Conditions of Forming Protein Complexes with GroEL Can Influence the Mechanism of Chaperonin-assisted Refolding*

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Boris M. Gorovits and Paul M. Horowitz‡

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‡To whom correspondence should be addressed: Dept. of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284

The interaction of GroEL with urea-unfolded dihydrofolate reductase (DHFR) has been studied in the presence of DHFR substrates by investigating the ability of GroES to release enzyme under conditions where a stable GroES-GroEL-DHFR ternary complex can be formed. In these circumstances, GroES could only partially discharge the DHFR if ADP was present in the solution and approximately half of the DHFR remained bound on the chaperonin. This bound DHFR could be rescued by addition of ATP and KCl into the refolding mixture. The stable ternary complex did not show any significant protection of bound DHFR against proteolysis by Proteinase K. These results are in contrast to those observed with the GroEL-DHFR complex formed by thermal inactivation of DHFR at 45 °C in which GroES addition leads to partial protection of bound DHFR. Thus, the method of presentation influences the properties of the bound intermediates. It is suggested that the ability of GroES to bind to the same side of the GroEL double toroid as the target protein and displace it into the central cavity depends on the way the protein-substrate is presented to the GroEL molecule. Therefore, the compact folding intermediate formed by thermal unfolding can be protected against proteolysis after GroES binds to form a ternary complex. In addition, structural changes within GroEL induced by the experimental conditions may contribute to differences in the properties of the complexes. The more open urea-unfolded DHFR binds on the surface of chaperonin and can be displaced into solution by the tighter binding GroES molecule. It is suggested that the state of the unfolded protein when it is presented to GroEL determines the detailed mechanism of its assisted refolding. It follows that individual proteins, having characteristic folding intermediates, can have different detailed mechanisms of chaperonin-assisted folding.

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GroES Can Displace DHFR from cis- and trans-Toroids of GroEL

**RESULTS**

**GroEL Can Arrest Spontaneous Refolding of Urea-unfolded Bovine DHFR**—Bovine DHFR was unfolded in 8 M urea. Dilution of the unfolded material to the buffer, containing no denaturant, results in fast unassisted refolding, detected as an appearance of enzymatic activity of the native protein. The kinetics of NADPH oxidation by dihydrofolate, catalyzed by native DHFR, have been used in this study to analyze the amount of successfully folded enzyme in solution. As shown in Fig. 1, introduction of GroEL to the refolding solution results in a decrease of the enzyme activity. A similar result has been reported for the mouse DHFR whose refolding can be completely arrested upon binding to GroEL (8). Fig. 1 demonstrates that almost 80% of DHFR can be captured at 1:1 ratio of GroEL to the unfolded DHFR whether or not ADP is present in solution.

The GroEL-DHFR complex slowly dissociates (data not shown and Ref. 8). Under conditions when enzyme substrates (NADPH and dihydrofolate) are present in the solution, considerable dissociation was detected after 1 h of incubation (data not shown). The mouse DHFR-GroEL complex has been reported to completely dissociate after 3 h of incubation under similar conditions (8). These data suggest that the enzyme exists in dynamic equilibrium between free and bound states.

As with several other proteins, the addition of ATP alone can lead to the release of fully active bovine DHFR from its complex with GroEL. These weakly bound proteins contrast with the class of tightly bound proteins, like rhodanese (2), 6-hydroxy-p-nicotine oxidase (15), and ornithine transcarbamylase (16). These latter proteins require the presence of the complete chaperonin system, including the co-chaperonin GroES, in order to be successfully released. The presence of GroES, although not required, does facilitate dissociation of DHFR from the GroEL-DHFR complex (8).

