Divalent Cations Can Induce the Exposure of GroEL Hydrophobic Surfaces and Strengthen GroEL Hydrophobic Binding Interactions

NOVEL EFFECTS OF Zn$^{2+}$ GroEL INTERACTIONS*

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Fluorescent and non-fluorescent probes have been used to show that divalent cations (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$) significantly increase hydrophobic exposure on GroEL, whereas monovalent cations (K$^+$ and Na$^+$) have little effect. Zn$^{2+}$ always induced the largest amount of hydrophobic exposure on GroEL. By using a new method based on interactions of GroEL with octyl-Sepharose, it was demonstrated that Zn$^{2+}$ binding strengthens GroEL hydrophobic binding interactions and increases the efficiency of substrate release upon the addition of MgATP and GroES. The binding of 4,4′-bis(1-anilino-8-naphthalenesulfonic acid) to GroEL in the presence of Zn$^{2+}$ has a $K_d = 1 \mu M$, which is similar to that observed previously for the GroEL 4,4′-bis(1-anilino-8-naphthalenesulfonic acid) complex. Urea denaturation, sedimentation velocity ultracentrifugation, and electron microscopy revealed that the quaternary structure of GroEL in the presence of Zn$^{2+}$ had a stability and morphology equivalent to unliganded GroEL. In contrast, circular dichroism suggested some loss in both α-helical and β-sheet secondary structure in the presence of Zn$^{2+}$. These data suggest that divalent cations can modulate the amount of hydrophobic surface presented by GroEL. Furthermore, the influence of Zn$^{2+}$ on GroEL hydrophobic surface exposure as well as substrate binding and release appears to be distinct from the stabilizing effects of Mg$^{2+}$ on GroEL quaternary structure.

In vitro studies have demonstrated that the information necessary for a polypeptide to attain its native conformation is contained within its amino acid sequence (1). However, the yield of folded protein in vitro is often quite low, in part because aggregation competes with folding. Recently, a highly conserved family of proteins, the Hsp60 molecular chaperones, has been suggested to be able to facilitate protein folding both in vitro and in vivo (2). The primary function of the molecular chaperones appears to be preventing aggregation of partially folded intermediates (3, 4). The Escherichia coli protein GroEL is homologous to the eukaryotic mitochondrial protein Hsp60, and it is among the best characterized molecular chaperones. GroEL is composed of 14 57-kDa subunits arranged in two stacked, seven-membered rings with 7-fold symmetry to form a cylinder with a central cavity (14-mer) (5). GroEL assists in vitro refolding of a number of proteins, and it has been shown to be able to interact with over half of the proteins from E. coli (6). The mechanistic features for the binding and release of substrate polypeptides by GroEL are not completely understood. The promiscuous nature of GroEL-polypeptide interactions suggests that a GroEL recognition motif is not specific for polypeptide sequence. Thus, a GroEL substrate protein recognition motif must include characteristics that are common in a variety of incompletely folded polypeptides that are not present in their native forms which interact weakly or not at all with the chaperonin.

A key feature of protein folding intermediates is the easy accessibility of hydrophobic residues that would typically be buried in the native conformation of the protein. It has been suggested that GroEL binds the compact folding intermediates (molten globules) of proteins produced in the course of folding (7), and recent calorimetric studies have demonstrated that hydrophobic interactions are an important driving force for the association of substrate proteins with GroEL (8). Further evidence for the importance of hydrophobic interactions came from mutational analysis (9). That study identified polypeptide binding sites on the inside surfaces of the GroEL apical domains (one of the three domains into which the monomer is folded), facing the central channel. Interestingly, all of the apical domain residues determined to be essential for substrate polypeptide binding were hydrophobic residues.

Paradoxically, when the accessibility of hydrophobic surfaces on GroEL was assayed with the hydrophobic probe bis-ANS, only 1.4 molecules of bis-ANS were bound per GroEL 14-mer (10). This implies that most of the hydrophobic residues used for polypeptide binding are either buried or that the negatively charged bis-ANS molecule does not have access to the hydrophobic residues, due to the high net negative charge on GroEL (−266 per 14-mer).

The importance of charge to GroEL interactions has been suggested by studies of polypeptide binding and the effects of ions on the quaternary structure of the chaperonin (11–13). In this report, we demonstrate the importance of divalent cations in altering the apparent amount of hydrophobic surface presented by GroEL. The binding of divalent cations (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$), but not monovalent cations (K$^+$ and Na$^+$), results in an increase in the amount of GroEL hydrophobic surface exposure, with zinc inducing the greatest effects.

