Effect of Free and ATP-Bound Magnesium and Manganese Ions on the ATPase Activity of Chaperonin $GroEL_{14}$[†]

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ABSTRACT: Hydrolysis of ATP by the $GroEL_{14}$ chaperonin oligomer is activated and modulated by Mg^{2+} or Mn^{2+} ions. Mg-ATP and Mn-ATP can serve as substrates of the reaction and bind in a positively cooperative manner to the same catalytic sites on $GroEL_{14}$, with similar binding constants in the micromolar range. In addition, millimolar amounts of Mg^{2+} and Mn^{2+} ions can further activate the $GroEL_{14}$-ATPase while interacting with low-affinity noncatalytic sites on the chaperonin. The extent of ATPase activation by Mn^{2+} is half of that by Mg^{2+} ions. When both Mg^{2+} and Mn^{2+} ions are present in the same reaction, Mn^{2+} behaves as a noncompetitive partial inhibitor of the Mg-dependent ATPase. This inhibition requires the presence of ADP in the catalytic site. The binding affinity of Mn-ADP to the site is significantly higher than that of Mg-ADP. A slower release of Mn-ADP from the catalytic site thus changes the rate-determining step of the $GroEL_{14}$-ATPase cycle. In the cell, the concentrations of Mg^{2+} and Mn^{2+} ions are such that both divalent ions may modulate chaperonin activity.

In *Escherichia coli* (*E. coli*), the GroE chaperonins mediate the folding of a large array of proteins (Viitanen et al., 1992). The core oligomer $GroEL_{14}$ spontaneously binds unfolded proteins thereby preventing protein misfolding and aggregation (Goloubinoff et al., 1989; Buchner et al., 1991). Depending on the nature of the folding protein and on parameters such as temperature and protein concentration, $GroEL_{14}$-bound proteins require Mg-ATP, K^+ ions, and the presence of co-chaperonin $GroES_7$ to dissociate from $GroEL_{14}$ in a folding-competent state (Goloubinoff et al., 1989; Viitanen et al., 1990; Martin et al., 1991; Buchner et al., 1991; Fisher, 1992; Schmidt et al., 1994a). The mechanism by which chaperonins release proteins in a form that is committed to folding remains largely obscure (Martin et al., 1991, 1993; Azem et al., 1994b; Schmidt et al., 1994b; Todd et al., 1994). The binding of ATP to $GroEL_{14}$ is cooperative (Gray & Fersht, 1991). The presence of the co-chaperonin $GroES_7$ increases the cooperativity of ATP binding (Viitanen et al., 1990; Bochkareva et al., 1992; Jackson et al., 1993) and decreases the rate of ATP hydrolysis by causing a shift in the rate-determining step of the reaction, from ATP hydrolysis to the dissociation of ADP from the active site (Todd et al., 1993, 1994). The role of ATPase in protein folding remains to be refined in a system where the characteristics of ATP hydrolysis by $GroEL_{14}$ can be modulated. Millimolar amounts of divalent ions such as Mg^{2+} and Mn^{2+} have been shown to have an effect on the molecular architecture of the $GroEL_{14}$ oligomers (Azem et al., 1994a). The purpose of this research is to further examine the effects of the divalent ions Mg^{2+} and Mn^{2+}, in both the micromolar and the millimolar range, on the kinetic parameters of the GroEL-ATPase activity and thus set the basis for future investigation of the effects by divalent ions on chaperonin-assisted protein folding *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Chaperonin Purification. $GroEL_{14}$ was purified from *E. coli* as described in Azem et al. (1994a). Protein concentration was determined by the Bio-Rad protein assay (Bradford, 1976). The standard curve of bovine serum albumin (Sigma) was corrected upward by a factor of 1.12, obtained from a total amino acid concentration analysis of pure GroEL. In this work, the concentration of GroEL is expressed as the molarity of the 57.3-kDa protomer and not of the 802-kDa $GroEL_{14}$ oligomer.

