Changing the Nature of the Initial Chaperonin Capture Complex Influences the Substrate Folding Efficiency*

(Received for publication, March 17, 1998, and in revised form, June 22, 1998)

Paul A. Voziyan, Bryan C. Tieman, Chee-Meng Low, and Mark T. Fisher‡
From the Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421

For the chaperonin substrates, rhodanese, malate dehydrogenase (MDH), and glutamine synthetase (GS), the folding efficiencies, and the lifetimes of folding intermediates were measured with either the nucleotide-free GroEL or the activated ATP-GroEL-GroES chaperonin complex. With both nucleotide-free and activated complex, the folding efficiency of rhodanese and MDH remained high over a large range of GroEL to substrate concentration ratios (up to 1:1). In contrast, the folding efficiency of GS began to decline at ratios lower than 8:1. At ratios where the refolding yields were initially the same, only a relatively small increase (1.6-fold) in misfolding kinetics of MDH was observed with either the nucleotide-free or activated chaperonin complex. For rhodanese, no change was detected with either chaperonin complex. In contrast, GS lost its ability to interact with the chaperonin system at an accelerated rate (8-fold increase) when the activated complex instead of the nucleotide-free complex was used to rescue the protein from misfolding. Our data demonstrate that the differences in the refolding yields are related to the intrinsic folding kinetics of the protein substrates. We suggest that the early kinetic events at the substrate level ultimately govern successful chaperonin-substrate interactions and play a crucial role in dictating polypeptide flux through the chaperonin system. Our results also indicate that an accurate assessment of the transient properties of folding intermediates that dictate the initial chaperonin-substrate interactions requires the use of the activated complex as the interacting chaperonin species.

The bacterial chaperonin GroEL, a member of the Hsp 60 chaperonin family, influences the successful protein folding in vitro (for review, see Fenton and Horwich (1)). The GroEL oligomer contains 14 identical 57-kDa monomers arranged in a complex of two toroid-like heptameric rings to form a cylindrical structure with two central cavities (2, 3). Through a mechanism involving multiple rounds of binding and dissociation of co-chaperonin GroES, coupled with the binding and hydrolysis of MgATP, GroEL cycles between a number of conformational states that have different affinities for the protein substrate (4, 5). The binding of GroES brings about a major conformational change in the chaperonin complex allowing for a brief encapsulation of the protein substrate inside the GroEL cavity where folding can continue (2, 6–8). A substantial amount of in vitro and in vivo evidence now indicates that protein folding is not necessarily completed within this enclosed environment. It has been found that, during chaperonin cycling, a large amount of protein is released into solution as non-native states that can rebind to the chaperonin (9–14). The continuous cycle of binding and release of the co-chaperonin and the protein substrate is controlled by the rate of ATP binding and hydrolysis. The GroEL/GroES complex is thought to have a half-life of about 20 s at 25 °C (14).

Despite the significant progress in the understanding of the GroE chaperonin structure and function, the molecular processes involved with the interactions between a folding polypeptide and the chaperonin complex during multiple rounds of substrate binding and release remains obscure. With regard to successful folding, the initial interaction of folding intermediates with the GroE chaperonin complex is clearly one of the most critical steps of the chaperonin mechanism. The binding interaction between the protein folding intermediates and the substrate binding sites on GroEL is governed mainly by hydrophobic interactions (15, 16), although electrostatic forces may also contribute to this binding (17). These general hydrophobic interactions are most likely responsible for the low substrate specificity of the GroE complex. This property partially explains the ability of chaperonins to productively interact with different folding intermediates and influence the folding of structurally diverse proteins. Because folding proteins may undergo very rapid compaction and hydrophobic collapse prior to binding to the chaperonins, it has been postulated that chaperonins may be able to bind to folding intermediates that possess a large amount of secondary structure, i.e. a molten globule state (18–20). Indeed, hydrogen-deuterium exchange experiments performed with GroEL-bound dihydrofolate reductase and analyzed by NMR or mass spectrometry have revealed that the bound structure has a significant amount of native-like characteristics (21, 22). Although earlier data suggested that the chaperonins may recognize specific secondary elements in proteins, recent crystallographic evidence indicates that GroEL preferentially binds to unstructured or extended regions in the folding protein (23). Even so, it appears that the chaperonin interacts with a wide range of folding intermediates whose structures vary from early collapsed forms to near native forms (11, 24–27). Interestingly, proton exchange experiments indicate that partial to complete unfolding can occur during long term substrate-chaperonin interactions (28). It has been suggested that chaperonins may function primarily by unfolding misfolded protein intermediates (5, 29–31) where the binding reaction between GroEL and the protein substrate would provide the free energy necessary for unfolding (29). However, in cases where the folded conformer is constrained by disulfide bonds, the protein may not be measurably.

