Binding of a Chaperonin to the Folding Intermediates of Lactate Dehydrogenase†

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ABSTRACT: When *Bacillus stearothermophilus* LDH dimer is incubated with increasing concentrations of the denaturant guanidinium chloride, three distinct unfolded states of the molecule are observed at equilibrium [Smith, C. J., et al. (1991) *Biochemistry 30*, 1028–1036]. The kinetics of LDH refolding are consistent with an unbranched progression through these states. The *Escherichia coli* chaperonin, GroEL, binds with high affinity to the completely denatured form and more weakly to the earliest folding intermediate, thus retarding the refolding process. A later structurally defined folding intermediate, corresponding to a molten globule form, is not bound by GroEL; neither is the inactive monomer. The complex between GroEL and denatured LDH is destabilized by the binding of magnesium/ATP (Mg/ATP) or by the nonhydrolyzable analogue adenylyl imidodiphosphate (AMP-PNP). From our initial kinetic data, we propose that GroEL exists in two interconvertible forms, one of which is stabilized by the binding of Mg/ATP but associates weakly with the unfolded protein. The other is destabilized by Mg/ATP and associates strongly with unfolded LDH. The relevance of these findings to the role of GroEL in vivo is discussed.

Molecular chaperones have been defined as a class of protein molecules required for the correct folding, assembly, and transport of newly synthesized proteins in a number of contexts [for examples, see Ellis and Hemmingsen (1989) and Wickner (1989)]. Chaperonins form a subclass of molecular chaperones that are found in prokaryotes, plastids, and mitochondria. Two chaperonins, the *Escherichia coli groE* gene products (the proteins GroEL and GroES), are necessary for the assembly of bacteriophages T4, T5, and λ (Georgopoulos & Hohn, 1978; Hohn et al., 1979; Hendrix, 1979; Tilly et al., 1981) and are strongly induced by heat shock and other conditions that apply stress to the cell (Neidhardt et al., 1987). GroEL is homologous to a number of eukaryotic chaperonins (Hemmingsen et al., 1988; Cheng et al., 1989), including the widely studied Rubisco subunit-binding protein, which aids the assembly of ribulose 1,5-bisphosphate carboxylase–oxygenase in plant chloroplasts. GroEL can modulate the folding/assembly of prokaryotic Rubisco, both in vivo and in vitro, (Goloubinoff et al., 1989a,b) and has been shown to bind to unfolded or partially folded forms of proteins (Bochkareva et al., 1988; Lecker et al., 1989; Laminet et al., 1990). Such observations have led to the conclusion that chaperonins interact with nonnative forms of proteins, perhaps to prevent nonspecific aggregation, to stabilize intermediates (thus functioning as a catalyst), or to stabilize proteins with hydrophobic signal sequences until they can bind to their specific carriers. To understand the mechanism of action of these ubiquitous proteins at the molecular level, in vitro studies will be essential. The ideal substrate for such a study is a protein whose folding pathway has already been studied and whose activity is readily determined in a continuous assay.

Early attempts to analyze the refolding pathways of proteins led to the conclusion that folding was a single-step highly cooperative process. More recently, an increasing number of multiple-step refolding pathways have been identified (Ptitsyn, 1981, 1985, 1987; Kuwajima, 1989), and equilibrium denaturation has revealed that proteins can exist in distinct partially folded states (Baum et al., 1989; Smith et al. 1991).

In this paper, we measure the rates of refolding of *Bacillus stearothermophilus* LDH and correlate the kinetic intermediates with the equilibrium states already observed (Smith et al., 1991). The effects of GroEL upon the folding kinetics of LDH are the assessed, and models are proposed for both the folding kinetics and the mechanism of action of GroEL in this process.

Materials and Methods

Preparation of Proteins. The cloned L-lactate dehydrogenase gene from *B. stearothermophilus* was expressed in *E. coli* TG2 cells from the pKK223-3 vector, as described previously (Barstow et al., 1986), and the protein was purified by using an oxamate affinity column followed by DEAE-Sepharose ion-exchange chromatography (Clarke et al., 1985a). The *E. coli* groEL gene (Fayet et al., 1986) was expressed in *E. coli* MC1061 cells from the pND5 plasmid (Jenkins et al., 1986). The protein was purified by a modification of the procedure described by Chandrasekhar et al. (1986) with a final anion-exchange step using a Mono-Q FPLC resin.[1] Protein concentration was estimated spectrophotometrically by assuming an absorption of 0.16 for a 1 mg/mL solution at 280 nm, calculated from the amino acid sequence.

