Effect of Divalent Cations on the Molecular Structure of the GroEL Oligomer†

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ABSTRACT: Structural analysis, by chemical cross-linking with glutardialdehyde (GA), and by urea denaturation, was carried out for the chaperonin oligomer $GroEL_{14}$ from *Escherichia coli*. The cross-linking reaction of $GroEL_{14}$ presents two phases: a rapid intralayer cross-linking reaction, which first occurs between the monomers of individual $GroEL_7$ heptameric rings, and a slow interlayer cross-linking reaction, which later occurs between the two stacked heptameric rings of the $GroEL_{14}$ oligomer. The biphasic behavior of the cross-linking reaction indicates that the surfaces of contact between GroEL monomers within individual heptameric rings are more extensive than the surfaces of contact between the two $GroEL_7$ rings of the oligomer. Millimolar amounts of the divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, or Zn^{2+}, but not of monovalent ions, increase the velocity of both intra- and interlayer cross-linking. Divalent cations increase the stability of the native $GroEL_{14}$ oligomer in urea. In contrast, Mg^{2+} activates ATP hydrolysis by $GroEL_{14}$, with an activation constant in the micromolar range, while Ca^{2+} does not significantly assist ATP hydrolysis. It is concluded that divalent cations affect the structure of $GroEL_{14}$, in particular the contacts between monomers within the $GroEL_7$ heptameric layers. The effect of divalent cations on the structure of the chaperonin molecule is quantitatively and qualitatively distinct from that of magnesium ions on the chaperonin ATPase activity.

Chaperonins, also called cpn60[1] and cpn10, belong to a ubiquitous class of sequence-related molecular chaperones in mitochondria, chloroplasts, and bacteria. In the cell, they are implicated in the folding of proteins (Hemmingsen et al., 1988; Goloubinoff et al., 1989a; Van Dyk et al., 1989) and in the molecular response to cellular stress (Martin et al., 1992; Horwich et al., 1993). *In vitro*, chaperonins assist the correct refolding of proteins by preventing protein aggregations [Goloubinoff et al., 1989b; Laminet et al., 1990; Buchner et al., 1991; for a review, see Hendrick and Hartl (1993)]. As determined by electron microscopy, cpn60 from bacteria (GroEL) and from the plant chloroplast (Rubisco binding protein) is an oligomer of 14 identical subunits of 57.3 kDa, with a structure of 2 stacked heptameric rings (Hendrix, 1979; Hohn et al., 1979; Pushkin et al., 1982; Saibil et al., 1993). The $GroEL_{14}$ oligomer recognizes and binds unfolded proteins (Goloubinoff et al., 1989b). Cpn60 from animal mitochondria, however, was purified as a single heptameric ring, capable of binding and assisting the refolding of unfolded proteins (Viitanen et al., 1992).

The molecular mechanism by which chaperonins assist the folding of many proteins remains largely unclear. Central to this issue is the molecular architecture of the $GroEL_{14}$ oligomer which can spontaneously interact with an unstable protein folding intermediate, to form a stable chaperonin/folding protein complex (Goloubinoff et al., 1989b). The step leading to the dissociation of this complex, and the subsequent correct refolding of the assisted protein, requires ATP and is coordinated by the co-chaperonin $GroES_7$ (Goloubinoff et al., 1989b; Martin et al., 1991). Monovalent ions, such as K^+ or NH_4^+, are essential for ATP hydrolysis by $GroEL_{14}$ and for chaperonin-assisted protein folding (Viitanen et al., 1990).

We show here that the divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} affects the contacts between GroEL monomers within the $GroEL_{14}$ oligomer. This effect is distinct from that of Mg^{2+} on ATP hydrolysis by the chaperonin.