ATPase activity . Unless otherwise indicated, the ATPase activity of $GroEL_{14}$ was measured at 37 °C, using 0.6−3.7 μM GroEL (protomers) in 50 mM Tris-HCl, pH 7.5, 2−10 mM KCl (as indicated), 0.1−1.0 mM [γ-^{32}P]ATP (0.001−0.05 Ci $mmol^{-1}$), and $MgCl_2$ and/or $MnCl_2$ (as indicated). The unhydrolyzed ATP was separated from radioactive inorganic phosphate by adsorption to 5% charcoal suspended in 20 mM H_3PO_4 according to Bais (1975). Typically, the spontaneous degradation of ATP did not exceed 1.5%.

Calculations. The data for the velocity of the ATPase were fitted to the Hill equation, using the nonlinear regression method of the Enzfitter computerized program according to Leatherbarrow (1987). The following constants were derived: $k_{cat} = V_{max}/[GroEL]$, $K' = [S]_{0.5}{}^n$, and n_H = Hill coefficient. Initial rates were calculated using a linear

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[1] Abbreviations: EC_{50}, concentration that causes 50% of the observed effect; $[S]_{50}$, concentration of the substrate at which the reaction rate is half of its maximal value; IC_{50}, concentration of inhibitor that causes 50% inhibition of the observed effect; kDa, kilodalton.

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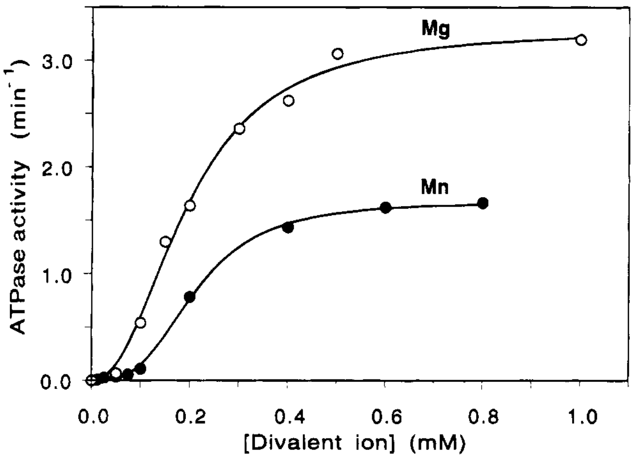

FIGURE 1: Mg^{2+} and Mn^{2+} activation of the $GroEL_{14}$-ATPase. The assay was performed as described in Experimental Procedures, using 1.12 μM GroEL, 0.5 mM ATP, and increasing amounts of Mg^{2+} (○) or Mn^{2+} (●). The reaction time was 15 min.

Table 1: Kinetic Constants of $GroEL_{14}$ ATPase.[a]

substrate components				Hill coefficient
variable	fixed	k_{cat} (min^{-1})	K' (mM)	(n_H)
Mg^{2+}	ATP	3.39 ± 0.22	0.022 ± 0.009	2.29 ± 0.25
Mn^{2+}	ATP	1.65 ± 0.04	0.020 ± 0.005	2.70 ± 0.76
ATP	Mg^{2+}	5.98 ± 0.18	0.047 ± 0.028	1.54 ± 0.26
ATP	Mn^{2+}	2.77 ± 0.10	0.036 ± 0.017	1.18 ± 0.13

[a] ATPase activity was measured as described in Experimental Procedures. The kinetic constants were calculated from eight different Mg/Mn concentration dependency curves and from six different ATP concentration dependency curves, using the Enzfitter computer program for nonlinear regression (Leatherbarrow, 1987).

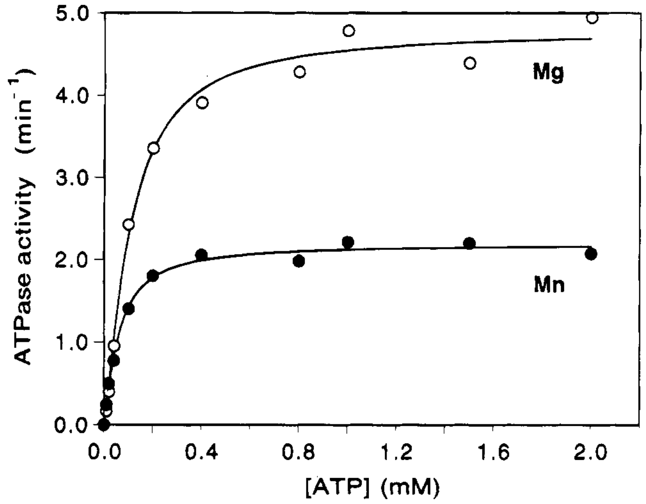

FIGURE 2: ATP concentration dependency of $GroEL_{14}$-ATPase in the presence of Mg^{2+} or Mn^{2+} ions (representative experiment). GroEL (1.12 μM) was incubated for 25 min in the presence of 3 mM Mg^{2+} (○) or Mn^{2+} (●) and increasing amounts of ATP (0–2 mM). The kinetic parameters from six repeated experiments are summarized in Table 1.

