Cooperative effects of potassium, magnesium, and magnesium-ADP on the release of *Escherichia coli* dihydrofolate reductase from the chaperonin GroEL

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Abstract

Previous investigation has shown that at 22 °C and in the presence of the chaperonin GroEL, the slowest step in the refolding of *Escherichia coli* dihydrofolate reductase (EcDHFR) reflects release of a late folding intermediate from the cavity of GroEL (Clark AC, Frieden C, 1997, *J Mol Biol* 268:512–525). In this paper, we investigate the effects of potassium, magnesium, and MgADP on the release of the EcDHFR late folding intermediate from GroEL. The data demonstrate that GroEL consists of at least two conformational states, with apparent rate constants for EcDHFR release that differ by four- to fivefold. In the absence of potassium, magnesium, and ADP, ~80–90% of GroEL resides in the form with the faster rate of release. Magnesium and potassium both shift the distribution of GroEL forms toward the form with the slower release rate, though cooperativity for the magnesium-induced transition is observed only in the presence of potassium. MgADP at low concentrations (0–50 μ M) shifts the distribution of GroEL forms toward the form with the faster release rate, and this effect is also potassium dependent. Nearly identical results were obtained with a GroEL mutant that forms only a single ring, demonstrating that these effects occur within a single toroid of GroEL. In the presence of saturating magnesium, potassium, and MgADP, the apparent rate constant for the release of EcDHFR from wild-type GroEL at 22 °C reaches a limiting value of 0.014 s⁻¹. For the single ring mutant of GroEL, the rate of EcDHFR release under the same conditions reaches a limiting value of 0.024 s⁻¹, suggesting that inter-ring negative cooperativity exists for MgADP-induced substrate release. The data suggest that MgADP preferentially binds to one conformation of GroEL, that with the faster apparent rate constant for EcDHFR release, and induces a conformational change leading to more rapid release of substrate protein.

Keywords: conformational changes; ligand binding; protein folding

GroEL is a member of the Hsp60 class of molecular chaperones that bind unfolded polypeptides and increase the yield of native proteins by decreasing the extent of aggregation (Fenton & Horwich, 1997; Sigler et al., 1998). The subunits of the tetradecameric GroEL complex are arranged as a double toroid of heptamers (Hendrix, 1979; Braig et al., 1994), and GroEL exhibits a weak ATPase activity (Hendrix, 1979), which in some cases is coupled

to the release of substrate protein (Fenton & Horwich, 1997). Each heptameric unit, or ring, exhibits positive cooperativity within the subunits with respect to MgATP binding and hydrolysis whereas the tetradecamer exhibits inter-ring negative cooperativity (Yifrach & Horowitz, 1995). This allows for the formation of a functionally asymmetric complex (Todd et al., 1994), with MgATP bound to one ring and MgADP bound to the second ring, that cycles between states with high (MgADP) or low (MgATP) affinity for non-native polypeptides.

To simplify the investigation of GroEL-mediated protein folding, a single ring mutant of GroEL (SR1) was previously constructed (Weissman et al., 1995). SR1 consists of a single toroid of heptamers and is capable of binding unfolded substrate proteins, hydrolyzing ATP, and binding GroES (Weissman et al., 1995; Llorca et al., 1997). In the presence of ATP and GroES, SR1 facilitates the productive refolding of aggregation-prone proteins in a manner similar to that of wild-type GroEL (Weissman et al., 1996; Rye et al., 1997). Thus SR1 can be used to determine whether inter-ring interactions contribute to GroEL-induced substrate refolding.

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Abbreviations: MTX, methotrexate; Bis-tris, bis[2-hydroxyethyl]imino tris[hydroxymethyl]methane; Tris, tris[hydroxymethyl]aminomethane; MOPS, 3-[N-morpholino]propane sulfonic acid; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; DTT, dithiothreitol; EcDHFR, *Escherichia coli* dihydrofolate reductase; SR1, the single ring mutant of GroEL.

Dihydrofolate reductase from *Escherichia coli* (EcDHFR), a monomeric protein of MW 17,680, refolds with essentially 100% efficiency from the denatured state (Touchette et al., 1986; Frieden, 1990). The native enzyme is known to exhibit conformational heterogeneity (Dunn et al., 1978; Cayley et al., 1981; Penner & Frieden, 1987), and we have shown that this results from the persistence of a late folding conformation in the native protein (Clark & Frieden, 1999a). When refolded from urea-containing buffer in the presence of GroEL, EcDHFR binds rapidly to the chaperonin as the unfolded conformation (Clark & Frieden, 1997), and the bound protein folds with rate constants similar to those in bulk solution until the formation of the final folding intermediate. The partitioning of the final non-native conformation between binding to GroEL and folding to the native conformation allows for a transient interaction with GroEL at lower temperature ($<30^{\circ}\text{C}$) or a stable interaction at higher temperature ($>30^{\circ}\text{C}$) (Clark & Frieden, 1999b).

We have used the release of the final folding intermediate of EcDHFR from GroEL to further investigate the effects of potassium, magnesium, and MgADP on GroEL function. It has previously been demonstrated that both potassium and magnesium are required for GroEL ATPase activity. Potassium may increase the affinity of GroEL for ATP (Todd et al., 1993), while magnesium is required for GroEL ATPase activity since the magnesium-liganded form of the nucleotide binds GroEL. In this paper we demonstrate that in the absence of potassium, magnesium, and ADP, GroEL consists of at least two conformational states that differ by four- to fivefold in their rates of release of EcDHFR. We refer to these forms of GroEL as slow and fast in regard to their apparent rate constants for the release of EcDHFR. In addition, we describe the effects of potassium, magnesium, and ADP on the distribution of the two GroEL forms.

Results

EcDHFR was initially unfolded in 5 M urea-containing buffer B (50 mM MOPS, pH 7.2, 1 mM DTT, 22°C). Refolding was initiated by rapidly diluting 11-fold into buffer B that contained GroEL (1 μM) so that the final concentration of EcDHFR was 1 μM . The refolding experiments have been described in detail (Clark & Frieden, 1997, 1999b). The final protein concentrations represent 2 mol of DHFR binding sites per mole of GroEL (Clark et al., 1996).

