THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 22, Issue of May 30, pp. 14080–14086, 1997
Printed in U.S.A.

How GroES Regulates Binding of Nonnative Protein to GroEL*

(Received for publication, December 23, 1996, and in revised form, March 17, 1997)

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At present, it is still enigmatic how the reaction cycle by which the *Escherichia coli* GroE chaperones mediate protein folding in the cell is coordinated with respect to the sequential order of binding and release of GroES, nucleotide, and nonnative protein. It is generally assumed that the asymmetric GroEL·GroES complex is the acceptor state for substrate protein. Nevertheless, this species is poorly understood in its binding characteristics for nucleotide and nonnative protein. We show here that this species has a high affinity binding site for nonnative protein. In addition to this, binding of nucleotide to one GroEL ring is strongly favored by GroES binding to the other ring. However, the slow rate of release of substrate protein from the unproductive trans-position kinetically favors the binding of a second GroES, thereby forming a symmetric $GroEL_{14}·(GroES_7)_2$ complex and simultaneously ensuring that substrate protein is sequestered in a position underneath GroES. Our results demonstrate that the intrinsic binding characteristics of the trans-bullet complex determine the sequence of events during the reaction cycle.

Molecular chaperonins such as GroE play an essential role in assisting non-native polypeptides to reach a biologically active conformation under nonpermissive conditions (1–3). They mediate protein folding in the cytoplasm of prokaryotes and in mitochondria and chloroplasts of eukaryotes. Their most prominent tasks seem to be the folding of newly synthesized proteins (4) and preventing irreversible aggregation of nonnative protein (5, 6).

In vitro, their protein folding activity can be demonstrated by the increase in the reactivation yield of numerous structurally different proteins after denaturation with chaotropes (7). Chaperone-mediated refolding of denatured protein allows to overcome highly unfavorable or even completely nonpermissive folding conditions. To achieve this function in the case of GroE, a chaperone oligomer of extraordinary structural complexity is required. Seven GroEL monomers are associated to a ring, forming a binding cavity for nonnative protein at the inner side of the central channel (8). Two such seven-membered rings are associated back to back building a cylindrical complex. The crystal structure of GroEL shows a tripartite structure of the GroEL monomer (9). The interactions of the equatorial domains provide the contacts between the individual subunits within a ring as well as the interface to the second ring of the complex. A small intermediate domain connects to the apical domain, which forms the ends of the cylinder. These ends are of outstanding functional importance since both binding of nonnative protein and of the co-chaperone GroES are located in this region.

The reversible interaction of GroEL with nonnative substrate protein via hydrophobic interactions (10–12) is controlled by the binding and hydrolysis of ATP. Loading of the nucleotide-binding sites of GroEL with ATP switches them to a low affinity state for denatured protein (12, 13). The importance of ATP-induced conformational changes for the functional cycle has been demonstrated using a GroE mutant in which domain movements were blocked by a cystine bridge (14). If GroES is present, the ordered association of nucleotide and GroES leads to the formation of an asymmetric, bullet-shaped GroES·GroEL complex, the three-dimensional structure of which has been elucidated by cryo-electron microscopy and image reconstruction (15). Hydrolysis of ATP in one ring releases GroES and nucleotide from the other ring in reverse order (16, 17). The order of binding is also of crucial importance in GroE-mediated folding of substrate protein. Association of a folding intermediate and subsequent binding of GroES to the same ring sequesters the polypeptide in a position under GroES (cis-bullet complex) and is a prerequisite for productive folding (18).

At present, it is enigmatic how the binding of nonnative protein, GroES, and ATP and their dissociation from GroE are integrated in the folding cycle. To analyze the acceptor state for denatured substrate protein, we determined the ability of GroEL, as well as that of the asymmetric GroEL·GroES bullet complex, to bind nonnative protein.

As a nonnative protein substrate, we used a slow folding mutant of MBP[1] since the unfolding of this protein is completely reversible and it can be monitored directly by following changes in tryptophan fluorescence. This allows quantitative analysis of the folding kinetics. The interaction of MBP with the chaperone SecB has been investigated in detail (19) and, recently, MBP has been used to characterize the effects of nucleotides on the binding characteristics of GroEL (12). Folding of MBP can be completely suppressed in the presence of GroEL. The protein released from GroEL in the presence or absence of nucleotide folds with kinetics similar to those of the spontaneous folding reaction. Thus, the rate-limiting folding step is not changed by binding to GroEL. Structural rearrangements may, however, occur on GroEL in the case of kinetically trapped proteins (see "Discussion"). Efficient release of MBP requires the loading of GroEL with nucleotides, which converts the high affinity binding site(s) for proteins into the low affinity state. Here, we used the release of MBP bound to GroEL to determine how GroES bound to one ring of GroEL influences the binding properties of the opposite ring. A major advantage

* This work was supported by grants from the Deutsche Forschungsgemeinschaft; Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie; the German-Israeli Science Foundation; and the Fonds der chemischen Industrie (to J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MBP, maltose-binding protein; RBP, ribose-binding protein; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

of MBP as a model substrate in this context is that it allows the performance of competition experiments with the structurally closely related RBP and mutants thereof (20), and thus the determination of microscopic on and off rates of the interaction between MBP and GroE complexes.

