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GrpE Accelerates Nucleotide Exchange of the Molecular Chaperone DnaK with an Associative Displacement Mechanism†

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ABSTRACT: The ATP hydrolysis and protein-binding and release cycle of the molecular chaperone DnaK is regulated by the accessory proteins GrpE and DnaJ. Here we describe a study of the formation of complexes between the molecular chaperone DnaK, its nucleotide exchange factor GrpE, and the fluorescent ADP analog N_8-[4-[(*N*′-methylanthraniloyl)amino]butyl]-8-aminoadenosine 5′-diphosphate (MABA-ADP) by equilibrium and stopped flow kinetic experiments. The catalytic cycle of the GrpE-stimulated nucleotide exchange involves a ternary DnaK·GrpE·ADP complex as well as the binary DnaK·GrpE and DnaK·ADP complexes. The equilibrium data of the interaction of GrpE with DnaK·ADP and the nucleotide-free DnaK can be described by a simple equilibrium system where GrpE reduces the affinity of ADP for DnaK 200-fold. However, transient kinetic studies revealed that the functional cycle of GrpE in addition includes at least two distinct ternary DnaK·GrpE·ADP complexes. Our data indicate that the initial weak binding of GrpE to DnaK·ADP is followed by an isomerization of the ternary complex which leads to weakening of nucleotide binding and finally to its rapid dissociation. The maximal stimulation for nucleotide exchange brought about by GrpE was found to be 5000-fold. We propose that this kinetically observed isomerization represents a structural change (opening) of the nucleotide binding pocket of DnaK that allows for fast nucleotide exchange.

The functions of molecular chaperones in cells include the facilitation of folding of proteins, assistance in the correct assembly or disassembly of oligomeric protein complexes (Ellis & Hemmingsen, 1989; McKay, 1993; Ellis, 1994; Frydman & Hartl, 1996; Hartl, 1996), and participation in protein transport across membranes (Pfanner et al., 1994; Stuart et al., 1994; Wickner, 1994). The mechanism currently considered to mainly contribute to the assistance of protein folding is the binding and release of unfolded or partially folded proteins (Hubbard & Sander, 1991; Schmid et al., 1994; Todd et al., 1994). This cycle of protein binding and release withdraws aggregation-prone protein species from free solution and therefore helps to prevent the bimolecular process of (normally irreversible) protein aggregation.

Some chaperones, for example the families of the Hsp70 and Hsp60 proteins, appear to use the chemical energy that becomes available upon hydrolysis of ATP to drive this binding and release cycle and possibly make it more efficient (McKay, 1993; Szabo et al., 1994; Hartl, 1996). This allows for control of the switch between high- and low-affinity states for substrate binding, since the state of the nucleotide (ATP versus ADP) determines the affinities and exchange rates of substrate proteins (Schmid et al., 1994; McCarty et al., 1995; Theyssen et al., 1996).

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The ATP-dependent Hsc70 system of the prokaryote *Escherichia coli* consists of three components, namely the principal member of the Hsp70 family, DnaK, which has a nucleotide and a peptide binding site, and the two cofactors or cohort proteins. These regulating accessory chaperones are the nucleotide exchange factor GrpE, with a molecular mass of 22 kDa, and the ATPase-stimulating protein DnaJ with a molecular mass of 41 kDa (Zylicz et al., 1987; Liberek et al., 1988).

The control of such a nucleotide hydrolysis cycle is a very general principle of many regulatory proteins, prominent examples being GTPases involved in regulation and signal transduction. DnaJ is known to stimulate the ATPase rate of DnaK, which is very low under unstimulated conditions, and GrpE acts as a nucleotide exchange factor which increases the nucleotide exchange rates dramatically for the DnaK chaperone system.

The understanding of the kinetic parameters of the interactions of GrpE and DnaJ are crucial for the functional and mechanistic understanding of the ATP-driven substrate binding and release cycle of DnaK. Here we describe the kinetic analysis of the interactions of the nucleotide exchange factor GrpE with its target ATPase DnaK. The use of a novel fluorescent nucleotide analog MABA-ADP[1] (Theyssen et al., 1996) allowed the direct measurement and definition of the dissociation constants and key rate constants of GrpE-mediated nucleotide exchange. The implications of these kinetic constants for the mechanism of nucleotide exchange and regulation of the overall catalytic cycle of DnaK are discussed.