* This work was supported by National Institutes of Health Grant GM43909. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Kansas Medical Center School of Medicine, 3901 Rainbow Blvd., Kansas City, KS 66160-7421. Tel.: 913-588-6940; Fax: 913-588-7440; E-mail: mfisher1@kumc.edu.
unfolded during its interaction with the chaperonin (26).

With a diverse set of chaperonin substrates, there is a high degree of variability in the chaperonin requirements for folding; for example, some proteins need GroES and ATP to fold with GroEL, while others simply require ATP (for review, see Jaenicke (32)). This variability depends on the individual properties of the different substrates that can influence chaperonin mechanism either directly or indirectly. Direct influences may result from binding interactions, where the folding substrate affects either the lifetime of the GroEL-GroES complex or the initial binding of GroES onto the GroEL-polypeptide complex. Indirect influences would depend more on the properties of the folding substrates prior to their binding to or after their release from the chaperonin system. For instance, it has been shown that the GroES requirement depends, in part, on the propensity of the substrate to spontaneously fold to its native structure, without the aid of chaperonins (33).

Most of the experimental work characterizing the chaperonin solution requirements has been done by first forming an initial complex between protein substrates and the nucleotide-free chaperonin (GroEL alone). Following the formation of the complex, folding was initiated by the addition of either ATP or GroES. This approach, however, gives virtually no information about the properties of the folding intermediates prior to their interaction with the chaperonins. Furthermore, the chaperonin species that has the highest affinity for polypeptide (GroEL alone) is not the major chaperonin capture complex in the cell. Because of the relatively high intracellular concentrations of ATP, ADP, and GroES, and the high affinity of GroEL for these components, it is more than likely that the lower affinity forms (ATP(ADP)GroEL-GroES) are the major chaperonin complexes that interact with the folding polypeptide in vivo (for review, see Fenton and Horwich (1)). Previous data by Horwich and co-workers indicated that the nature of the chaperonin capture complex may influence the protein folding reaction (6). In our current study, we have examined the folding propensity and kinetic aspects of the initial interactions between protein substrates and different chaperonin complexes. Glutamine synthetase (GS), rhodanese, and MDH, were chosen because each has different chaperonin requirements for efficient folding. While GS and rhodanese require at least a stoichiometric amount of the chaperonin for successful folding, MDH folds efficiently with only one-tenth of this amount of the chaperonin (29). Furthermore, rhodanese and MDH absolutely require GroES for folding and are commonly defined as representative chaperonin substrates, whereas GS can fold with only GroEL and ATP (37). Our data indicate that the folding efficiencies and capture kinetics of each protein depended differently on the nature of the initial capture complex. Our results suggest that the substrate-specific difference in the flux through the chaperonin system is, in part, determined by the initial interactions between protein folding intermediates and chaperonin. This flux is more accurately described when protein folding is examined with the physiologically relevant activated chaperonin complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutamate, ATP, EDTA, DTT, NAH, and ketomalic acid were purchased from Sigma. Urea and guanidinium HCl, both >99% pure, were from ICN Biochemical (Aurora, OH) and Fisher, respectively. Bovine liver rhodanase and pig heart mitochondrial MDH was purchased from Sigma. GS was purified from *Escherichia coli* as described previously (34). All the other chemicals were of the highest purity available.

