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Increased Efficiency of GroE-assisted Protein Folding by Manganese Ions*

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This study addresses the role of ATP-bound and free Mg^{2+} and Mn^{2+} ions in the activation and modulation of chaperonin-assisted refolding of urea-denatured malate dehydrogenase. As compared with Mg^{2+}, Mn^{2+} ions caused a significant increase in the rate of GroE-assisted malate dehydrogenase refolding and, concomitantly, a decrease in the rate of ATP hydrolysis. Moreover, Mn^{2+} increases the affinity of GroES for GroEL, even in the presence of saturating amounts of Mg^{2+}. Chemical cross-linking showed that lower concentrations of Mn-ATP as compared with Mg-ATP are needed to form both asymmetric $GroEL_{14}GroES_7$ and symmetric $GroEL_{14}(GroES_7)_2$ particles. The manganese-dependent increase in the rate of protein folding concurred with a specific increase in the amount of symmetric $GroEL_{14}(GroES_7)_2$ particles detected in a chaperonin solution. Thus, Mn^{2+} is a cofactor that can markedly increase the efficiency of the chaperonin reaction *in vitro*. Mn^{2+} ions can serve as an important tool for analyzing the molecular mechanism and the structure of chaperonins.

In *Escherichia coli*, chaperonins GroEL and GroES assist the folding and assembly of a large array of proteins (Goloubinoff *et al.*, 1989a; Viitanen *et al.*, 1992; Martin *et al.*, 1992; Horwich *et al.*, 1993). Purified $GroEL_{14}$ and $GroES_7$ oligomers assist the correct refolding of nonnative polypeptides by preventing protein aggregation (Goloubinoff *et al.*, 1989b; Buchner *et al.*, 1991). Nonnative proteins are proposed to interact with hydrophobic surfaces on the $GroEL_{14}$ core chaperonin (Pelham, 1986; Goloubinoff *et al.*, 1989b, 1991; Fenton *et al.*, 1994). Under "nonpermissive" conditions (Schmidt *et al.*, 1994), co-chaperonin $GroES_7$ is required for the release of the bound protein from the chaperonin in a state that is committed to the correct folding pathway (Goloubinoff *et al.*, 1989b; Martin *et al.*, 1991; Mendoza *et al.*, 1991).

A full understanding of the molecular mechanism by which GroEL and GroES chaperonins assist the refolding of a nonnative protein requires further analysis of the correlations between the affinity of ATP, ADP, $GroES_7$, and unfolded proteins for the $GroEL_{14}$ and $GroEL_{14}GroES_7$ oligomers during the protein folding reaction. In addition, the energy requirement of the protein folding reaction needs further investigation. Under nonpermissive conditions, protein folding absolutely depends on ATP hydrolysis (Goloubinoff *et al.*, 1989b; Schmidt *et al.*, 1994). Refolding of one protomer of rhodanese or Rubisco was estimated to require the hydrolysis of approximately 130–100 ATP molecules (Martin *et al.*, 1991; Azem *et al.*, 1995). However, ATP hydrolysis does not reciprocally depend on protein folding, because $GroEL_{14}$ alone or in association with $GroES_7$ hydrolyzes ATP even in the absence of unfolded proteins (Hendrix, 1979). An increase in the rate of ATP hydrolysis by $GroEL_{14}$ has been observed in the presence of guanidium HCl denatured proteins (Martin *et al.*, 1991). However, this increase has been attributed to the guanidium HCl rather than to the unfolded proteins (Todd and Lorimer, 1995). The protein folding process was therefore suggested to take advantage of, rather than activate, a futile cycle of ATPase by the chaperonin (Todd *et al.*, 1993, 1994; Martin *et al.*, 1993). Nevertheless, five GroEL mutants with reduced ATPase but unaffected protein folding activities have been reported (Fenton *et al.*, 1994), pointing out that the energy cost of the chaperonin folding reaction can be lowered. Therefore, the possibility remains that the *in vitro* chaperonin reaction is not optimal and can be improved by mutagenesis or by cofactors.

In this study, we describe such a cofactor, Mn^{2+} ions. Even in the presence of excess Mg^{2+}, Mn^{2+} ions decrease the rates of ATP hydrolysis while at the same time increasing the rate of protein folding and the affinity of GroES for GroEL. Thus, Mn^{2+} significantly increases the efficiency of the chaperonin reaction.