EXPERIMENTAL PROCEDURES

Cloning and Expression of GroEL Chaperonins from Escherichia coli. The *groES* gene was amplified by polymerase chain reaction and cloned within the *Nco*I–*Hin*dIII restriction sites of plasmid pSE420 (Invitrogen Inc.). *Nco*I and *Eco*RI restriction sites were engineered within second and third codons of the *groES* gene. The *groEL* gene was appended to the *groES* gene by substituting the 500 bp *Sac*II fragment of the above construct with a 4500 bp *Sac*II fragment from plasmid pKT200 (Bloom et al., 1986), encoding for the last 60 codons of GroES, the intergenic *groES-groEL* sequences [as in Hemmingsen et al. (1988)], the complete *groEL* gene, and ~2.5 kilobases of the original sequences which are 3′ to the *groEL* gene from *E. coli*.

Purification of GroEL. The chaperonin proteins were overexpressed in *E. coli* containing plasmid pSESac2. Cell paste (~5 g) was diluted in 10 mL of ice-cold 50 mM Tris, pH 7.5, containing 1 mg/mL lyzozyme, 10 mM $MgCl_2$, 1 mM KCl, 1 mM EDTA, 2.6 mM PMSF, and 20 μg/mL leupeptin (Sigma). Cells were ruptured using three consecutive passes through a French pressure cell (3000 psi). After centrifugation (30000*g*, 30 min, 4 °C), the supernatant was applied on a Fractogel EMD DEAE-650 (M) column (10 mL, Merck), equilibrated with 50 mM Tris, pH 7.5, 0.1 mM EDTA, and 0.1 mM DTT, and eluted with a linear gradient

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; cpn60, chaperonin-60; cpn10, chaperonin-10; DTT, dithiothreitol; EC_{50}, concentration that causes 50% of the observed effect; EDTA, ethylenediaminetetraacetic acid; GA, glutardialdehyde; kcal, kilocalorie; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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of 0–500 mM KCl (60 mL) to provide fractions enriched in GroEL (340 ± 40 mM KCl). Fractions were subsequently diluted 3-fold in 50 mM Tris, pH 7.5, 0.1 mM EDTA, and 0.1 mM DTT, reapplied on the anion-exchange Mono-Q HR 5/5 column (1 mL, Pharmacia), and eluted on a linear gradient (25 mL) of 0–500 mM KCl. GroEL-enriched fractions were diluted in 3 volumes of 20 mM sodiumphosphate buffer (pH 8.2) and reapplied on a Mono-Q column, and a linear gradient of 0–500 mM sodiumphosphate was applied. GroEL-enriched fractions (350 ± 50 mM sodiumphosphate) were concentrated by centrifugation on Amicon concentrators (Centricon-30) to 400 μL and applied to a gel filtration column (10 mL, Superose 6B, Pharmacia), equilibrated in Tris, pH 7.5, 0.1 mM DTT, and 0.05% sodiumazide. $GroEL_{14}$ was collected as a single symmetric peak with an approximate molecular mass of 800 ± 150 kDa.

Conditions for Protein Cross-Linking. Cross-linking of native $GroEL_{14}$ oligomers (8–16 μM GroEL protomers) was carried out at 25 °C in 50 mM triethanolamine, pH 7.5, and 0.1% (w/v) glutardialdehyde (Sigma). The type and amounts of divalent ions, as well as the time of reaction, are specified in the text. Unless specified otherwise, the cross-linking reaction was stopped by the addition of one-third volume of 1 M Tris–glycine (pH 8.8), 4% SDS, and 10% 2-mercaptoethanol. Samples were boiled for 2 min prior to electrophoresis.

Electrophoresis. SDS gel electrophoresis of cross-linked proteins was carried out in tubes containing 3.3% polyacrylamide according to Weber et al. (1972) and in slab gels containing a gradient of polyacrylamide (3–30%), using the Laemmli buffer system (Laemmli, 1970). Gels were stained by Coomassie Brilliant Blue R-250 (Sigma).