The E. coli chaperonins, GroEL and GroES, were isolated from lysates of cells containing overexpression plasmids (gifts of Dr. George Lorimer and Dr. Edward Eisenstein) and purified as described earlier (35, 36). Because GroEL or GroES does not contain tryptophan, the loss of the tryptophan indole absorption, assayed by second derivative analysis of absorption spectra, was used as a criterion for purity of the preparations, in addition to analysis by SDS-polycrylamide gel electrophoresis (37). No contribution from tryptophan-containing contaminants was detected.

**Denaturation of Protein Substrates**—GS and rhodanese were denatured in solutions containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM DTT, and 8 M urea. Denaturation was performed for 4 h (GS) or 2 h (rhodanese) at 0 °C (11, 37). For rhodanese, unfolding buffer also contained 50 mM sodium thiouisulfate (11). Denaturation of MDH was followed by the measurements of intrinsic tyrosine fluorescence (at 25 °C) as a function of denaturant concentration and incubation time. By these criteria, MDH was completely unfolded after incubation in 50 mM TEA (pH 7.5), 50 mM KCl, 20 mM MgCl2, 10 mM DTT containing 4 M guanidinium HCl for 0.5 h at 0 °C.

**Refolding of Protein Substrates**—Refolding of the denatured proteins was initiated by a rapid 100-fold dilution into 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 50 mM KCl, 0.5 mM EDTA, 10 mM DTT (for GS and rhodanese) or 50 mM TEA (pH 7.5), 50 mM KCl, 20 mM MgCl2, 10 mM DTT (for MDH) at 37 °C, followed by incubation at this temperature. For rhodanese, the refolding buffer also contained thiouisulfate to stabilize rhodanese against irreversible inactivation in the presence of oxygen (38). To assess differences in the chaperonin affinities and their potential effects on rescuing the folding protein, either nucleotide-free or activated chaperonin complexes were added to the refolding protein in question (4). These chaperonin complexes were generated by altering the order of addition of GroEL, GroES, and ATP. When folding was initiated in the presence of GroEL and GroES alone, with no nucleotide present, the chaperonin with the highest affinity (i.e. GroEL) was the only species capable of interacting with the folding protein. The release of the protein from the capture complex was initiated by addition of ATP to the solution after a 10-min incubation. In a different set of experiments, GroEL, GroES, and ATP were first mixed to generate the ATP-GroEL-GroES complex. Within 1 min after formation, this chaperonin species was rapidly mixed with denatured protein to initiate refolding.

To measure the irreversible misfolding rates, either the nucleotide-free or activated chaperonin complexes were added to the samples at different times after the initiation of spontaneous refolding. After folding is complete, the activities for each kinetic time point (time of chaperonin addition) were determined. Unless otherwise stated, final concentrations of chaperonins in all incubations were 1 μM GroEL oligomers and 2 μM GroES oligomers. The concentrations of all the substrate proteins are presented in terms of monomer concentrations.

**Measurements of Protein Enzymatic Activity**—GS activity was determined by the glutamyl transferase assay (39). Rhodanese activity was assayed by monitoring thiocyanate formation after 10 min at 25 °C (40). MDH activity was measured by diluting an aliquot from the folding mixture into 1 mM ketomalic acid, 0.2 mM NADH, 10 mM DTT, and 50 mM TEA, pH 7.5, and following the oxidation of NADH at 340 nm (29).