MATERIALS AND METHODS

Chaperonin Purification—$GroEL_{14}$ was purified as described by Azem *et al.* (1994a). $GroES_7$ was purified according to Todd *et al.* (1993) with small modifications. In this article, the concentration of chaperonins is expressed as the molarity of the 57.3-kDa GroEL protomer and of the 10.0-kDa GroES protomer.

Chaperonin Activity—Mitochondrial malate dehydrogenase (mMDH)[1] from pig heart (Boehringer Mannheim) was used as a substrate in the GroE-assisted protein folding reaction. mMDH (18 μM) was denatured at 25 °C for 3–4 h in 6 M urea containing 1 mM EDTA and 10 mM dithiothreitol. Renaturation was initiated by a 70-fold dilution of the urea-treated mMDH into 50 mM triethanolamine, pH 7.5, 20 mM KCl, 10 mM dithiothreitol, 2 mM phosphoenolpyruvate, pyruvate kinase (20 μg/ml, Sigma), and $GroEL_{14}$, $GroES_7$, ATP, $MgAc_2$, or $MnAc_2$, as indicated. Pyruvate kinase was found to be equally active in the presence of Mn^{2+} and/or Mg^{2+} ions (not shown). Because no spontaneous refolding of urea-denatured mMDH occurs at 37 °C, all the chaperonin-assisted refolding assays of mMDH were performed at 37 °C.

Malate Dehydrogenase Activity—The activity of mMDH was assayed at 25 °C in 150 mM potassium phosphate buffer, pH 7.5, 10 mM dithiothreitol, 0.5 mM oxaloacetate, and 0.28 mM NADH (Sigma). The time-dependent oxidation of NADH by mMDH was monitored at 340 nm. The refolding activity was expressed in nanomolar mMDH refolded per micromolar GroEL monomer per minute (units). The enzymatic activity of mMDH is not affected by the concentrations of the Mg^{2+} and Mn^{2+}

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[1] The abbreviations used are: mMDH, mitochondrial malate dehydrogenase; $MgAc_2$, magnesium acetate; $MnAc_2$, manganese acetate; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

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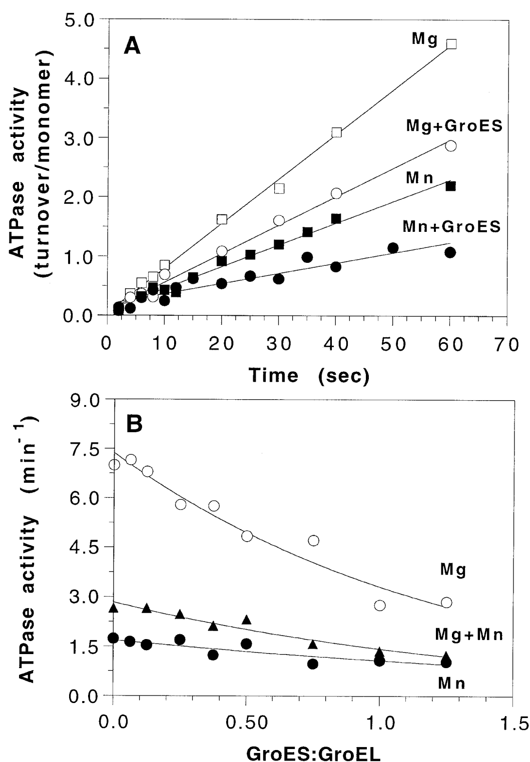

FIG. 1. **Effect of Mn^{2+} and GroES on the GroEL ATPase.** *A*, time-dependent hydrolysis of ATP. GroEL (3 μM protomers) was incubated at 25 °C with (*circles*) or without (*squares*) GroES (9 μM protomers) in the presence of 0.2 mM [γ-^{32}P]-ATP, 20 mM KCl, and 4 mM $MgAc_2$ (○ and □) or $MnAc_2$ (● and ■). The initial rates of the ATPase activity, expressed as the number of turnovers per GroEL monomer, were derived from a linear regression analysis. *B*, GroES-dependent hydrolysis of ATP. GroEL (2 μM) was incubated for 17 min at 37 °C with increasing concentrations of GroES in the presence of 1 mM ATP and 10 mM $MgAc_2$ (○) or 2 mM $MnAc_2$ with ( ) or without (●) 10 mM $MgAc_2$.

ions investigated here (not shown). Despite its mammalian origin, dimeric mMDH undergoes inactivation when incubated at 37 °C in a purified and diluted form for more than 30 min. However, this effect was negligible in the presence of GroEL, GroES, and ATP and within the time range of the protein folding assays described in this article.

