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A polypeptide bound by the chaperonin groEL is localized within a central cavity

(protein folding)

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ABSTRACT **Chaperonins are oligomeric protein complexes that play an essential role in the cell, mediating ATP-dependent polypeptide chain folding in a variety of cellular compartments. They appear to bind early folding intermediates, preventing their aggregation; in the presence of MgATP and a cochaperonin, bound polypeptides are released in a stepwise manner, associated with folding to the native state. Chaperonin complexes appear in the electron microscope as cylindrical structures, usually composed of two stacked rings, each containing, by negative staining, an electron dense central "hole" ≈6.0 nm in diameter. We sought to identify the site on the *Escherichia coli* chaperonin groEL, where the "molten globule"-like intermediate of dihydrofolate reductase (DHFR) becomes bound, by examining in the scanning transmission electron microscope complexes formed between groEL and DHFR molecules bearing covalently crosslinked 1.4-nm gold clusters. In top views of the groEL complexes, gold densities were observed in the central region; in side views, the densities were seen at the end portions of the cylinders, corresponding to positions within the individual rings. In some cases, two gold densities were observed in the same groEL complex. We conclude that folding intermediates are bound inside central cavities within individual chaperonin rings. In this potentially sequestered location, folding intermediates with a compact conformation can be bound at multiple sites by surrounding monomeric members of the ring; localization of folding within the cavity could also facilitate rebinding of structures that initially fail to incorporate properly into the folding protein.**

Recent studies indicate that in the living cell newly synthesized and newly translocated polypeptides are folded to their native conformations with the assistance of molecular chaperones (for review, see ref. 1–4). One class of chaperone, the Hsp70 family, appears to bind nascent polypeptide chains with extended conformations, via hydrophobic residues, acting to prevent aggregation. A second class of chaperone, the Hsp60 class, whose members are also referred to as chaperonins, may act subsequently, binding polypeptides that have already acquired secondary structure but lack native tertiary structure. Chaperonins are found as oligomeric protein complexes, usually composed of two stacked rings. They appear in the electron microscope as cylindrically shaped structures and are typically ≈14 nm in diameter and ≈16 nm in height (5–9), a size nearly that of a ribosome. Chaperonins have been shown both *in vivo* and *in vitro* to mediate ATP-dependent polypeptide chain folding (see refs. 1–4). So far, they have been identified as two structurally and evolutionarily related families of components, one family with members in the bacterial cytoplasm (groEL) (10, 11) and in endosymbiotically derived organelles, chloroplasts (ribulose-bisphosphate carboxylase binding protein) (12) and mitochondria (Hsp60) (13), and the second family with members in thermophilic archaebacteria (TF55) and the eukaryotic cytosol (TCP1 complex) (14, 15).

Studies carried out both in intact cells and with the purified chaperonin complexes *in vitro* suggest that binding of unfolded proteins by these components prevents aggregation and maintains the bound proteins in productive intermediate conformations that are at least in some cases collapsed "molten globule"-like forms (16–26). In the presence of cochaperonin and ATP hydrolysis, bound polypeptides are released from chaperonin complexes associated with folding to native conformation. At least the initial stages of folding appear to occur while the polypeptide chain remains associated with the complex (21).

Little is known about the nature of interaction between unfolded polypeptides and chaperonin complexes. A fundamental question concerns where polypeptides localize in relation to the double ring structures. Here we have addressed this question by direct inspection in the scanning transmission electron microscope (STEM) of a polypeptide tagged with a gold cluster bound to the *Escherichia coli* chaperonin groEL.