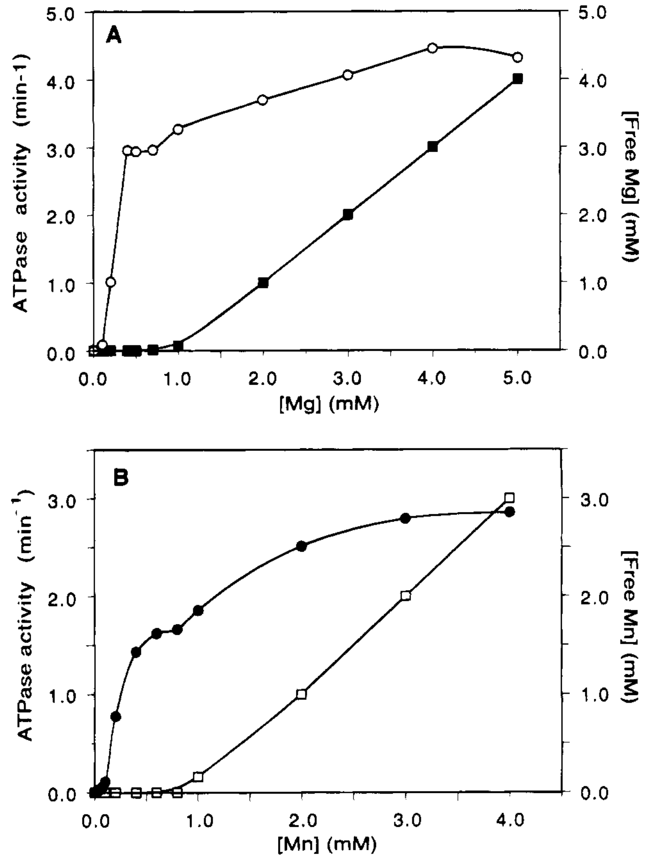

FIGURE 3: $GroEL_{14}$-ATPase activation by free Mg^{2+} and Mn^{2+} ions. GroEL (3.7 μM for Mg^{2+} and 1.12 μM for Mn^{2+}) was incubated for 25 min as in Figure 1, in the presence of 1 mM ATP and increasing amounts of Mg^{2+}(○) or Mn^{2+}(●). The fraction of free ions was calculated according to Walaas (1958). The predicted concentrations of free Mg^{2+} (■) and free Mn^{2+} (□) are plotted on the right abscissa of panels A and B, respectively.

regression of at least four time points. The concentrations of free Mn^{2+} and Mg^{2+} in the presence of various ATP concentrations were calculated according to the equilibrium constants established by Walaas (1958).

RESULTS

$GroEL_{14}$ Can Hydrolyze Mg-ATP or Mn-ATP. Both Mn^{2+} and Mg^{2+} ions are able to activate the hydrolysis of ATP by $GroEL_{14}$ (Figure 1A). However, the average k_{cat} value of the Mn-ATPase is half that of the Mg-ATPase (Table 1). The activation constants (K′) for Mn-ATP and Mg-ATP are similar (20 and 22 μM, respectively). The Hill transformation [see Segel (1975)] of the data shows that both Mg^{2+} and Mn^{2+} bind $GroEL_{14}$ in an allosteric manner (not presented), with at least three (possibly up to 14) binding sites for divalent cations per $GroEL_{14}$ that positively cooperate with one another ($n_H = 2.3$ and 2.7 for Mg^{2+} and Mn^{2+}, respectively) (Table 1).