MATERIALS AND METHODS

Reagents and Proteins—All reagents used were analytical grade. The chaperonin, GroEL, was purified from lysates of cells containing the

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1 The abbreviations used are: bis-ANS, 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); TNS, 6-(p-toluidinyl)naphthalene-2-sulfonic acid; BSA, bovine serum albumin.
Either MgCl$_2$ or ZnCl$_2$ was added to two of these samples. A fourth vortexed and then centrifuged for 30 s. The supernatants were removed, mixture also contained 10 mM MgCl$_2$, 10 mM ZnCl$_2$, 5 mM KCl, and 5 mM ATP in 50 mM Tris-HCl (pH 7.0). The GroEL concentrations refer to monomers, and the salt concentrations are 10 mM, unless stated otherwise. All of the concentrations given are final concentrations. Bis-ANS fluorescence experiments in the presence of salts were performed with an excitation wavelength of 397 nm and an emission wavelength of 500 nm on either SLM 500c or SLM 48000s fluorometer. Each sample contained 2 $\mu$m GroEL and 20 $\mu$m bis-ANS and one of the following salts: NaCl, KCl, CaCl$_2$, MnCl$_2$, MgCl$_2$, or ZnCl$_2$. To determine a dissociation constant for bis-ANS in the presence of GroEL (2 $\mu$m) and ZnCl$_2$, a titration was performed over a bis-ANS concentration range of 0–25 $\mu$m.

To assess the effects of high ionic strength, fluorescence spectra were acquired for samples containing bis-ANS (20 $\mu$m), GroEL (2 $\mu$m), and the required concentrations of ZnCl$_2$, with and without NaCl (100 mM). To assess the reversibility of the effect of zinc binding, the fluorescence spectrum of bis-ANS (20 $\mu$m) and GroEL (2 $\mu$m) was acquired with and without ZnCl$_2$ (5 mM), followed by the addition of EDTA (8 mM). To determine the association of MgCl$_2$ and GroEL, samples of GroEL (10 $\mu$m) and bis-ANS (10 $\mu$m) in the presence and absence of ZnCl$_2$ (5 mM) were titrated with MgCl$_2$ (0–20 mM) and the fluorescence spectra acquired.

A titration of bis-ANS with ethanol was performed to measure the fluorescence intensity changes as a function of the shift of the fluorescence wavelength maximum. Specifically, samples of bis-ANS (1 $\mu$m) were prepared with increasing concentrations of ethanol (0–95%), decreasing the dielectric constant of the solvent. The wavelength maximum of the bis-ANS fluorescence at each ethanol concentration was converted to frequency and plotted against the fluorescence intensity at the respective frequency maximum.

Nile Red fluorescence measurements were performed with an excitation wavelength of 550 nm and an emission wavelength of 680 nm. Three samples were prepared containing Nile Red (10 $\mu$m) and GroEL (2 $\mu$m). Either MgCl$_2$ or ZnCl$_2$ was added to two of these samples. A fourth sample was prepared containing only Nile Red (10 $\mu$m). TNS fluorescence measurements were performed with an excitation wavelength of 315 nm and an emission wavelength of 445 nm. Three samples were prepared containing TNS (10 $\mu$m) and GroEL (2 $\mu$m). Either MgCl$_2$ or ZnCl$_2$ was added to this sample. A fourth sample was prepared containing only TNS (10 $\mu$m).

**GroEL Octyl-Sepharose Binding**—A slurry of octyl-Sepharose was made in 25% ethanol, and 300-$\mu$l aliquots were added to 1.5-mL Eppendorf tubes and centrifuged for 30 s. The supernatants were removed, and the octyl-Sepharose in each tube was washed with 400 $\mu$L of 50 mM Tris-HCl (pH 7.0), containing 10 mM MgCl$_2$, and incubated for 30 min. The samples were vortexed, and the octyl-Sepharose was centrifuged for 30 s. The supernatants were discarded, and the procedure was repeated three times. Following octyl-Sepharose equilibration, 100-$\mu$l samples containing GroEL (10 $\mu$m), ZnCl$_2$, and BSA (15 $\mu$m) were then added to two of these samples. Four 5-$\mu$l aliquots of Octyl-Sepharose resin, GroES (15 $\mu$m) was subsequently added to the appropriate incubation mixture of GroEL, MgATP, and divalent cation.