MATERIALS AND METHODS

Coupling Reactions and Methotrexate-Agarose Affinity Chromatography. Gold clusters (200 nmol; diameter, 1.4 nm) (27) were incubated with 20 mmol of iodoacetic acid N-hydroxysuccinimide ester for 45 min at 23°C in 400 μl of buffer containing 0.1 M sodium phosphate (pH 7.5) and 20% (vol/vol) dimethylformamide. The activated gold clusters were separated from the crosslinker by gel filtration on GH25 in 0.1 M sodium phosphate, pH 8.0/1 mM EDTA/10% (vol/vol) isopropanol. Chicken dihydrofolate reductase (DHFR) (20 nmol) (Sigma) was added to the activated clusters in a 2-ml vol and incubated overnight at 4°C. The reaction products were applied to a Superdex 75 column and the void volume (4 ml) in turn was incubated overnight with methotrexate-agarose (0.3 ml of hydrated resin) (Sigma) at 4°C in a buffer containing 50 mM potassium phosphate (pH 5.6) (28). The resin was then washed twice with 1 ml of 10 mM potassium phosphate (pH 5.6), twice with 1 ml of 50 mM ammonium bicarbonate (pH 8.5), and then eluted by incubation with 1.2 ml of 200 μM dihydrofolate in 50 mM ammonium bicarbonate (pH 8.5). The elution products were concentrated by lyophilization.

Incubations of Gold–DHFR Adduct (Au26DHFR) and Preparation for Microscopic Examination. To test whether the 26-kDa elution product (Au26DHFR) could be refolded from 6 M guanidine hydrochloride (Gdn·HCl), ≈1 μg was first incubated for 20 min at 20°C in 6 μl of 6 M Gdn·HCl and then diluted 1:60 into 360 μl of buffer containing 50 mM potassium phosphate (pH 7.4). The mixture was then adjusted to pH 5.6

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Abbreviations: STEM, scanning transmission electron microscopy; DHFR, dihydrofolate reductase; Gdn·HCl, guanidine hydrochloride.

by addition of KH_2PO_4, incubated overnight with 0.1 ml of methotrexate agarose resin as described above, washed twice with 0.3 ml of 10 mM potassium phosphate buffer (pH 5.6), twice with 0.3 ml of 50 mM ammonium bicarbonate (pH 8.5), and then eluted as described above in a vol of 0.25 ml and concentrated by lyophilization. For incubation with groEL, the Au26DHFR product, 0.1–0.2 μg of DHFR as judged by SDS/PAGE and silver staining, was incubated first with 6 M Gdn·HCl for 20 min at 20°C in a final vol of 3 μl and then diluted into 180 μl containing groEL (5 μg) prepared as described (21) in 50 mM ammonium acetate (pH 7.4). After 10 min, the mixture was filtered in a Centricon 100 (Amicon). Three microliters of the retained high molecular size products were then spotted onto thin carbon-coated holy grids and freeze-dried as described (29). In a control preparation, the filtration products were first incubated with 1 mM MgATP. Samples were either negatively stained with uranyl acetate or freeze-dried as described (29).

Microscopic Examination. Specimens were transferred to the STEM and examined under vacuum at −150°C as described (29). Unstained specimens were first scanned at ×125,000 to detect orientation of the groEL particles and then scanned at ×500,000 to visualize the gold clusters. Particles with shapes recognizable as top or side views were scored for location of gold clusters. Statistical analysis was carried out on localization of gold clusters in relation to groEL. For top and side views, a simple χ^2 analysis with three categories was performed; P values were determined with 2 degrees of freedom.

RESULTS

Coupling of 1.4-nm Gold Clusters to DHFR. Gold clusters with a diameter of 2.7 nm, each composed of a 1.4-nm-diameter core of 67 gold atoms surrounded by an organic shell (27), were covalently coupled to chicken DHFR (30) (Fig. 1*A*). After gel filtration to separate DHFR molecules from unbound gold, the products were examined in SDS/polyacrylamide gel. A collection of species of molecular size ranging from 14 to >45 kDa was observed (Fig. 1*B*, lane 1). The presence of species migrating more rapidly than the input DHFR (≈21 kDa; lane 4) indicated that proteolysis had occurred to some extent during the reactions, contributing to heterogeneity. Species migrating more slowly than the input DHFR are likely to comprise gold adducts, whose heterogeneity could be explained at least in part by attachment of more than one DHFR molecule to gold clusters containing more than one amino group. Less likely is the possibility that more than one gold cluster has been added to some of the DHFR molecules—this would necessarily involve reaction at sites other than the single cysteine at position 11 in DHFR.