MATERIALS AND METHODS

Materials. ADP (disodium salt) was purchased from Pharma Waldhof Mannheim. Tris (ultrapure) was purchased from Boehringer Mannheim. Bovine serum albumin (BSA) was purchased from Sigma. $MgCl_2 \cdot 6H_2O$ was purchased from Aldrich, and EDTA and KCl were purchased from Fluka.

Proteins and Nucleotides. Recombinant nucleotide-free DnaK and the fluorescent nucleotide analog MABA-ADP were prepared as described previously (Theyssen et al., 1996). Isolation of recombinant GrpE was as follows. The 22 kDa protein was purified at 4 °C by ion exchange chromatography on an EMD-DEAE column (MERCK, 2.6 × 60 cm) with a gradient of 0.025 to 0.5 M KCl in 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTE, 2 mM EDTA, 2 mM NaN_3, and 10% v/v glycerol (buffer A). After dialysis (twice) at 4 °C against 10 mM KP_i (pH 7.5), 0.5 mM EDTA, and 10% v/v glycerol, the pooled fractions (SDS−PAGE) were applied to a hydroxylapatite column (MERCK, 37 mL) and eluted with a gradient of 0.01 to 0.5 M potassium phosphate buffer (pH 7.0). This was followed by a purification step by using a Superdex 200 gel filtration column (Pharmacia, 320 mL) with buffer A and 200 mM KCl at 2 mL/min. The protein was then applied to a Resource Q ion exchange column (Pharmacia, 6 mL) and eluted with a gradient of 0.025 to 0.3 M KCl in buffer A. After analysis by SDS−PAGE, the peak fractions containing proteins with a molecular mass of 22 kDa were pooled and again dialyzed twice at 4 °C against the storage buffer [20 mM Tris/HCl (pH 7.5), 25 mM KCl, 1 mM $MgCl_2$, and 2 mM DTE]. The proteins could be stored at a concentration of >15 mg/mL at −80 °C for over 6 months in the storage buffer without appreciable loss of activity.

Fluorescence Measurements. Fluorescence measurements were performed with a SLM "Smart" 8000 Photon-Counting Spectrofluorimeter. The excitation wavelength was 360 nm, and the emmission wavelength was 440 nm. Stopped flow measurements were performed with a Hi-Tech Scientific SF-61 single mixing apparatus. Unless stated otherwise, the buffer was 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTE, and 100 mM KCl (standard buffer), and the temperature of the cuvette was held at 25 °C using a water bath. Dissociation constants in simple binary systems were determined with a quadratic equation as described (Reinstein et al., 1990).

RESULTS

Equilibrium Studies of the DnaK·GrpE·ADP System. The characterization of interactions of DnaK with GrpE and ADP in a quantitative and time-resolved way requires a spectroscopic signal. Addition of GrpE to DnaK did not cause any observable change in the intrinsic tryptophan signal of DnaK. However, we could make use of the fluorescence signal of the novel nucleotide analog N_8-[4-[(*N*′-methylanthraniloyl)-amino]butyl]-8-aminoadenosine 5′-diphosphate (MABA-ADP) as a spectroscopic probe. This ADP analog was shown to be comparable to ADP in all kinetic and thermodynamic parameters tested so far (Theyssen et al., 1996) and is thus an ideal model nucleotide for a spectroscopic probe. The fluorescence of 1 μM MABA-ADP increases by approximately 100% upon addition of 1 μM DnaK (Figure 1A). The following titration of the so-formed DnaK·MABA-ADP complex with GrpE decreases the MABA-ADP fluorescence signal since GrpE causes nucleotide release, as shown below. It is important to note that after volume correction the final fluorescence signal does not reach the initial signal of DnaK-free MABA-ADP (indicated by the horizontal line). However, the addition of excess unlabeled ADP (100 μM) results in reversion to this initial signal. This is indicative of the formation of a ternary DnaK·GrpE·MABA-ADP complex at high GrpE concentrations, meaning that there is residual affinity of MABA-ADP for the DnaK·GrpE complex. The formation of a ternary complex of DnaK, GrpE, and a spin-labeled nucleotide analog was observed previously by ESR spectroscopy (Neuhofen et al., 1996).