**RESULTS**

**Folding of Substrate Proteins after the Initial Interaction with Nucleotide-free and Activated Chaperonin Complexes**—At the molar ratio of 2.1 (1 μM GroEL oligomer to 0.5 μM GS subunit), the refolding of GS with activated complexes showed a significant decrease in both folding rate and final yield, compared with refolding with nucleotide-free complex (Fig. 1A). Following a 5-fold increase in the molar ratio of GroEL to GS where the concentration of the substrate protein was lowered, the reactivation rates and yields for both preparations became very similar (Fig. 1D). In the presence of the nucleotide-free complex, the refolding yields of GS were independent of protein concentration (Fig. 2). With the activated complexes, the yields generally increased as GS concentration increased (Fig. 2). When the GS subunit concentration was lowered to 0.1 μM, the renaturation yields rose to the same levels as measured with the nucleotide-free GroE complexes (Fig. 2). A similar tendency was observed for GS yields with the nucleotide-free and the activated complexes when the substrate protein concentration remained constant at 0.5 μM and the chaperonin
concentration increased to 5 μM GroEL, 10 μM GroES (data not shown). Thus, folding with the activated complex requires a high GroEL to GS molar ratio (>10) to compensate for the decreased affinity for polypeptide as well as a reduction in the number of substrate binding sites. In these experiments, we changed the order of addition of nucleotide and protein substrate to induce the formation of initially different substrate-chaperonin complexes. Because GS itself is capable of binding ATP, it was important to ensure that the presence of the nucleotide did not affect spontaneous refolding of the enzyme. No difference in the kinetics of spontaneous refolding of GS was detected between the samples containing either 5 mM ATP or no ATP after 2 h (data not shown).

Over the same concentration range used for GS, the refolding yields of rhodanese were independent of protein concentration when either the nucleotide-free or the activated complexes were present (Fig. 3). Furthermore, even at the lowest chaperonin to rhodanese molar ratio (2:1), the refolding rates with both complexes were identical (data not shown). Thus, in contrast to what was observed with GS, the chaperonin-assisted refolding of rhodanese remained the same with either the nucleotide-free or activated complexes. Only when protein concentration was increased to 1.5 μM did the refolding yields of rhodanese with both chaperonin complexes simultaneously decline to about 70% of native activity (data not shown). Like rhodanese, the folding yields of MDH with either nucleotide-free or activated chaperonin complexes remained high and did not decline within the substrate concentration range from 0.1 to 0.5 μM MDH (Fig. 3). Refolding yields of MDH remained unchanged even when protein concentration was increased to 1.5 μM (data not shown). These latter results support an earlier observation that an optimal refolding of MDH can occur at chaperonin to substrate molar ratios as low as 1:10 (29).

Our data demonstrate that GS reactivation is sensitive to the nature of the chaperonin capture complex over a substrate concentration range of 0.1 to 0.5 μM, while rhodanese and MDH are unaffected over the same range. These results imply that a portion of the folding GS monomers are unable to interact with the activated chaperonin as the concentration of GS increases. We have reasoned that a decrease in the interaction between the chaperonin complex and the folding protein could be among the most probable causes of the observed decline in GS yields. To investigate this possibility, we determined the time frame in which each folding protein substrate can productively interact (i.e. fold to native structure) with the different chaperonin complexes.

Irreversible Misfolding of Substrate Proteins in the Presence of Various Chaperonin Complexes

**Fig. 1.** Refolding of GS in the presence of activated and nucleotide-free complexes. Denatured GS was rapidly diluted 100-fold into refolding buffer to the final monomeric concentration of either 0.5 μM (A) or 0.1 μM (B). Renaturation kinetics of the protein in the presence of either nucleotide-free (●) or activated (■) chaperonin complexes was followed at 37 °C as described under “Experimental Procedures.” Final concentrations of GroEL, GroES, and ATP were 1 μM, 2 μM, and 5 mM, respectively. The degree of GS renaturation was assessed by measuring activity of the protein using a glutamyl transferase assay. The calculation of recovered activity was based on the activity of the native protein incubated under the same conditions.