In the experiments described here, we have focused only on the longer time courses, which reflect the partitioning of a late folding intermediate, called I_3 , between rebinding to GroEL and folding to the native form (Clark & Frieden, 1997). The apparent rate constants that are observed represent a combination of release from and rebinding to GroEL as well as refolding to the native structure. The apparent rate of refolding for EcDHFR at 22°C is slower in the presence of GroEL compared to refolding in buffer alone (Clark & Frieden, 1997), reflecting the slow release of the I_3 folding intermediate from GroEL. At 22°C , greater than 90% of EcDHFR is released from the chaperonin and refolds to the native state.

Effects of magnesium on the release EcDHFR from GroEL

The slow release of the late folding intermediates, I_3 , from GroEL has been described in detail (Clark & Frieden, 1997, 1999b). We show here (Fig. 1) that magnesium alone affects the rate of release of EcDHFR from GroEL. While release is slow at all concentra-

Fig. 1. Refolding of EcDHFR (1 μM) in the presence of GroEL (1 μM) in buffer B (50 mM MOPS, pH 7.2, 1 mM DTT, 22°C) as a function of magnesium. **A:** Curve 1; 0 KCl and 0 MgSO_4 ; curve 2; 0 KCl and 15 mM MgSO_4 . The continuous lines represent fits of the data to a two exponential equation, as described in Materials and methods. **B:** Relative amplitude of the slow (■) and fast (●) phase of EcDHFR release from GroEL from experiments like those described in A, performed in the absence of potassium. The continuous lines represent fits of the data to the Hill equation, as described in Materials and methods. Parameters obtained from the fits are summarized in Table 1. **C:** Relative amplitude of the slow (■) and fast (●) phase of EcDHFR release from GroEL from experiments like those described in A, performed in the presence of 100 mM KCl. The continuous lines represent fits of the data to the Hill equation, as described in Materials and methods.

tions of magnesium, the half-time increased from ~ 50 s in the absence of magnesium (Fig. 1A, curve 1) to ~ 250 s in the presence of 15 mM magnesium (Fig. 1A, curve 2). At all magnesium concentrations, the data were best fit by a two exponential equation. The apparent rate constants determined from the fits were 0.01 and 0.003 s^{-1} and were invariant at all concentrations of magnesium. The relative amplitudes associated with the two phases, however, were dependent on the magnesium concentration. In the absence of magnesium, for example, the amplitude of the fast phase ($k_{\text{app}} \sim 0.01\text{ s}^{-1}$) comprises about 80–90% of the total change in amplitude. In the presence of 15 mM MgSO_4 , the amplitude of the slow phase ($k_{\text{app}} \sim 0.003\text{ s}^{-1}$) comprises about 80–90% of the total amplitude change.

Time-dependent data similar to that shown in Figure 1A were obtained as a function of magnesium concentration from 0 to 15 mM. In all cases, the data were well fit to a two exponential equation, with apparent rate constants determined from the fits of $\sim 0.01 \text{ s}^{-1}$ (fast release) and $\sim 0.003 \text{ s}^{-1}$ (slow release). The relative amplitudes of the two phases are shown in Figure 1B. In the absence of magnesium, the amplitude of the fast phase predominates, representing $\sim 95\%$ of the total change in amplitude. The relative amplitude of the fast phase decreases, while the relative amplitude of the slow phase increases, with increasing concentrations of magnesium (Fig. 1B). At 15 mM magnesium, the slow phase represents $\sim 90\%$ of the total change in amplitude. The amplitudes shown in Figure 1B were fit to the Hill equation (continuous lines in Fig. 1B), as described in Materials and methods. The Hill coefficient determined from the fits was 0.9 ± 0.2 , demonstrating the transitions that occur are noncooperative. The midpoint of the transition, as determined from the fits, is $3.6 \pm 1.1 \text{ mM}$ magnesium. The parameters obtained from the fits of the data are summarized in Table 1.

Similar experiments were done in buffer A (50 mM potassium phosphate, pH 7.2, 100 mM KCl, 1 mM DTT) to examine the magnesium-induced transition in the presence of potassium. As for the data described above, the data obtained in the presence of 100 mM KCl were well fit to a two exponential equation. While the apparent rate constants (~ 0.01 and $\sim 0.003 \text{ s}^{-1}$) were independent of the magnesium concentration, the relative amplitudes of the fast and slow phases were not (Fig. 1C). In agreement with the data obtained in the absence of KCl, the fast phase predominates at low magnesium concentrations ($\sim 90\%$ of the total change in amplitude). While the relative amplitude of the fast phase decreases with an increase in magnesium concentration, the transition appears to be cooperative, in contrast to the results obtained in the absence of KCl. The data were fit to the Hill equation (continuous lines in Fig. 1B), as described in Materials and methods. The Hill coefficient determined from the fits was 3.5 ± 0.9 , and the midpoint for the transition, $K_{0.5}$, was $4.9 \pm 0.2 \text{ mM MgSO}_4$ (Table 1).

The experiments described in Figure 1A–C reflect only the dissociation of the late folding intermediate of EcDHFR, I_3 , from GroEL. We interpret the two phases to demonstrate the presence of two conformational states of GroEL. The conformational states release EcDHFR with apparent rates that differ by four- to five-fold. While the transition from the fast form to the slow form of GroEL occurred between 3.5–5 mM magnesium, the transition is

cooperative only in the presence of potassium. The data suggest that magnesium binds to GroEL and drives the conformational equilibrium toward the slow-releasing conformation. Because conformational changes in GroEL are very fast relative to the rate of protein folding (Yifrach & Horovitz, 1998), the effects of magnesium observed here are on the relative amplitudes of the slow- and fast-releasing conformations of GroEL, rather than the apparent rate constants for slow and fast release.