Measurements of the Refolding of LDH. LDH was denatured from concentrated stock solutions by the addition of the appropriate concentration of guanidine hydrochloride (GuHCl) in 5 mM β-mercaptoethanol (BME) and 50 mM triethanolamine-hydrochloride (TEA-HCl), pH 7.0, to give

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[1] S. Woods and A. Aufder Mauer, unpublished results.

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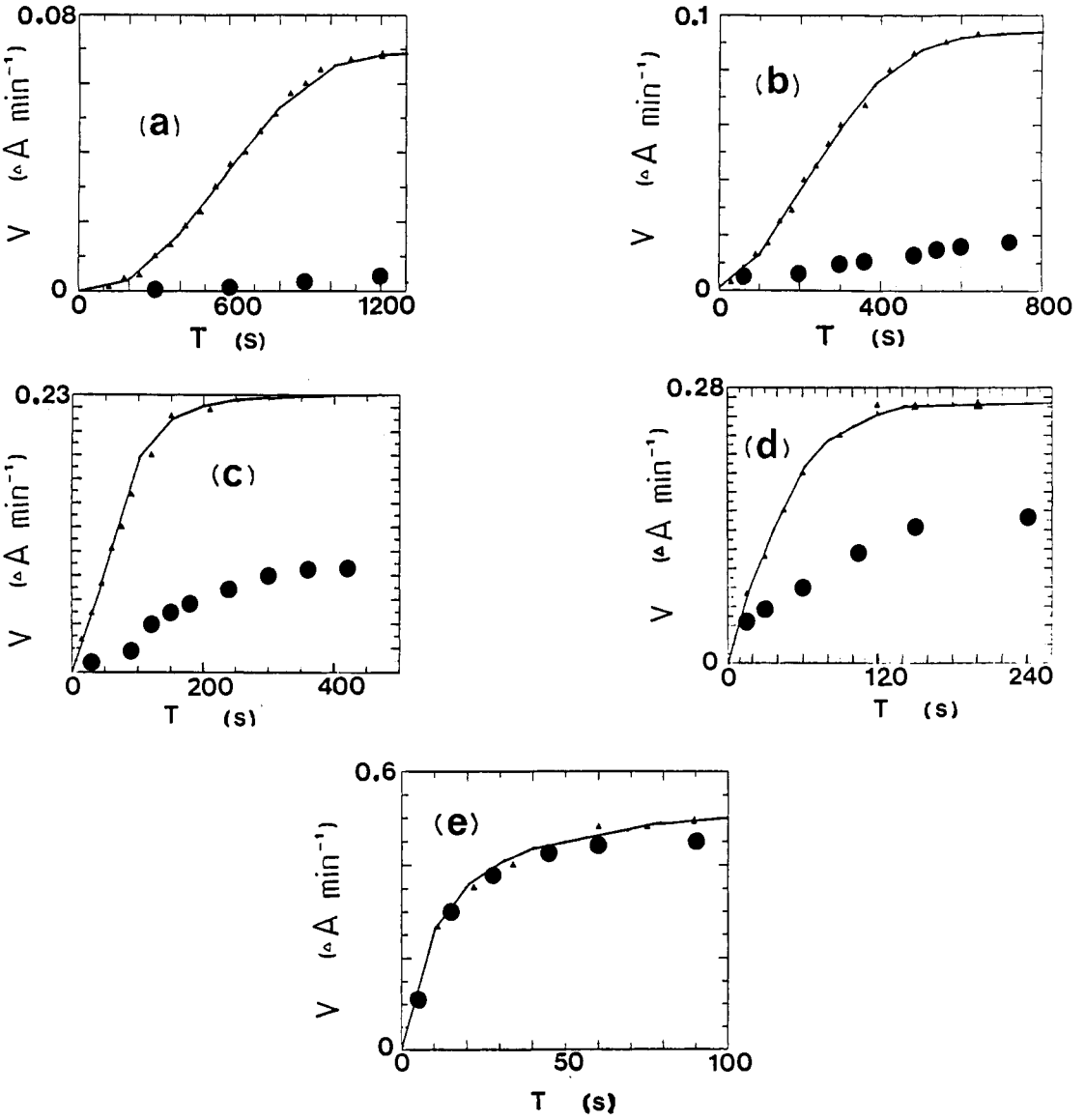