In order to access the individual dissociation constants that characterize the interactions of GrpE with DnaK but also with the DnaK·MABA-ADP complex, we performed titration series such as that described above (Figure 1B) at different initial conditions. The data were analyzed with a global fit procedure that uses the information of all data sets simultaneously and calculates the expected fluorescence signal according to the equilibrium system shown in Scheme 1. In this scheme, K is DnaK, A is MABA-ADP, and E is GrpE. The dissociation constants for this system are as follows:

$$K_d^{equ}1 = \frac{[K][A]}{[K\cdot A]};\ K_d^{equ}2 = \frac{[K][E]}{[K\cdot E]};\ K_d^{equ}3 = \frac{[K\cdot A][E]}{[K\cdot E\cdot A]};$$

$$K_d^{equ}4 = \frac{[K\cdot E][A]}{[K\cdot E\cdot A]}$$

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; Tris, tris-(hydroxymethyl)aminomethane; DTE, dithioerythritol; MABA-ADP, N_8-[4-[(*N*′-methylanthraniloyl)amino]butyl]-8-aminoadenosine 5′-diphosphate; K, DnaK; E, GrpE; A, MABA-ADP.

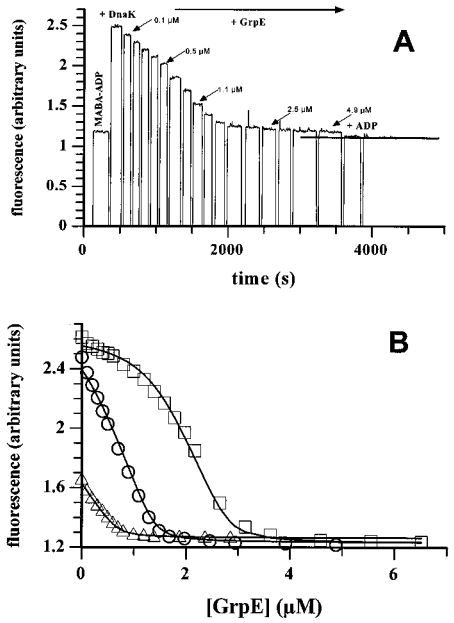
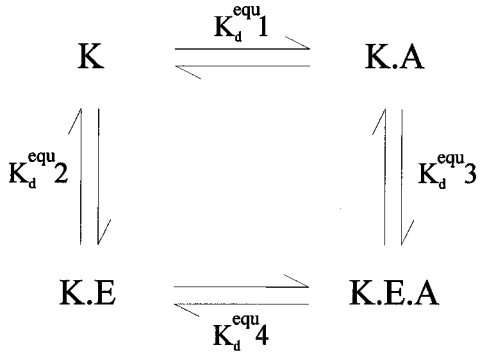


FIGURE 1: Titration of the DnaK·MABA-ADP complex with the nucleotide exchange factor GrpE. (A) The fluorescence signal of the nucleotide analog MABA-ADP (1 μM) increased upon addition of DnaK (1 μM). Addition of GrpE (up to 5 μM) to this preformed DnaK·MABA-ADP complex and subsequent release of the nucleotide then decreased the signal. The initial fluorescence of the free nucleotide could only be reached after addition of excess unlabeled ADP (100 μM) which is indicative of the formation of a ternary DnaK·GrpE·MABA-ADP complex. The experimental conditions were as follows. The signal was monitored constantly in a fluorimeter over the whole equilibrium titration. After each step, the reaction mixture was allowed to reach equilibrium as can be seen by the constant fluorescence signal in 100 mM KCl, 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTE, and 2 mM EDTA at 25 °C. The excitation wavelength was 340 nm, and the emission wavelength was 440 nm. The values shown in this figure are not volume corrected. (B) Equilibrium titrations of preformed DnaK·MABA-ADP complexes at different initial concentrations: (□) 2 μM DnaK and 1 μM MABA-ADP, (○) 1 μM DnaK and 1 μM MABA-ADP, and (△) 0.5 μM DnaK and 2 μM MABA-ADP. After volume correction, the data were analyzed with a global fit procedure (see text) that uses the information of all three data sets simultaneously to give $K_d^{equ}2 = 0.001 \pm 0.0003$ μM and $K_d^{equ}3 = 0.22 \pm 0.1$ μM (see Scheme 1). The known $K_d^{equ}1$ (0.09 μM) was held constant. All experiments were performed as described in part A. The fluorescence intensity of 2 μM free MABA-ADP was scaled to the signal obtained with 1 μM free MABA-ADP.