We present a model of how GroES regulates the interaction of the trans-ring with nucleotide, nonnative protein, and GroES.

EXPERIMENTAL PROCEDURES

Purification of Proteins—GroEL and GroES were purified from the *Escherichia coli* strain JM 109 TZ 136 bearing the multicopy plasmid DHαpOF 39 (21) as described previously (22). The concentrations of both chaperones were determined spectrophotometrically by using the following extinction coefficients: $E_{280}^{0.1\%} = 0.2$ for GroEL (12) and $E_{276}^{0.1\%} = 0.142$ for GroES (22). To determine GroEL concentrations, the absorbance spectrum was corrected for light scattering of the solution due to the particle size of GroEL. Furthermore, the GroEL concentrations were confirmed by the results of the binding of MBP Y283D to GroEL showing two independent binding sites (12).

Mature MBP Y283D and RBP A27T were purified according to previously published protocols (12). The concentration of MBP Y283D and RBP A27T were determined spectrophotometrically using extinction coefficients of $E_{280}^{0.1\%} = 1.94$ and $E_{280}^{0.1\%} = 0.55$, respectively.[2] The molecular masses are 799,932 Da for $GroEL_{14}$, 72,709 Da for $GroES_7$, 40,659 Da for MBP Y283D, and 28,504 Da for RBP A27T.

Fluorescence Measurements—Folding assays of MBP Y283D were performed in a Spex FluoroMax spectrofluorimeter. Tryptophans were excited with an excitation wavelength of 295 nm, and emission was recorded at 344 nm. The bandwidths for excitation and emission were set to 2.5 and 8.5 nm, respectively. All experiments were performed in a thermostatted cuvette under constant stirring. The temperature for all experiments was set to 25 °C. Our standard folding conditions used throughout the experiments were 20 mM Tris/HCl, pH 7.6, 200 mM KCl, 5 mM $MgCl_2$.

MBP Y283D was denatured in 4 M guanidinium chloride, 50 mM Tris/HCl, pH 7.6, for at least 1 h at room temperature. Denatured MBP Y283D was diluted 200-fold in renaturation buffer, giving a final concentration of 25 nM. The residual concentration of guanidinium chloride was 20 mM. A slow folding mutant of RBP A27T (CP632) was denatured for at least 4 h at room temperature in 3.3 M urea. Stock solutions of denatured RBP A27T were 480 μM.

Competition experiments were performed as follows (*cf.* Ref. 12). Prior to addition of denatured MBP Y283D, the following GroEL or $GroEL_{14}{\cdot}GroES_7$ complexes were formed (see also "Results"). 1) The high affinity state of $GroEL_{14}$ was analyzed in the absence of nucleotide; 2) the low affinity state of $GroEL_{14}$ was formed at concentrations of 1 mM ATPγS and 5 mM ADP, respectively; 3) the high affinity site of the trans-ring of the $GroEL_{14}{\cdot}GroES_7$ complex was populated in the presence of 5 μM ATPγS or 10 μM ADP (25 nM GroEL and 80 nM GroES); and 4) the low affinity site of the trans-ring of the $GroEL_{14}{\cdot}GroES_7$ complex was created in the presence of 100 μM ATPγS and 200 μM ADP, respectively.

After forming these species, 25 nM denatured MBP Y283D was added to start the reaction. To avoid interference with the reassociation reaction, denatured RBP A27T was added as a competitor 30–60 s later. The final concentration of the competitor was 5 μM (200-fold excess over MBP Y283D), yielding a residual concentration of 30 mM urea. Binding of GroES to GroEL is not affected by the final concentration of denaturant, since the same number of binding sites for MBP can be determined both in its presence or absence (see "Results").

Since the amount of denatured RBP A27T is constantly reduced by spontaneous folding, additional denatured RBP A27T was added every 200 s.

Data Analysis—To calculate the concentrations of high and low affinity binding sites, numerical simulation of the data assuming a rebinding equilibrium was performed with the program KINSIM (24). The following mechanism file was used for the simulation of the titration of the binding sites with different nucleotides (Scheme 1).

$$1)\ MBP_{den.} + GroEL_{high\ aff.} \leftrightarrow MBP_{den.} \cdot GroEL_{high\ aff.}$$

$$2)\ MBP_{den.} + GroEL_{low\ aff.} \leftrightarrow MBP_{den.} \cdot GroEL_{low\ aff.}$$

$$3)\ MBP_{den.} \rightarrow MBP_{nat.}$$

SCHEME I

The different species in the mechanism are as follows. $MBP_{den.}$ is a MBP Y283D folding intermediate; $MBP_{nat.}$ is a native MBP Y283D molecule; $GroEL_{high\ aff.}$ is a high affinity site of GroEL, irrespective whether it refers to a GroEL double ring or an asymmetric bullet; $GroEL_{low\ aff.}$ is a low affinity site of GroEL, irrespective whether it refers to a GroEL double ring or an asymmetric bullet; $\leftrightarrow$ represents a binding-rebinding equilibrium; $\rightarrow$ represents an irreversible reaction.