A Gold Adduct with a Native-like Conformation, Au26-DHFR, Purified by Methotrexate Affinity Chromatography, Can Refold to Its Native-like State Following Dilution from 6 M Gdn·HCl. Gold-labeled DHFR species that retained a sufficiently native-like conformation to contain a substrate binding pocket were selected by substrate affinity chromatography on methotrexate-agarose (28). Only one of the applied species was recovered, with a relative migration of ≈26 kDa (Fig. 1*B*, lane 2). The apparent size of this species suggests that it is composed of an intact DHFR polypeptide with a single gold cluster attached, presumably at Cys-11. The presence of gold clusters was confirmed by examination of the sample in the STEM (at ×500,000, gold densities were regularly observed; data not shown). A localized haziness surrounding the clusters was presumed to be contributed by the DHFR polypeptide. The gold adduct, termed Au26-DHFR, was tested for the ability to refold to its native-like state by first unfolding it in 6 M Gdn·HCl and then diluting the mixture 1:60 into an aqueous buffer. When the diluted

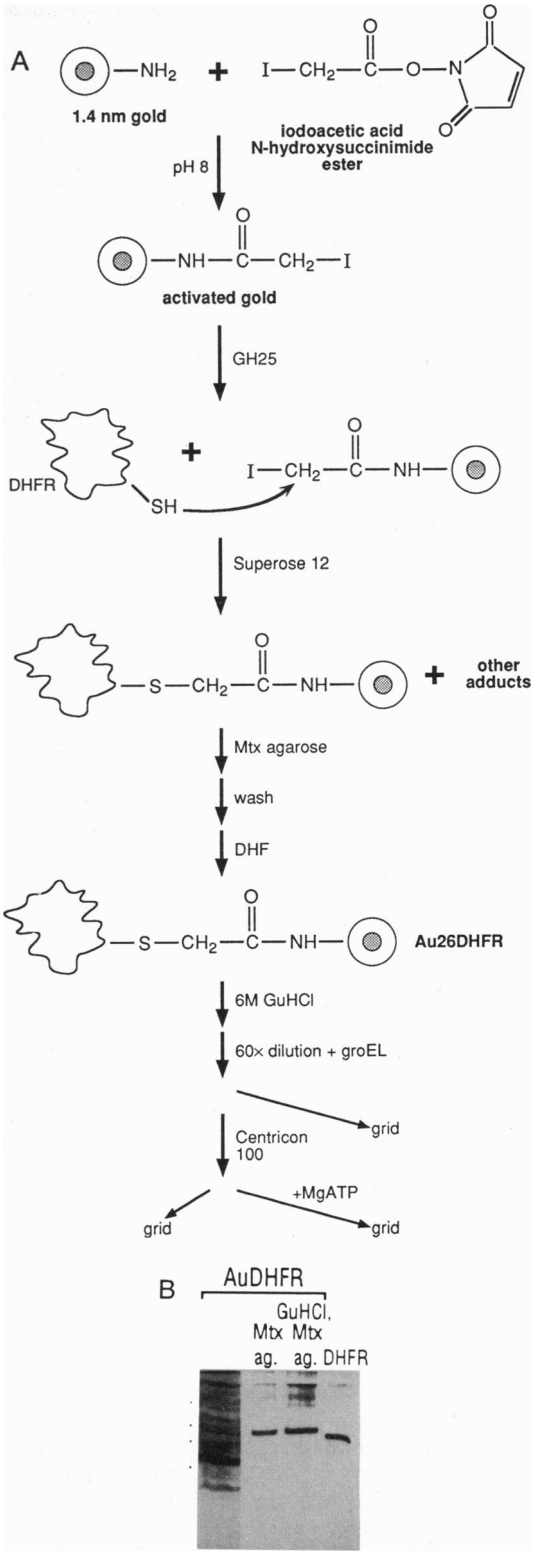

FIG. 1. Preparation and examination of gold-labeled DHFR and SDS/PAGE analysis of gold adducts. (*A*) Chemical crosslinking of 1.4-nm gold clusters to DHFR, purification by substrate affinity chromatography of an adduct with a native-like conformation (Au26DHFR), and preparation and examination of complexes of adduct with groEL. (*B*) Silver-stained SDS/polyacrylamide gel showing products of gold coupling (lane 1), gold adduct isolated by substrate affinity chromatography (Au26DHFR) (lane 2), and gold adduct after dilution from denaturant and substrate affinity chromatography (lane 3). Species are compared with input chicken DHFR (lane 4). Size marker dots (top to bottom) correspond to 45, 29, 21, and 14 kDa. Mtx, methotrexate; ag., agarose; GuHCl, Gdn·HCl.