Scheme 1



K_d^{equ} indicates that these constants were obtained with equilibrium titration measurements as opposed to being obtained with time-resloved stopped flow measurements. This distinction was necessary since some of these dissociation constants are composed of two individual steps as shown below. The dissociation constant for the interaction of MABA-ADP with DnaK was determined previously ($K_d^{equ}1 = 0.09$ μM; Theyssen et al. (1996), and $K_d^{equ}4$ is determined by $K_d^{equ}4 = (K_d^{equ}1K_d^{equ}3)/K_d^{equ}2$. The complete equilibrium system was solved numerically with the Newton Raphson algorithm (Press et al., 1989) with the assumption that K·A and K·E·A give the same fluorescence yield for bound MABA-ADP. The dissociation constants that described the set of data best according to a nonlinear least-squares optimization were as follows: $K_d^{equ}2 = 0.001 \pm 0.0003$ μM and $K_d^{equ}3 = 0.22 \pm 0.1$ μM. The known $K_d^{equ}1$ (0.09 μM) was held constant, and $K_d^{equ}4$ was calculated to be 20 μM (see also Table 1).

Table 1: Kinetic and Dissociation Constants for the Interaction of DnaK, MABA-ADP, and GrpE Determined by Transient Kinetics and Equilibrium Experiments[a]

	K_d	rate constant	k_+	k_- (s^{-1})
$K_d1 = K_d^{equ}1$	0.09 μM[b]	1	0.36×10^6 M^{-1} s^{-1}[b]	0.022[b]
$K_d2K_d5 = K_d^{equ}2$	0.001 μM	2		
K_d3	20 μM	3		
$K_d3K_d7 = K_d^{equ}3$	0.22 μM	5		
$K_d6 \approx K_d^{equ}4$	20 μM	6	$\geq 50 \times 10^6$ M^{-1} s^{-1}	≥ 1000
K_d7	0.011	7	127 s^{-1}	1.4

[a] (See Schemes 1 and 2). Dissociation constants named (K_d^{equ}) were measured with fluorescence equilibrium titrations. K_d7 was derived from $K_d7 = K_d^{equ}3/K_d3$, and k_{-7} was derived from $k_{-7} = K_d7k_{+7}$. [b] Theyssen et al. (1996).

Titration of a DnaK·GrpE Complex to MABA-ADP. To evaluate the reliability of the constants obtained from the procedure above, the dissociation constant $K_d^{equ}4$ was measured independently. Different concentrations of a preformed DnaK·GrpE complex (with a 4-fold excess of GrpE) were added to MABA-ADP (data not shown). The increase of the fluorescence signal (observed with a fluorimeter) of the nucleotide analog under these conditions is mainly caused by the formation of a ternary DnaK·GrpE·MABA-ADP complex since the excess of GrpE and its high affinity favor the formation of this ternary complex versus the binary DnaK·MABA-ADP complex. It should be noted that because of the prohibitively high protein concentrations required it was not possible to reach saturation in this measurement. The data were analyzed with a quadratic equation (Reinstein et al., 1990) that describes a simple binding mechanism to give $K_d^{equ}4 = 30 \pm 3$ μM for the interaction of MABA-ADP with the DnaK·GrpE complex. This agrees reasonably well with the calculated $K_d^{equ}4$ of 20 μM as described above. In principle, measurements of the formation of the DnaK·GrpE complex would provide a direct determination of $K_d^{equ}2$. However, since the affinity of GrpE for DnaK is very high, these measurements would require radiolabeled proteins to gain the required sensitivity which are not available at the time being.