This mechanism reflects that folding of denatured MBP Y283D is completely blocked after association with GroEL and that folding intermediates free in solution fold to the native state with the spontaneous folding rate. Furthermore, an MBP Y283D folding intermediate can associate to a low and a high affinity binding site, depending on whether the respective ring is titrated with nucleotide or not. For both the high and the low affinity GroEL binding sites, the microscopic association and dissociation rates were determined by competition experiments as described previously (12), and these parameters were included in the simulation. The competition experiments were performed for the GroEL double ring and the asymmetric bullet, respectively.

For the ADP-jump experiments, simulating the transition from the high affinity state to the low affinity state of the trans-ring in the asymmetric bullet, the following mechanism was used (Scheme II).

$$1)\ MBP_{den.} + GroEL_{high\ aff.} \leftrightarrow MBP_{den.} \cdot GroEL_{high\ aff.}$$

$$2)\ MBP_{den.} + GroEL_{low\ aff.} \leftrightarrow MBP_{den.} \cdot GroEL_{low\ aff.};$$
$$MBP_{den.} \cdot GroEL_{high\ aff.} \rightarrow GroEl_{low\ aff.} + MBP$$

$$3)\ MBP_{den.} \rightarrow MBP_{nat.}$$

SCHEME II

Since all MBP Y283D molecules were stably associated with the high affinity site of the trans-bullet, the concentration of the $MBP_{den.}{\cdot}GroEL_{high\ aff.}$ complex was set to 25 nM in the beginning of the reaction. Upon binding of ADP, this complex is converted to a low affinity complex and MBP Y283D folding intermediates formerly bound to the trans-ring are released. These folding intermediate may then fold to the native state with the spontaneous folding rate, or they may be rebound transiently by a low affinity complex, which is characterized by the on and off rates obtained from the competition experiments.

Analysis of the cooperativity in the transition of the GroEL binding sites from a high affinity state to a low affinity state was performed by fitting the relative percentage of low affinity states to the Hill equation: $x_0 = x_{max} \times K[S]^n/(1 + K[S]^n)$, where x_0 and x_{max} are the minimal and maximal percentages, [S] is the concentration of nucleotide, K is the apparent nucleotide binding constant, and n is the Hill coefficient.

RESULTS

The Affinity of a GroEL Ring to Substrate Protein Is Exclusively Controlled by the Binding of Nucleotide—The first step in GroE-mediated protein folding is the entry of nonnative protein in the reaction cycle. However, none of the potential acceptor states has been analyzed previously with respect to their protein binding properties. Therefore, we characterized both GroEL and the asymmetric bullet as potential acceptor states for binding nonnative protein using a mutant of maltose-binding protein, MBP Y283D, as a nonnative substrate. This protein directly reports the number of binding sites on GroEL, as well as the strength of the interaction (12). Association of MBP Y283D to a high affinity binding site of GroEL leads to the formation of a very tight complex (K_D 1.0×10^{-11} M), in which the folding of MBP Y283D is completely suppressed (12). For GroEL, binding of nucleotide to one ring is sufficient to convert its high affinity binding site for nonnative protein to a low affinity state. The conformational change induced by nucleotide binding leads to an increase in the dissociation equilibrium constant for nonnative protein by 3 orders of magnitude (12). Consequently, in the low affinity state complete folding of MBP Y283D is not inhibited by GroEL.

Binding of nucleotide to GroEL occurs in a cooperative manner involving one ring at a time (25). Therefore, concomitant changes in affinity can be directly tested by the binding assay for denatured MBP Y283D, which allows a quantitative description of this reaction, since all the microscopic rate con-

[2] L. Randall, personal communication.

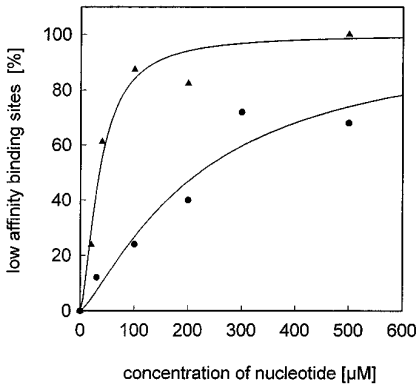

FIG. 1. **Hill plot for the transition of GroEL binding sites from the high affinity state to the low affinity state.** 12.5 nM GroEL, corresponding to 25 nM binding sites, was titrated with ATPγS (△) or ADP (●). 25 nM denatured MBP Y283D was added, and kinetic traces were recorded. The concentrations of high and low affinity sites was determined as described under "Experimental Procedures." The data points were fitted to the Hill equation to account for the cooperative binding of nucleotide to GroEL (*solid line*). The Hill coefficients were determined to be 1.6 and 1.2 for ATPγS and ADP, respectively.