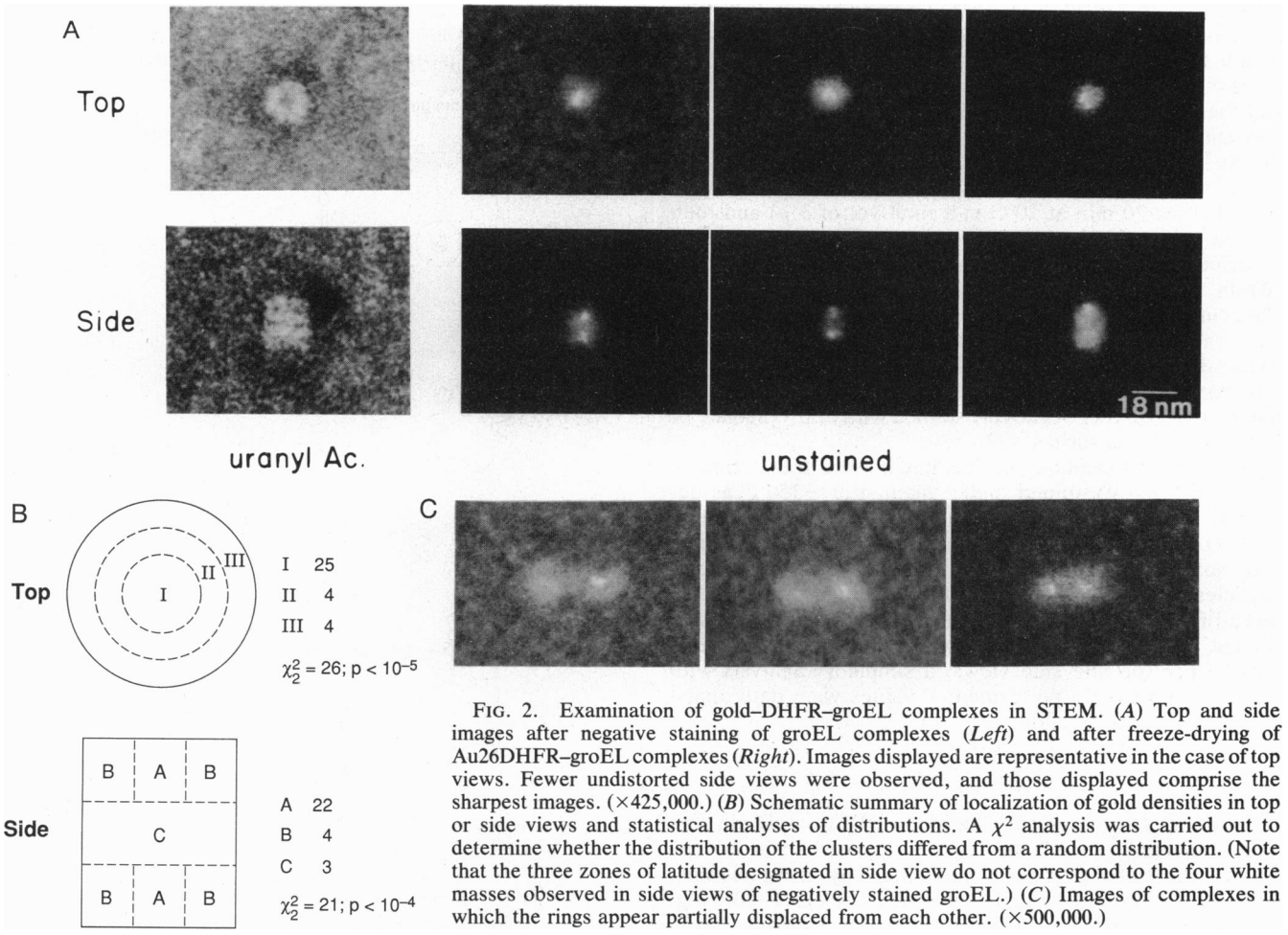

FIG. 2. Examination of gold–DHFR–groEL complexes in STEM. (*A*) Top and side images after negative staining of groEL complexes (*Left*) and after freeze-drying of Au26DHFR–groEL complexes (*Right*). Images displayed are representative in the case of top views. Fewer undistorted side views were observed, and those displayed comprise the sharpest images. (×425,000.) (*B*) Schematic summary of localization of gold densities in top or side views and statistical analyses of distributions. A χ^2 analysis was carried out to determine whether the distribution of the clusters differed from a random distribution. (Note that the three zones of latitude designated in side view do not correspond to the four white masses observed in side views of negatively stained groEL.) (*C*) Images of complexes in which the rings appear partially displaced from each other. (×500,000.)