The ATP concentration dependency curves of the ATPase in the presence of Mg^{2+} or Mn^{2+} confirm a 2-fold difference in the k_{cat} values (Figure 2). However, k_{cat} and K′ values are nearly twice as high, and the Hill coefficients are lower, as compared to the constants derived from the ion concentration dependency curves (Table 1). This difference could result from the presence of an excess of either ion over ATP, causing an additional activation of the chaperonin ATPase (see below).

Free Mn^{2+} or Mg^{2+} Ions Activate the $GroEL_{14}$-ATPase Activity. In a solution of 1 mM ATP and 0.8 mM Mg^{2+} or Mn^{2+}, all divalent ions are associated with ATP. Above 0.8 mM, the association constants for Mg-ATP and Mn-ATP (Walaas, 1958) predict an increasing fraction of free ion in the solution. A deflection in the ATPase activity curve near 0.8 mM is observed for Mg^{2+} (Figure 3A) and for Mn^{2+} (Figure 3B), which correlates with the appearance of

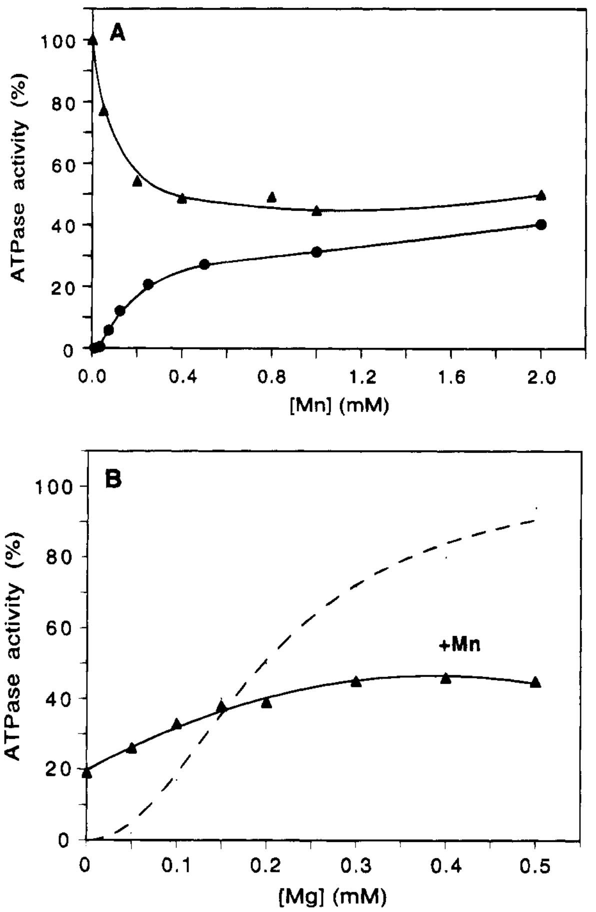

FIGURE 4: Inhibition of GroEL-ATPase by Mn^{2+}. GroEL (1.12 μM) was incubated for 15 min with 0.5 mM ATP (A) in the presence of increasing concentrations of Mn^{2+} (0−2 mM), without (●) or with 10 mM Mg^{2+} (▲), or (B) in the presence of increasing concentrations of Mg^{2+} (0−0.5 mM), without (dashed curve as in Figure 1) or with 0.1 mM Mn^{2+} (▲).

free ion in the solution. Thus, Mg^{2+} or Mn^{2+} ions that are not bound to ATP have an additional activatory effect on the $GroEL_{14}$-ATPase. The effective ion concentration that causes half of the maximal ATPase activation is 2.6 ± 0.6 mM for Mg^{2+} and 2.3 ± 1.0 mM for Mn^{2+} (calculated from three and five experiments, respectively). This ionic concentration is 2 orders of magnitude higher than that necessary to saturate the catalytic sites with either Mg-ATP or Mn-ATP, suggesting that divalent ions bind to additional noncatalytic activatory sites with relatively low affinity.