FIGURE 1: LDH refolding from different states of denaturation. For each experiment 20 nM LDH was allowed to refold in the assay buffer described in the text, and the reaction velocity (V) was plotted against time. The enzyme was preincubated in the following denaturing conditions: (a) 4.0 M GuHCl, (b) 3.5 M GuHCl, (c) 3.0 M GuHCl, (d) 2.5 M GuHCl, and (e) 2.0 M GuHCl (▲). In each case the renaturation was repeated in the presence of 0.4 μM GroEL (●). In the absence of GroEL, the lines through the data represent the fit to the model described in Scheme I.

final LDH concentrations of 2.0 μM with respect to subunits. BME is required to prevent slow oxidative disulfide bridge formation, which renders the LDH denaturation irreversible over a period of hours (Smith et al., 1991). Protein solutions in denaturant were incubated for 3 h to allow the unfolding process to reach equilibrium.

Unless otherwise stated, refolding was initiated by a 100-fold dilution of the denatured LDH to a final concentration of 20.0 nM in a 1-mL cuvette containing 10.0 mM pyruvate, 0.2 mM NADH, 5 mM BME, and 50 mM TEA-HCl, pH 7.0. These concentrations are sufficient to saturate the enzyme with respect to both substrates. The residual concentration of GuHCl (up to 0.04 M) is insufficient to destabilize the enzyme (Smith et al., 1991).

The absorbance at 340 nm was monitored as refolding progressed, providing a direct measure of NADH consumption by the refolded enzyme. Tangents to curves of NADH concentration versus time were taken to be proportional to the concentration of refolded LDH; to safeguard against loss of saturation, the reaction was only followed until half the NADH was oxidized. Product and substrate inhibition are known to be negligible under these conditions (Clarke et al., 1985b).

RESULTS

Folding Mechanism of LDH. Figure 1a–e shows time courses for the emergence of LDH activity after equilibrium denaturation to a range of unfolded states. In the absence of GroEL, the rate of refolding from the fully denatured state at 4.0 M GuHCl (see Figure 1a) is most simply described by a scheme in which the random coil undergoes two slow unimolecular rearrangements followed by a bimolecular step in which folded but inactive monomers associate to give active dimers:

Scheme I

$$2U_1 \xrightarrow{k_1} 2U_2 \xrightarrow{k_2} 2U_3 \xrightarrow{k_3} D$$

where U_n are unfolded states and D is the active dimer

The FACSIMILE numeric integration program (Curtis & Sweetenham, 1985) used to model the refolding process produced the following rate constants for this scheme: $k_1 = 3$

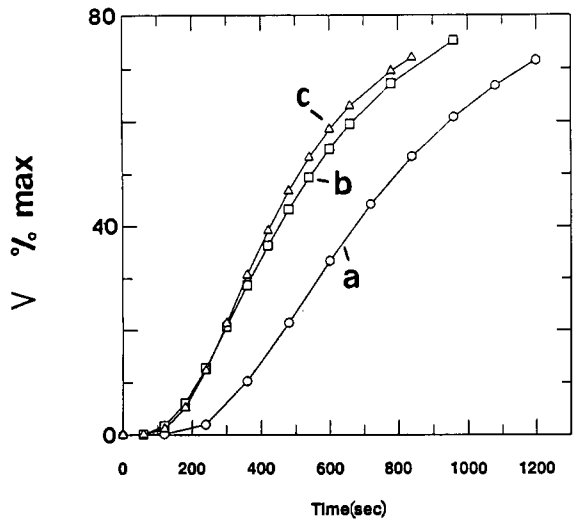


FIGURE 2: Concentration dependence of LDH refolding. Samples of LDH were denatured at 4.0 M GuHCl and refolded in assay buffer as described under Materials and Methods. LDH concentrations were (a) 20, (b) 40, and (c) 80 nM. Reaction velocity (V) was plotted as a percentage of the maximum (which is achieved after 2 h of incubation).

$\times 10^{-3}$ s^{-1}, $k_2 = 15 \times 10^{-3}$ s^{-1}, and $k_3 = 1 \times 10^6$ M^{-1} s^{-1}.

The fitted line superimposed on the results in Figure 1a shows the close agreement between the above model and the experimental results.

We justify this model on the following grounds: (1) Attempts to reduce the number of unimolecular steps to one produced an extremely poor fit in which the residual sum of the squares between the model and real data increased by a factor of 10. (2) The fully denatured protein is monomeric (Smith et al., 1991), whereas the active form at low protein concentration is a dimer; therefore, a bimolecular step is obligatory. (3) The model predicts that, by increasing the protein concentration, the observed refolding rate will increase to a point where it becomes determined only by the two unimolecular steps (i.e., the product of the bimolecular rate constant k_3 and the concentration of U_3 becomes much greater than either k_1 or k_2). This prediction is confirmed by inspection of the results shown in Figure 2. At higher concentrations of LDH, refolding ceases to get faster and the bimolecular rate becomes invisible. At this point the profile still shows a lag phase (i.e., it is sigmoidal), demonstrating that there remains more than one slow unimolecular step.