Effects of potassium on EcDHFR release from GroEL

The data described in Figure 1 suggest that GroEL undergoes a cooperative transition that is induced by magnesium, although this occurs only when potassium is bound to the chaperonin. It is not clear from the data, however, whether the magnesium-bound form of GroEL could undergo a cooperative transition induced by potassium. This possibility was addressed in experiments similar to those described in Figure 1A, in which EcDHFR was refolded in buffer B (no KCl) in the presence of GroEL, and magnesium was added to a final concentration of 15 mM (Fig. 2, open circles) or was left out of the experiment (Fig. 2, closed circles). KCl was added to final concentrations between 0 and 1 M, and the apparent rate of release of EcDHFR was monitored as described in Figure 1. In contrast to the effects of magnesium, the data demonstrated that potassium affects the apparent rate of release of EcDHFR from the fast phase. There was little effect on the relative amplitudes of the fast and slow phases or on the apparent rate of the slow phase (data not shown). Shown in Figure 2 are the apparent rate constants for the release of EcDHFR from the fast phase as a function of the concentration of KCl, in the presence and absence of 15 mM MgSO_4 . At concentrations of potassium below 500 mM, the time-dependent data were best fit to a two exponential equation. The data demonstrate that in the absence of MgSO_4 , potassium decreased the apparent rate constant for the fast phase (Fig. 2, closed symbols). At concentrations of potassium greater than 500 mM, the time-dependent data were best fit to a single exponential equation, and the apparent rate constant was similar to the slow rate of release observed in Figure 1 ($\sim 0.002 \text{ s}^{-1}$).

It is not clear which form of GroEL is prevalent at 500 mM to 1 M KCl, so it is uncertain what this single slow phase represents. While the apparent rate constant for release from the slow form may also decrease at the high concentrations of potassium, we cannot distinguish this very slow rate in the stopped-flow instrument. With this caveat in mind, the apparent rate constants deter-

Table 1. Effect of magnesium, potassium, and ADP on wild-type GroEL^a

Effector	Parameter fit	$K_{0.5}$	n^H
Mg^{2+} (with KCl)	Amplitude fast release	$4.9 \pm 0.2 \text{ mM Mg}^{2+}$	3.5 ± 0.9
Mg^{2+} (no KCl)	Amplitude fast release	$3.6 \pm 1.1 \text{ mM Mg}^{2+}$	0.9 ± 0.2
KCl (no Mg^{2+})	Rate fast release	$122 \pm 26 \text{ mM KCl}$	0.9 ± 0.1
Low ADP (with KCl)	Amplitude fast release	$11 \pm 1 \text{ } \mu\text{M ADP}$	3.1 ± 0.9
Low ADP (no KCl)	Amplitude fast release	$\sim 1,080 \text{ } \mu\text{M ADP}$	N/D ^b
High ADP (with KCl)	Rate fast release	$269 \pm 51 \text{ } \mu\text{M ADP}$	2.1 ± 0.8

^aThe effects of magnesium, potassium, and ADP on the parameters ($K_{0.5}$ and n^H) derived from fitting changes in either amplitude or apparent rate of fast EcDHFR release from wild-type GroEL to the Hill equation, as described in Materials and methods.

^bN/D = not determined.

Fig. 2. Refolding of EcDHFR (1 μ M) in the presence of GroEL (1 μ M) in buffer B (50 mM MOPS, pH 7.2, 1 mM DTT, 22°C) as a function of potassium. The data, like those described in Figure 1A, were fit to a two exponential equation, and the apparent rate constant for the fast phase k_{app} is shown. The data obtained at KCl concentrations >500 mM were well fit to a single exponential. The data for the closed circles (●) included 0 MgSO₄ in the buffer while those for the open circles (○) included 15 mM MgSO₄ in the buffer. The data with 0 MgSO₄ (●) were fit to the Hill equation (continuous line), as described in Materials and methods. Parameters obtained from the fits are summarized in Table 1. The data for 15 mM MgSO₄ (○) were fit to a linear equation.

mined in the absence of magnesium (Fig. 2, closed circles) were fit to the Hill equation (continuous line in Fig. 2), as described in Materials and methods. The Hill coefficient obtained from the fits is 0.9 ± 0.1 , demonstrating that the effect of potassium is non-cooperative. The midpoint of the transition ($K_{0.5}$) is 122 ± 26 mM KCl (see Table 1). Because the effect of potassium was noncooperative, the $K_{0.5}$ also represents an apparent dissociation constant for KCl (in the absence of magnesium). When MgSO₄ (15 mM) was included in the buffer (Fig. 2, open symbols), the majority of the total amplitude change was due to the slow phase, consistent with the data shown in Figure 1. The apparent rate of refolding was ~ 0.002 s⁻¹ in the absence of KCl and varied little as a function of the KCl concentration. These data demonstrate that potassium, in the absence of magnesium, decreases the apparent rate constant for fast EcDHFR release, with little effect on the relative distribution of the two forms of GroEL, and this effect occurs noncooperatively. In the presence of magnesium (15 mM), 80–90% of the total amplitude change is due to slow release of EcDHFR from GroEL, so the effect of potassium is not observed.

In control experiments (data not shown), EcDHFR was refolded in buffer B without GroEL at KCl concentrations between 0 and 500 mM. There was little effect on the apparent rate constant for the final folding step under these conditions (0.04 s⁻¹ at 0 KCl vs. 0.036 s⁻¹ at 500 mM KCl). In similar control experiments (data not shown), EcDHFR was refolded in buffer B without GroEL, and the final concentration of MgSO₄ was varied between 0 and 15 mM. Under these conditions there was no difference in the apparent rate constant for the final folding reaction (0.04 s⁻¹ at all concentrations of magnesium), demonstrating that the results described for potassium and magnesium are due to specific effects of the ions on the chaperonin.

It is not clear from the results described in Figures 1 and 2 whether the binding of potassium or magnesium to GroEL affect

interactions that occur within a single ring of GroEL (intra-ring), or whether the ions affect interactions that occur between the two rings (inter-ring). Either of these possibilities could result in conformational changes in GroEL, which lead to altered binding properties of protein substrates. To address these possibilities, we examined the interactions of EcDHFR with a mutant of GroEL, called SR1, which forms only a single toroid. SR1 has been shown to bind unfolded protein and to facilitate the refolding of aggregation-prone proteins in the presence of ATP and the co-chaperonin GroES (Weissman et al., 1996; Rye et al., 1997).