**Fig. 2.** Concentration dependence of GS refolding. Refolding of denatured GS was initiated by a rapid 100-fold dilution into the buffer containing either nucleotide-free (●) or activated (■) chaperonin complexes. After 16 h at 37 °C, GS activity was measured using glutamyl transferase assay. Spontaneous refolding of the protein (▲) was determined in the absence of chaperonins.
of Nucleotide-free and Activated Chaperonin Complexes—
Measurement of the kinetics of irreversible misfolding is a
convenient experimental approach for the characterization of
the initial interactions between chaperonins and protein fold-
ing intermediates (18, 29). Introduction of the chaperonin com-
plexes at different times following the initiation of spontaneous
refolding allows one to probe how rapidly the protein folding
intermediates lose their ability to interact with the chaper-
onins. In our current study, we examined the rate with which
the folding intermediates of the three protein substrates lose
their ability to interact with either the nucleotide-free (GroEL
alone) or an activated (ATP-GroEL-GroES) complex. These
chaperonin species exhibit significantly different affinities for
protein substrates. For example, according to the estimates by
Clarke and co-authors, the affinities between the two chaperon-
in complexes and lactate dehydrogenase may differ by as
much as 60 fold (4). It is important to note that ADP-GroEL-
GroES complex has a significantly higher affinity for protein
folding intermediates compared with ATP-GroEL-GroES com-
plex (4). To ensure that the activated complex remains in
ATP-bound form throughout the initial interaction, the com-
plex was prepared separately for every experimental point, 1
min prior to the initiation of refolding. Since ATP hydrolysis
rate of GroEL at 37 °C is approximately 26.6 min⁻¹ per oli-
gomer,² only about 0.5% of the total ATP was hydrolyzed to
ADP within this 1-min time period under our experimental
conditions before refolding was initiated. Because rhodanese is
highly susceptible to oxidation (38), control experiments were
performed in the presence of an activated oxygen scavenger
system (42) in an oxygen-depleted buffer under argon atmosphere.
For the duration of our experiments, no difference in the mis-
folding kinetics was observed between these oxygen-depleted
controls and those samples containing oxygen and 10 mM DTT
(data not shown).

For all three protein substrates, a significant portion of the
activity could not be recovered by either chaperonin complex
after an approximate 10 s delay in chaperonin addition (Figs.
4-6). For GS however, the kinetics of irreversible misfolding
within this time interval was significantly faster when the
activated complex was used to rescue protein folding interme-
diates compared with the nucleotide-free complex, with the
rates differing by more than 8-fold (Fig. 4 and Table I). In
contrast, irreversible misfolding of rhodanese was virtually
identical with both chaperonin complexes (Figs. 5 and Table I).
MDH showed a slight decline (1.6-fold) in the rates of misfold-
ing with activated complex compared with nucleotide-free com-
plex (Fig. 6 and Table I). Throughout these experiments, a
molar ratio of chaperonin to protein substrates was maintained
at 10:1 (0.1 μM protein substrate) to ensure the identical cap-
ture efficiency by either nucleotide-free or activated chaperonin
complexes at time zero. When the addition of chaperonins was
performed in the presence of an activated oxygen scavenger
system (42), refolding of GS was initiated by a rapid 100-fold dilution into refolding
buffer at 37 °C to a final protein concentration of 0.1 μM. At the
indicated times either nucleotide-free (●) or activated (□) chaperonin
complexes were added to the refolding mixture. The final concentrations of
GroEL, GroES, and ATP were 1 μM, 2 μM, and 5 mM, respectively.
Renaturation of the protein was allowed to proceed to completion at
37 °C before its activity was determined. Activity corresponding to
spontaneous refolding (no chaperonins) was subtracted from each data
point. The data represent the combined results of three independent
experiments.

![Graph showing kinetics of irreversible misfolding of GS.](image)

**TABLE I**
Rates of irreversible misfolding of substrate proteins in the presence of
nucleotide-free and activated chaperonin complexes

| Substrate protein | Rate constants |
|-------------------|---------------|
|                   | With nucleotide-free complex | With activated complex |
| GS                | 0.23 ± 0.11 | 1.85 ± 0.84 |
| Rhodanese         | 0.72 ± 0.13 | 0.73 ± 0.15 |
| MDH               | 0.30 ± 0.06 | 0.49 ± 0.67 |

Terminals and the nucleotide-free chaperonin complexes. As
the chaperonin addition was delayed for longer time intervals,
the recoverable activity continued to decline, eventually ap-
proaching the levels observed during spontaneous folding (not
shown).