To assay the competition between GroES and octyl-Sepharose for GroEL binding sites, increasing concentrations of GroES were incubated with GroEL (1:1, 2:1, 3:1, and 4:1; GroES 7-mer/GroEL 14-mer) before addition to the octyl-Sepharose resin. The GroES/GroEL incubation mixture also contained 10 mM MgCl$_2$, 10 mM ZnCl$_2$, 5 mM KCl, and 5 mM ATP in 50 mM Tris-HCl (pH 7.0). The protein content in the supernatant was analyzed by either 12% SDS-polyacrylamide gel electrophoresis or by BCA protein assay. Gel images were captured electronically, and the optical densities of the GroEL bands were quantified using the Image Master software. Gel images (version 1.0) were analyzed to determine the ratio of GroEL tetradecamers to GroEL dimers.

**RESULTS**

**GroEL Hydrophobic Exposure in the Presence of Divalent Cations**—The influence of cations on the exposure of hydrophobic surfaces on GroEL was probed using the fluorescence associated with bis-ANS binding. Fig. 1 shows the effects on the bis-ANS fluorescence when salts were added to separate solutions of GroEL (2 $\mu$m) and bis-ANS (20 $\mu$m). The bottom three spectra show there is little influence of the monovalent cations (K$^+$ and Na$^+$). However, the addition of MgCl$_2$, CaCl$_2$, MnCl$_2$, or ZnCl$_2$ resulted in an increase in the fluorescence intensity, indicating complete reversibility of the Zn$^{2+}$ induced hydrophobic exposure is not simply a result of increased ionic strength, since the fluorescence spectrum of bis-ANS + GroEL in the presence of 100 mM NaCl was equally responsive to the addition of Zn$^{2+}$.

The neutral fluorescent probe Nile Red, was also used to probe for the presence of hydrophobic sites on GroEL (data not shown). Although Nile Red is not as environmentally sensitive a probe as bis-ANS, the data still followed the trend seen with bis-ANS. Specifically, the addition of divalent cations, but not monovalent cations, resulted in an increase in the fluorescence intensity of Nile Red. For example, the fluorescence intensity increased 5% in going from GroEL to GroEL + MgCl$_2$, whereas there was no change upon the addition of KCl or NaCl. The

**Cation-induced GroEL Hydrophobic Exposure**

The urea dissociation of GroEL tetradecamers was detected by intrinsic tyrosine fluorescence and bis-ANS fluorescence spectroscopy. The urea dissociation of GroEL tetradecamers was detected by intrinsic tyrosine fluorescence and bis-ANS fluorescence. GroEL tyrosine fluorescence was excited at a wavelength of 274 nm, and the emission was detected at a wavelength of 310 nm. Bis-ANS fluorescence was excited at a wavelength of 397 nm, and the emission was detected at 500 nm. To follow the dissociation of GroEL tetradecamers by tyrosine fluorescence and bis-ANS fluorescence, an identical range of urea concentrations (0–6 M) was covered for three different GroEL conditions. The three GroEL conditions used for tyrosine fluorescence measurements were as follows: GroEL (15 $\mu$m), GroEL + Mg$^{2+}$, and GroEL + Zn$^{2+}$. The conditions for bis-ANS (10 $\mu$m) measurements were GroEL (1 $\mu$m), GroEL + MgCl$_2$, and GroEL + ZnCl$_2$.

**GroEL Light Scattering**—Kinetics of GroEL aggregation were followed by 90° light scattering using an SLM 500c fluorometer by setting both the excitation and emission wavelengths at 400 nm. The protein samples were GroEL (1 $\mu$m), GroEL + ZnCl$_2$, and BSA (15 $\mu$m) + ZnCl$_2$. Buffer blanks were collected and subtracted from the light scattering data collected for each protein sample.

**Circular Dichroism Spectroscopy**—Circular dichroism spectra of GroEL (5 $\mu$m) at three different ZnCl$_2$ concentrations (0, 1, and 10 mM) were collected on a Jasco J500C spectropolarimeter from 250 to 180 nm, at 25 °C. Buffer blanks were collected and subtracted from the appropriate sample. A spectral bandwidth of 2 nm, a digitizing interval of 0.1 nm and a 2-s time constant were used for all samples. Ellipticity ($\theta$) is expressed as degrees cm$^2$ (dmol amino acid)$^{-1}$. The spectra were fit using the k2d program of Andrade et al. (16) utilizing a neural network algorithm.