**GroES Can Partially Discharge DHFR from the Complex with GroEL in the Presence of ADP**—Fig. 2 shows that GroEL can arrest approximately 72% of the spontaneous folding of DHFR (open squares, Fig. 2). Addition of GroES to the preformed GroEL-DHFR complex results in partial discharge of the active enzyme (closed squares, Fig. 2). The amount of the released protein increases up to about 1:1 the ratio of GroES to GroEL-DHFR complex. Subsequent addition of GroES does not result in any considerable change of the free enzyme concentration. The maximum level of DHFR released (65%) represents about 50% of the total protein that had been captured by GroEL (Fig. 2). The remaining half of the captured enzyme could be released upon addition of ATP and KCl, producing a 95% yield of active protein. As a control, a similar titration of GroES to the GroEL-DHFR complex with no nucleotide present in the solution was carried out. Since the GroEL-GroES interaction is not possible under this condition (data not shown and Ref. 4), no significant discharge of the DHFR over a control sample was observed within the time of this experiment.

**Digestion Analysis of the GroEL-DHFR-GroES Complex**—The presence of a nucleotide is not required for the binding of an unfolded protein-substrate to GroEL (Fig. 1; Ref. 2). Therefore, a random distribution of the bound nucleotide (ADP) relative to the site of the unfolded polypeptide binding on two GroEL toroids can result in two distinct types of GroES-GroEL...
Proteinase K was added (lanes 2–3) and methylsulfonyl fluoride to 3 mM. Samples were analyzed on the 12% SDS Laemmli gel system followed by silver staining. The arrows show GroEL (1) and DHFR (2).

interactions (4). Thus, GroES binding can occur on the same toroid (cis) where protein-substrate is bound or on the opposite site (trans) of the GroEL oligomer. These two complexes can be distinguished by using protease digestion analysis as was reported earlier (4). In the present study, two different methods were used to form the GroEL-DHFR complex. In the first case, DHFR was unfolded in 8 M urea for 15 h before dilution in the presence of GroEL. In the second case, the complex was formed by heating together GroEL and native DHFR at 45 °C for 10 min as reported earlier (7). Complexes were purified using size exclusion chromatography, and fractions containing GroEL were collected and concentrated. Solutions were supplemented with ADP and GroES (when needed). Digestion analysis was performed using Proteinase K (final concentration 0.2% w/w). Two different results were observed. While unfolded DHFR can be partially (42 ± 5%) protected by GroEL-GroES interaction if the GroEL-DHFR complex was preformed by heat denaturation (Fig. 3, lane 7), no protection could be detected if the complex was formed by addition of urea-unfolded protein (Fig. 3, lane 3). The positive controls, containing GroEL-DHFR complexes, formed by using urea or heat-denatured enzyme and consequently treated with ADP and GroES, but not Proteinase K, are shown in lanes 1 and 6, respectively. These results suggest that, due to the considerable differences in the protein conformation between chemically and thermally unfolded protein, the resulting GroEL-protein complexes have different properties, leading to the different mechanism of the GroES-facilitated protein release.

**FIG. 3.** Proteolytic analysis of the DHFR-GroEL complexes. Complexes between DHFR and GroEL were formed with urea-denatured (lanes 1, 2, and 3) and heat-denatured DHFR (lanes 4, 5, 6, and 7). Complexes were purified on a Superose 12 gel filtration column. Fractions containing GroEL were collected and concentrated to the final GroEL concentration of 0.35 mM. All samples were supplied with 2 mM ADP, and 0.7 μM GroES was added if necessary (lanes 1, 3, 6, and 7). Proteinase K was added (lanes 2, 3, 5, and 7) to a final concentration of 0.2% of the total protein (w/w), and the solution was incubated for 10 min at room temperature. The reaction was stopped by adding phenylmethylsulfonyl fluoride to 3 mM. Samples were analyzed on the 12% SDS Laemmli gel system followed by silver staining. The arrows show GroEL (1) and DHFR (2).

