentration of 30 mM. Unless otherwise stated, the standard refolding buffer conditions were 20 mM Tris-HCl (pH 7.2 at 40 °C), 5 mM $MgCl_2$, 200 mM KCl, 200 μM ATP.

Fluorescence measurements

Folding assays of MBP were performed in a thermostatted cuvette (2 ml sample volume) under constant stirring in a Spex FluoroMax 2 spectrofluorimeter. Tryptophan fluorescence of MBP was excited at 295 nm and refolding was monitored by an increase of the fluorescence signal at the emission maximum of native MBP (345 nm). At the standard MBP concentration of 50 nM, the excitation and emission bandwidths were 2.1 nm and 10.6 nm, respectively. To guarantee long-term stability of the signal, all fluorescence kinetics were measured in the reference mode, where the light beam is split into two parts, one going through the sample chamber, the second being a reference for lamp intensity. Therefore, the fluorescence signal is given as cps (counts per second, detected by the photon counting sample detector) divided by μA (current signal provided by the reference detector).

Maltose-binding to native MBP was measured by recording fluorescence emission spectra (excitation at 295 nm) before and after addition of maltose to a final concentration of 5 mM. The resulting spectra were corrected for the change in volume caused by the addition of the maltose solution. Maltose-binding was indicated by a red-shift of the MBP fluorescence emission and by small decrease in the signal amplitude (Miller *et al*., 1983).

All fluorescence data were corrected for buffer fluorescence and the small fluorescence background resulting from GroE.

Size-exclusion HPLC

Complexes between MBP and GroE proteins were analyzed by HPLC size-exclusion chromatography followed by ''on-line'' detection of MBP fluorescence (excitation at 295 nm, emission at 345 nm) using a Tosohaas TSK4000 PW analytical column (flow-rate 0.75 ml/minute) and a Jasco FP-920 fluorescence detector. Samples were injected onto the column (100 μl sample volume) under refolding conditions with respect to protein concentrations and buffer conditions. Column and running buffer were thermostatted in a water bath. Peak areas

were integrated using the Borwin chromatography software (Jasco).

Nucleotide analysis

ATP hydrolysis and the purity of commercially available nucleotides were analyzed by reversed-phase HPLC. Nucleotides were separated with a Hypersil-ODS column (Bischoff) using 100 mM potassium phosphate (pH 7.3), 25 mM tetrabutylammonium bromide, 18% methanol as running buffer at a flow-rate of 2 ml/minute. Samples were prepared under the buffer and protein concentrations used for refolding assays. ATP hydrolysis was quenched by addition of 2 µl of 1 M $HClO_4$ to 20 µl aliquots. After one minute on ice, 28 µl of 2 M potassium acetate was added, the sample was centrifuged for two minutes at 18,000 ***g*** and 20 µl of the supernatant was applied to the column.

ATPase quench during refolding

EDTA or EGTA (50 µl of 400 mM stock solutions) or five or ten units of apyrase (Sigma, grade VI) were added to refolding samples of 2 ml directly into the stirred cuvette to quench hydrolysis of ATP by GroEL. The refolding traces were corrected for volume changes and signal changes due to inner filter effects occuring when solutions are added during the kinetics or due to background fluorescence from apyrase.

Electron microscopy and image analysis

GroEL (25 nM) and GroES (200 nM) were mixed under standard refolding conditions as indicated. Urea (30 mM) was present in all samples. For the apyrase quench experiment denatured MBP (50 nM) was added to GroEL and GroES, ATP was added (200 µM) after 60 seconds, and apyrase corresponding to ten units in refolding samples was added 5 or 30 seconds later. The samples were applied to carbon-coated grids and negatively stained with 3% (w/v) uranyl acetate. Electron micrographs were recorded at 120 kV at a magnification of 45,000× with a Philips CM12. For image processing, negatives were digitized (pixel size 0.3 nm) using an Eikonics 412 CCD camera. Particles were aligned, averaged, and subjected to a classification procedure based on eigenvector-eigenvalue analysis (Frank & van Heel, 1982; Hegerl, 1996; Saxton, 1996).

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