**Sedimentation Analysis**—GroEL samples (5.0 $\mu$m) at three different ZnCl$_2$ concentrations (2.5, 5.0, and 10 mM) were subjected to sedimentation velocity using a Beckman TLS analytical ultracentrifuge with 12-mm double sector cells in a four-hole Ti-60 rotor with a rotor speed of 18,000 rpm. The scans were analyzed by the method of van Holde and Weischat (17) using the Ultrascan ultracentrifuge data collection and analysis program (B. Demeler, Missoula, MT). All data were corrected to standard conditions.

**Electron Microscopy**—Two 5 $\mu$m GroEL (50 $\mu$l) samples were prepared with ZnCl$_2$ and incubated for 30 min. The samples (5 $\mu$l) were then placed on carbon-coated grids and immediately washed (three times) with 15 $\mu$L of H$_2$O. The samples were then negatively stained with 8 $\mu$L of 1% (w/v) uranyl acetate and dried by touching the edge of each grid with a piece of filter paper. The micrographs were examined and photographed in a Phillips 301 transmission electron microscope.

**Urea Dissociation of GroEL Followed by Intrinsic Tyrosine Fluorescence and Bis-ANS Fluorescence Spectroscopy**—The urea dissociation of GroEL tetradecamers was detected by intrinsic tyrosine fluorescence and bis-ANS fluorescence. GroEL tyrosine fluorescence was excited at a wavelength of 274 nm, and the emission was detected at a wavelength of 310 nm. Bis-ANS fluorescence was excited at a wavelength of 397 nm, and the emission was detected at 500 nm. To follow the dissociation of GroEL tetradecamers by tyrosine fluorescence and bis-ANS fluorescence, an identical range of urea concentrations (0–6 M) was covered for three different GroEL conditions. The three GroEL conditions used for tyrosine fluorescence measurements were as follows: GroEL (15 $\mu$m), GroEL + Mg$^{2+}$, and GroEL + Zn$^{2+}$. The conditions for bis-ANS (10 $\mu$m) measurements were GroEL (1 $\mu$m), GroEL + MgCl$_2$, and GroEL + ZnCl$_2$.

The neutral fluorescent probe Nile Red was also used to probe for the presence of hydrophobic sites on GroEL (data not shown). Although Nile Red is not as environmentally sensitive a probe as bis-ANS, the data still followed the trend seen with bis-ANS. Specifically, the addition of divalent cations, but not monovalent cations, resulted in an increase in the fluorescence intensity of Nile Red. For example, the fluorescence intensity increased 5% in going from GroEL to GroEL + MgCl$_2$, whereas there was no change upon the addition of KCl or NaCl. The
fluorescence intensity increased 18% upon the addition of ZnCl$_2$ with a slight shift of the fluorescence maximum to a shorter wavelength. Thus, although there were considerable differences in the fluorescence intensity changes between the two probes, the fluorescence changes using Nile Red were in the same direction as those seen with bis-ANS. This difference was attributed to the environmental sensitivity of the two probes. For example, in going from H$_2$O to 50% ethanol (v/v), the bis-ANS fluorescence intensity increased 328-fold, whereas the Nile Red fluorescence only increased 2.6-fold.

A third fluorescent molecule, TNS, was used to probe for the presence of hydrophobic sites on GroEL (data not shown). TNS is negatively charged, and its sensitivity to apolar environments is intermediate between bis-ANS and Nile Red. The addition of MgCl$_2$ to GroEL resulted in a 2.2-fold increase in the TNS fluorescence intensity, an effect similar to that observed with bis-ANS (2.8-fold increase, Fig. 1). The addition of ZnCl$_2$ to GroEL resulted in a 5-fold increase in the TNS fluorescence and a 15-nm blue shift in the emission maximum.