Scanning of Protein Gels. Scanning was performed directly on gels using an Ultrascan-XL scanner (LKB). The shape of well-separated electrophoretic species from uniform tube gels (Weber et al., 1972) fit a Gaussian function (Darawshe et al., 1991) that translates more faithfully to quantitative numbers than the shape of electrophoretic species obtained from gradient gels (Azem, unpublished results). Accordingly, the quantitative estimation of cross-linked protein products was performed from uniform tube gels. The fraction of protein in each electrophoretic species was determined by Gaussian fitting. Gradient gels of polyacrylamide were preferentially used for the identification of the cross-linked species (Figure 2).

Terminology. For the sake of clarity, we refer in this work to [GroEL]*n* as a covalently cross-linked species, with a migration of *n* multiples of GroEL monomers, as revealed by denaturing SDS–polyacrylamide gels, regardless of the oligomeric state of the native complex in which this cross-linked species may have existed. We refer to $GroEL_m$ as a native GroEL oligomer of *m* subunits, regardless of the possible covalent cross-links that may or may not join the various subunits of this native complex.

ATPase Activity. Hydrolysis of ATP by $GroEL_{14}$ was measured using 1 μM protomers of GroEL and 0.5 mM [γ-^{32}P]-ATP (0.005 Ci mmol^{-1}) in 50 mM Tris-HCl, pH 7.5, containing 2 mM KCl and Mg^{2+}, Ca^{2+}, or Zn^{2+} as specified. Similar results were obtained using triethanolamine, instead of Tris-HCl, as the buffer of reaction. At the end of the incubation period (10–20 min, 37 °C), unhydrolyzed ATP was separated from the radioactive inorganic phosphate by absorption on 5% activated charcoal in 20 mM H_3PO_4 according to Bais (1975). The P_i resulting from spontaneous, GroEL-independent ATP hydrolysis was subtracted. The data were fitted to Hill equations using a computer program by Enzfitter for nonlinear regressions (Leatherbarrow. 1987).

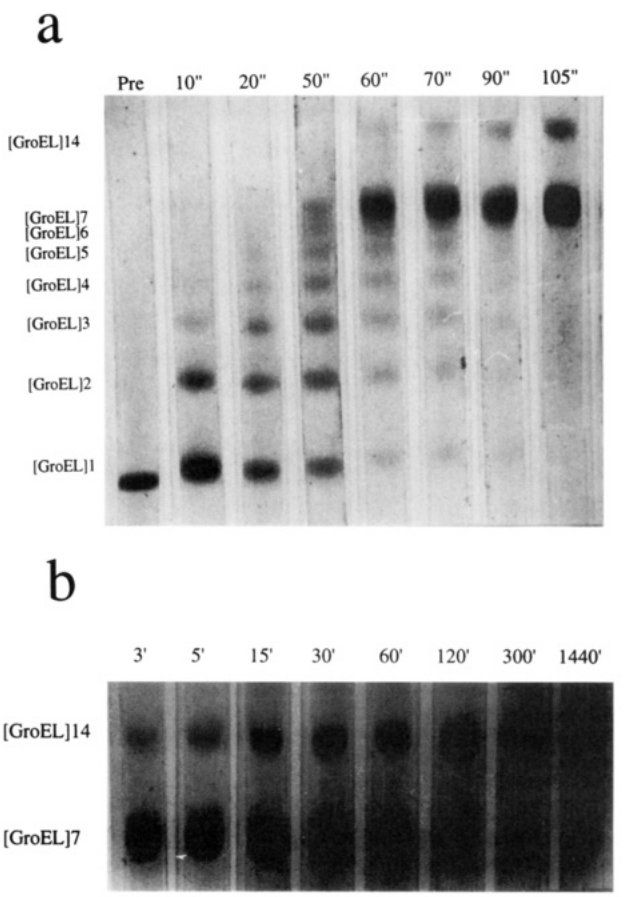

FIGURE 1: Cross-linking of the $GroEL_{14}$ oligomer with glutardialdehyde. 17 μM GroEL was exposed to GA (0.1% v/v), in the presence of 10 mM $MgCl_2$, for various periods of time at 25 °C and submitted to uniform SDS–polyacrylamide gel electrophoresis (tube, 3.2%). (a) Fast phase of cross-linking. (b) Slow phase of cross-linking.