Refolding of EcDHFR in the presence of SR1

EcDHFR was initially unfolded in 5 M urea-containing buffer A (50 mM potassium phosphate, pH 7.2, 100 mM KCl, 1 mM DTT, 22°C). In experiments analogous to those described for wild-type GroEL, refolding of EcDHFR was initiated by rapidly diluting 11-fold into buffer A that contained SR1 so that the final concentration of EcDHFR was 1 μ M. The final concentrations of SR1 were varied between 0 and 2 μ M. In the presence of SR1, an initial increase in fluorescence was observed in the first second of data collection, with an apparent rate that was faster than the initial phase of EcDHFR refolding alone (data not shown). These results are similar to those described for wild-type GroEL (Clark & Frieden, 1997) and have been interpreted as the binding of unfolded EcDHFR to GroEL, which results in a increase in fluorescence for the bound species. At longer times (1–400 s), a decrease in fluorescence was observed that is slower than the rate of EcDHFR refolding alone (Fig. 3). The apparent rate becomes slower as the concentration of SR1 is increased. In the case of SR1, in contrast to wild-type GroEL, the changes in apparent rates saturate at equal concentrations of EcDHFR and SR1 (1 μ M), indicating that the binding stoichiometry is 1 mol of EcDHFR per mole of SR1 heptamer. Similar experiments for wild-type GroEL demonstrated a binding

Fig. 3. Refolding of EcDHFR (1 μ M) in the presence of varying concentrations of SR1 in buffer A (50 mM potassium phosphate, pH 7.2, 100 mM KCl, 1 mM DTT, 22°C). The final concentration of SR1 was (bottom to top) 0, 0.1, 0.25, 0.5, 1, and 2 μ M (not distinguishable from 1 μ M).

stoichiometry of 2 mol of EcDHFR per mole of GroEL tetradecamer (Clark et al., 1996; Clark & Frieden, 1997). As with the wild-type GroEL, the fluorescence of EcDHFR in the presence of SR1 eventually (>500 s, data not shown) returns to that observed for EcDHFR alone. The data shown in Figure 3 represent the release of I_3 from the chaperonin, which results in kinetic partitioning between rebinding to SR1 and folding to the native form. Under these conditions, the partitioning favors formation of native EcDHFR, and this results in a transient interaction with the chaperonin. A transient interaction between EcDHFR and wild-type GroEL at 22 °C has been described in detail (Clark & Frieden, 1997, 1999b; Frieden & Clark, 1997). These data suggest that SR1 is an excellent model for the interaction of EcDHFR with a single toroid of GroEL.

Effects of magnesium on the release of EcDHFR from SR1

To determine whether the interaction of EcDHFR with SR1 is dependent on the concentration of magnesium, EcDHFR ($1\ \mu\text{M}$) was refolded in buffer B (no KCl) in the presence of SR1 ($1.5\ \mu\text{M}$), and in the presence or absence of 15 mM magnesium. The results are shown in Figure 4. As described for the wild-type GroEL, we have focused on the long time courses (1–1,000 s) to examine the partitioning of a late folding intermediate (I_3) between rebinding to SR1 and folding to the native form. As shown in Figure 4A, refolding of EcDHFR in the presence of SR1 was faster in the absence of magnesium. The apparent half-time for refolding was ~ 50 s in the absence of magnesium (Fig. 4A, curve 1) compared to ~ 200 s in the presence of 15 mM magnesium (Fig. 4A, curve 2). The data were best fit to a two exponential equation, and the apparent rate constants determined for the two phases were ~ 0.01 and $\sim 0.003\ \text{s}^{-1}$. In the absence of magnesium, ~ 90 to 95% of the total change in amplitude was due to the fast phase (Fig. 4A). In contrast, when magnesium (15 mM) was present, $\sim 80\%$ of the total change in amplitude was due to the slow phase.

In separate experiments, EcDHFR was refolded in buffer B (no KCl) in the presence of SR1 as described above, and the concentration of MgSO_4 was varied between 0 and 15 mM. To examine whether the effects of magnesium on the release of EcDHFR from SR1 were dependent on potassium, as observed for the wild-type GroEL, the experiments were performed in the absence (Fig. 4B, closed circles) or presence (Fig. 4B, open circles) of 100 mM KCl. The data obtained were similar to those described in Figure 4A, and the amplitudes of the fast phase ($\sim 0.01\ \text{s}^{-1}$) of EcDHFR release, determined from fits of the data to a two exponential equation, are shown in Figure 4B. The data are similar to the effects of magnesium and potassium on the release of EcDHFR from wild-type GroEL (compare Figs. 1, 4). There is a decrease in the relative amplitude of the fast phase (Fig. 4B) and a concomitant increase in the relative amplitude of the slow phase (data not shown) with increasing concentrations of magnesium. There was little effect on the apparent rate constants associated with the two phases over this range of magnesium concentrations (data not shown).

In the presence of 100 mM KCl (Fig. 4B, open symbols), the decrease in the apparent amplitude of the fast phase appeared to be cooperative. The data were fit to the Hill equation (continuous lines in Fig. 4B), as described in Materials and methods, and the Hill coefficient determined from the fits was 2.9 ± 0.5 . This demonstrates that the transition induced by magnesium is cooperative in the presence of potassium. The midpoint for the transition ($K_{0.5}$)

Fig. 4. Refolding of EcDHFR ($1\ \mu\text{M}$) in the presence of SR1 ($1.5\ \mu\text{M}$) in buffer B (50 mM MOPS, pH 7.2, 1 mM DTT, 22 °C) as a function of magnesium. **A:** Curve 1; 0 KCl and 0 MgSO_4 ; curve 2; 0 KCl and 15 mM MgSO_4 . The continuous lines represent fits of the data to a two exponential equation, as described in Materials and methods. **B:** Relative amplitude of the fast phase of EcDHFR release from SR1 from experiments like those described in A. Open symbols represent data collected in buffer B + 100 mM KCl, whereas closed symbols represent data collected in buffer B without KCl. The continuous lines represent fits of the data to the Hill equation, as described in Materials and methods. Parameters obtained from the fits are summarized in Table 2.

was $2.8 \pm 0.2\ \text{mM}\ \text{MgSO}_4$ (see Table 2). In the absence of potassium (Fig. 4B, closed symbols), the decrease in the relative amplitude of the fast phase was noncooperative, and the midpoint of the transition ($K_{0.5}$) was also somewhat lower (1.4 mM magnesium). The parameters obtained from the fits are summarized in Table 2. The data for the effects of magnesium and potassium on the interactions of EcDHFR with SR1 are similar to those described for wild-type GroEL (compare Figs. 1, 4). The data demonstrate that the transitions in GroEL from a form with slow release to a form with fast release of substrate protein occur within the heptameric unit (i.e., are due to intra-ring rather than inter-ring interactions). In addition, the data demonstrate that the two phases observed here are not due to different rates of release from the two GroEL rings (fast release from one ring and slow release from the opposite ring).