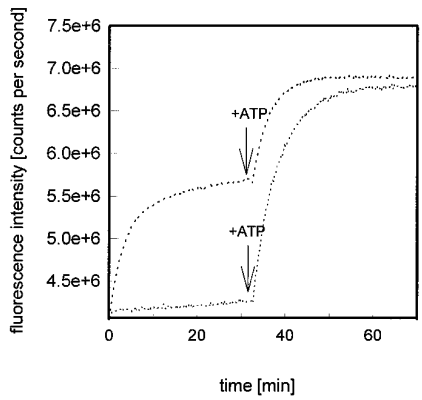

FIG. 2. **GroES forms a stable asymmetric complex with GroEL in the presence of ADP.** 25 nM MBP Y283D were denatured and then diluted into a solution containing GroEL (12.5 nM) (*lower curve*). In a second experiment, 25 nM denatured MBP Y283D was added to a solution containing 12.5 nM GroEL, 80 nM GroES, and 10 μM ADP (*upper curve*). ATP (1 mM) was added after folding of uncomplexed MBP Y283D was complete.

stants for association and dissociation of MBP Y283D and GroEL can be obtained by a competition assay (12). As expected, titration of GroEL with increasing amounts of nucleotide leads to a decrease of high affinity binding sites, which is reflected in a higher amount of folding of MBP Y283D. For GroEL alone, an increase in the concentration of ATPγS resulted in a cooperative transition of the substrate binding sites in both rings to the low affinity state with a midpoint at 40 μM ATPγS (Fig. 1). The Hill coefficient for the allosteric transition was 1.6. This is in good agreement with data by Gray and Fersht (26) and Yifrach and Horovitz (25). For ADP, the midpoint of the transition occurs at much higher nucleotide concentrations, reflecting its weak binding to GroEL. The midpoint of the transition is at 200 μM, and the two binding sites for substrate protein are completely shifted to the low affinity state at ADP concentrations above 2 mM. Similarly, conformational changes were reported for a pyrene-labeled GroEL upon binding of ADP (17, 27). Thus, independent methods monitoring conformational changes of GroEL confirm the validity of the transitions observed.

The Trans-ring in the Asymmetric GroEL·GroES Complex Is Regulated by GroES in the Cis-position—The co-chaperone GroES is an essential component for GroE-mediated protein folding under nonpermissive conditions (18, 28–30). In the presence of nucleotide and GroES, an asymmetric bullet-shaped GroEL·GroES particle is formed. The binding of GroES to GroEL is very tight, with a dissociation constant for this binary complex of 0.3–0.5 nM (27). Therefore, the asymmetric bullet should be present exclusively under our experimental conditions. This could be directly demonstrated in the MBP binding assay. Since two independent binding sites for MBP Y283D have been reported (12), addition of nucleotide and GroES to GroEL prior to substrate protein should result in a reduction of the available binding sites to one. As shown in Fig. 2 in the presence of low concentrations of nucleotide and excess GroES, indeed about only one half of the fluorescence amplitude can be detected (Fig. 2). The rate constant of this folding reaction is identical to the rate constant observed in the absence of chaperones. This indicates that half of the molecules did not interact with GroEL, because one binding site in the $GroES_7$·ADP_7·GroEL14 complex is blocked by GroES. The second half of the amplitude for the MBP Y283D folding reaction could be recovered by the addition of ATP (Fig. 2). This proved that half of the molecules were indeed trapped by the asymmetric bullet, with GroES and nonnative substrate protein associated to opposite ends of the cylinder (trans-bullet). Since folding of MBP Y283D could be suppressed completely in the presence of equimolar concentrations of the asymmetric bullet and MBP Y283D, we conclude that the trans-ring existed in a nucleotide-free, high affinity state for substrate protein. Competition experiments with denatured RBP A27T confirmed the tight binding of nonnative protein to the trans-ring. In the presence of an excess of denatured RBP A27T, about the same off rate of MBP Y283D folding intermediates was measured as for a high affinity binding site of GroEL alone (Table I). Since residual amounts of denaturant introduced in the folding assay may disturb the stability of the GroEL·GroES complex (31), we measured the number of binding sites in the presence of corresponding amounts of urea and found again one binding site per asymmetric complex (data not shown). We therefore conclude that GroES was indeed associated with GroEL during the competition assay.

From published data (*e.g.* Refs. 16 and 17), it is evident that, in the presence of ADP, GroEL and GroES form a stable complex in which GroES does not exchange during a time course of hours. The experiments described above show that nonnative protein is stably bound in the trans-position of the GroEL·GroES complex with an affinity comparable to that previously determined for GroEL (12). It is therefore possible to create a well defined static bullet complex loaded with substrate protein in trans as a starting point for experiments to analyze the regulation of the trans-ring by GroES. This is an important unresolved question for understanding the molecular mechanism of protein folding mediated by the GroE double ring complex.

First, we performed titration experiments with ADP and ATPγS. In these experiments, the influence of GroES bound to one GroEL ring on nucleotide binding to the opposite GroEL ring could be tested. Interestingly, we found that the affinity for both ATPγS and ADP to the trans-ring was about 1 order of magnitude higher in the asymmetric complex compared with GroEL alone (Fig. 3). For ATPγS, the trans-ring was converted to the low affinity state for protein between 5 μM and 30 μM, while for ADP the respective transition was between 20 μM and 100 μM. The Hill coefficients for nucleotide binding increased from 1.6 to 4.4 for ATPγS and from 1.2 to 3.7 for ADP, respectively.