The binding of GroEL to octyl-Sepharose permits the assessment of hydrophobic binding that is independent of fluorescent probes. Octyl-Sepharose is a cross-linked beaded agarose that contains octyl groups (~50 μmol per ml of swollen Sepharose) attached by an ether linkage. To assay GroEL binding, suspensions of the octyl-Sepharose resin (300 μl) were added to 1.5-ml Eppendorf tubes, and the resin was equilibrated with the appropriate buffer (as described under “Materials and Methods”). Following equilibration of the resin, 100-μl samples containing GroEL (0.7 μM 14-mer) and either a mono- or divalent cation (10 mM) were added to the appropriate resin suspension and mixed. Sepharose HB, a gel filtration agarose bead that contains no hydrophobic groups attached to it, was used as a control to determine the extent to which GroEL was binding to the agarose portion of the resin.

Protein released from the octyl-Sepharose resin was quantified from SDS gels of aliquots of the resin supernatants as described under “Materials and Methods.” Comparisons of GroEL binding between controls using HB Sepharose with samples using octyl-Sepharose revealed a 44% difference in binding. This supports the contention that GroEL binds to octyl-Sepharose by hydrophobic interactions. Both K$^+$ and Na$^+$ weaken GroEL hydrophobic interactions, resulting in a 5 and 8% loss in binding, respectively. The divalent cations Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$, on the other hand, strengthen the hydrophobic interactions of GroEL, resulting in a 6, 8, 14, and 25% increase in binding, respectively.

It was also observed that a 1:1 ratio of the substrate protein rhodanese:GroEL (14-mer) could compete with the octyl-Sepharose resin for GroEL hydrophobic binding sites. For example, in the absence of rhodanese, 45% of the GroEL was bound to the octyl-Sepharose. However, at a concentration of 1 μM rhodanese, less than 2% of the GroEL was bound to the octyl-Sepharose. Subsequent trials of this experiment indicated variability of the rhodanese competition results, possibly due to the excess hydrophobic exposure on complexes formed between GroEL and the molten globule folding intermediate of rhodanese (18). It was demonstrated by Martin et al. (18), using the hydrophobic probe 1,8-ANS, that the rhodanese-GroEL complex presents a large amount of hydrophobic surface in contrast to the minimal hydrophobic surfaces present on either rhodanese or GroEL alone. Therefore, to avoid the experimental ambiguities of both GroEL and GroEL-rhodanese complexes binding to the octyl-Sepharose resin, the co-chaperonin protein GroES was used to assay competition with octyl-Sepharose. It is known from the recent GroEL-GroES crystal structure that upon binding of GroES to GroEL, the substrate polypeptide binding elements of GroEL become involved in the GroES binding interactions (19). Thus, an experiment was carried out in which preformed GroES-EL complexes were incubated for 1 min before addition to the octyl-Sepharose resin. The ratios of the GroES-EL complexes were 1:1, 2:1, 3:1, and 4:1, which demonstrated a 50, 52, 80, and 97% decrease in octyl-Sepharose binding, respectively. This suggests that the octyl-Sepharose and GroES heptamer compete for binding sites on the GroEL 14-mer, although it cannot be concluded that the identical sites are involved.

These data show that divalent cations can play a role in modulating hydrophobic surfaces on GroEL. Independent of the method used to measure the presence of GroEL hydrophobic surface(s), Zn$^{2+}$ always induced the largest amount of hydrophobic surface on GroEL. Titration curves for Zn$^{2+}$ and Mg$^{2+}$ (data not shown) were generated in the presence of 2 μM GroEL and 20 μM bis-ANS and yielded dissociation constants of Zn$^{2+}$ ~4 mM and Mg$^{2+}$ ~10 mM. The bis-ANS fluorescence for the Mg$^{2+}$ titration became saturated at a concentration of 30 mM Mg$^{2+}$ and the fluorescence intensity never approached that observed with the Zn$^{2+}$ titration. Thus, the binding of the divalent cations to GroEL, as reported by bis-ANS fluorescence emission spectra. Emission spectra of bis-ANS from bottom to top are GroEL + NaCl (10 mM), + KCl (10 mM), + MgCl$_2$ (10 mM), + CaCl$_2$ (10 mM), + MnCl$_2$ (10 mM), and + ZnCl$_2$ (10 mM). The spectra of GroEL + NaCl and + KCl are overlapping. The spectra of GroEL + CaCl$_2$ and + MnCl$_2$ also overlap.
measurements, appears to occur at low affinity sites within GroEL. From these data, it might be conjectured that the ability of each divalent cation to modulate GroEL hydrophobic surfaces may be a reflection of their binding strength to GroEL.