Table 2. Effect of magnesium, potassium, and ADP on single ring GroEL (SR1)^a

Effector	Parameter fit	$K_{0.5}$	n^H
Mg ²⁺ (with KCl)	Amplitude fast release	2.8 ± 0.2 mM Mg ²⁺	2.9 ± 0.5
Mg ²⁺ (no KCl)	Amplitude fast release	1.4 ± 0.1 mM Mg ²⁺	1.5 ± 0.5
Low ADP (with KCl)	Amplitude fast release	7 ± 1 μ M ADP	3.2 ± 0.8
Low ADP (no KCl)	Amplitude fast release	~ 125 μ M ADP	N/D ^b
High ADP (with KCl)	Rate fast release	42 ± 3 μ M ADP	3.6 ± 0.9

^aThe effects of magnesium, potassium, and ADP on the parameters ($K_{0.5}$ and n^H) derived from fitting changes in either amplitude or apparent rate of fast EcDHFR release from SR1 to the Hill equation, as described in Materials and methods.

^bN/D = not determined.

Effects of MgADP on EcDHFR release from GroEL and SR1

To determine the effect of ADP on these reactions, EcDHFR was refolded in buffer B in the presence of GroEL, 100 mM KCl, and 15 mM MgSO₄, and the final concentration of ADP was varied between 0 and 2 mM. The release of EcDHFR from GroEL was measured at longer time courses as described for Figures 1–4, and the data were best fit to a two exponential equation with apparent rates of EcDHFR release of ~ 0.01 s^{−1} (fast release) and ~ 0.003 s^{−1} (slow release). In the absence of magnesium (not shown), ADP alone (0–1 mM) had no effect on the distribution of slow and fast-releasing forms of GroEL, indicating that the magnesium-liganded form of ADP binds GroEL. In the presence of magnesium (and absence of ADP), $\sim 80\%$ of the change in amplitude was due to the slow phase, as described above, indicating that these solution conditions favor the form of GroEL that releases EcDHFR slowly. At low concentrations of MgADP (0–50 μ M, Fig. 5A, circles), the relative amplitude of the fast phase increases with a concomitant decrease in the amplitude of the slow phase (amplitude data for the slow phase not shown), and the transition appears to be cooperative. The Hill coefficient obtained from fits of the data to the Hill equation was 3.1 ± 0.9 with a midpoint of the transition ($K_{0.5}$) of 11 ± 1 μ M ADP (summarized in Table 1).

It is not clear from the data with wild-type GroEL (Fig. 5A) whether MgADP binds to a single ring in the double toroid (intra-ring effects) or whether the conformational changes that result from nucleotide binding are in the ring opposite bound nucleotides (inter-ring effects). To examine these issues, we carried out experiments with SR1 similar to those described for wild-type GroEL. EcDHFR was refolded in buffer B in the presence of 100 mM KCl, 15 mM MgSO₄, and SR1, as described above for wild-type GroEL, and the data were fit to a two exponential equation. Under these conditions the slow phase (~ 0.003 s^{−1}) predominates ($\sim 90\%$ of the total change in amplitude, see Fig. 4B). Titration of MgADP at final concentrations between 0 and 20 μ M resulted in a shift in the distribution of relative amplitudes toward the fast phase (~ 0.01 s^{−1}) of release (Fig. 5A, squares). As observed for wild-type GroEL, the transition in SR1 appeared to be cooperative. The Hill coefficient obtained from fits of the data to the Hill equation was 3.2 ± 0.8 , and the midpoint of the transition ($K_{0.5}$) was 7 ± 1 μ M ADP (Table 2). The Hill coefficient is very similar to that obtained for wild-type GroEL and indicates that the transition observed for GroEL in the presence of MgADP occurs within a heptameric unit of the tetradecamer. Currently we cannot distinguish whether the transition occurs in both heptameric rings of the tetradecamer, although the data suggest that if both heptameric rings are involved then the transitions would occur without inter-ring negative cooperativity.

While low concentrations of MgADP (0–50 μ M) resulted in a shift of the GroEL conformations toward the form with the faster rate of release of EcDHFR, there was no effect on the apparent rate constants for the slow or fast phase. In contrast, higher concentrations of MgADP (50 μ M to 1 mM) affected the apparent rate constant for the fast phase. As shown in Figure 5B, the rate constant for the fast phase of release of EcDHFR increased from approximately 0.005 to 0.014 s^{−1} as the concentration of MgADP was increased to 1 mM (Fig. 5B, circles). In comparable experiments with SR1, the rate constant for the fast phase increased from approximately 0.008 to 0.024 s^{−1} at MgADP concentrations between 20 and 100 μ M (Fig. 5B, squares). The apparent rates were fit to the Hill equation, and the Hill coefficients obtained from the fits were 2.1 ± 0.8 (wild-type GroEL) and 3.6 ± 0.9 (SR1), with midpoints of the transitions ($K_{0.5}$) of ~ 270 μ M MgADP (wild-type GroEL) and 42 μ M MgADP (SR1). In these experiments, there was no effect of MgADP (0–1 mM) on the apparent rate constant for the slow phase either from wild-type GroEL or SR1 (data not shown). In addition, experiments done in the absence of MgSO₄ or in the presence of 2 mM EDTA (data not shown) demonstrated that magnesium was required to observe the effect of ADP on the apparent rate constants. This indicates that MgADP is the form of nucleotide bound by the chaperonin.