To analyze the profiles generated by the complete renaturation of partially unfolded forms of LDH (Figure 1b–e), covering the range 3.5–2.0 M GuHCl, we applied the principle of Occam's razor. With the model and rate constants obtained above, the data were fitted by varying only the initial equilibrium distribution of the LDH molecule between the U_1, U_2, and U_3 intermediates. This strategy assumes no change in the mechanism of refolding. As the denaturant concentration is lowered, the sole property of the system that alters is the equilibrium distribution of intermediates around distinct transitions. Using this method, we obtained close agreement between the model and the results (see Figure 1).

The deduced initial concentrations of the intermediates versus [GuHCl] are shown in Figure 3 (bottom). To seek a correlation between the kinetically determined distribution of intermediates across these equilibrium transitions and the structural state of the protein, we compared these results to those derived from fluorescence studies (Smith et al., 1991).

Figure 3 (top) shows the fluorescence of a single genetically inserted tryptophan at position 147 in the protein sequence,

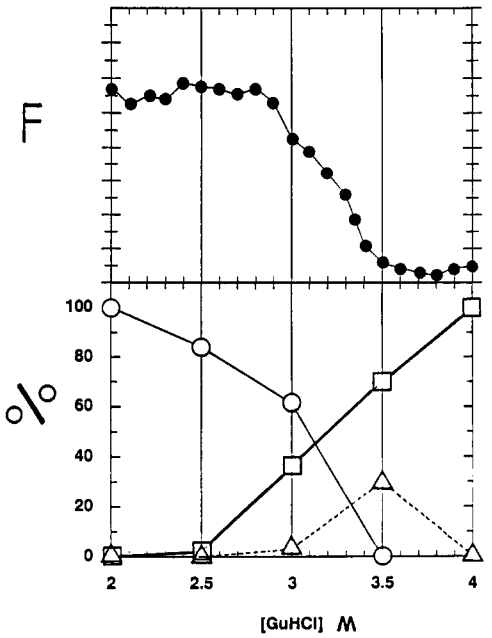


FIGURE 3: Correlation between a fluorescence profile for LDH unfolding and the populations of folding intermediates. (top) Fluorescence intensity (F) (excitation 295 nm; emission 345 nm) of a single genetically inserted tryptophan residue at amino acid position 147 in LDH [see Barstow et al. (1986)] a function of GuHCl concentration. The fluorescence scale is arbitrary. (bottom) Deduced percentage equilibrium populations of intermediates at given GuHCl concentrations as described in *Folding Mechanism of LDH*, under Results; (□) U_1, (△) U_2, and (○) U_3.

as the protein is incubated at GuHCl concentrations between 2 and 4 M. This reporter was chosen as it is extremely sensitive to conformational transitions between these concentrations. The fluorescence result shows a broad transition in structure between 2 and 4 M GuHCl that reflects a change from the "molten globule" state (60% of the native α-helix still intact) to the random coil (Ptitsyn, 1987; Smith et al., 1991). It can be seen that the distribution of the kinetically defined intermediates U_1 and U_3 correlates well with this fluorescence transition. We therefore identify U_1 as random coil and U_3 as the molten globule. The species U_2, however, corresponds to a state that is maximally stable at intermediate GuHCl concentrations but at no point is the dominant species. These results show that the molten globule state is reached in a two-step reaction with a distinct, but only moderately stable, intermediate. The fluorescence profile supports this conclusion in that the broad decay in structure between 2.2 and 4.0 M GuHCl shows evidence of comprising a double transition (Smith et al., 1991).

Binding of the GroEL Protein to Folding Intermediates in the Absence of Nucleotides. When LDH is refolded from the random coil state at 4.0 M GuHCl, the presence of the chaperonin vastly reduces the rate of recovery of activity (Figure 1a). This effect is reminiscent of that seen when pre-β-lactamase is a substrate for GroEL. As LDH is refolded from progressively less denatured states (i.e., from lower GuHCl concentrations), the retardation effect becomes less pronounced. At 2.0 M (Figure 1e) the presence of the chaperonin has no effect on refolding. From these results, we can conclude that the chaperonin cannot bind to the molten globule form of the LDH protein but binds tightly to either the random coil (U_1), or the first intermediate (U_2), or both.