A bis-ANS binding study was performed in the presence of GroEL and ZnCl₂ (Fig. 2) and yielded a $K_d = 1 \, \mu M$, a value similar to previously published bis-ANS dissociation constants ($10^4$). Several attempts were made to determine the number ($n$) of bis-ANS binding sites/GroEL 14-mer in the presence of Zn²⁺. However, aggregation of GroEL at the higher protein concentrations needed to determine the fluorescence of bound bis-ANS prevented accurate determination of the number of bis-ANS binding sites/GroEL 14-mer. This aggregation of GroEL observed experimentally may be attributed to the large increase in GroEL hydrophobic surface exposure in the presence of Zn²⁺.

To estimate the environment of the bis-ANS binding site(s) on GroEL, the fluorescence of bis-ANS bound to GroEL was compared with the fluorescence of bis-ANS (1 μM) in increasing ethanol concentrations (0–85% v/v). The rationale for this approach is that as the effective dielectric constant of the solvent (e.g., Kosower Z factor) decreases (i.e., increasing ethanol), the bis-ANS quantum yield (i.e., fluorescence intensity) increases, and the wavelength maximum shifts to shorter wavelengths. If there is an increase in the number of bis-ANS molecules bound with no change in the binding environment, the observed intensity would increase with no change in the wavelength maximum. Thus, information can be deduced about the bis-ANS binding environment by comparing the ratios of fluorescence intensities of bis-ANS in ethanol concentrations that give the same wavelength maximum observed for bis-ANS in the presence of Zn²⁺.

From these experiments indicate no competition between the two ligands, and they suggest that independent binding sites exist on GroEL for MgCl₂ and ZnCl₂.

Zn²⁺ Strengthens GroEL Hydrophobic Binding Interactions and Improves the Efficiency of GroEL Release upon the Addition of MgATP and GroES—The use of octyl-Sepharose as a GroEL substrate permits the assessment of substrate (octyl-Sepharose) binding and release by monitoring the GroEL protein concentration. Less than 50% of the GroEL is bound in the absence of any ligands (Fig. 3). The addition of either MgCl₂ (10 mM) or MgCl₂ (10 mM) + ZnCl₂ (10 mM) resulted in a 25% and 25% increase in GroEL octyl-Sepharose binding, respectively. The addition of ATP increased GroEL binding in the presence of MgCl₂ (10 mM) and decreased GroEL binding in the presence of MgCl₂ (10 mM) + ZnCl₂ (10 mM). Further addition of GroES to these samples resulted in the partial release of GroEL (6% of the GroEL bound) in the presence of MgCl₂ (10 mM) and complete release of bound GroEL in the presence of MgCl₂ (10 mM) + ZnCl₂ (10 mM). This demonstrated that Zn²⁺ not only supports GroEL function by strengthening hydrophobic interactions with substrate, but it also improves the efficiency of substrate release in the presence of MgATP and GroES.

Structural Characterization GroEL-Zn²⁺—Functionally active GroEL is a homotetradecameric protein made of two stacked 7-subunit rings. In the absence of ligands, the GroEL tetradecamer must be perturbed, e.g., with 2.5 M urea, to observe a large increase in hydrophobic exposure when probed with bis-ANS. The ability of Zn²⁺ to modulate large changes in GroEL hydrophobic surface exposure was characterized further.

Intrinsic tyrosine fluorescence, bis-ANS fluorescence, and sedimentation velocity ultracentrifugation were used to follow the urea dissociation of GroEL 14-mers into monomers. It has been demonstrated in urea denaturation experiments, which follow intrinsic GroEL tyrosine fluorescence (Fig. 4), that Mg²⁺ (filled circles) stabilizes the GroEL 14-mer ($U_{1/2} = 3.35$ M) when compared with the unliganded (filled diamonds) GroEL 14-mer to monomer transition ($U_{1/2} = 1.9$ M). The parameter $U_{1/2}$ is defined as the urea concentration at the transition midpoint of the protein denaturation curve. The addition of Zn²⁺ does not shift the GroEL urea transition midpoint to higher urea concentrations ($U_{1/2} = 2.1$ M), as would be expected for 14-mer stabilization, but remains coincident with unliganded GroEL (Fig. 4, filled squares). The urea dissociation of GroEL 14-mers...
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FIG. 3. The binding and release of GroEL from octyl-Sepharose. The data are plotted as percent GroEL bound to the octyl-Sepharose (as determined by BCA protein assay) for a given set of GroEL incubation conditions and normalized to a GroEL control that contained no Sepharose resin. The concentrations of the ligands used were 10 mM MgCl₂ or 10 mM MgCl₂ + 10 mM ZnCl₂, 10 mM ATP, and 10 μM GroES.