In experiments similar to those described in Figures 5A and 5B, the two phases of release of EcDHFR were examined under solution conditions in which MgSO₄ (15 mM) was present but KCl was absent (in buffer B). As shown in Figure 5C, while the relative amplitude of the fast phase increases as the MgADP concentration is increased from 0 to 2 mM, the midpoint of the transition is very different in the absence of potassium. For wild-type GroEL, the midpoint of the transition is increased from 11 μ M to $\sim 1,080$ μ M MgADP (compare Fig. 5A, circles, to Fig. 5C, circles). For SR1 the midpoint of the transition is increased from 7 to ~ 125 μ M (compare Fig. 5A, squares, to Fig. 5C, squares). In the absence of potassium, there was no effect of MgADP (0–1 mM) on the apparent rate constants for fast or slow phases of release of EcDHFR either from wild-type GroEL or SR1. Because ADP binding to GroEL was not directly measured in these experiments, it is not possible to determine whether potassium affects ADP binding to GroEL or conformational changes that take place after ADP binding to the chaperonin.

Discussion

Consistent with our previous paper (Clark & Frieden, 1997) that GroEL interacts transiently with EcDHFR at 22 °C, we now find

Fig. 5. Relative amplitudes and apparent rate constants for EcDHFR release from wild-type GroEL and SR1 as a function of MgADP concentration from refolding experiments performed in buffer B (50 mM MOPS, pH 7.2, 1 mM DTT, 22 °C), in the presence of 15 mM MgSO_4 and either 100 mM KCl (A and B) or 0 KCl (C). The continuous lines represent fits of the data to the Hill equation, as described in Materials and methods. Parameters obtained from the fits are summarized in Table 1 (GroEL) or Table 2 (SR1). Where the value in the tables is given as N/D, there were not enough points to obtain an accurate number. **A:** Relative amplitude of the fast phase of EcDHFR release from wild-type GroEL (●) and SR1 (■) as a function of MgADP concentration (0–100 μM). **B:** Apparent rate constant for the fast phase of EcDHFR release from wild-type GroEL (●) and SR1 (■) as a function of MgADP concentration (0–1000 μM). **C:** Relative amplitude of the fast phase of EcDHFR release from wild-type GroEL (●) and SR1 (■) as described in A, except that experiments were performed in the absence of potassium.

that the single ring mutant of GroEL (SR1) also interacts transiently with EcDHFR at 22 °C. At higher temperatures the interaction between GroEL and EcDHFR is stable (Clark & Frieden,

1999b), suggesting that GroEL may act to bind aggregation-prone folding intermediates in a temperature-dependent manner. These results also demonstrate the importance of using bacterial proteins to study GroEL function, as differences in the rates of folding of bacterial and mammalian isoforms of DHFR lead to different interactions with the chaperonin (Clark & Frieden, 1999b).

In the absence of potassium, magnesium, and nucleotides, both a fast phase ($\sim 0.01 \text{ s}^{-1}$) and slow phase ($\sim 0.003 \text{ s}^{-1}$) are observed for the release of EcDHFR from GroEL and SR1. We have interpreted these results as demonstrating the existence of two conformations of GroEL. Each conformation releases substrate proteins with apparent rates that differ by four- to fivefold. Magnesium shifts the relative distribution of the two forms toward that with the slower apparent rate constant for release of EcDHFR, without changing the apparent rate constants for the slow or fast forms. In contrast, potassium (below 500 mM) decreases the apparent rate constant for the fast phase, without affecting the relative amplitudes of the two phases, and this occurs noncooperatively. The magnesium-induced transition between fast and slow conformations of GroEL is cooperative only in the presence of potassium. Nearly identical results were obtained with a single ring mutant of GroEL (SR1), demonstrating that magnesium and potassium affect intra-ring, rather than inter-ring, interactions. The conformational changes in GroEL, in turn, affect the binding of substrate protein.

The data indicate that a separate site for magnesium, not related to the MgATP binding site, exists on GroEL. The crystal structure of GroEL complexed with MgATP shows that two metal ions are present in the nucleotide binding site of GroEL. One of these metal ions is the Mg^{2+} associated with MgATP, while the other site is not further discussed (Sigler et al., 1998). In addition, a previous study found that both Mg^{2+} and Mn^{2+} activate GroEL ATP hydrolysis at concentrations far above the levels necessary to saturate the MgATP site (Diamant et al., 1995). The effective magnesium concentration that resulted in half maximal ATPase activity in that study was $2.6 \pm 0.6 \text{ mM}$, very similar to the $K_{0.5}$ values (3.5–5 mM) for the transition between fast and slow releasing forms of GroEL observed in our study. Two different studies have found that magnesium stabilizes the structure of GroEL, as measured by glutaraldehyde cross-linking and stability in urea (Azem et al., 1994) or protection from proteolysis (Gibbons & Horowitz, 1996).

Hydrolysis of ATP by GroEL requires the presence of K^+ , Rb^+ , or NH_4^+ (Viitanen et al., 1990), presumably because high affinity binding of ATP is dependent upon the presence of potassium (Todd et al., 1993). Our study shows that in the absence of nucleotide, potassium is itself an effector of GroEL function. Potassium decreases the apparent rate of EcDHFR release from the fast-releasing form of GroEL, and at concentrations greater than 500 mM may shift the distribution toward the form of GroEL with the slower rate of release. The transitions induced by potassium are noncooperative. At potassium concentrations greater than 500 mM, it is not clear whether the release of EcDHFR occurs from the slow-releasing form or from the fast-releasing form since the data could be fit to a single exponential equation with an apparent rate constant equal to that for slow release of EcDHFR. The magnesium-induced transition between fast and slow-releasing conformations of GroEL is cooperative only in the presence of potassium, suggesting that potassium modulates the binding of magnesium to GroEL and is itself a weak effector of GroEL conformation. Further studies are necessary to fully understand the effects of potassium on GroEL, and to determine whether this effect is specific for potassium or a general effect of monovalent ions.