ATPase Activity—The hydrolysis of ATP by chaperonins was measured as described by Diamant *et al.* (1995). Unless specified otherwise, the temperature of the assay was 37 °C. Unhydrolyzed [γ-^{32}P]ATP was separated from released γ-$^{32}P_i$ by adsorption on activated charcoal as in Bais (1975).

Cross-linking of GroEL-GroES Oligomers—Native $GroEL_{14}$ and $GroES_7$ in 50 mM triethanolamine, pH 7.5, 20 mM KCl, 2 mM phosphoenolpyruvate, 20 μg/ml of pyruvate kinase (Sigma), ATP and $MgAc_2$ or $MnAc_2$, as indicated, was reacted with 0.22% glutaraldehyde for 60 min at 37 °C. The cross-linking reaction was terminated by the addition of ⅓ volume of 1 M Tris-glycine, pH 7.5, 4% SDS, and 10% β-mercaptoethanol and boiling for 3 min. Samples were separated on 2.8% acrylamide gel (tubes) and stained with Coomassie Brilliant Blue. Quantitation of cross-linked species was by densitometry as described in Azem *et al.* (1994a, 1994b).

RESULTS

Mn^{2+} and GroES Inhibit GroEL ATPase in an Additive Manner—In the presence of 4 mM Mn^{2+}, the rate of ATP hydrolysis by the GroEL alone was half the rate in the presence of 4 mM Mg^{2+} (2.21 and 4.53 min^{-1}, respectively) (Fig. 1*A*). The addition of a 3-fold molar excess of GroES increased the inhibition of the GroEL ATPase to 77% (1.04 min^{-1}). In comparison, under the same conditions, GroES caused an inhibition of 36% in the presence of Mg^{2+} alone (2.89 min^{-1}). Thus, GroES and Mn^{2+} ions inhibit the GroEL-ATPase in an additive manner.

In the presence of saturating concentrations of ATP (1 mM) and Mg^{2+} (10 mM), increasing concentrations of GroES caused a maximal inhibition of 60% of the GroEL-ATPase activity (Fig. 1*B*). Remarkably, the addition of 2 mM Mn^{2+} further increased the inhibition of the ATPase by GroES to 82%, approaching the level of inhibition in the presence of 2 mM Mn^{2+} alone (85%). Thus, Mn^{2+} acts as an inhibitor of the magnesium-dependent ATPase activity of GroEL in the presence of GroES.

The Rate of Protein Folding Is Higher in the Presence of Mn^{2+}—The initial rate of mMDH refolding in the presence of Mn^{2+} (2 mM) and a limiting amount of ATP (22.5 μM) was 5-fold higher than in the presence of the same amount of Mg^{2+} ions (Fig. 2*A*). Because an ATP regeneration system was present, the reaction could be carried out for long periods of time (1 h), in which case the difference in the rates decreased. The activity of the regeneration system was not different in the presence of Mg^{2+} or Mn^{2+} ions, as determined by the direct measurement of pyruvate kinase activity (not shown). The time curve patterns of mMDH refolding were similar whether ADP or ATP was used in the reaction (not shown), demonstrating that the ATP regeneration system was fully active under the conditions of the assay. The sigmoidal shape of the mMDH refolding curve in the presence of either Mg^{2+} (2 mM) or Mn^{2+} (2 mM) is consistent with a consecutive uni-bimolecular reaction mechanism (Miller *et al.*, 1993).

As in the case of the GroEL ATPase, divalent ions are essential to the protein folding activity of GroE chaperonins. In contrast to the 2-fold inhibition of the chaperonin ATPase by Mn^{2+} (Fig. 1), the manganese-dependent rate of mMDH folding was twice as high as the magnesium-dependent rate of protein folding (Fig. 2*B*). Moreover, Mg^{2+} and Mn^{2+} activated mMDH refolding in a biphasic manner. This suggests that chaperonin-dependent protein folding is activated by micromolar amounts of either Mg-ATP or Mn-ATP and also by millimolar amounts of free Mg^{2+} or Mn^{2+}, as in the case of the $GroEL_{14}$ ATPase (Diamant *et al.*, 1995).