RESULTS

Identification of Cross-Linked Species. When exposed to glutardialdehyde (GA), native $GroEL_{14}$ oligomers are predicted to show on an SDS–polyacrylamide gel at least 14 cross-linked products, ranging from 57.3 to 803 kDa in molecular mass. We observed, however, only 8 cross-linked products, further identified as multiples of 1, 2, 3, 4, 5, 6, 7, and 14 of the GroEL monomer (Figure 1a,b). In Figure 2, the identification of the various GroEL cross-linked products was based on the linear correlation that is found between the logarithmic expression of the predicted molecular mass of the cross-linked species [GroEL]1, [GroEL]2, [GroEL]3, [GroEL]4, [GroEL]5, [GroEL]6, [GroEL]7, and [GroEL]-14 (M) (normalized by the molecular weight of the monomer; M_p = 57 300) and the logarithm of the acrylamide concentration predicted on a 3–30% gradient SDS–polyacrylamide gel, at the distance of migration of each of the observed products (T%) (Lambin, 1978).

Kinetics of $GroEL_{14}$ Cross-Linking. The cross-linking reaction of native $GroEL_{14}$ with GA is biphasic. The fast cross-linking phase is characterized by the appearance of seven electrophoretic species, corresponding to [GroEL]1–6 and [GroEL]7. In the presence of Mg^{2+} ions, over 85% of the proteins accumulate within minutes, in the [GroEL]7 form (Figure 1a). The slow cross-linking phase that follows is characterized by the appearance of a [GroEL]14 species at

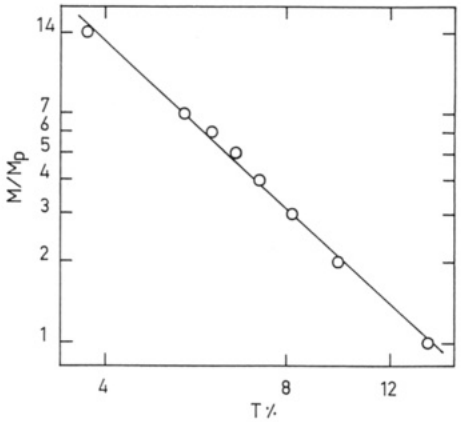

FIGURE 2: Migration patterns of GroEL cross-linked species and their identification. Native $GroEL_{14}$ oligomer was cross-linked for 60 s as described in Figure 1 and then separated by electrophoresis on a 3–30% gradient gel of SDS–polyacrylamide. The logarithm of the acrylamide concentration (T%) at the migration position found for each individual cross-linked species correlates linearly with the logarithm of 1, 2, 3, 4, 5, 6, 7, and 14 multiples of the 57.3-kDa GroEL monomer (M/M_p).

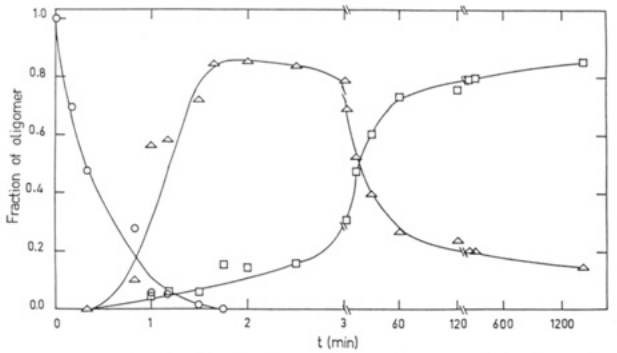

FIGURE 3: Kinetic distribution of GroEL cross-linked species. Fraction of [GroEL]1 (circles), [GroEL]7 (triangles), and [GroEL]-14 (squares) species as a function of the time of exposure to GA, obtained from a densitometer scan of the gel shown in Figure 1.