In its low affinity state, the trans-bullet showed almost no affinity for denatured protein. The folding rates of MBP Y283D

TABLE I
Microscopic on and off rates for nonnative protein and GroEL or the asymmetric GroEL·GroES complex

The microscopic on and off rates were determined via a competition assay with RBP A27T (see "Experimental Procedures" and Ref. 12). The affinity of a GroE binding site for MBP Y283D folding intermediates is reflected in the ability of the respective GroE species to retard the folding of MBP Y283D compared to the spontaneous folding reaction. In the presence of the competitor protein, the off rate can be directly determined. The respective on rates were obtained by a kinetic simulation of the reaction.

Complex	On rate	Off rate	K_{diss}
	$M^{-1}\cdot s^{-1}$	s^{-1}	M
GroEL + ADP			
High affinity state	$1.0 \cdot 10^{8}$	$7.5 \cdot 10^{-4}$	$7.5 \cdot 10^{-12}$
Low affinity state	$3.0 \cdot 10^{5}$	$1.7 \cdot 10^{-3}$	$5.7 \cdot 10^{-9}$
GroEL + ATPγS			
High affinity state	$1.0 \cdot 10^{8}$	$7.5 \cdot 10^{-4}$	$7.5 \cdot 10^{-12}$
Low affinity state	$8.0 \cdot 10^{5}$	$1.0 \cdot 10^{-3}$	$1.3 \cdot 10^{-9}$
$GroES_7$·ADP·$GroEL_{14}$ complex			
High affinity state	$1.0 \cdot 10^{8}$	$9.7 \cdot 10^{-4}$	$9.7 \cdot 10^{-12}$
Low affinity state	$1.0 \cdot 10^{5}$	$2.8 \cdot 10^{-3}$	$2.8 \cdot 10^{-8}$
$GroES_7$·ATPγS_7·$GroEL_{14}$ complex			
High affinity state	$1.0 \cdot 10^{8}$	$5.0 \cdot 10^{-4}$	$5.0 \cdot 10^{-12}$
Low affinity state	$2.0 \cdot 10^{5}$	$2.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-8}$

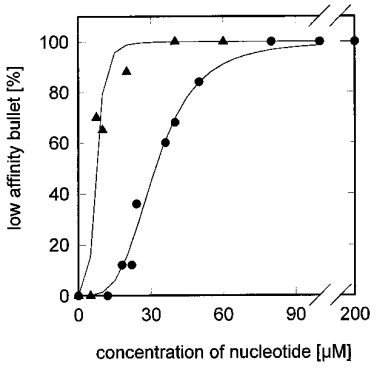

FIG. 3. **Hill plot for the titration of the trans-ring of the asymmetric GroEL·GroES complex.** 25 nM of the asymmetric bullet was preformed with 25 nM GroEL, 80 nM GroES, and increasing concentrations of ATPγS (▲) or ADP (●). 25 nM denatured MBP Y283D was added to this asymmetric bullet, and folding was recorded by monitoring changes in fluorescence. The data were fitted to the Hill equation (*solid lines*). The Hill coefficients were determined to be 4.4 and 3.7 for ATPγS and ADP, respectively.

in the presence of the low affinity bullet were almost identical to the spontaneous folding reaction. Surprisingly, in comparison to the low affinity sites of GroEL, the dissociation equilibrium constant was further increased by more than a factor of 5 (Table I). Thus, GroES bound in cis weakens the interaction of substrate bound to GroEL in the trans-ring.

From the data presented above, we conclude that the trans-ring of the asymmetric bullet exists in a low and high affinity state, depending on whether it is occupied with nucleotide or not. Interaction of GroES with one GroEL ring increases the affinity and cooperativity of nucleotide binding to the other ring considerably. This results in a shift for the transition from a high affinity state to the low affinity state to much lower nucleotide concentrations compared with GroEL. Thus, the notion of two distinct states determining the interaction with nonnative protein is also valid for the trans-ring, while the cis-ring is stably associated with GroES. In addition, GroES influences the trans-ring further by increasing the dissociation equilibrium constant for nonnative protein. The markedly enhanced binding of nucleotide to the trans-ring makes the formation of a symmetric GroE-species with respect to nucleotide binding more favorable. This is in contrast to the empty GroEL ring, where symmetric binding of nucleotide is strongly hindered by the negative cooperativity of the GroEL rings (25).

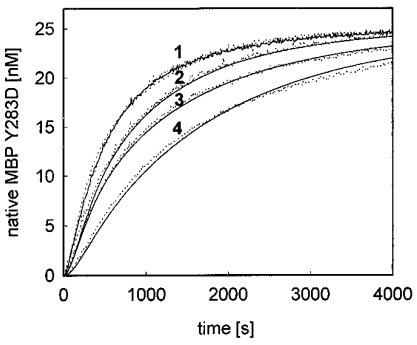

FIG. 4. **Folding kinetics of MBP Y283D after release from the trans-ring via an ADP-jump.** 25 nM of the asymmetric bullet with a high affinity state in the trans-ring was created by incubating 25 nM GroEL and 80 nM GroES in the presence of 20 μM ADP. Then the trans-ring was loaded with 25 nM MBP Y283D and the suppression of folding was controlled for 5 min. Subsequently, ADP was added to titrate the trans-ring to a low affinity state. 250 μM (*1*), 500 μM (*2*), 1 mM (*3*), and 2 mM ADP (*4*) were used, and the resulting folding reaction was monitored by fluorescence. The traces of the release reaction were simulated using the following mechanism: $GroES_7$·ADP_7·GroEL14·MBP $Y283D_{den.}$ + 7 ADP → $GroES_7$·ADP_7·GroEL14·ADP_7 + MBP $Y283D_{den.}$ (*solid line*); MBP $Y283D_{den.}$ → MBP $Y283D_{nat.}$ In all jump experiments, the complete fluorescence amplitude for MBP Y283D was recovered, as addition of ATP at the end of the reaction did not result in a further increase in fluorescence.