ATP Supports mMDH Refolding More Efficiently in the Presence of Mn^{2+}—In the presence of low concentrations of ATP and of an active ATP regeneration system, Mn^{2+} supported the chaperonin-dependent refolding of mMDH more efficiently than Mg^{2+} (Fig. 2*C*). Thus, 11 μM ATP in the presence of Mn^{2+} could drive half of the maximal rate of the mMDH refolding. In contrast, 38 μM ATP were necessary to drive the same half-maximal rate of mMDH refolding in the presence of the same amount of Mg^{2+}.

Mn^{2+} Increases the ATP-dependent Binding of GroES to GroEL—The binding of GroES to GroEL in the presence of Mn^{2+} or Mg^{2+} as a function of ATP concentration was measured after chemical cross-linking (Fig. 3). In the presence of 2 mM Mg^{2+}, at least 25 μM ATP was required to drive the complete binding of one $GroES_7$ to $GroEL_{14}$ (Fig. 3*A*, *lane 4*), but 75 μM ATP did not suffice to initiate the binding of a second $GroES_7$ to the $GroEL_{14}GroES_7$ particle (Fig. 3*A*, *lane 5*). In contrast, in the presence of 2 mM Mn^{2+}, the maximal binding of one $GroES_7$ to $GroEL_{14}$ was obtained in the presence of as little as 2 μM ATP (Fig. 3*B*, *lane 2*), and 25 μM ATP sufficed to enable most of the binding of a second $GroES_7$ to $GroEL_{14}GroES_7$ particles (Fig. 3*B*, *lane 4*).

Increased affinity of GroES for GroEL in the presence of Mn^{2+} as compared with Mg^{2+} was demonstrated in Fig. 4. In the presence of excess Mg^{2+} (20 mM), higher concentrations of GroES were required to drive the chaperonin-dependent refolding of mMDH than in the presence of the same amount of Mg^{2+}, supplemented with 10-fold less Mn^{2+} (2 mM). Thus, in the presence of Mg^{2+} alone, a GroES/GroEL molar ratio ($R_{S/L}$) of 1.1 could drive half of the maximal rate of mMDH refolding,

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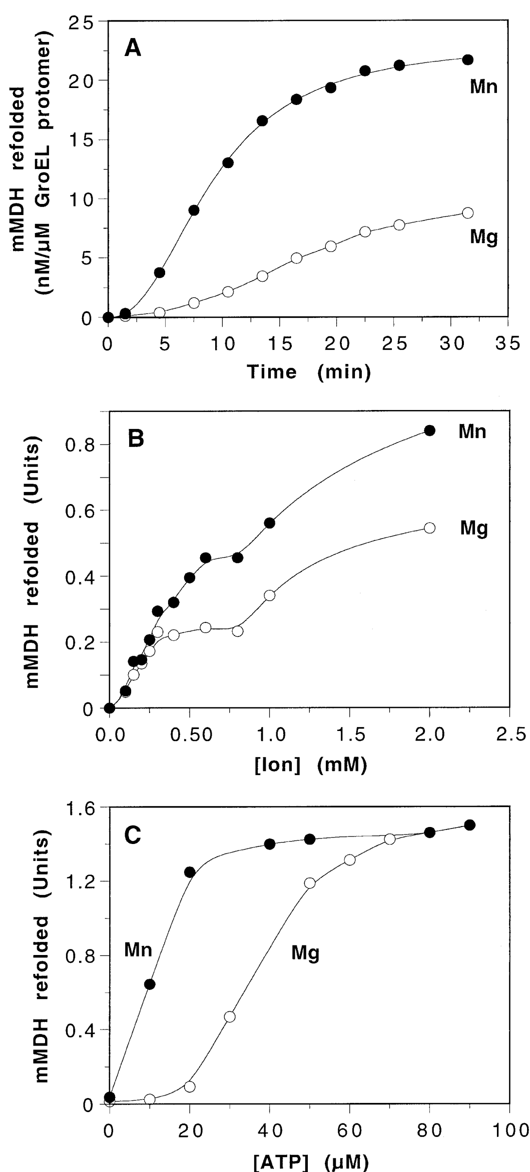