Mn^{2+} Inhibits the Mg-ATPase of $GroEL_{14}$. Figure 4A shows that even in the presence of a 153-fold excess of Mg^{2+} (10 mM), Mn^{2+} can inhibit the Mg-ATPase activity of $GroEL_{14}$ by 25%. Interestingly, as concentrations of Mn^{2+} increase, ATP hydrolysis either in the absence or in the presence of excess Mg^{2+} approaches the same value, which corresponds to half that of the uninhibited Mg-ATPase. The IC_{50} for the Mn-dependent inhibition of the Mg-ATPase is 65 μM, which is 3.5 times lower than the EC_{50} for the activation of ATPase by Mn^{2+} alone (234 μM). Figure 4B further demonstrates that Mn^{2+} acts as a partial inhibitor. When the Mg^{2+} concentration is below 65 μM, 100 μM Mn^{2+} results in a net activation of the ATPase. However, when the Mg^{2+} concentration exceeds 65 μM, Mn^{2+} has an inhibitory effect which competes with the activatory effect of either ion. As the concentration of Mg^{2+} increases, the extent of inhibition by Mn^{2+} is not reduced as would be expected if Mn^{2+} was a simple competitor of Mg-ATP.

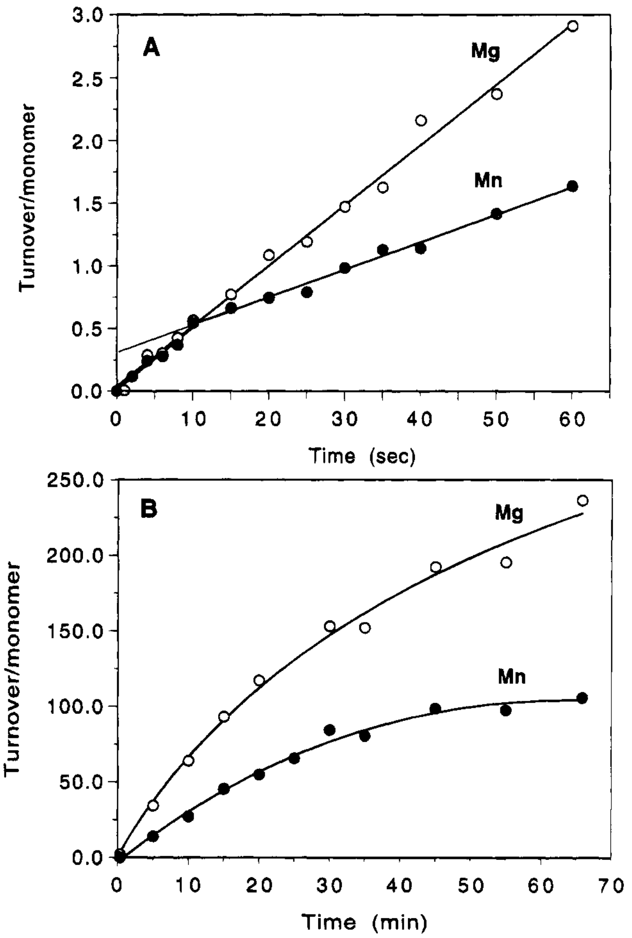

FIGURE 5: Time course of Mg^{2+}- and Mn^{2+}-activated $GroEL_{14}$-ATPase. (A) Initial and steady-state rates: GroEL (3.36 μM) was incubated at 25°C with 0.2 mM ATP, 10 mM KCl, and 4 mM Mg^{2+} (○) or Mn^{2+} (●). The lines were calculated by linear regression analysis, for two time intervals independently (0−10 and 10−60 s). (B) Long term rates: GroEL (1.12 μM) was incubated at 37°C with 0.5 mM ATP, 1 mM KCl, and 10 mM Mg^{2+} (○) or 4 mM Mn^{2+} (●).

This inhibition by Mn^{2+} is reversible, since $GroEL_{14}$ molecules that were preincubated with an excess of Mn^{2+} (5 mM) and subsequently dialyzed against Mg^{2+} (20 mM) recovered full, uninhibited Mg-dependent ATPase activity (not shown).

The First Turnover of the ATPase Cycle Occurs at a Similar Rate in the Presence of Mg^{2+} or Mn^{2+}. In the presence of Mg^{2+} alone, the first turnover occurs at the same rate as the following turnovers of the enzyme (0.096 s^{-1}, Figure 5A). In the presence of Mn^{2+}, however, the rates of the second and subsequent turnovers become inhibited by half (0.038 s^{-1}). This indicates that the Mn-induced inhibition depends on the presence of ADP in the catalytic site. The extrapolation of the Mn steady-state rate to $t = 0$ suggests that, in the presence of Mn^{2+}, only seven of the 14 catalytic sites can hydrolyze ATP at a time.