**DISCUSSION**

The initial interactions of protein folding intermediates with
the chaperonins depend on the rate of chaperonin cycling as
well as the folding rate of substrate proteins. With the assump-
tion that the kinetic properties of the protein substrates (i.e.
viewing the properties of chaperonin complexes and folding
intermediates as a function of time) are critical for under-
standing of chaperonin mechanism, we have compared folding of
three structurally diverse proteins with different chaperonin
complexes. One of these substrates, GS, was isolated from the
bacterium *E. coli* and is a potential natural substrate for the
GroE chaperonins (35, 43). The two other substrates, rho-
danese and MDH, both isolated from mitochondria, are the
model substrates most frequently used in the study of protein
folding with the GroE chaperonins. All three proteins are well
established chaperonin substrates that have substantially dif-
frent chaperonin requirements for the efficient folding. In
addition, GS, a dodecamer, and MDH, a dimer, initially exist as
subunits that interact with the chaperonin for short or long
time periods, respectively (29, 41).² Monomeric rhodanese un-

² M. T. Fisher, unpublished data.
Spontaneous refolding of MDH was initiated by a rapid 100-fold dilution into refolding buffer at 37 °C to the final protein concentration of 0.1 mM. At the indicated times either nucleotide-free (●) or activated (■) chaperonin complexes were added to the refolding mixture. The final concentrations of GroEL, GroES, and ATP were 1 mM, 2 mM, and 5 mM, respectively. Renaturation of the protein was allowed to proceed to completion at 37 °C before its activity was determined. Activity measured after the completion of spontaneous refolding (no chaperonins) was subtracted from each data point. Data represent the combined results of three independent experiments.

Substrate Interactions with Various Chaperonin Complexes

Collectively, our data reveal that the kinetic “sampling” of ATP is estimated to be in the millimolar range (47), the activated (GroEL-GroES-ATP or ADP) capture complex is more likely to participate in the binding of protein substrates under physiological conditions. In addition to its decreased affinity for protein substrates (4), this complex has a reduced number of protein binding sites compared with GroEL alone. Half of these binding sites become occupied by GroES, resulting in an asymmetric bullet-shaped complex (2).

It is clear from our experiments that the kinetics of the initial interactions of protein folding intermediates with chaperonin complexes depends on the nature of the substrate protein. The conclusion is based on differences in kinetics of irreversible misfolding observed with the same substrate in the presence of different chaperonin complexes. This experimental design provides an internal reference point to ensure that the observed differences are related to chaperonin-substrate interactions rather than to the different individual folding and assembly paths of the structurally diverse protein substrates. Interestingly, the refolding of the E. coli protein, GS, was the most sensitive to the change in chaperonin affinity. When nucleotide-free chaperonin complexes were used, the initial misfolding kinetics of GS was the slowest among the proteins studied (Table I). This indicates that the nucleotide-free chaperonin complex and GS folding intermediates interact more efficiently over a longer period of time. With the activated chaperonin complex, the irreversible misfolding rate of GS increases dramatically. It is conceivable that the disparity in GS folding yields results from the differences in formation of successful encounter complex between the substrate and the chaperonin. However, since the concentration of the chaperonins in our experiments were adjusted so the initial (time 0) overall folding efficiency was the same for both nucleotide-free and activated complexes, we tend not to favor this possibility. Instead, we suggest that a smaller population of the off-pathway GS intermediates is recognized by the activated chaperonin complexes compared with the nucleotide-free complexes. Since numerous folding intermediates may be in rapid equilibrium, the preferential binding of one particular intermediate population could result in the partitioning of other populations onto chaperonin complex due to mass action effects. Thus, in the kinetic sense, GS may not be adequately captured by the activated complex because of a slower rate of conformational transition to a form that binds to the chaperonin. Alternatively, a direct (conformational) effect of substrate binding on the affinity and cycling of chaperonin complex may be, in part, responsible for the observed differences in misfolding kinetics. The folding intermediates of both MDH and rhodanese lose their ability to interact with the nucleotide-free complex at a faster rates than GS. However, unlike GS, there is little or no change in the misfolding kinetics when the activated chaperonin complex is present suggesting that, for MDH and rhodanese, similar populations of the folding intermediates interact with equal or near equal efficiency with either the activated or nucleotide-free chaperonin complexes. The observation that GS misfolds more rapidly with the activated chaperonin than do other substrates adds a new aspect of substrate variability to the chaperonin mechanism. At present, it is not known if these findings reflect a more general tendency of preferential interaction of the activated complex with the stringent chaperonin substrates compared with nonstringent substrates. In any case, for an adequate folding and assembly of GS (a so-called nonstringent substrate) in a cellular environment at limiting chaperonin concentrations, the parallel participation of the DnaK system may be necessary (48, 49). Experiments designed to address this question are currently underway.