FIG. 2. **Effect of Mn^{2+} on chaperonin-assisted protein folding.** *A*, time-dependent protein folding. Urea-denatured mMDH was diluted 70-fold to a final monomer concentration of 257 nM in buffer containing GroEL (2 μM protomers), GroES (6 μM protomers), 2 mM $MgAc_2$ (○) or $MnAc_2$ (●), and ATP (22.5 μM). Aliquots were assayed for mMDH activity at the indicated time points. *B*, protein refolding depends on Mg^{2+} or Mn^{2+} concentrations. Denatured mMDH was diluted as in *A* in buffer containing GroEL (2 μM), GroES (3 μM), ATP (0.75 mM), and increasing concentrations of $MgAc_2$ (○) or $MnAc_2$ (●). Samples were incubated for 8 min and assayed for mMDH activity. *C*, ATP concentration-dependent refolding. Denatured mMDH was diluted as in *A* in a buffer containing GroEL (2 μM), GroES (6 μM), $MgAc_2$ (2 mM) (○) or $MnAc_2$ (●), and increasing concentrations of ATP. Samples were incubated for 8 min and assayed for mMDH activity.

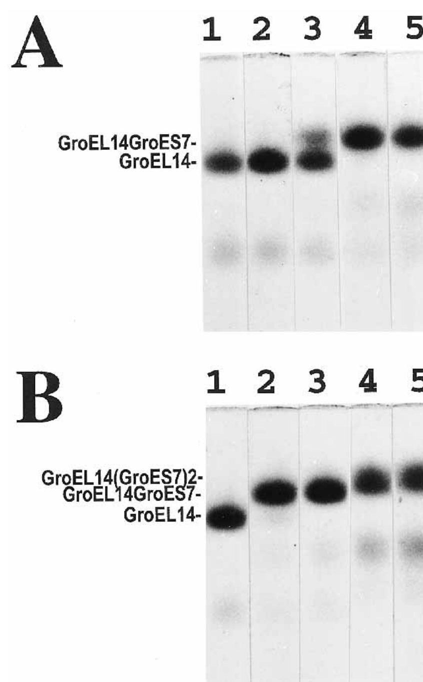

FIG. 3. **ATP concentration dependence of GroES binding to GroEL.** GroEL (3 μM) was first incubated as in Fig. 2*C* with GroES (9 μM) in the presence of 0, 2, 6, 25, and 75 μM ATP (*lanes 1–5*, respectively) and $MgAc_2$ (*A*) or $MnAc_2$ (*B*) and then cross-linked with glutaraldehyde and separated on SDS-polyacrylamide gels (see "Materials and Methods").

whereas in the presence of Mg^{2+} and Mn^{2+}, a $R_{S/L}$ of only 0.75 was able to drive the same effect (Fig. 4*A*).

Under the same conditions, the effect of Mn^{2+} on the chaperonin ATPase activity in the presence of increasing amounts of GroES was measured (Fig. 4*B*, *inset*). The number of ATP molecules hydrolyzed per refolded mMDH (Fig. 4*B*) was found to be about 20 times higher than in the case of rhodanese (Martin *et al.*, 1991) or Rubisco (Azem *et al.*, 1995).

In addition, regardless of the nature of the divalent ions, an increase in GroES concentration caused a decrease of the energy cost of the folding reaction. Thus, in the presence of Mg^{2+} alone, the reaction was 74-fold less efficient at $R_{S/L} = 0.4$ than at $R_{S/L} = 2.0$. Remarkably, Mn^{2+}, even in the presence of a 10-fold excess of Mg^{2+}, increased the efficiency of the reaction, particularity when GroES was substoichiometric to GroEL. For example, at $R_{S/L} = 0.4$, the efficiency of the reaction was 21-fold higher in the presence of 2 mM Mn^{2+} and 20 mM Mg^{2+} than in the presence of 20 mM Mg^{2+} alone (Fig. 4*B*, *inset*). However, Mn^{2+} improved the chaperonin efficiency by 3-fold for all $R_{S/L} \geq 1.0$.

Mn^{2+} Ions Increase the Amount of Symmetric $GroEL_{14}(GroES_7)_2$ Particles—Chemical cross-linking confirmed that GroES has a higher affinity for GroEL in the presence of Mn^{2+} and a 10-fold excess of Mg^{2+}, as compared with the presence of Mg^{2+} alone. In the presence of Mn^{2+} and Mg^{2+}, less GroES was necessary to populate by half the chaperonin solution with $GroEL_{14}(GroES_7)_2$ particles ($EC_{50} = 0.75$) than in the presence of Mg^{2+} alone ($EC_{50} = 1.1$) (Fig. 4*C*). A corresponding decrease by Mn^{2+} ions of the GroES concentration necessary to drive protein folding activity at half the maximal rate was observed (Fig. 4*A*). Thus, whereas Mn^{2+} ions increase the affinity of $GroES_7$ for both $GroEL_{14}$ (not shown) and $GroEL_{14}GroES_7$ particles (Fig. 4*C*), the manganese-dependent increase in the rate of protein folding correlates with the particular increase in the affinity of $GroES_7$ for the asymmetric $GroEL_{14}GroES_7$ chaperonin hetero-oligomer.