Bound Protein Is Released by the Transition of the Trans-ring from the Low to the High Affinity State—Next, we determined how the reaction cycle proceeds after formation of the asymmetric bullet with nonnative protein bound in trans. Therefore, in addition to studying the transition in binding affinity under equilibrium conditions, we tried to directly demonstrate the conformational switch by an ADP-jump experiment. To do this, we formed the asymmetric bullet in the presence of low concentrations of nucleotide and bound nonnative MBP Y283D to the high affinity site of the GroEL trans-ring. Under these conditions, folding was completely suppressed. Addition of ADP allowed us to titrate the trans-ring with ADP and to study the kinetics of the change in affinity.

Analysis of the kinetic trace of the reaction showed a lag phase indicating two consecutive steps (Fig. 4). We attributed the first step of this reaction to a slow conformational change in GroEL switching the initial $GroES_7$·ADP_7·$GroEL_{14}$·MBP Y283D complex to a $GroES_7$·ADP_{14}·$GroEL_{14}$ complex. Simultaneously with this conformational change, bound MBP Y283D is released and the trans-ring is now in a low affinity state. A simulation of the reaction based on our model showed very good agreement with the experimental data (Fig. 4).

To obtain the rate constants of the slow conformational change, we simulated the apparent reaction kinetics for the different concentrations of nucleotide. The rate constant of the conformational switch to the low affinity state, which is accompanied by the immediate release of protein, was strongly dependent on the final concentration of ADP. It changed by a factor of 10 from 8.4×10^{-4} s^{-1} to 1.0×10^{-2} s^{-1} over a concentration range of 250 μM ADP to 1 mM ADP (Fig. 5). Thus, the concentration of ADP necessary for inducing the conformational switch of the trans-ring of the GroEL·GroES complex in the presence of bound substrate protein is about 10 times higher than the one that promotes the formation of the low affinity state in equilibrium in the absence of nonnative protein. This shows that nucleotide and substrate protein bound to GroEL are indeed allosteric antagonists. Thus, substrate protein bound to GroEL stabilizes the high affinity state and slows down the conformational change to the low affinity state.

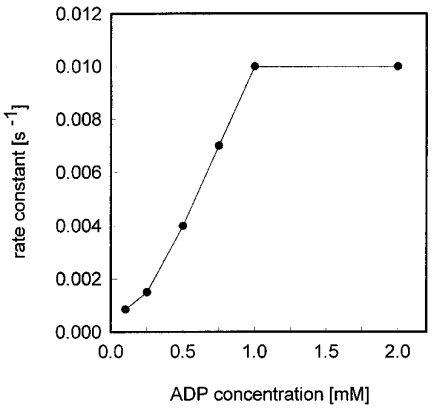

FIG. 5. **Rate constants for the slow conformational change leading to the release of MBP Y283D from GroEL.** The rate constants for the isomerization reaction of the trans-ring to the low affinity state, which represent the first step of the release mechanism, were plotted against the ADP concentration. The constants were obtained from a simulation; the kinetic traces are shown in Fig. 4.

DISCUSSION

The Affinity of a GroEL Ring to Substrate Protein Is Exclusively Controlled by the Binding of Nucleotide—GroE-mediated folding reactions are still enigmatic with respect to the precise sequence of the partial reactions occurring while nonnative protein is associated to GroEL. In particular, the role of GroES in the reaction cycle is poorly understood. Therefore, we set out to characterize the influence of GroES on the binding of nucleotide and nonnative proteins to GroEL (Fig. 6).

GroEL is able to bind nucleotide in a weakly cooperative manner. Binding of nucleotides induces a conformational change, as indicated by fluorescent changes of a pyrene dye attached to GroEL (27) and susceptibility to proteolytic cleavage (32). An important question in this context is whether the conformational change is correlated to an equivalent change in the binding affinity for nonnative protein. To test this, we used a quantitative binding assay, reporting the high and low affinity sites for nonnative protein in GroEL.

For the GroEL ring, our binding assay revealed that the weakly cooperative binding of ATPγS converts both binding sites of GroEL to a low affinity state. This conformational switch is complete at a concentration of about 100 μM ATPγS. For ADP, the transition is uncooperative. Very high concentrations (>2 mM ADP) are needed to titrate both GroEL rings to the low affinity state. Thus, binding of nucleotide indeed controls the change in affinity of GroEL to nonnative protein, which is directly monitored by our binding assay in equilibrium.