FIG. 4. GroEL urea dissociation followed by intrinsic tyrosine fluorescence. The intrinsic tyrosine fluorescence of GroEL (filled squares), GroEL + 10 mM Mg²⁺ (filled circles), and GroEL + 10 mM Zn²⁺ (filled diamonds) is plotted as a function of the urea concentration.

in the presence of Zn²⁺ was also followed by bis-ANS binding. The maximal bis-ANS fluorescence occurs at a urea concentration of approximately 2.6 M urea, which again is equivalent to that seen with unliganded GroEL (13). Finally, the sedimentation velocity of GroEL (5 μM) in three Zn²⁺ concentrations (2.5, 5.0 and 10 μM) was analyzed, and the sedimentation coefficients (s₂₀,₀) were as follows: 21.8, 22.5, and 22.7 S, respectively, compared with the native GroEL 14-mer which gave 22 S. Thus, Zn²⁺ did not appear to stabilize the quaternary structure of GroEL as seen with Mg²⁺.

When the secondary structure of GroEL in the presence of Zn²⁺ was followed by circular dichroism, an interesting result was observed. Fig. 5 shows the CD spectra of 5 μM GroEL (Fig. 5, bottom spectrum), 5 μM GroEL + 1 mM Zn²⁺ (Fig. 5, middle spectrum), and 5 μM GroEL + 10 mM Zn²⁺ (Fig. 5, top spectrum). GroEL is a highly helical protein (45%), and the CD spectrum of GroEL is indicative of this helicity. The CD spectra of GroEL were fit using a neural network analysis program as noted under “Materials and Methods” (16). The secondary structural predictions for the fit of the CD data for GroEL alone were in close agreement with the crystal structure of GroEL. For example, the fitted values yielded 43% α-helix, 22% β-sheet, and 35% other structure, compared with the actual percentages of 45% α-helix, 16% β-sheet, and 39% other from the crystal structure (24). Fits of the CD spectra of GroEL in going from 0 to 10 mM Zn²⁺ (Fig. 5) indicate that the presence of Zn²⁺ resulted in a 19% decrease in α-helix and a 59% decrease in β-sheet. Although the possibility cannot be ruled out that aggregation of GroEL might contribute to the difference in the GroEL CD spectra ± Zn²⁺, the aggregation of GroEL involves less than 5% of the total protein. This percent of GroEL aggregation was estimated for each GroEL sample by measuring the difference of the absorbance plateaus obtained during sedimentation velocity ultracentrifugation. For example, the change in the absorbance plateau measured at the beginning of centrifugation compared with the plateau at the end of centrifugation for each Zn²⁺ condition described above was less than 5%.

The possibility that Zn²⁺ may be causing the oligomerization of GroEL 14-mers was further addressed by light scattering and transmission electron microscopy. The light scattering data for GroEL (1 μM) indicated that there is no aggregation of the 14-mer in this concentration range (Fig. 6, middle curve), but the addition of Zn²⁺ to GroEL (Fig. 6, top curve) did appear to result in some aggregation of the GroEL 14-mer, possibly due to the increase in hydrophobic surface or a change in electrostatic repulsion. However, this aggregation of GroEL represented a small fraction of the total protein concentration according to sedimentation velocity analysis (see above). The chelation of Zn²⁺ with an equivalent concentration of EDTA completely reversed the aggregation observed in Fig. 6, whereas the presence of 5 mM ATP + 5 mM Mg²⁺ prevented the Zn²⁺-induced aggregates from forming. The protein BSA (1 μM) (Fig. 6, bottom curve) did not aggregate in the presence of Zn²⁺, suggesting that this effect of Zn²⁺ on GroEL reflects a specific interaction. It is important to note that the large fluorescence changes seen with GroEL + Zn²⁺ are instantaneous within the time of manual mixing, and therefore, they were detected much earlier than the accumulation of any GroEL aggregates. Transmission electron microscopy was used to follow any aggregation of GroEL in the presence of Zn²⁺. The morphology seen in the negatively stained images of GroEL + Zn²⁺ and the morphology of the GroEL 14-mers did not differ significantly from each other or from previously published images, and no large aggregates were apparent on the grids (data not shown).