Stopped Flow Time-Resolved Studies of the DnaK·GrpE·ADP System. The important regulatory role of a nucleotide exchange factor is determined not only by how much it weakens the interaction of its target protein with nucleotide but also by the acceleration of nucleotide exchange it brings about. While these two effects are related, time-resolved measurements are needed to quantitate the kinetic properties. We therefore measured the nucleotide exchange rate in a

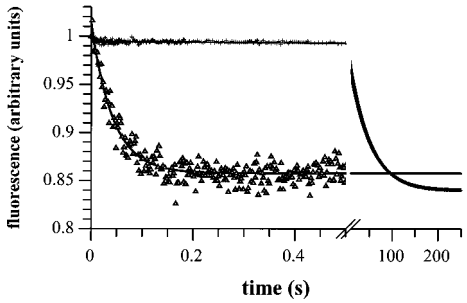


FIGURE 2: Time course of the displacement of MABA-ADP from a DnaK·MABA-ADP complex in the absence and presence of GrpE. In a stopped flow experiment, DnaK (0.5 μM) and MABA-ADP (2 μM) were mixed with 200 μM ADP (to make the dissociation of MABA-ADP irreversible) in the absence (+) or presence of GrpE (△, 5 μM). The data can be described by a single-exponential equation with a k_{off} = 0.023 s^{-1} (+) or 23 s^{-1} (△, 5 μM). The stimulation of nucleotide dissociation is thus 1000-fold at 5 μM GrpE. The signal for the dissociation was the change of the fluorescence of MABA-ADP upon dissociation from DnaK. Excitation was at 340 nm, and emmission was measured with a cutoff filter at 455 nm.

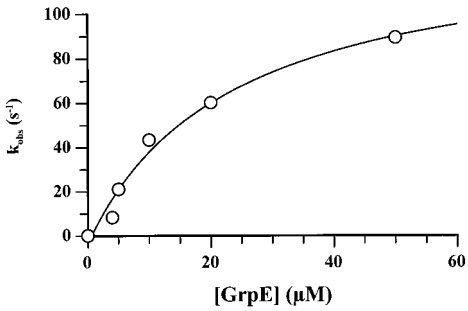
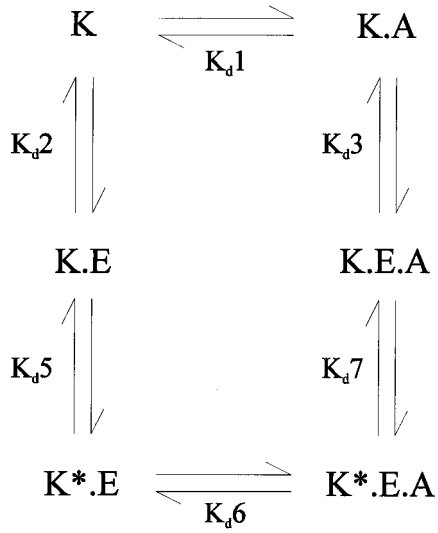


FIGURE 3: Dependence of the observed dissociation rate constant of MABA-ADP on GrpE concentration. Stopped flow experiments as described in Figure 2 were performed at various GrpE concentrations, and the resulting rate constant (k_{obs}) is plotted versus the GrpE concentration in the mixing chamber. The data can be described by a hyperbolic curve with a K_d of 20 ± 4 μM and an amplitude of 127 ± 9 s^{-1} for the maximal GrpE-stimulated nucleotide release. The maximal stimulation of GrpE-mediated nucleotide exchange is thus 5000-fold.

stopped flow experiment (Figure 2). A complex of DnaK and MABA-ADP was formed by mixing 0.5 μM DnaK and 2 μM MABA-ADP in syringe A of the instrument. This complex was then mixed with 200 μM ADP in the presence or absence of 5 μM GrpE. In both cases, the data represent the time dependence of the displacement of MABA-ADP by ADP and follow single-exponential curves with a k of 0.023 s^{-1} in the absence and a k of 23 s^{-1} in the presence of GrpE. This shows that the dissociation rate constant of MABA-ADP is accelerated 1000-fold in the presence of GrpE.

To determine the maximal acceleration brought about by GrpE, the observed dissociation rate constant was measured as a function of the GrpE concentration (Figure 3). A replot of the observed rate constant (k_{obs}) versus the GrpE concentration follows a hyperbolic curve with a K_d of 20 ± 4 μM and an amplitude of 127 ± 9 s^{-1}. This shows that the maximal stimulation of GrpE-induced nucleotide exchange is 5000-fold. The most straightforward interpretation of the hyperbolic form of this replot is that GrpE binds to the DnaK·MABA-ADP complex in a two-step binding mechanism (Bagshaw et al., 1974). The first step would be fast with a K_d3 of 20 μM (see also Scheme 2), followed by a

Scheme 2



conformational change with a k_{+7} of 127 s^{-1} to give K*·E·A, the DnaK species that allows for fast nucleotide exchange.