the expense of the [GroEL]7 species. After 24 h of exposure to GA, over 85% of the protein is in the [GroEL]14 form (Figure 1b). The kinetics of the two phases, based on a densitometric evaluation of the protein patterns in Figure 1, are shown in Figure 3. The fast phase displays the disappearance, within minutes, of [GroEL]1, while [GroEL]7 becomes the predominant species ([GroEL]2–6 are not represented). The slow phase displays the disappearance, within hours, of [GroEL]7, while [GroEL]14 reciprocally becomes the predominant species.

Effect of Divalent Cations on Cross-Linking of the $GroEL_{14}$ Oligomer. Cross-linking by GA of the native $GroEL_{14}$ oligomer is accelerated by Mg^{2+} ions (Figure 4a). A similar effect is observed with saturating amounts (10 mM) of other divalent cations such as Ca^{2+}, Mn^{2+}, or Zn^{2+}, but not with the monovalent cations Na^+, K^+, Rb^+, or Li^+ (not shown). In the absence of Mg^{2+}, the [GroEL]7 species accumulates with a $t_{1/2}$ which is about 3 times greater than that of the reaction in the presence of 10 mM Mg^{2+} (Table 1). Both the fast and the slow cross-linking phases are accelerated by Mg^{2+} ions. Half of the [GroEL]7 species is obtained at 2.9 mM Mg^{2+} (Figure 4b). It is not possible to assess the precise $t_{1/2}$ of the slow cross-linking reaction in the absence of Mg^{2+}, since even after 24 h of exposure to GA most of the protein remains in the [GroEL]7 form.

Effect of Divalent Cations on the ATPase Activity of $GroEL_{14}$. ATP hydrolysis by $GroEL_{14}$ is Mg^{2+}-dependent