The Role of the Asymmetric Bullet as an Acceptor State for Nonnative Protein—The role of GroES and especially its ability to dramatically increase the yield of protein reactivation under nonpermissive conditions compared with GroEL alone has been most intriguing (29). Despite its important role in providing the lid for the GroEL cavity (18), forming a sequestered space for productive folding, GroES is also essential in coordinating the reaction cycle. Due to the high affinity of GroES for GroEL, the asymmetric bullet is the prevailing species if nucleotide is present in solution (27), and therefore it is most likely the acceptor state for denatured protein (18, 30). However, to be able to integrate this complex in the reaction cycle of GroE-mediated folding, it is most important to investigate the nucleotide and protein binding characteristics of the $GroEL_{14}$·$GroES_7$ complex.

In the presence of a GroE bullet with a high affinity trans-ring, folding of MBP Y283D is inhibited due to the binding of nonnative protein in the trans-position. Equimolar amounts of the asymmetric bullet to MBP Y283D are required for complete suppression of folding, since, compared with GroEL (12), only half of the binding sites were available for nonnative protein. The binding affinity of the trans-bullet complex for nonnative substrate protein and the stability of the interaction are the same as determined for high affinity binding sites of GroEL. Thus, if the trans-ring is not titrated with nucleotide, GroES bound to the opposite ring does not influence protein binding.

GroES Associated with GroEL Increases the Affinity for Nucleotide and the Dissociation Equilibrium Constant for Nonnative protein in the Trans-ring—A regulatory effect of GroES transmitted via GroEL subunits to the other GroEL ring has not been investigated or discussed previously. In the present models, the trans-ring cannot actively participate in the folding cycle, except for the initial binding of protein. Accordingly, in a next step GroES has to be released from the opposite ring and rebind to the ring associated with nonnative protein to create again a cis-conformation, which seems to be required to actively control folding and release in combination with ATP hydrolysis in the opposite ring.

However, binding of GroES to one GroEL ring showed two marked effects on the other ring. First, it enhances association of nucleotide to the trans-ring about 10-fold and promotes the complete transition of the high affinity state to the low affinity state in a very narrow range of nucleotide concentration. In contrast, in the empty GroEL ring, association of 14 nucleotides is hindered by strong negative cooperativity between the rings (25). Second, binding of GroES to the opposite ring strongly influences the dissociation of bound substrate protein from the trans-ring (13). In the presence of this low affinity bullet, MBP Y283D can fold with almost no retardation in the apparent folding kinetic compared with spontaneous folding.

Both results have significant implications for the coordination of the GroE reaction cycle. Importantly, binding of nucleotide to the substrate-loaded trans-ring is promoted by GroES bound to the opposite ring. Therefore, this ring will exist in an ATP-liganded state for a comparatively long time, because the unimolecular conformational change to the low affinity state is slow, especially with substrate protein stabilizing the trans-ring in the high affinity state. The formation of a symmetrical football-shaped $GroEL_{14}$·$(GroES_7)_2$ particle is therefore most likely, since association of ATP is sufficient for binding of GroES (33) and binding is very fast compared with the time scale of the conformational switch (17). Furthermore, GroES binds to GroEL with the same probability, irrespective, whether it is associated with nonnative protein or not (18). As demonstrated above, the symmetric $GroEL_{14}$·$(GroES_7)_2$ complex is not only a kinetically reasonable intermediate due to the regulating function of the first GroES bound, it would also significantly economize the folding cycle, since a productive cis complex is formed with high efficiency. In addition to this, the conversion of the trans-ring to the low affinity state is accompanied by protein release.

Implications for a GroE Reaction Cycle—The data presented above provide insight into important aspects of the GroE reaction cycle. The first question to be answered is how nonnative substrate protein is included in the GroE-mediated folding cycle. A reaction cycle may start with a symmetric $GroEL_{14}$·$(GroES_7)_2$ complex, which is highly accumulated in the presence of ATP (34, 35). After hydrolysis and release of nucleotide in one GroEL ring, an asymmetric bullet with a high affinity state in the trans-ring is created, since nucleotide and GroES are exchanged in this reaction (16). Substrate protein may therefore bind to this ring. In the presence of high concentrations of ATP, the trans-ring will be immediately loaded with ATP. However, although the binding of ATP is very fast, the