The inability of GroEL to retard the rate of refolding of the molten globule form of LDH is consistent with the proposal that the interaction between GroEL and its substrate proteins is predominantly hydrophobic in nature. In the molten globule

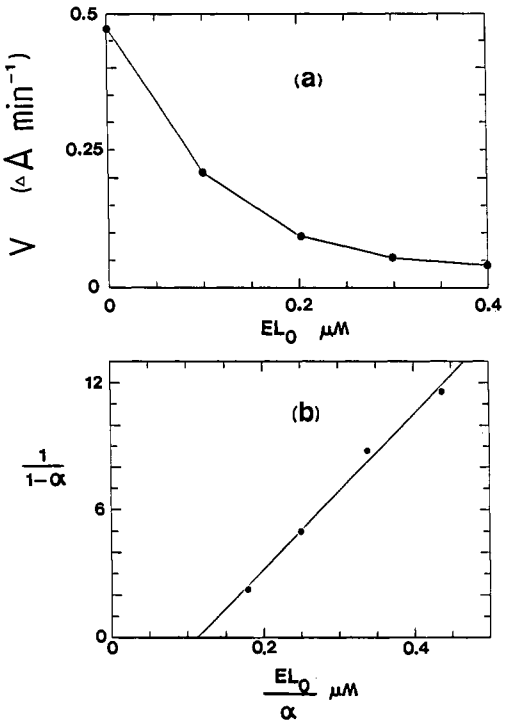


FIGURE 4: Analysis of the binding of apo-GroEL to fully unfolded LDH. (a) Samples of fully unfolded LDH (at 4.0 M GuHCl) were diluted into assay buffer to give a final enzyme concentration of 100 nM. The assay buffer contained differing concentrations of GroEL, as shown on the horizontal axis. The vertical axis describes LDH activity 240 s after refolding is initiated and so defines the proportion of LDH not associated with the chaperonin. (b) These results were plotted according to the transform $1/(1 - \alpha) = 1/K_d \times EL_0/\alpha - 1/LDH_0$, where α is the fractional saturation of LDH by GroEL, K_d is the dissociation constant, EL_0 is the total concentration of GroEL, and LDH_0 is the total concentration of LDH. The intercept of this graph on the X-axis gives the concentration of LDH that is available to bind to GroEL, the slope gives the binding constant.

state hydrophobic side chains are no longer freely accessible, and the chaperonin is demonstrably unable to bind this structure.

Stoichiometry of the GroEL–LDH Interaction. The GroEL oligomer is extremely large, consisting of 14 subunits and having a molecular mass in excess of 800 kDa. To determine the number of binding sites for the unfolded LDH molecule of this complex, we allowed the enzyme to fold in the presence of increasing concentrations of GroEL. Figure 4a shows the LDH activity recovered after 240 s of refolding from the random coil state. From this experiment, the concentration of GroEL-bound enzyme at a given time can be determined, and the proportional saturation (α) can be deduced. The transformed data, shown in Figure 4b, give an intercept on the horizontal axis of 0.11 μM GroEL complex in an experiment where the LDH concentration is 0.10 μM, yielding a stoichiometry of 1 LDH molecule per GroEL complex. The apparent dissociation constant for this complex is 0.02 μM, a value that is over-estimated since the data cannot be analyzed at zero time.

Effect of Nucleotides on the GroEL–LDH Complex. The results shown in Figure 5 demonstrate that preincubation of Mg/ATP with GroEL reduces the GroEL-mediated inhibition of LDH refolding. However, an extension of the lag phase is evident from the refolding profiles, suggesting that a degree of interaction does occur between the GroEL–Mg/ATP complex and refolding LDH. More surprisingly, the nonhydrolyzable analogue of ATP, adenylyl imidodiphosphate (AMP-PNP), has the same effect when incubated with GroEL