DISCUSSION

Since the first *in vitro* assay for the chaperonin-assisted refolding of a nonnative protein, Rubisco, by $GroEL_{14}$, $GroES_7$, and Mg-ATP (Goloubinoff *et al.*, 1989b), various additional co-factors have been described. Thus, K^+ or ammonium ions are essential for ATP hydrolysis and GroE-assisted protein folding (Viitanen *et al.*, 1990; Todd *et al.*, 1993). ATP analogues inhibit protein folding in the presence of chaperonins (Staniforth *et al.*, 1994; Miller *et al.*, 1993). Here, we present evidence that ATP-bound and free Mg^{2+} and Mn^{2+} ions affect the affin-

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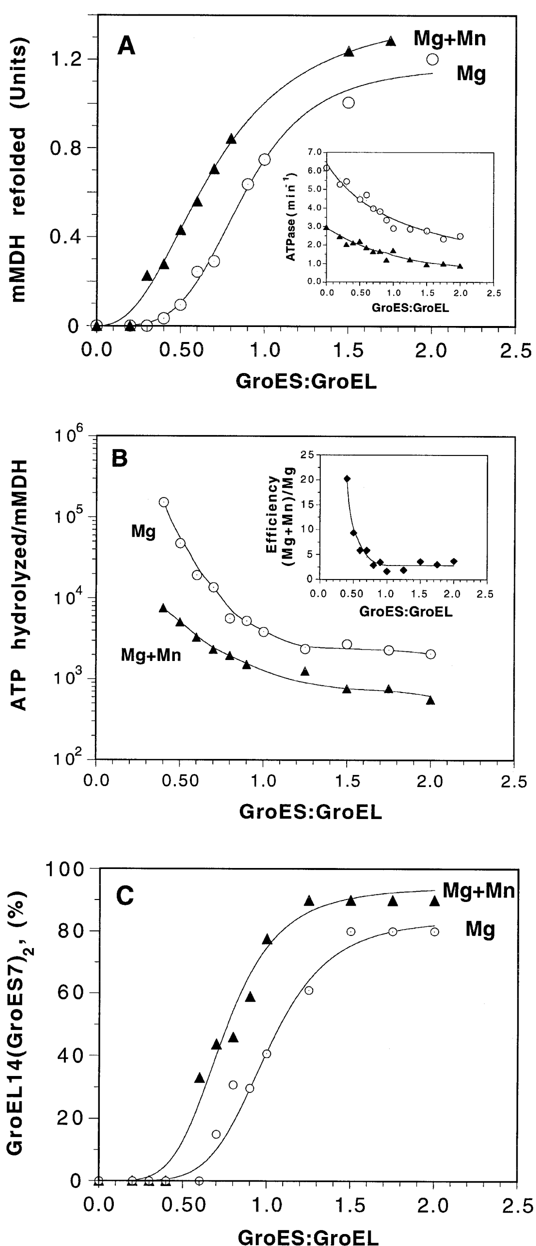

FIG. 4. **Mn^{2+} ions increase the affinity of $GroES_7$ to $GroEL_{14}$.** Urea-denatured mMDH was diluted as in Fig. 2 in buffer containing increasing amounts of GroES, GroEL (1.75 µM), ATP (0.75 mM), and $MgAc_2$ (20 mM) or $MgAc_2$ (20 mM) supplemented by $MnAc_2$ (2 mM). *A*, GroES-dependent protein refolding activity and chaperonin ATPase activity (*inset*) in the presence of $MgAc_2$ and $MnAc_2$ ( ) or in the presence of $MgAc_2$ alone (○). *B*, GroES-dependent energy cost of chaperonin reaction expressed as the number of ATP molecules hydrolyzed per refolded mMDH in the presence of $MgAc_2$ and $MnAc_2$ ( ) or in the presence of only $MgAc_2$ (○). *Inset*, GroES-dependent relative efficiency of chaperonin activity in the presence of $MgAc_2$ and $MnAc_2$ compared with the efficiency in the presence of $MgAc_2$ alone (♦). *C*, GroES-dependent formation of $GroEL_{14}(GroES_7)_2$ chaperonin hetero-oligomers in the presence of $MgAc_2$ and $MnAc_2$ ( ) or in the presence of only $MgAc_2$ (○) measured by cross-linking with glutaraldehyde and SDS electrophoresis as in Fig. 3 (gel not shown).