Collectively, our data reveal that the kinetic “sampling” of
different folding intermediates by chaperonins, a process that is likely to occur in the cellular environment, dictates the flux of protein substrates through the chaperonin system during folding. The observed differences in misfolding kinetics reflect the substrate-specific variations in the lifetimes of dynamic folding intermediate structures, the differences in the global properties of these intermediates, such as size, degree of transient exposure of hydrophobic and charged surfaces, or even formation of transient aggregates. Our experiments also establish a time interval when these differences could be observed, up to about 10 s from the initiation of refolding. Whether these findings could be generalized for the wider range of the chaperonin substrates remains to be determined. Even so, our comparative studies indicate that it is necessary to examine multiple substrates before conclusions about the particular aspects of the chaperonin mechanism can be drawn.

REFERENCES

1. Fenton, W. A., and Horwich, A. L. (1997) *Protein Sci.* 6, 743–760
2. Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R., and Saibil, H. R. (1997) *Nature* 382, 265–266
3. Boisvert, D. C., Wang, J., Otwinowski, Z., Horwich, A. L., and Sigler, P. B. (1996) *Nat. Struct. Biol.* 3, 170–177
4. Staniforth, R. A., Burston, S. G., Atkinson, T., and Clarke, A. R. (1994) *Biochem. J.* 306, 651–658
5. Corrales, F. J., and Fersht, A. R. (1996) *Cell* 85, 221–231
6. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) *Cell* 83, 577–587
7. Roseman, A., Chen, S., White, H., Braig, K., and Saibil, H. (1996) *Cell* 87, 241–251
8. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) *Nature* 388, 741–750
9. Weissman, J. S., Kashi, Y., Fenton, W. A., and Horwich, A. L. (1994) *Cell* 78, 693–702
10. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1994) *Science* 265, 659–666
11. Smith, K. E., and Fisher, M. T. (1995) *J. Biol. Chem.* 270, 21517–21523
12. Burston, S. G., Weisman, J. S., Farr, G. W., Fenton, W. A., and Horwich, A. L. (1996) *Nature* 383, 96–99
13. Fassler, R., Schaeff, E. C., Schumacher, R. J., Sondek, S., and Horwich, A. L. (1995) *Cell* 80, 927–937
14. Ranson, N. A., Burston, S. G., and Clarke, A. R. (1997) *J. Mol. Biol.* 266, 656–664
15. Zhu, R., and Pluckthun, A. (1994) *J. Mol. Biol.* 242, 165–174
16. Lin, S., Schwar, F. P., and Eisenstein, E. (1995) *J. Biol. Chem.* 270, 1011–1014
17. Perrett, S., Zahn, R., Stenberg, G., and Fersht, A. R. (1997) *J. Mol. Biol.* 269, 892–901

18. Golubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature* 342, 884–889
19. Martín, J., Langer, T., Beteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) *Nature* 352, 36–42
20. Viitanen, P. V., Gatenby, A. A., and Lorimer, G. H. (1992) *Protein Sci.* 1, 363–369
21. Robinson, C. V., Mayhew, M., Hartl, F. U. and Radford, S. E. (1996) *Proc. Natl. Acad. Sci.* 93, 1080–1085
22. Buckle, A. M., Zahn, R., and Fersht, A. R. (1997) *Proc. Natl. Acad. Sci.* U. S. A. 94, 3571–3575
23. Baecher, A. M., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lung, P., and Clarke, A. R. (1991) *Biochemistry* 30, 9195–9200
24. Schmidt, M., and Buchner, J. (1992) *J. Biol. Chem.* 267, 16829–16833
25. Lill, E., and Buchner, J. (1995) *Proc. Natl. Acad. Sci.* U. S. A. 92, 8100–8104
26. Gorovits, B. M., and Horowitz, P. M. (1997) *J. Biol. Chem.* 272, 32–35
27. Zahn, R., Perrett, S., Stenberg, G., and Fersht, A. R. (1996) *Science* 271, 642–645
28. Ranson, N. A., Dunster, N. J., Burston, S. G., and Clarke, A. R. (1995) *J. Mol. Biol.* 250, 581–586
29. Todd, M. J., Lorimer, G. H., and Thirumalai, D. (1996) *Proc. Natl. Acad. Sci.* U. S. A. 93, 4030–4034
30. Chan, H. K., and Dill, K. A. (1996) *Proteins* 24, 345–351
31. Jaenicke, R. (1993) *Curr. Top. Cell Regul.* 24, 221–225
32. Frey, H., and Fersht, A. R. (1997) *Biochemistry* 36, 11663–11673
33. Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H., and Viitanen, P. V. (1994) *J. Biol. Chem.* 269, 10304–10311
34. Fisher, M. T., and Stadtman, E. R. (1992) *J. Biol. Chem.* 267, 1872–1880
35. Fisher, M. T. (1994) *J. Biol. Chem.* 269, 13629–13636
36. Eisenstein, E., Reddy, P., and Fisher, M. T. (1998) *Methods Enzymol.* 290, 119–135
37. Fisher, M. T. (1992) *Biochemistry* 31, 3955–3963
38. Moorthy, B. A., and Horowitz, P. M. (1988) *Biochem. Biophys. Acta* 956, 30–38
39. Wollf, C. A., Shapiro, B. M., and Stadtman, E. R. (1966) *Arch. Biochem. Biophys.* 116, 177–180
40. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem.* 266, 13044–13049
41. Fisher, M. T., and Yuan, X. (1994) *J. Biol. Chem.* 269, 29598–29601
42. Horowitz, P. M., Butler, M., and McClure, G. D., Jr. (1992) *J. Biol. Chem.* 267, 23596–23600
43. Fisher, M. T. (1998) *Biochemistry (Moscow)* 63, 382–402
44. Landry, S., Jordan, R., McMacken, S., and Gierasch, L. (1992) *Nature* 355, 455–457
45. Schulz, R., Danner, M., and Scholz, R. (1993) *J. Biol. Chem.* 268, 2767–2772
46. Clark, A. C., and Frieden, C. (1997) *Proc. Natl. Acad. Sci.* U. S. A. 94, 8100–8104
47. Lehninger, A. L. (1982) *Principles of Biochemistry*, p. 373, Worth Publishers, New York
48. Greager, A., Nuider, E., Komissarova, N., Gaitanaris, G. A., Gottesman, M. E., and Nikiforov, V. (1992) *Proc. Natl. Acad. Sci.* U. S. A. 89, 10341–10344
49. Buchberger, A., Schröder, H., Hester, H. J., and Bukau, B. (1996) *J. Mol. Biol.* 261, 328–333