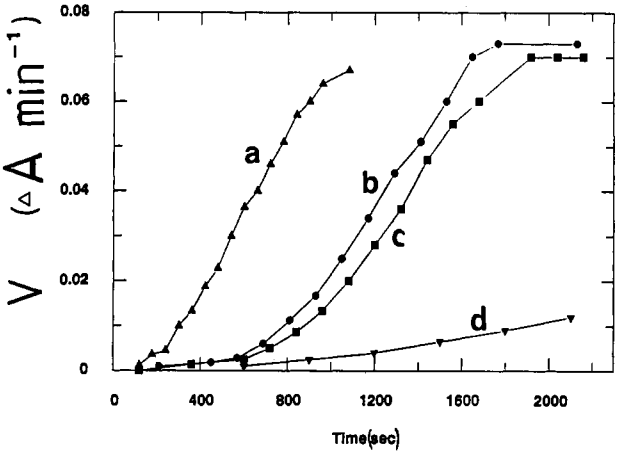


FIGURE 5: LDH refolding: the effects of GroEL and nucleotides. LDH, 20.0 nM, was renatured as described in the legend to Figure 1. Included in the buffer were (a) 10 mM $MgCl_2$, (b) 0.4 μM GroEL, 10 mM $MgCl_2$, and 2.7 mM ATP, (c) 0.4 μM GroEL, 10 mM $MgCl_2$, and 2.7 mM AMP-PNP, and (d) GroEL and 10 mM $MgCl_2$.

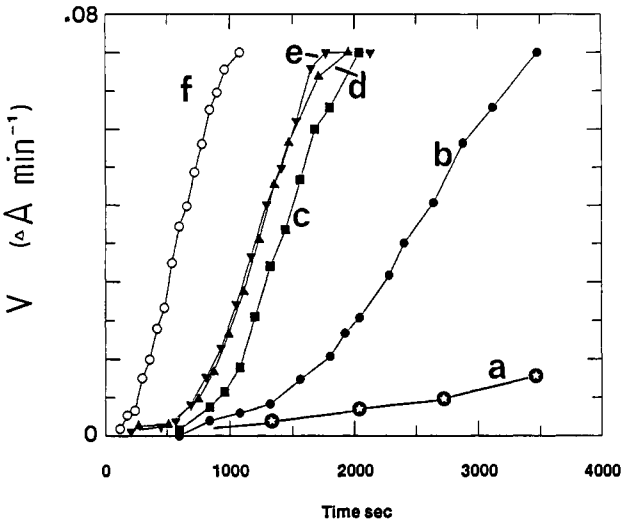


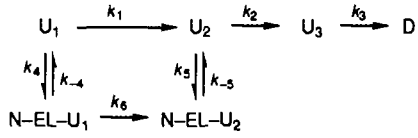
FIGURE 6: Dependence of the GroEL–LDH interaction on the concentration of nucleotide. The experiment described in the legend to Figure 5 was repeated at varying concentrations of AMP-PNP. GroEL, 0.4 μM, and 10 mM $MgCl_2$ were included in all assays in addition to (a) nothing, (b) 0.3 mM AMP-PNP, (c) 0.9 mM AMP-PNP, (d) 2.7 mM AMP-PNP, and (e) 9.1 mM AMP-PNP. The control assay (f) contained 9.1 mM AMP-PNP and 10 mM $MgCl_2$ but no GroEL.

in the presence of Mg^{2+}. These results contrast with those of other investigators (Bochkareva et al., 1988; Goulobinoff et al., 1989b) who conclude that ATP hydrolysis is obligatory in releasing protein from the chaperonin. Preincubation of GroEL with Mg/ADP and/or orthophosphate had no effect on refolding (results not shown).

The nucleotide concentration dependence of this process was examined by incubating GroEL at a range of Mg/AMP-PNP concentrations and then adding unfolded LDH. The results, shown in Figure 6, demonstrate that the effect saturates at about 1 mM AMP-PNP and has a half-maximum (with respect to the slope of the linear portion of the curve) at about 0.3 mM. Even saturating levels of nucleotide still show a delay in refolding over the control assay, demonstrating that it is unable to prevent protein–protein interaction completely. These values for concentration dependence correspond closely with the apparent K_m value for ATP observed in the chaperonin mediated reconstitution of Rubisco activity (Goulobinoff et al., 1989b).

Having observed that the GroEL–nucleotide complex is able to retard the folding process to a modest degree (see Figure 6), we wished to define the nature of its interaction with unfolded LDH. To do this, we attempted to fit the observed refolding curve shown in Figure 5, where GroEL is preincubated with 8.1 mM AMP-PNP, to

Scheme II



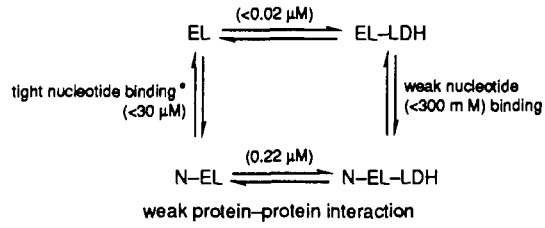
Although this model contains eight rate constants, three of these (k_1–k_3) have been measured in previous experiments (see Scheme I) and so can be directly compared. Secondly, k_3, k_4, and k_5 are all collision-controlled steps and are assumed to occur with the same rate constant.