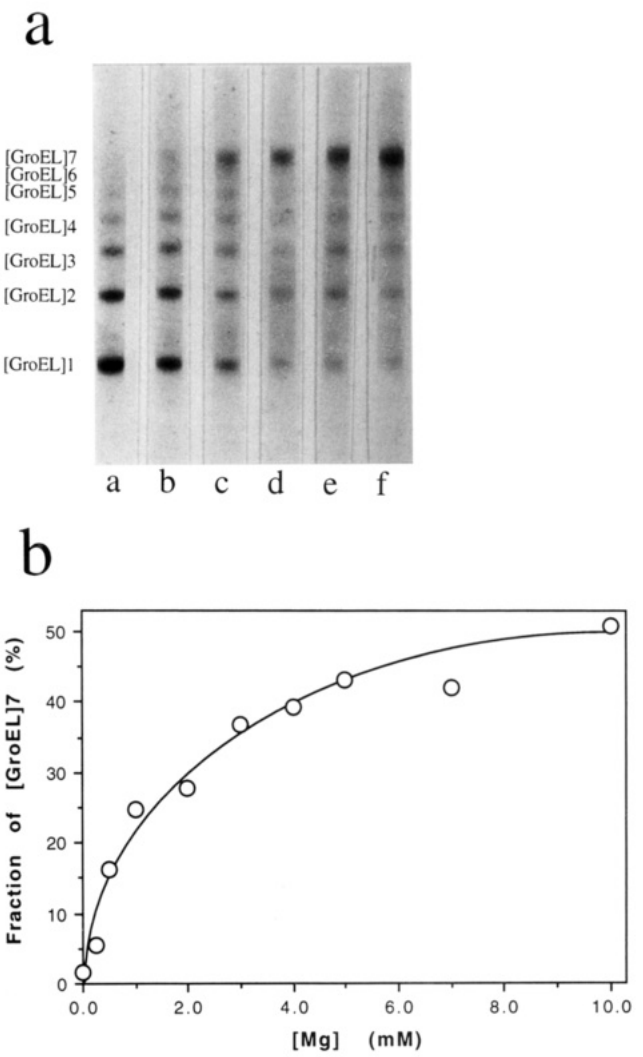

FIGURE 4: Effect of Mg^{2+} ions on the formation of [GroEL]7 cross-linked species. $GroEL_{14}$ oligomer was exposed to GA for 60 s in the presence of increasing concentrations of Mg^{2+} ions. (**a**) Uniform 3.2% SDS–polyacrylamide gel: (a) 0 mM; (b) 0.25 mM; (c) 1 mM; (d) 3 mM; (e) 5 mM; (f) 10 mM $MgCl_2$. (**b**) Fraction of [GroEL]7 cross-linked species as a function of [Mg^{2+}], obtained from a densitometric scan of (a).

Table 1: Effect of Mg^{2+} on Kinetic Parameters of Cross-Linked Species in $GroEL_{14}$[a]

species	Mg^{2+} (10 mM)	$t_{1/2}$ appearance	$t_{1/2}$ disappearance
[GroEL]1	+		22 s
[GroEL]1	–		72 s
[GroEL]7	+	67 s	17 min
[GroEL]7	–	210 s	>24 h
[GroEL]14	+	17 min	
[GroEL]14	–	>24 h	

[a] Parameters with Mg^{2+} are from Figure 3. Parameters without Mg^{2+} are from data not shown.

(Figure 5). The sigmoidal shape of the curve indicates that the binding of Mg^{2+} to $GroEL_{14}$, probably as Mg-ATP, is cooperative with the following constants: $V_{max} = 5.8 \pm 0.01$ min^{-1}, $K' = 22 \pm 9$ μM, and $n_H = 2.29 \pm 0.25$. These data indicate that at least 3 Mg-binding sites are strongly cooperative in the $GroEL_{14}$ molecule or that more than 3 (possibly up to 14) Mg-binding sites are weakly cooperative. Unlike the case of the cross-linking reaction, the activation by Ca^{2+} ions alone (2 mM) results in less than 2.5% of the equivalent Mg-dependent ATPase activity. The ATPase activity in the

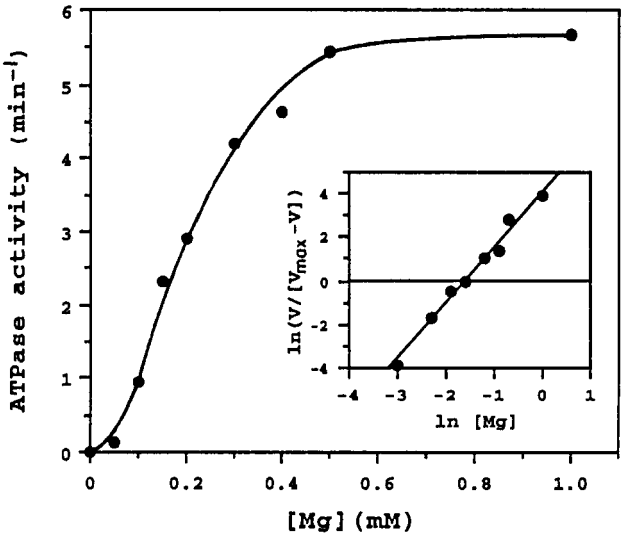

FIGURE 5: Effect of [Mg^{2+}] on the ATPase activity of GroEL. The assay was performed as described under Experimental Procedures, in the presence of 1 μM GroEL, 0.5 mM ATP, and increasing concentrations of Mg^{2+}. The insert shows a Hill plot of the data presented. The kinetic constants were derived by a nonlinear regression computerized program (Enzfitter; Leatherbarrow, 1987).