Mn^{2+} Increases the Affinity of ADP to the Catalytic Site. When $GroEL_{14}$ in the presence of Mg^{2+} alone is allowed to hydrolyze over 50% of the ATP, the rate of the ATPase is reduced by 60% (0.060 s^{-1}, Figure 5B). In contrast, when $GroEL_{14}$ in the presence of Mn^{2+} alone was allowed to hydrolyze less than 25% of the ATP, the ATPase already

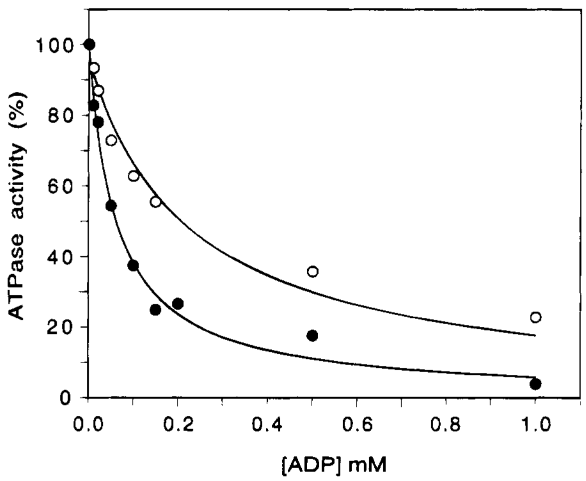

FIGURE 6: ADP inhibition of GroEL Mg- and Mn-dependent ATPase. GroEL (1.12 μM) was incubated for 6 min with 0.1 mM ATP, 1 mM Mg^{2+} (○) or Mn^{2+} (●), and increasing amounts of ADP (0−1 mM). The curves are the best computerized fits (99%) of the data to the equation predicting a simple competitive inhibition (Segel, 1975).

appeared fully inhibited. This suggests that the inhibition by ADP of the $GroEL_{14}$-ATPase is promoted by Mn^{2+} ions.

The inhibitory effect of ADP on the ATPase activity of $GroEL_{14}$ was measured in the presence of Mn^{2+} or Mg^{2+} ions. Figure 6 shows that Mn-ADP is a more potent inhibitor than Mg-ADP: the IC_{50} values are 36 and 205 μM for Mn-ADP and Mg-ADP, respectively.

DISCUSSION

Mg^{2+} is an essential component of the substrate complex of the $GroEL_{14}$-ATPase. Azem et al. (1994a) showed a Mg concentration dependence curve of the $GroEL_{14}$-ATPase. We show here that Mn^{2+} can mimic in some respects, and modify in others, the Mg-dependent ATPase activity of $GroEL_{14}$. Both Mg^{2+} and Mn^{2+} ions bind to the catalytic sites as Mg-ATP and Mn-ATP complexes, with high affinity and in a strongly cooperative manner. In addition, Mg^{2+} and Mn^{2+} can further activate the $GroEL_{14}$-ATPase by interacting as free ions with low-affinity, noncatalytic sites. Azem et al. (1994a) reported that millimolar amounts of either Mg^{2+}, Mn^{2+}, Ca^{2+} or Zn^{2+} enhance the velocity of chemical cross-linking between the subunits of $GroEL_{14}$. This was attributed to the effect of divalent ions on contacts between the GroEL monomers within the $GroEL_{14}$ oligomer. Possibly, the additional activatory effect on the ATPase by millimolar amounts of free Mg^{2+} or Mn^{2+} ions that we report here is an expression of the same effect of divalent ions on the quaternary structure of the chaperonin oligomer.

Mn-ATP is hydrolyzed by $GroEL_{14}$ at about half the rate of Mg-ATP. This observation can be attributed to any combination of less efficient binding, decreased rate of hydrolysis of Mn-ATP in the catalytic site, and slower release of Mn-ADP from the catalytic site. Since the activation constants for Mn-ATP and Mg-ATP are similar, a less efficient binding of Mn-ATP than Mg-ATP is unlikely to justify the difference in rates.