In Scheme 2, the following abbreviations were used: K = DnaK, E = GrpE, A = MABA-ADP, and K* = the DnaK isomer that allows for fast nucleotide exchange. All K_d values are defined in the direction of K*·E·A dissociating to the individual components; the rate constants are accordingly defined by the subscript + for all steps leading to K*·E·A. The rate constant leading from K·E·A to K*·E·A is defined as k_{+7}, for example.

Although it was not shown directly, we think it is very likely that the K·E complex exists in two different conformations (K·E and K*·E) also in the absence of nucleotide. In principle, the rate-limiting step with 127 s^{-1} could also be assigned to the dissociation of nucleotide (k_{-6} in Scheme 2). However, in this case, the calculated association rate constant for the binding of MABA-ADP to the DnaK·GrpE complex would be 127 s^{-1}/20 μM (≈6 × 10^6 M^{-1} s^{-1}) which appears to be at least 1 order of magnitude too slow for the diffusion-controlled binding of nucleotide to a presumably open binding pocket. To clarify this point, we performed two sets of stopped flow experiments. First, the accessibility of the nucleotide binding site of the DnaK·GrpE complex was probed by adding MABA-ADP (2−15 μM) to this preformed complex (2 μM DnaK and 4 μM GrpE, data not shown). Even at low MABA-ADP concentrations (but high enough to allow for complex formation), the binding was too fast to be measured in the dead time of the stopped flow experiment of 2 ms. This sets a lower limit for k_{-6} of 1000 s^{-1}.

The second set of stopped flow experiments measured the dissociation of MABA-ADP (with 1 mM ADP as a chase) from a preformed DnaK·MABA-ADP·GrpE complex at different concentrations of GrpE (2 μM DnaK, 2 μM MABA-ADP, and 2−15 μM GrpE). Again, the dissociation was too fast to be observed by stopped flow. What could be observed was a time trace with a minor amplitude (decreasing to 4% of the expected full signal for MABA-ADP dissociation at high GrpE concentrations) with a rate constant of 150 s^{-1}. This observation is consistent with fast dissociation of MABA-ADP from K*·E·A with k_{-6} of >1000 s^{-1} and subsequent isomerization of a small amount (determined by K_d7) of K·E·A to K*·E·A with 130−150 s^{-1} followed again

by fast dissociation of MABA-ADP. It is not consistent with k_{-6} being the rate-limiting step with 130 s^{-1} since in this case the amplitude of the observed signal should increase with increasing concentrations of GrpE. The experiments described above show that the nucleotide binding site of DnaK is highly accessible in the presence of GrpE. The equilibrium and rate constants determined by these measurements are summarized in Table 1.

Interestingly, no fast phase oflp;&-1q the fluorescence change was observed at high GrpE concentrations, indicating that the formation of the K·E·A complex from K·A and E is spectroscopically silent. This suggests that the initial binding of GrpE does not change the environment of MABA-ADP bound to DnaK such that the fluorescent group would be affected. This is in support of a mechanism where GrpE affects nucleotide binding of DnaK indirectly as described below.

Efficiency of Chaperone Action Dependent on the GrpE/DnaK Ratio. It was shown before that, in addition to DnaK and ATP, the accessory proteins DnaJ and GrpE are necessary to allow efficient refolding of firefly luciferase after denaturation (Schröder et al., 1993; Buchberger et al., 1994). To determine the optimal ratio of GrpE to DnaJ, we performed the luciferase refolding assay with constant concentrations of DnaK and DnaJ and various GrpE concentrations (Figure 4A). A plot of the yields of refolding versus the GrpE/DnaJ ratio (Figure 4B) shows that the refolding efficiency reaches a maximum at about 2.5 GrpE/DnaJ. Interestingly, the yield of refolded luciferase drops at a ratio of 5 to 1 or higher which we interpret as clear evidence that the balance of ATPase stimulation and acceleration of nucleotide exchange by DnaJ and GrpE, respectively, is of crucial importance for the functionality of DnaK-assisted protein folding.