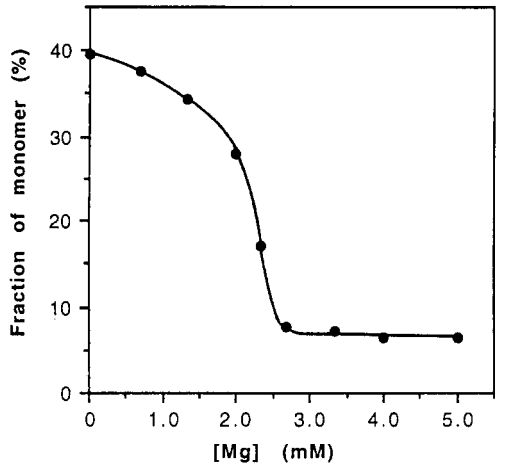

FIGURE 6: [Mg]-dependent stability of $GroEL_{14}$ in urea. $GroEL_{14}$ (3.3 μM) in 50 mM triethanolamine (pH 7.5), 10 mM KCl, 1 mM DTT, and 2% glycerol was incubated in 2.5 M urea for 24 h at 25 °C, in the presence of increasing amounts of $MgOAc_2$ (0–5 mM). Electrophoresis of the samples was carried out in a uniform 7% polyacrylamide gel, using the Laemmli buffer system, without SDS. After the gel was stained, the fraction corresponding to monomeric $GroEL_1$ was quantified by a scanner.

presence of Mg^{2+} and Ca^{2+} (2 mM each) is 65% of that in the presence of Mg^{2+} alone. Zn^{2+} could not be evaluated as an effector of $GroEL_{14}$ ATPase because of the lack of ATP stability in the presence of this cation (data not shown).

Effect of GA on the ATPase Activity of $GroEL_{14}$. Within half a minute of exposure to GA as in Figure 1a, all ATPase activity is lost (data not shown). Thus, the ATPase site on GroEL appears as a primary target for chemical modification by GA, which becomes modified before initiation of the rapid intralayer cross-linking reaction.

Divalent Ions Increase the Stability of $GroEL_{14}$ in Native Buffer and in Urea. When a submicromolar concentration of $GroEL_{14}$ was incubated for several days in triethanolamine (50 mM, pH 7.5) at 4 °C, cross-linking with GA revealed that the oligomer has entirely dissociated into GroEL monomers. The dissociation was completely prevented when incubation prior to the cross-linking was in the presence of 10 mM $MgCl_2$ (data not shown).

In the absence of Mg^{2+} ions, incubation of $GroEL_{14}$ oligomers in 2.5 M urea results in the apparent dissociation of 40% of the oligomers into monomers (Figure 6). The presence of 2.1 mM Mg^{2+}, during the incubation, reduces the fraction of GroEL monomer to 20%, while the presence of more than 2.7 mM Mg^{2+} keeps the fraction of monomeric GroEL to a low of 6%. Mn^{2+} ions have the same effect on the stability of $GroEL_{14}$ in urea (data not shown).

DISCUSSION

During the first cross-linking phase of native $GroEL_{14}$ oligomers by GA, a species with an apparent molecular weight of seven GroEL monomers accumulates at the expense of monomers and of transient di-, tri-, tetra-, penta-, and hexameric cross-linked species. In agreement with Hendrix (1979), who first showed by electron microscopy that $GroEL_{14}$ is organized in two stacked heptameric rings, possibly with an apparent 2-fold axis of symmetry across the equatorial plan of the molecule (Saibil et al., 1993), the kinetic behavior of the [GroEL]7 species correlates with predicted kinetic values for the cross-linking of a homogeneous heptameric ring structure (Azem and Daniel, submitted for publication). We conclude that the fast cross-linking phase corresponds to the joining by GA of specific lysine or arginine residues, from neighboring GroEL monomers within the same $GroEL_7$ ring. The absence of transient species accounting for 8, 9, 10, 11, 12, and 13 multiples of the monomer demonstrates that interlayer cross-linking occurs only after the intralayer cross-linking is completed.