**DISCUSSION**

Several different mechanisms have been suggested for the function of the GroEL-GroES system (4, 6). The release of the bound polypeptide from its complex with GroEL is one of the central steps that such a mechanism must address. Several aspects of these mechanisms are based on the ability to form relatively stable complexes involving GroEL in the presence of ADP. It was demonstrated (7) that ternary complexes containing DHFR, GroEL, and GroES could be formed by thermal denaturation and that a significant fraction of the bound DHFR was protected against Proteinase K digestion. This was taken to imply that GroES could bind in one of two ways to the complex. First, GroES could bind over the central cavity of GroEL on the same toroid as DHFR (cis), thus capping the complex and ejecting the DHFR into the chamber formed from the central cavity in the GroEL toroid and the overlying GroES dome; or second, GroES could bind on the toroid opposite to the bound DHFR (trans) leaving the DHFR on the surface. In this model, the DHFR in the cis complex is protected from Proteinase K, while that in the trans complex can be proteolyzed.

The present results are consistent with the observations for the thermally denatured protein, but the results are quite different for the urea-unfolded DHFR. While nearly 50% of DHFR can be protected by GroEL-GroES interaction in the former case, no protection was detected in the case of urea-unfolded protein-substrate (Fig. 3). Thus, it is clear that the complexes formed in these different ways have different characteristics. In contrast to the thermally produced complex, the urea complex can be partially discharged by GroES, and the undischarged part of the bound DHFR is sensitive to Proteinase K digestion. The considerable conformational differences between heat- and chemically unfolded protein can explain this phenomenon. DHFR in 8 M urea is likely to be fully unfolded, and the collapsed states that form on dilution could bind extensively to the binding sites on one of the two GroEL toroids. These extensive surfaces and the rapid collapse of the DHFR on dilution would favor binding close to the surface of the chaperonin. This surface location would be favored by the fact that the protein is introduced to a relatively unperturbed GroEL molecule. Since protein binding and GroES binding appear to involve the same or similar sites, peripheral binding of DHFR would lead to a weak complex in which cis GroES binding could release DHFR into the bulk solution by direct displacement. The trans ternary complex would be relatively stable in the presence of ADP. Thus, urea-unfolded DHFR would form a complex with GroEL, and GroES would release 50% by direct displacement and leave 50% as a trans complex that is susceptible to Proteinase K.

Thermally produced DHFR-GroEL complexes are different from complexes formed using urea-denatured DHFR. Thermal experiments that demonstrate the complex are performed by heating DHFR together with GroEL at 45 °C, so that thermal effects on both DHFR and GroEL must be considered. Bovine DHFR has a thermal transition with a midpoint at −50 °C (17), so the 45 °C incubation does not lead to fully heat-denatured protein. In addition, many proteins, even after complete thermal denaturation, still contain significant ordered structures that can be unfolded further (18). In addition, GroEL itself has been reported to undergo two thermal transitions between 25 and 35 °C (19), so that temperature-induced formation of complexes influences the structure of GroEL. This suggestion is supported by the observation that there are proteolytic bands that must come from GroEL after proteolytic treatment of the thermally induced complexes (Fig. 3). It is possible, then, that heating DHFR in the presence of GroEL results in small, partially unfolded structures that can bind inside of the perturbed GroEL cavity. Then, the subsequent addition of GroES can result in forming a capped cis complex as described previously. The heating together of DHFR and GroEL is important, since the control experiments that have been carried out in the present study showed that no complexes between GroEL and DHFR can be formed when GroEL is preheated at 45 °C for 10 min and then added to native DHFR at 25 °C (data not shown). When DHFR was heated in the absence of GroEL, a precipitate formed, and the protein that remained in the solution could not be captured by GroEL at 25 °C.

The present data support the concept that the ability of the unfolded polypeptide to form a stable complex with GroEL at different steps of its folding pathway can influence the detailed mechanism of its assisted refolding. This would be in keeping with suggestions that chaperonin-facilitated folding can be modulated by the properties of both the protein-substrate and the GroEL (20–22).
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