ity of GroES for GroEL and the rates of chaperonin ATPase and of protein folding.

Micromolar amounts of ATP-bound Mg^{2+} or Mn^{2+} have been previously shown to activate ATP hydrolysis by $GroEL_{14}$ (Azem *et al.*, 1994a; Diamant *et al.*, 1995). In addition, millimolar amounts of free Mg^{2+} and Mn^{2+} were shown to stabilize the quaternary structure of the $GroEL_{14}$ (Azem *et al.*, 1994a) and further activate the ATPase of $GroEL_{14}$ (Diamant *et al.*, 1995). Free Mn^{2+} was also suggested to interact with a high affinity allosteric site on $GroEL_{14}$, inhibiting the ATPase activity by half, even in the presence of a large excess of Mg^{2+} ions (Diamant *et al.*, 1995).

We show here that Mn^{2+} ions and $GroES_7$ use distinct mechanisms to inhibit the ATPase activity of the $GroEL_{14}$ core oligomer. Despite this inhibition, the rate of chaperonin-dependent refolding of mMDH was higher in the presence of Mn^{2+} and an excess of Mg^{2+} than in the presence of Mg^{2+} ions alone. This implies that the efficiency of the chaperonin reaction can be dramatically improved *in vitro* by a co-factor, such as Mn^{2+}.

GroES directly controls the energy cost of the chaperonin reaction. Thus, at $R_{S/L} = 0.5$, the refolding of a mMDH molecule required the hydrolysis of 20 times more ATP molecules than at $R_{S/L} = 1.25$ (Fig. 4*B*). This difference is reduced to 2.9-fold when 2 mM Mn^{2+} is added to 20 mM Mg^{2+}. Chemical cross-linking revealed that at $R_{S/L} = 0.5$, the chaperonin solution was populated by a majority of asymmetric $GroEL_{14}GroES_7$ particles (Azem *et al.*, 1994b, 1995). In contrast, at $R_{S/L} = 1.25$, the chaperonin solution was populated by a majority of symmetric $GroEL_{14}(GroES_7)_2$ particles (Fig. 4*C*).

Although Mn^{2+} ions increase the affinity of $GroES_7$ for both $GroEL_{14}$ and $GroEL_{14}GroES_7$ particles, we found that the rate of mMDH refolding precisely correlates with the amount of symmetric $GroEL_{14}(GroES_7)_2$ particles in the solution, confirming that the formation of symmetric $GroEL_{14}(GroES_7)_2$ particles is rate-determining for the protein folding reaction (Azem *et al.*, 1995). Consistent with the observation that Mn^{2+} increases the affinity of $GroES_7$ for the $GroEL_{14}GroES_7$ particle, protein folding was dramatically improved, especially under substoichiometric amounts of $GroES_7$, when the symmetric particle was limiting. Thus, Mn^{2+} improves the efficiency of protein folding by two means: increasing the formation of symmetric $GroEL_{14}(GroES_7)_2$ particles and reducing the rates of ATP hydrolysis.

Both the protein substrate and GroES have been suggested to go through mechanistically coupled cycles of binding/release until the folding protein has lost its affinity for GroEL (Martin *et al.*, 1993). Furthermore, the binding/release cycle of GroES has been shown to be coupled with the ATPase cycle (Todd *et al.*, 1994). We found that Mn^{2+} ions decreased the rate of ATP hydrolysis but increased the affinity of GroES for GroEL. From such a behavior, Mn^{2+} should inhibit the ATP-dependent cycle of GroES binding/release on GroEL. However, we found that Mn^{2+} increased the rates of protein folding. We conclude that conditions may exist *in vitro* were the two cycles of protein and GroES binding/release are not necessarily coupled mechanistically. We are now investigating the effect of Mn^{2+} on the rates of GroES exchange on GroEL during protein folding.

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