DISCUSSION

GroEL binds a variety of polypeptides in their non-native states (3, 20–21). This implies that the primary sequence of a substrate polypeptide does not supply the information required for recognition. Thus, binding must occur by a more general mechanism of molecular recognition. A common feature of unfolded and misfolded polypeptides is the solvent exposure of hydrophobic residues that would be buried in the native state. It is generally accepted that GroEL polypeptide interactions occur by hydrophobic association. Several studies have described these interactions and stated the importance of GroEL
hydrophobic binding of substrate polypeptides (8, 22–23). The GroEL crystal structure (24), complemented by GroEL mutational analysis (9), has made it possible to suggest the substrate polypeptide binding sites on GroEL. These sites are within flexible segments containing hydrophobic residues in the apical domains of the GroEL 14-mer. However, when hydrophobic surfaces on unliganded GroEL are probed with bis-ANS, only a few molecules of bis-ANS bind per GroEL 14-mer (10). This result raises an interesting question. If GroEL substrate polypeptide binding occurs by hydrophobic association in the apical domains of GroEL, where are all the hydrophobic sites on GroEL?

The answer to this question may be that hydrophobic sites on GroEL are buried until the appropriate ligands signal their exposure. For example, several studies have demonstrated that GroEL quaternary structure changes after the addition of adenine nucleotides and divalent cations (12, 25). Thus, it could be conjectured that subsequent to these quaternary changes, there is an exposure of GroEL hydrophobic surfaces. It is also possible that the substrate polypeptide itself might be able to trigger GroEL hydrophobic surface(s). It was shown in an experiment using a positively charged amphipathic peptide that the binding of the peptide to GroEL induced hydrophobic surfaces on GroEL (26). Thus, ligand-induced exposure of hydrophobic surfaces on GroEL may be a mechanism to present hydrophobic binding surfaces to substrate polypeptide. Furthermore, the interaction of a positively charged protein with GroEL has also been described as an effector of GroEL substrate polypeptide binding (11). In that study, it was demonstrated by point mutations within the protein chymotrypsin inhibitor 2 that positive charges on the protein confer tighter binding to GroEL, whereas negative charges on chymotrypsin inhibitor 2 diminish binding (11).

It has been demonstrated in this study that hydrophobic surface(s) on GroEL can be significantly increased upon the addition of divalent cations. The fluorescent probes bis-ANS, Nile Red, and TNS were used to demonstrate that additions of divalent cations (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$) but not monovalent cations (K$^+$ and Na$^+$) result in an increase in the fluorescence intensity of each probe. The presence of hydrophobic surfaces on GroEL was also measured by the non-fluorescent method using octyl-Sepharose (Fig. 3). The results of that experiment also demonstrate an increase in GroEL hydrophobic surfaces in the presence of divalent cations. This indicates that, independent of the method of hydrophobic measurements, the effects of divalent cations on GroEL hydrophobic surface exposure still occur. One of the most interesting findings in this study is the contribution of Zn$^{2+}$ in strengthening GroEL sub-
strate binding and increasing the efficiency of substrate release upon the addition of MgATP and GroES.

Magnesium has been previously shown to stabilize the quaternary structure of GroEL (12, 13). The present work shows that zinc does not stabilize the GroEL quaternary structure (Fig. 4), but it does appear to influence the secondary structure of GroEL (Fig. 5). In contrast, there was no detectable change in the GroEL secondary structure in the presence of magnesium (data not shown). These results suggest that Zn\(^{2+}\) binding can affect the secondary structure in a region that does not significantly contribute to the global stability of GroEL as reported by the methods used in this study.

Thus, it appears that interactions with divalent cations can modulate the exposure of hydrophobic surfaces presented by GroEL, with Zn\(^{2+}\) being the most effective. It is possible that GroEL has a low affinity metal binding site (12) or that the divalent cations act in a more general fashion by shielding the negative charge on GroEL. For example, due to the high net negative charge on GroEL (~266 per 14-mer), it may be that cationic interactions of salts and/or cationic amphipathic secondary structure on substrate polypeptides are required for a coordinated exposure of buried hydrophobic binding sites on GroEL. Thus, the binding of divalent cations to GroEL may be a way of fine-tuning the exposure of hydrophobic residues for the binding of unfolded substrate polypeptides.

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