In modeling the results to Scheme II, we concluded that binding of GroEL to both U_1 and U_2 is required for good fit to the observed data. Further the N-EL-U_1 → N-EL-U_2 conversion fits with such a slow rate constant that it can be neglected in the model.

The rate constants fitted to Scheme II are $k_1 = 5.9 \times 10^{-3}$ s^{-1}, $k_2 = 5.1 \times 10^{-3}$ s^{-1}, k_3, k_4, and $k_5 = 0.5 \times 10^6$ M^{-1} s^{-1}, $k_{-4} = 0.11$ s^{-1}, $k_{-5} = 0.82$ s^{-1}, and $k_6 = 9.9 \times 10^{-9}$ s^{-1}.

The values of k_1, k_2, and k_3 show good agreement with the figures obtained in the absence of GroEL. The constants k_{-4} and k_{-5} define the rates at which GroEL dissociates from U_1 and U_2, respectively, and the binding constants for these interactions are 0.22 and 1.64 μM, respectively. These binding constants show that even the more stable of the GroEL–nucleotide–LDH complexes is over 10 times weaker than the unliganded GroEL–LDH complex (see Figure 4). As a summary of these results, we propose the following simple scheme for the interactions between GroEL, ATP (AMP-PNP), and LDH:

Scheme III: Tight Protein–Protein Interaction



For this scheme, N represents either ATP or its analogue AMP-PNP. The tight interaction* between free GroEL and ATP is dictated by the thermodynamic cycle. The role of ATP turnover would be to convert the EL–N species back into a form that can associate tightly with an unfolded protein.

In the these experiments, nucleotide was preincubated with GroEL to allow it to associate with the chaperonin in the absence of a protein substrate. We next sought to discover the effect of adding Mg/AMP-PNP to the preformed GroEL–LDH complex. The results (see Figure 7) show that LDH is rapidly released and refolds.

Effect of the GroEL Protein on Yields of Active LDH. When LDH is incubated at 4 M GuHCl and allowed to refold in the conditions described in Figure 1, only 18–20% of the original activity is recovered. The inclusion of 0.4 μM GroEL and 2.7 mM ATP/10 mM Mg^{2+} has no effect on this yield (see Figure 5), but, on omitting the nucleotide, the recovery of active protein is increased to 48–53%. We attribute this improvement in yield to the tight interaction between unliganded GroEL and unfolded LDH. Because of the slow dissociation rate of this complex, the effect is to reduce the concentration of free unfolded LDH during renaturation and so prevent loss of enzyme activity due to hydrophobic aggregation of these unstructured chains.

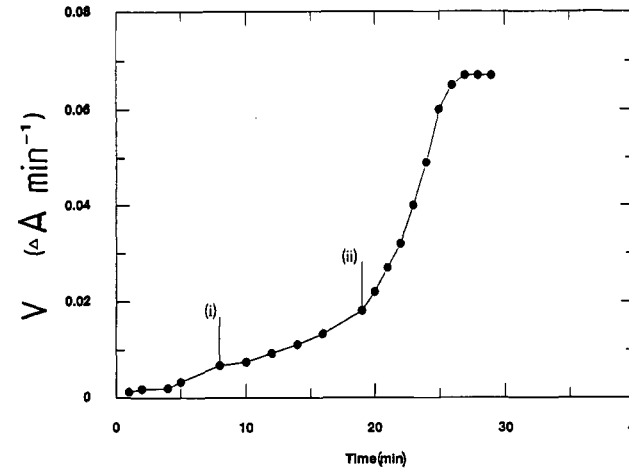
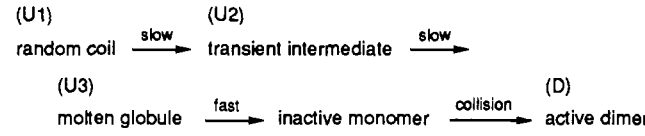


FIGURE 7: Time course of refolding: the release of bound LDH by AMP-PNP. Fully denatured 20 nM, was renatured in assay buffer containing 0.4 μM GroEL. After 960 s (i) 10 mM $MgCl_2$ was added and after 2280 s (ii) 2.7 mM ATP-PNP was added.