Under dilute conditions, such as those used in our system, glutardialdehyde is in a monomeric form (Kawahara et al., 1992) and may covalently link two neighboring residues standing in the range of 8 Å apart (Peters & Richards, 1977). The 30-fold difference between the approximate $t_{1/2}$ values of the fast and slow cross-linking phases can be interpreted as if the contacts between the two $GroEL_7$ rings within the $GroEL_{14}$ oligomer are much less extensive than the contacts between GroEL monomers within the individual heptameric rings. Averaging pictures from electron microscopy of $GroEL_{14}$ complexes confirms the presence of fewer contacts between the two $GroEL_7$ layers than between monomers of the same layer (Saibil et al., 1993). A loose packing between layers of the molecule, particularly in the absence of divalent cations, could account for the report of a cpn60 from bovine mitochondria which was purified in the form of a single heptameric ring (Viitanen et al., 1992).

Millimolar amounts of divalent cations increase the velocity of both intra- and interlayer cross-linking reactions, suggesting that divalent cations cause structural changes in the proteins, bringing surfaces of subunits into better contacts with one another. This observation is confirmed independently by equilibrium experiments in urea. In 2.5 M urea, 2.7 mM Mg^{2+} fully stabilizes $GroEL_{14}$ oligomers (Figure 6). In addition, we found that 10 mM Mg^{2+} increases the EC_{50} of the dissociation reaction from 1.85 to 2.55 M urea (not shown). In solution, this difference corresponds to a gain of free energy of least of 7.35 kcal/mol of GroEL, which is 17% of the total free energy of the molecule (Pace, 1986). In addition, the presence of 10 mM Mg^{2+} ions can prevent a diluted solution of $GroEL_{14}$ from dissociating into monomers. We conclude that divalent cations strongly affect the affinity of GroEL monomers for one another within the $GroEL_{14}$ complex.

It is remarkable that the monovalent cations Na^+, K^+, Rb^+, and Li^+, even in high amounts (50 mM), do not influence the kinetics of either fast or slow cross-linking reactions. This

suggests that the structure of the molecule is less dependent on the ionic strength of the solution than on specific charge interactions that exist between the surfaces of the protein subunits. In addition, or alternatively, structural changes in the molecule, leading to increased contacts between protein surfaces, could be under the specific control of divalent cations.

The effect of divalent cations on the molecular stability of native $GroEL_{14}$ is not only quantitatively but also qualitatively different from the specific requirement for Mg^{2+} ions for the ATPase activity of the chaperonin. While a concentration as low as 500 μM Mg^{2+} fully saturates the ATPase reaction, neither Ca^{2+} nor Zn^{2+} can substitute for Mg^{2+} in the reaction.

Modulation of protein structure by millimolar amounts of divalent cations was reported for high molecular weight oligomeric hemoglobins (Kapp et al., 1984) and hemocyanins (Vannoppen-ver Eecke & Lontie, 1973). Since the physiological concentrations of Ca^{2+} are in the nanomolar range and those of Mn^{2+} are in the micromolar range (De Médicis et al., 1986), this raises the possibility that Mg^{2+} plays an essential role in the stability of structure of many oligomer proteins in the cell. De Médicis et al. (1986) reported concentrations of Mg^{2+} in *E. coli* as high as 50 mM, during logarithmic growth. However, this level can drop below 20 mM during the stationary phase. Since the protein concentration in the cell is in the range of 300 mg/mL, one would expect most of the Mg^{2+} ions to be in close association with proteins. Any significant decrease in the concentration of Mg^{2+} ions in the cell, as observed during the late-logarithmic and stationary phases (De Médicis et al., 1986), could have consequences on the structure and the function of proteins.

It is tempting to speculate that by varying the concentration of Mg^{2+} ions cells can modulate the protein structure and activity, in particular of chaperonins, to suit different physiological conditions.

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