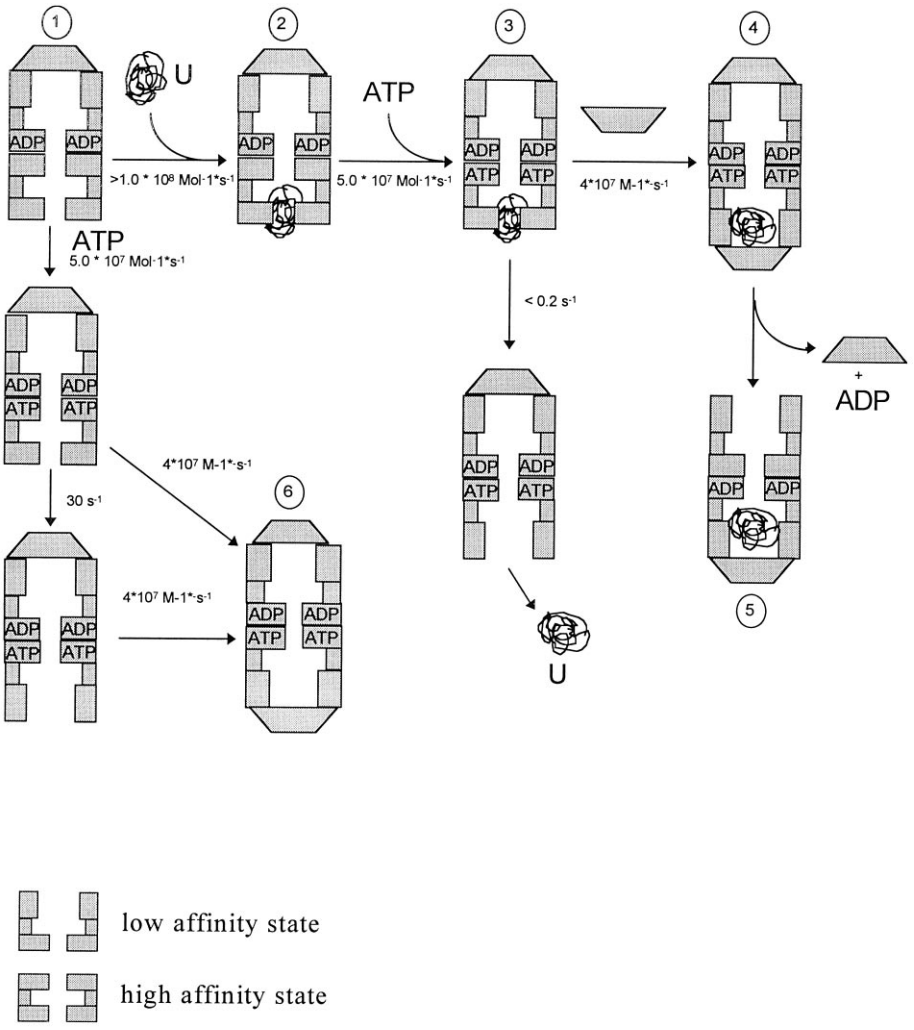

FIG. 6. **Scheme for a reaction cycle of GroEL.** An asymmetric GroEL·GroES complex with a high affinity binding site (*1*) binds both nonnative protein (*2*) and ATP (*3*) in a diffusion-controlled reaction. However, since the conformational switch to the low affinity state is a relatively slow isomerization reaction, substrate binding under cellular concentrations occurs with a high probability. After ATP is bound to the GroEL ring, GroES may associate to the GroEL ring, forming either a productive football (*4*) or an empty $GroEL_{14}$·$(GroES_7)_2$ complex (*6*). Hydrolysis of ATP in this ring discharges GroES and ADP of the opposite ring and simultaneously restores an asymmetric GroEL·GroES complex that has a high affinity binding site (*5*).

isomerization to the low affinity state is a relatively slow conformational change occurring at a rate of 30 s^{-1} in the presence of 2 mM ATP (27). With cellular GroEL concentrations of 2.5 μM (3), the bimolecular association of denatured protein and GroEL occurring at a diffusion-controlled rate of 1.0×10^8 M × s^{-1} (12), is about 10 times faster than the unimolecular isomerization reaction leading to the low affinity state. Furthermore, the rate of bimolecular association reactions may very well be underestimated, since the collision frequency of molecules is probably much higher due to excluded solvent effects caused by the high concentration of macromolecules in the cytoplasm (36). Therefore, the binding of denatured protein is likely to occur before the ring switches to a low affinity state. However, once protein is bound to GroEL, the ability to undergo a conformational change is dramatically slowed down, since the high affinity state is stabilized by bound protein. The very slow conversion of this species, which is in the range of 1 s, provides time for the association of ATP, which is promoted by GroES in the trans-position. Association of ATP (before its hydrolysis to ADP) is able to stimulate the interaction of GroEL with GroES (33); thus, in a next step, a second GroES is bound forming a symmetric football-shaped complex. This association promotes a coordinated upward and outward movement of the apical domains (15) initiating productive folding in the cis-position. Otherwise, an (unproductive) release of the bound folding intermediate from the trans-ring into bulk solution would occur. It is not likely that GroES initially bound to the opposite ring dissociates spontaneously, since this is also a very slow reaction (17). Instead, hydrolysis of ATP in the ring with substrate protein bound in a position underneath GroES triggers exchange of GroES and nucleotide from the opposite ring. At the same time, a trans-bullet is restored, which now provides a novel high affinity binding site. This would be the start of a new binding cycle.

During the interaction with the GroE complex, especially kinetically trapped nonnative proteins as shown recently for Rubisco (37) and for MBP Y283D at higher temperatures (35) seem to be unfolded to an extent that allows the protein to start the folding process again from an untrapped conformation. Unfolding by GroEL and binding of completely unfolded protein have recently been demonstrated directly by NMR techniques (38, 39). Generally, GroEL has the ability to interact with proteins at different stages of folding. In this scenario, unfolding may be restricted to folding intermediates with low intrinsic stability (40, 41).

Taken together, our results suggest a highly regulated interplay in the GroEL toroid, which allows protein folding to proceed efficiently under nonpermissive conditions. GroES binding to one GroEL ring not only provides a passive lid for the GroEL cavity, it also tunes the other ring to accept nonnative protein and nucleotide.

Acknowledgments—We thank Linda Randall for providing strains and information and Martina Beissinger for critically reading the manuscript.

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