Discussion

The GroEL protein is a member of a subgroup of molecular chaperones termed the chaperonins. These proteins are constitutively abundant in bacterial cells, chloroplasts, and mitochondria, and their concentrations increase after certain forms of cell damage owing to the "heat-shock" response. The demonstrable functions of chaperonins are in aiding the assembly of large oligomeric protein structures and in acting as transient carrier for proteins destined for transport across membranes [for a review, see Ellis and Hemmingsen (1989)]. The two *E. coli* proteins encoded by the *groE* locus (GroEL and GroES) are the best studied of the chaperonins. In this work, we attempt to simplify our experimental system by observing the effects of the GroEL protein alone on a well-characterized substrate that has defined folding intermediates. The folding pathway of LDH can be summarized



The first conclusion we draw is that the chaperonin can only bind tightly to the unfolded LDH molecule (U_1) and more weakly to the first and transient intermediate (U_2). There are three other defined structural forms of LDH: the molten globule (U_3) (a collapsed yet mobile structure in which core residues are accessible to solvent but 60% of the native α-helix has formed), the inactive monomer (which is in most respects the same to the native fold, except for the exposure of the hydrophobic intersubunit contact area to the solvent), and the active dimer. We found no evidence that GroEL binds to any of these latter three structures.

This observation underlines the lack of specificity of chaperonin–substrate interactions; GroEL will bind to proteins entirely unrelated to LDH, such as cyanobacterial Rubisco and pre-β-lactamase. The results also show that GroEL does not act as a "folding catalyst". If this were true, we would expect it to bind most strongly to either folding intermediates

or to "transition states" between them. The GroEL–LDH interaction, in fact, arrests the folding process: it is only dissociation of the complex that allows the enzyme to resume folding, and it does so at the same rate as in the absence of GroEL (see Figure 6). In other words, folding does not occur while LDH is bound to the surface of the chaperonin (also note the negligible magnitude of k_6 in Scheme II). The other striking result [confirming the findings of Laminet et al. (1990) but using a completely different experimental model and technique] is the stoichiometry of the interaction. The results under *Stoichiometry of the GroEL–LDH Interaction* demonstrate that only a single LDH chain can be accommodated per 14-mer of GroEL. Given the "double ring" quaternary structure of the protein, it is probable that the unfolded LDH chain occupies the single central hole in the chaperonin.

As in the case of other interactions between unfolded proteins and GroEL, the binding of magnesium and ATP induces the release of the protein substrate. However, in this case, we show that nucleotide hydrolysis is not a prerequisite of the dissociation step. The unhydrolyzable analogue AMP-PNP also promotes dissociation.

The observations presented here suggest the following model to connect the molecular properties of GroEL with its cellular function. The common property of unfolded proteins and of proteins with hydrophobic signal sequences is their very sparing solubility. Once precipitated as a disordered aggregate, the function of most proteins is irretrievably lost. In the case of folding proteins, the fraction of molecules that undergo correct intramolecular rearrangements before ordered intermolecular assembly, compared to that which is lost through random aggregation of unfolded chains, is determined by the concentration of free unfolded protein. The lower the concentration, the greater is the yield of correctly folded and assembled protein. This point has recently been demonstrated by Buchner et al. (1991) in the refolding of citrate synthase and is supported by the results under *Effect of the GroEL Protein on Yields of Active LDH*. It would be equally true of a relative insoluble and/or unstable preprotein with a hydrophobic signal sequence. Here the chaperonin would serve to sequester the free protein prior to its interaction with the transmembrane transport machinery thus preventing aggregation.

Interpretation of the cellular role of ATP binding and hydrolysis in such systems is less straightforward. Our results show that unfolded LDH interacts tightly with apochaperonin and that ADP has no effect on the stability of this complex. On binding ATP (or AMP-PNP) there is a marked reduction in affinity for LDH.

The chaperonin in acting to prevent hydrophobic aggregation must interact with its substrates with a stability similar to that between aggregated proteins. Thus, the role of ATP would seem to be linked with the need to provide a rapid dissociation pathway for the chaperonin–protein complex. Since chaperonins are nonspecific in their protein affinities, this dissociation mechanism probably entails a gross conformational rearrangement of the chaperonin (for example, to bury hydrophobic binding surfaces).

Since ATP hydrolysis is not required for protein release, the implied mechanism is analogous to that of the actin–myosin interaction and of the G-proteins. In muscle, the energy to dissociate the protein–protein complex is provided by the ATP-binding energy, and ATP hydrolysis is then used to return the system to its initial conditions.

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