The results described in Figures 1–4 are consistent with the mechanism shown in Scheme 1. The scheme proposes two conformations of GroEL, called GroEL_s and GroEL_f. When neither potassium nor magnesium is present, the form that predominates in solution is the conformation with the faster rate of release of EcDHFR, GroEL_f. When magnesium binds in the absence of potassium (front face of the cube in Scheme 1), the equilibrium favors the slow form of the chaperonin [GroEL_s(Mg²⁺)], and the transition from GroEL_f to GroEL_s(Mg²⁺) occurs noncooperatively (Hill coefficient ~ 1). However, when pre-incubated with 150 mM potassium, the potassium-liganded form is present at the start of the experiment [GroEL_f(K⁺)]. The transition from GroEL_f(K⁺) to GroEL_s(Mg²⁺)(K⁺) (back face of the cube in Scheme 1) is cooperative, with a Hill coefficient of ~3.5. When potassium is added in the absence of magnesium, the potassium-liganded form of the chaperonin has a slower rate of release than the unliganded form (top face of the cube in Scheme 1), and the transition from GroEL_f(K⁺) → GroEL_s(K⁺) requires high concentrations of potassium (>500 mM) and is not cooperative. When the chaperonin is pre-incubated with magnesium so that the magnesium-liganded form is present at the start of the experiment [GroEL_s(Mg²⁺)], there is no further change in amplitudes or rates upon the addition of KCl. This is likely because the conformational equilibrium already favors GroEL_s, thus the transition of GroEL_s(Mg²⁺) → GroEL_s(Mg²⁺)(K⁺) is not observable. Overall, the transition of the liganded forms of GroEL (GroEL_f → GroEL_s) is cooperative in the presence of potassium, although it is not yet clear whether there is a preferred order to the binding of potassium and magnesium.

There were two effects of ADP observed in the experiments. First, at low concentrations, ADP shifted the distribution of GroEL forms toward the form with the faster rate of release of EcDHFR (Fig. 5A). The midpoint for the transition ($K_{0.5}$) was similar for wild-type GroEL (11 μ M) and SR1 (7 μ M), demonstrating that MgADP binds tightly to both GroEL and SR1. At these concentrations of ADP, there was no effect on the apparent rate constant for the release of EcDHFR. Second, at higher concentrations of ADP, the apparent rate constant for the fast phase increased

(Fig. 5B). The limiting values for the fast phases were 0.014 and 0.024 s⁻¹, for wild-type GroEL and SR1, respectively, in the presence of saturating concentrations of potassium, magnesium, and MgADP. In addition, the midpoint of the transition ($K_{0.5}$) was 270 μ M ADP for wild-type GroEL and 42 μ M ADP for SR1. In the absence of potassium (Fig. 5C), much higher concentrations of ADP are necessary to complete the transition between slow and fast releasing forms of GroEL. It has previously been demonstrated that the binding of ATP to GroEL is weaker in the absence of potassium (Todd et al., 1993), thus our results may reflect weaker binding of ADP to GroEL. Alternatively, potassium may affect the transition between slow and fast releasing forms of GroEL. Because nucleotide binding was not measured directly in these experiments, it is not possible to determine which mechanism is responsible for the potassium dependence of the ADP-induced transition.

The results described in Figure 5 are consistent with the model shown in Scheme 2. This scheme is essentially an extension of Scheme 1 in which the bottom face of the cube for Scheme 1 is now the top face of the cube in Scheme 2. At low concentrations of magnesium and in the presence of potassium, the GroEL_f(Mg²⁺)(K⁺) form of the chaperonin is favored, and this form has a faster rate constant for the release of EcDHFR. At high concentrations of magnesium, with potassium present, the GroEL_s(Mg²⁺)(K⁺) form is favored, and this form has a slower rate constant for the release of EcDHFR. As described in Scheme 1, the transition from GroEL_f(Mg²⁺)(K⁺) to GroEL_s(Mg²⁺)(K⁺) is cooperative, with a Hill coefficient of ~3.5. Although the slow form of GroEL is favored at high concentrations of magnesium, low concentrations of ADP (<50 μ M) facilitate the transition to the fast form. This indicates that ADP binds preferentially to the fast form [GroEL_f(Mg²⁺)(K⁺)] (back face of the cube in Scheme 2). In the absence of potassium (front face of the cube in Scheme 2), ADP appears to bind preferentially to the fast form as well, although much higher levels of ADP (>500 μ M) are necessary for this reaction to go to completion. Our data are consistent with the proposed two step binding models for nucleotides (Jackson et al., 1993; Lin & Eisenstein, 1996; Yifrach & Horovitz, 1998) in which

Scheme 1. A model for the effects of potassium and magnesium on the conformation of GroEL. Potassium and magnesium affect the equilibrium between GroEL conformations with fast (GroEL_f) and slow (GroEL_s) apparent release rates for EcDHFR late folding intermediate (see text).

Scheme 2. A model for the effects of MgADP binding on the conformation of GroEL. MgADP binds preferentially to the conformation of GroEL that releases EcDHFR late folding intermediate with a fast (GroEL_f) apparent release rate (see text).

a weak encounter complex of GroEL with nucleotides is followed by a cooperative conformational change that increases the affinity for nucleotide binding. The ADP-induced conformational changes observed in our experiments lead to an increase in the apparent rate for release of EcDHFR. Subsequent conformational changes that occur as a result of the binding of ADP, which increase the rate of EcDHFR release from the fast form, are not shown in this scheme.

In a previous study, it was demonstrated that the apparent rate of folding for rhodanese, mediated by SR1, was similar in the presence of MgADP or MgATP (Hayer-Hartl et al., 1996). This was quite surprising since the apparent rate of folding for rhodanese bound to wild-type GroEL is much faster in the presence of MgATP when compared to MgADP (Hayer-Hartl et al., 1996). The authors speculated that these results may be due to increased conformational flexibility in SR1 compared to wild-type GroEL. Our results indicate that the loss of inter-ring interactions in SR1, which contribute to negative cooperativity in wild-type GroEL, make the ADP-induced rapid release of substrate proteins more favorable in SR1. At the concentrations of ADP used in these experiments (0–1 mM), the conformational change that leads to faster release of EcDHFR may not go to completion for wild-type GroEL. Since inter-ring negative cooperativity has been demonstrated for ATP-induced conformational changes in GroEL (Yifrach & Horovitz, 1996), it is reasonable to assume that it may occur for ADP-induced conformational changes.