mixture was applied to methotrexate affinity chromatography, the Au26DHFR species was quantitatively recovered (Fig. 1*B*, lane 3), indicating that the attached gold cluster does not interfere with refolding of the DHFR polypeptide to a native-like state. It therefore seemed likely that Au26DHFR would be able to form a folding intermediate(s), similar to that of unmodified DHFR, that could be recognized and stabilized by the chaperonin groEL.

Examination of Au26DHFR Bound to groEL in STEM. Au26DHFR was diluted from Gdn·HCl into buffer containing groEL, and the mixture was filtered on a Centricon 100 membrane to separate Au26DHFR–groEL from unbound Au26DHFR. The retained fraction was then applied to thin carbon-coated grids, freeze-dried, and examined in the STEM. Examination of individual fields at ×125,000 revealed shapes characteristic of groEL, with top views appearing as circular discs ≈14 nm in diameter and side views appearing as rectangles ≈14 × ≈16 nm. When these same structures were examined at ×250,000 and ×500,000, their shapes were preserved, with top and side views remaining recognizable in comparison with negatively stained groEL particles that had been previously examined (Fig. 2*A Left*). At ×500,000 magnification, gold densities could be observed associated with many of the structures. The densities were found exclusively in association with the particles; in contrast, in preparations in which groEL was not first separated from unbound Au26DHFR, gold densities were observed throughout the fields (data not shown). In an additional control study, if gold clusters alone (nonactivated) were incubated directly with groEL and the filtered mixture was applied to grids, no clusters were observed associated with the groEL particles, indicating that binding of Au26DHFR occurs through the DHFR moeity. Clusters also failed to be observed if MgATP was added to the Au26DHFR/groEL mixture prior to the filtration step (data not shown), suggesting that Au26DHFR can be released from groEL in a manner similar to that of unmodified DHFR (21). MgATP-dependent release was more directly observed by examining comigration in a nondenaturing gel of Au26DHFR molecules that had been prepared with [^{35}S]methionine-radiolabeled human DHFR. After incubation with groEL, ^{35}S-labeled Au26DHFR was readily observed at the position of groEL, but after incubation of the ^{35}S-labeled Au26DHFR–groEL mixture with MgATP, the radiolabeled protein failed to enter the gel (data not shown), characteristic of monomeric DHFR.

In top-down views of two independent preparations of Au26DHFR–groEL, gold clusters were observed mostly within the central portion of the discs (Fig. 2 *A* and *B Upper*). While the gold clusters appeared off center in several cases, they never appeared outside the perimeter of the disc. This observation is corroborated by recent top views obtained after negative staining of groEL–polypeptide complexes that indicated reduced electron density of the central hole, suggesting the presence of polypeptide (31). In side views, the gold clusters in most cases were positioned at the end thirds of the cylinder, at or near the central long axis (representing the axis of symmetry, perpendicular to the plane of the rings) (Fig. 2 *A* and *B Lower*). Clusters were only rarely observed in the equatorial third of the cylinders and were never seen outside the perimeter. For both top and side views, a statistical analysis of the distribution of the densities supported our interpretation of the nonrandom distribution (Fig. 2*B*). The observations, taken at a 90° angle from each other, position the gold particles within the individual rings of the groEL

complex, as distinct from localization either at the outer surface or in the equator.