We showed here that, during the first turnover, hydrolysis occurs at the same rate for Mn-ATP as for Mg-ATP. Thus, less efficient hydrolysis of Mn-ATP than Mg-ATP does not appear to be the cause for the lower rates of the Mn-dependent ATPase. The fact that the subsequent turnovers are inhibited in the presence of Mn^{2+} ions suggests that Mn-ADP remains in the active site longer than Mg-ADP. A direct inhibition experiment confirmed that Mn-ADP is a more potent inhibitor of the ATPase than Mg-ADP. Thus, the rate-limiting step of the ATPase cycle for the Mn-activated $GroEL_{14}$ is the slow dissociation of ADP from the catalytic site, and not the ATP hydrolysis, as in the case of the Mg-activated $GroEL_{14}$ (Todd et al., 1993).

In addition, the inhibition imposed by low concentrations of Mn^{2+} on the Mg-ATPase activity of $GroEL_{14}$ cannot be overcome by high concentrations of Mg^{2+}. This indicates that the Mn-induced inhibition is due not only to a simple competition between Mn-ATP and Mg-ATP, or between Mn-ADP and Mg-ATP, for the same catalytic sites but to the effect of Mn^{2+} through an additional noncatalytic site.

Initial velocity measurements of the $GroEL_{14}$-ATPase suggest that the binding of Mn^{2+} promotes the transition of half of the sites to a form which is transiently incapable of hydrolyzing either Mg- or Mn-ATP, whereas the seven remaining sites appear to function similarly in the presence of either ion. Alternatively, Mn^{2+} equally affects the rate of ATP hydrolysis of all 14 sites, once the first turnover has occurred. Todd et al. (1993) have shown that in the presence of $GroES_7$ 7 mol of ADP is trapped on $GroEL_{14}$ in a form that is not readily exchangeable, while the seven remaining sites turn over ATP at the same rate as in the absence of $GroES_7$. The overall decrease of 50% in the rate of ATP hydrolysis by the oligomer was accompanied by a change in the rate-limiting step of the ATPase cycle from the ATP hydrolysis to the dissociation of Mg-ADP from the catalytic site. We find here that Mn^{2+} ions affect the rate-limiting step of the ATPase cycle in a similar manner, by increasing the affinity of Mn-ADP to the site.

We have shown here that both Mg^{2+} and Mn^{2+} ions exert multiple effects on the function of the $GroEL_{14}$ ATPase. As part of the substrate complex with ATP, Mg^{2+} and Mn^{2+} ions are essential activators of the GroEL ATPase. In addition, free Mg^{2+} or Mn^{2+} ions can bind to low-affinity activatory sites on $GroEL_{14}$, which can be correlated with changes in the quaternary structure of the chaperonin (Azem et al., 1994a). Finally, Mn^{2+} inhibits ATP hydrolysis in a noncompetitive, ADP-dependent manner. The effect of Mn^{2+} ions on chaperonin-assisted protein folding is now under investigation.

Activation and inhibition by Mg^{2+} and Mn^{2+} ions is not unique for the ATPase of $GroEL_{14}$. Similar effects of divalent ions were reported for other ATPases such as the heat-shock protein ClpB from *E. coli* (Woo et al., 1992), the CF_1 of spinach chloroplast F_1 ATPase (Hochman et al., 1976), and an ATPase from sheep kidney medulla (Gantzer & Grisham, 1979). In the case of the CF_1 ATPase, Mg^{2+} is a potent inhibitor when ADP is tightly bound to the catalytic site (Guerrero et al., 1990).

Escherichia coli cells may contain between 20 and 40 mM Mg^{2+} ions and between 0.1 and 0.3 mM Mn^{2+} ions. The concentration of Mg^{2+} significantly decreases during the transition from logarithmic to stationary growth (De Medicis et al., 1986), which corresponds to a starvation stress (Ulitzur & Kuhn, 1988). Furthermore, this transition is accompanied by an even sharper decrease in cellular Mn^{2+} and an increase in GroEL concentrations (up to 500 μM; Hemmingsen et al., 1988). It is tempting to speculate that quantitative and qualitative changes in the cellular balance between Mg^{2+} and Mn^{2+} ions during stress modulate the activity of the GroE chaperonins and thus control one of the molecular mechanisms by which cells protect themselves and recover from stress.

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