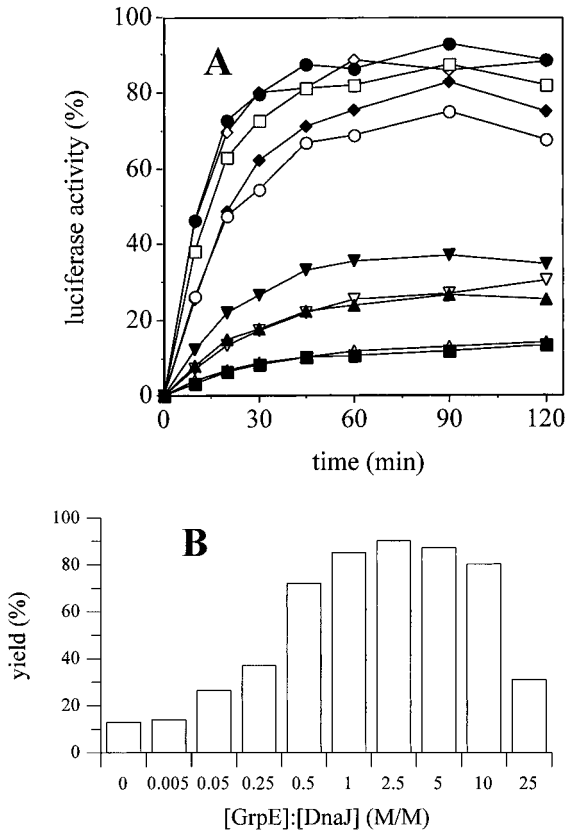


FIGURE 4: Determination of the GrpE dependence of luciferase refolding. (A) Time course of luciferase refolding reaction containing varying amounts of GrpE. Chaperone-dependent refolding of guanidinium hydrochloride-denatured firefly luciferase was performed at 30 °C as described (Buchberger et al., 1994). The final concentrations of luciferase (80 nM), DnaJ (160 nM), and DnaK (800 nM) were held constant, and GrpE was present at the following molar ratios relative to DnaJ: GrpE/DnaJ = 0 (■), 0.005 (△), 0.05 (▲), 0.25 (▼), 0.5 (○), 1 (□), 2.5 (●), 5 (◇), 10 (◆), and 25 (▽). Luciferase activity was normalized to the activity of a nondenatured control sample. Each data point is the average of two parallel reactions, with the standard deviation error bars being typically smaller than the plot symbols. (B) Luciferase refolding yield plotted as a function of the GrpE/DnaJ ratio. The refolding yield was estimated from the plateau phase of the curves in part A.

DISCUSSION

Interactions of DnaK, GrpE, and Nucleotide. The dissociation constant of 1 nM for the DnaK−GrpE interaction ($K_d^{equ}2$ in Scheme 1) agrees well with the observation that a stable complex of DnaK and GrpE can be observed by native gel electrophoresis (Buchberger et al., 1994) and ultracentrifugation (Schönfeld et al., 1995). The affinity of MABA-ADP with a $K_d^{equ}1$ of 0.09 μM (Theyssen et al., 1996) is reduced 200-fold in the presence of GrpE to a $K_d^{equ}4$ of 20 μM. Detailed thermodynamic balance requires that in turn the affinity of GrpE is also decreased 200-fold in the presence of excess nucleotide. This effect was actually observed when GrpE was first purified, since it was recognized that the addition of ATP disrupts the formerly stable DnaK·GrpE complex (Zylicz et al., 1987). In summary, the binding of GrpE and the binding of ADP to DnaK are mutually destabilized in the ternary complex by a factor of 200 but are not completely exclusive (classically competitive) so that a ternary complex can be observed under appropriate concentration conditions.

Mechanism of Nucleotide Exchange. The formation of the ternary DnaK·ADP·GrpE complex as indicated by equilibrium studies is a requirement for accelerated nucleotide dissociation. If GrpE would merely compete with ADP for the same binding site, the exchange mechanism would be dissociative with the consequence that the apparent nucleotide affinity is reduced, but no acceleration is brought about. Instead, the formation of a ternary complex shows that the displacement mechanism is associative. This means that the binding sites of GrpE and ADP are either distinct or only partially overlapping. The mechanism by which GrpE accelerates nucleotide exchange is presumably the following. GrpE rapidly forms a weak association complex with the binary DnaK·ADP complex with a K_d3 of 20 μM. This complex then undergoes a conformational change at a rate of 127 s^{-1} (k_{+7}) that opens the nucleotide binding site, the function of GrpE being to lower the energy barrier for this switch in nucleotide binding site accessibility.