Materials and methods

Materials

Methotrexate (MTX), MTX-agarose, fast-flow DEAE-sepharose, fast-flow Q-sepharose; Reactive Red 120 agarose (type 3000-CL), Sephadex G-200, Bis-tris, Tris, MOPS (sodium salt), ATP (disodium salt), ADP (sodium salt), and DTT were from Sigma (St. Louis, Missouri). Biogel A5M was from Biorad (Hercules, California). Ultrapure urea was from ICN (Costa Mesa, California). Dibasic and monobasic potassium phosphate and magnesium chloride were from Fisher (Fair Lawn, New Jersey). Magnesium sul-

fate (puratronic) was from Alfa Aesar (Ward Hill, Massachusetts). KCl and NaCl were from Mallinckrodt (Paris, Kentucky). Stock urea solutions that were 10 M in urea were made in buffer A (50 mM potassium phosphate, pH 7.2, 100 mM KCl, 1 mM DTT) or in buffer B (50 mM MOPS, pH 7.2, 1 mM DTT) and quantitated as described previously (Pace et al., 1989). GroEL and EcDHFR were purified and quantitated as described (Ahrweiler & Frieden, 1991; Fisher, 1992; Hoeltzli & Frieden, 1994; Clark et al., 1998). The concentrations shown here for GroEL are those of the 14-mer.

Purification of single ring GroEL

Single ring GroEL (SR1) was produced by site-directed mutagenesis of the plasmid pHOG1 (Clark et al., 1996) bearing wild-type GroELS, to produce the plasmid pHOG1SR1 with the R452E, E461A, S463A, and V464A mutations described previously (Weissman et al., 1995). The mutations were confirmed by sequencing. SR1 was purified from *E. coli* BL21 (DE3) in a manner identical to that described for wild-type GroEL (Clark et al., 1998), except that the protein was eluted twice from the reactive red column in order to obtain SR1 free from contaminating tryptophan fluorescence. In addition, ATP was not included in the buffers. The concentrations shown here for SR1 are those of the 7-mer. The molecular weight of SR1 was estimated using a calibrated Sephadex G-200 sizing column (48 × 1 cm), which separates proteins in the range of 5,000–600,000 Da. The column was run at room temperature, allowing flow by gravity (~1 mL/min) and collecting 0.25 mL fractions. Wild-type GroEL or SR1 (200 μL of 7.5 mg/mL) was loaded onto the column and run in buffer A. Wild-type GroEL eluted in the void volume (13.0 mL), while SR1 co-eluted with the apoferritin (MW 443,000) MW marker (14.25–14.50 mL).

Purification of ADP

Nucleotides obtained from Sigma were further purified by ion-exchange chromatography using fast-flow Q-sepharose resin at room temperature. Approximately 200 mg of nucleotide was dis-

solved in 5 mL of buffer C (50 mM Tris-HCl, pH 8, 200 mM NaCl) and was loaded at a flow rate of 5 mL/min onto a Q-sepharose column (19 × 2.6 cm) that had been pre-equilibrated with buffer C. The column was washed with buffer C (30 mL) at a flow rate of 5 mL/min, and the nucleotide was eluted using a linear salt gradient (total volume of 1 L) from 200 to 600 mM NaCl (50 mM Tris-HCl, pH 8). Typically, ADP eluted at ~275 mM NaCl. The gradient was run at a flow rate of 4 mL/min, and 16 mL fractions were collected. The A_{260} was monitored for each fraction, and fractions within the major peak were pooled. The nucleotide was precipitated in 10 mL aliquots by the addition of three volumes of 100% ethanol and incubation overnight at -20°C . The pellets were collected by centrifugation at 10,000 RPM for 20 min (SS-34 rotor, 4°C), washed three times with 70% ethanol (cooled to -20°C), and allowed to dry. The pellets were dissolved either in buffer A or buffer B and stored at -20°C .

Stopped-flow fluorescence studies

DHFR (11 μM) was initially unfolded in 5 M urea-containing buffer A or buffer B. Refolding of EcDHFR in the presence of GroEL or SR1 was initiated by rapidly diluting EcDHFR 11-fold (SX.17MV stopped-flow spectrometer, Applied Photophysics Ltd., Leatherhead, UK) into buffer A or buffer B that contained GroEL or SR1. This experimental protocol has been described in detail (Clark & Frieden, 1997, 1999b). The final urea concentration was 0.45 M, and the final protein concentrations were 1 μM for both EcDHFR and GroEL. One should note that 1 μM GroEL 14-mer represents 2 μM binding sites for EcDHFR (Clark et al., 1996). The fluorescence emission was monitored using an excitation wavelength of 295 nm and a cutoff filter of 305 nm. For all experiments, the temperature was maintained at 22°C using a circulating water bath.

Data analysis

Data obtained from the stopped-flow experiments were fit to the sum of two exponentials ($A_t = A_{\infty} + \sum A_i k_i$). The amplitudes and apparent rate constants determined from the fits were replotted as a function of the concentrations of KCl, MgSO_4 , or ADP as indicated in the figures. The amplitudes (A_i) and apparent rate constants (k_{app}) were fit to Equations 1 or 2:

$$k_{app} = k_0 + (k_{\max} [S]^{n_H}) / (K_{0.5}^{n_H} + [S]^{n_H}) \quad (1)$$

$$A = A_0 + (A_{\max} [S]^{n_H}) / (K_{0.5}^{n_H} + [S]^{n_H}); \quad (2)$$

based on the Hill equation (Hill, 1910) in which k_0 and A_0 refer to the initial apparent rate constants or amplitudes, respectively; $k_{\max}$ and $A_{\max}$ refer to the maximum apparent rate constants or amplitudes, respectively; n_H refers to the Hill coefficient; $K_{0.5}$ refers to the substrate concentration that promotes one-half maximal change in amplitude or rate constant, that is, the midpoint of the curve; and S refers to the concentrations of KCl, MgSO_4 , or ADP, as indicated in the figures.

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