In some top views, it appeared that two or perhaps three gold clusters were present in the same groEL complex (Fig. 2*A*). Considering that only single gold clusters were observed when Au26DHFR was applied alone to the grids, it appeared unlikely that this was due to aggregation of the gold clusters or of Au26DHFR itself. Rather, the possibility that two molecules of Au26DHFR could be simultaneously bound by a single complex was supported by the observation of gold densities in both end portions of the same cylinder in a number of side views (Fig. 2*A*). In addition, a number of extended shapes were observed in the preparation that are likely, based on previous negative staining studies, to be composed of two rings from the same groEL complex that had been partially separated during preparation (Fig. 2*C*). In several of these views, gold densities were observed simultaneously in both of the rings. We conclude that groEL can bind two DHFR polypeptides simultaneously *in vitro*. Whether this also occurs *in vivo* is unknown. A number of studies indicate that the single ring groES heptamer binds asymmetrically to groEL, one ring apposing one end of the groEL cylinder (19, 21, 31–34). If this association is stable in the cell, it may dispose only one cavity of groEL to entry of a polypeptide, presumably the contralateral cavity. Alternatively, if there is an order of interaction *in vivo*, with the polypeptide binding prior to groES, either cavity would be available, and either or both cavities could offer a site for folding. Considering this possibility, we note that folding can apparently take place in the same ring to which groES binds—a single ring mammalian hsp60 complex has recently been shown to be able to reconstitute a sequence of binding and groES-mediated folding of a polypeptide *in vitro* (35).

DISCUSSION

The observations reported here concerning localization of DHFR bound to groEL relied on scoring the position of covalently attached gold clusters whose cores were positioned ≈2.7 nm from a side chain of DHFR (27). Thus, the polypeptide itself could be somewhat removed from the site of the gold cluster. This would not be likely to affect the conclusion that DHFR is localized within the central cavities of the rings, except in a situation in which gold clusters would localize within the cavities while the polypeptide chains themselves would reside outside the ends of the cylinders. We cannot exclude this possibility but note that the aperture in groEL, ≈6.0 nm in diameter, is probably sufficient to accommodate even side-by-side entry of a 2.5- to 3.0-nm globular DHFR intermediate plus a 2.7-nm gold cluster.

The localization of polypeptide folding intermediates within the central cavities of chaperonin rings could have several potential advantages: (*i*) Intermediates with a compact conformation (21, 36, 37) could be bound at multiple sites by surrounding monomeric members of the ring. In contrast, binding at an outer, convex, surface of a ring would not be likely to afford such extensive interaction with a compact globule. (*ii*) Within the cavities of the rings, folding intermediates could in principle be physically removed from the bulk solution where other proteins, including nonnative, aggregation-prone forms, may reside in high concentration. This idea is attractive as a means of diminishing the chance of aggregation, but it is somewhat open to question given observations that bound polypeptides remain accessible to small proteases (21). (*iii*) As suggested by Creighton (38), chain folding carried out at least initially within the cavities of the rings, associated with ATP-dependent release controlled by groES (21), could be facilitated by the opportunity for the folding chain to be rebound by the surrounding monomers or by the members of the adjacent ring if a more native-like conformation is not initially achieved. (*iv*) With polypeptide binding sites at groEL positioned within a cavity, homotypic interactions between groEL rings themselves would be minimized.

Accompanying the foregoing advantages is an inherent limit to the volume of a polypeptide entering the cavity of groEL. Allowing for a cylindrical cavity within groEL ≈6.0 nm in diameter and ≈7.0 nm in height, and assuming a specific density of 2.3 Å^3 per Da based on the packing in protein crystals (39), the cavity within groEL could accommodate a 90-kDa globular protein. Thus, the majority of the cytosolic monomeric subunits of *E. coli* could be accommodated. Larger protein monomers could be bound in a domainwise fashion or other components could be involved with mediating their folding to native form. The size of the groEL aperture and cavity might be most significant with respect to dictating the conformation of proteins that could be bound by groEL. The size constraints could demand that only collapsed intermediates, which occupy relatively less volume than the extended forms of polypeptides, would have access to the binding sites within groEL. This could conceivably allow similar hydrophobic properties of nonnative proteins to be recognized by both groEL and hsp70 (40, 41), but, in the case of groEL, only polypeptides that have proceeded beyond an extended conformation to a collapsed state (37) could have access to the binding sites. While the present study provides insight into the geometry of polypeptide chain binding by chaperonins, the nature of binding sites within the chaperonins and the sites recognized in folding intermediates nevertheless remain to be established.

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