It has been shown that a highly conserved surface loop (amino acids 28−33) of the ATPase domain of DnaK is involved in GrpE binding (Buchberger et al., 1994). This loop is remote from the nucleotide binding site, consistent with the proposal that GrpE does not directly interact with the active site. It was proposed recently that GrpE lowers the affinity of ATP by disturbing the interaction of Mg^{2+} with the nucleotide and strengthening the interaction of Mg^{2+} with DnaK (Skowyra & Wickner, 1995). However, the 200-fold reduction of the affinity of ATP caused by GrpE in the absence of Mg^{2+} (Skowyra & Wickner, 1995) matches the 200-fold reduction of affinity of ADP we measured in the

presence of Mg^{2+}, making it rather unlikely that Mg^{2+} plays a major part in the acceleration of nucleotide exchange. Furthermore, these conclusions were derived from rather indirect steady state ATPase measurements.

It was postulated that one of the interactions responsible for stabilization of a closed form of the nucleotide binding pocket is salt bridges formed between subdomains from the nucleotide binding cleft (Buchberger et al., 1994). Mutations of these residues should disturb the equilibrium between the closed and open forms and thus result in faster dissociation rate constants of the nucleotide. The availability of MABA-ADP as a spectroscopic probe will now allow the direct measurement of the dissociation rate constants of these mutants so that we could test this hypothesis. We propose that the 5000-fold acceleration of nucleotide exchange brought about by GrpE under saturating conditions is caused by GrpE-induced opening of the nucleotide binding pocket. In principle, this opening could be caused by disruption of interactions that favor the closed form or interactions of GrpE and DnaK that occur preferentially with the open form.

Implications for the Chaperone Cycle. Two constants that are crucial for the understanding of the regulated DnaK ATPase and peptide release cycle are the degrees of stimulation of nucleotide exchange by GrpE and of ATPase activity by DnaJ. The maximal stimulation of nucleotide exchange by GrpE is 5000-fold, as reported in this study. DnaJ was reported to stimulate the ATPase reaction at least 200-fold in single-turnover experiments (Karzai & McMacken, 1996), and we were able to measure in an independent investigation a stimulation of 1200-fold in single-turnover quench flow experiments (D. Klostermeier, S. H. Thrall, T. Laufen, B. Bukau, and J. Reinstein, unpublished results). In comparison, the stimulation of nucleotide exchange for the Ras-related protein Ran by the nucleotide exchange factor RCC1 is (2×10^4)-fold (Klebe et al., 1995b), and GTPase stimulation of Ran by RanGAP1 is 10^5-fold (Klebe et al., 1995a). In the force-generating actomyosin system, the stimulation of ADP release from myosin by actin is 400-fold which gives a dissociation rate constant of 400 s^{-1} which has to be compared to 100 s^{-1} for the intrinsic ATPase activity of myosin (Lymn & Taylor, 1971; Geeves, 1989). It appears that the two counteracting activities of NTP hydrolysis and NDP release are balanced such that their maximal stimulation factors are comparable in magnitude. The drop of the yield of luciferase refolding when the GrpE/DnaJ ratio exceeds 6/1 (Figure 4B) indicates the importance of this balance. Interestingly, the ratio of GrpE to DnaJ was also found to be 6 in *E. coli* after heat shock (42 °C) treatment (Neidhardt & VanBogelen, 1987).

In summary, the hydrolysis cycle of regulatory or force-generating proteins can be characterized by (mainly two) functional states that are determined by the state of nucleotide occupancy. In the case of DnaK or Hsc70, the ATP state would represent the on-state with fast peptide or protein substrate exchange rates. The ADP state on the other hand represents the off-state that locks the peptide and is virtually inaccessible due to the very slow rate constant of substrate association (Schmid et al., 1994; Ha & McKay, 1995; McCarty et al., 1995; Gamer et al., 1996; Takeda & McKay, 1996; Theyssen et al., 1996). GrpE facilitates the switch to the ATP state by allowing for fast ADP dissociation with 5000-fold stimulation and thus ATP rebinding. DnaJ on the other hand accelerates the switch to the ADP state by accelerating the rate of hydrolysis of DnaK 1200-fold.

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