

Chaperonin GroE and ADP facilitate the folding of various proteins and protect against heat inactivation

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Abstract

In the presence of ADP, the molecular chaperones GroEL and GroES from *Escherichia coli* not only facilitated the refolding of various proteins, but also prevented their irreversible heat inactivation in vitro. Without nucleotides the refolding reactions were arrested by GroEL. Addition of GroES and ADP to the reaction mixture initiated the refolding reactions and the enzyme activities were regained efficiently. The presence of GroE (GroEL and GroES) and ADP also protected against heat inactivation of native enzymes at various temperatures. These findings suggest that in the presence of GroES, nucleotide binding is an important event in the mechanism of GroEL-facilitated protein folding.

Key words: Chaperonin; GroE; Molecular chaperone; Protein folding; Protein heat stability

1. Introduction

Protein folding and unfolding in vivo is affected by nonproductive aggregation under stressful conditions such as high temperature [1]. In living cells, however, there is a system that prevents these 'off-pathways' and allows correct protein folding to proceed efficiently. The molecular chaperones are responsible for this efficient control of off-pathways. Recently, the chaperonins, members of the molecular chaperone family, have also been shown to facilitate the in vitro folding of many proteins. Chaperonin GroE from *Escherichia coli* is one of the most extensively studied chaperonins. GroE is composed of two types of subunits; GroEL (14-mer), which forms two toroidal heptameric rings with a 57-kDa protein subunit [2] and GroES (7-mer), which forms a heptameric ring with a 10-kDa protein subunit [3].

GroE protein not only facilitates protein folding but also prevents heat denaturation of proteins in vitro in the presence of ATP [4–7]. Recently we reported that in the presence of GroES, not only ATP but also ADP, CTP, and UTP were very effective for GroEL-facilitated folding of tryptophanase [8] and enolase [9]. As ADP was not hydrolyzed by GroEL, it was postulated that nucleotide binding is important in dissociating folding intermediates from the chaperonin. In order to ascertain whether this hypothesis is reliable, we studied the refolding reactions of various proteins in the presence of chaperonin GroE and ADP, and found that GroE and ADP were also effective in protecting proteins from heat inactivation.

The enzymes used were glucose dehydrogenase (GLUCDH; tetramer, subunit MW = 26,300) from *Bacillus* species, lactate dehydrogenase (LDH; dimer, subunit MW = 32,000) from *Staphylococcus* species, malate dehydrogenase (MDH; dimer, subunit MW = 27,000) from *Thermus* species, and Taka-amylase A (TAA; monomer, MW = 54,000) from *Aspergillus oryzae*. The quaternary structure of these enzymes range from a monomeric state to a tetrameric state. Extra-cellular TAA is also distinguished from the other enzymes by the presence of 4 disulfide bonds located in its tertiary structure. We found that GroE and ADP not only facilitated the refolding of all these enzymes, but also prevented the irreversible heat inactivation of LDH and GLUCDH in vitro.

2. Materials and methods

2.1. Proteins

GroEL and GroES proteins were purified from a GroE-overproducing strain, *E. coli* DH1/pKY206 according to the method of Kubo et al. [9]. LDH from *Staphylococcus* species, GLUCDH from *Bacillus* species, and MDH from *Thermus* species were obtained from Amano Pharmaceutical Co., LTD, and TAA from *Aspergillus oryzae* was purified from the commercial product 'Takadiastase Sankyo' and crystallized according to the method of Akabori et al. [10]. The concentration of GroEL was determined spectrometrically on a Hitachi U-2000 spectrometer, using the absorption coefficient of $A_{1\text{cm}}^{1\%} = 2.36$ at 277 nm [8]. The concentrations of all other enzymes and GroES were determined by the method of Bradford [11], using bovine serum albumin as a standard.

2.2. Enzyme assay

The activities of LDH and MDH were assayed by measuring the decrease in absorption at 340 nm of NADH in 0.1 M Tris-HCl buffer (pH 7.8) at 25°C, with pyruvate and oxaloacetate as substrates, respectively. The activity of GLUCDH was measured by monitoring the increase of NAD⁺ in 0.1 M Tris-HCl buffer (pH 8.0) at 25°C using β -D-glucose as a substrate. TAA activity was assayed according to the method of Bernfeld [12] at 30°C.

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Abbreviations: GLUCDH, glucose dehydrogenase; Gdn-HCl, guanidine hydrochloride; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; TAA, Taka-amylase A.

2.3. Refolding assay

Enzymes were extensively unfolded in 4 or 6 M Gdn-HCl and appropriate aliquots were subsequently diluted into refolding buffer (50 mM MOPS-KOH, pH 7.0, containing 10 mM KCl and 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$). The concentrations of enzyme and Gdn-HCl during the refolding reaction were 1–4 $\mu\text{g}/\text{ml}$ and 30–50 mM, respectively, and the refolding temperature was 25°C. A fivefold molar excess of GroEL and GroES oligomer relative to refolding enzyme protomer and 2 mM nucleotide were selectively added to the refolding mixture. Refolding yield was determined as the percentage ratio of the activity of the refolding enzyme relative to that of native enzyme.

3. Results and discussion

3.1. Refolding of various proteins in the presence of GroEL, GroES, and ADP

The chaperonin-facilitated refolding characteristics of LDH, GLUCDH, MDH, and TAA were studied (Fig. 1). The activity of each of these enzymes was easily assayed with small aliquots during the refolding reaction. Fig. 1a shows a typical refolding pattern of LDH in the presence of GroE. Without nucleotides, the refolding reaction of each enzyme was prevented by adding the chaperonin, indicating that GroEL can bind the refold-

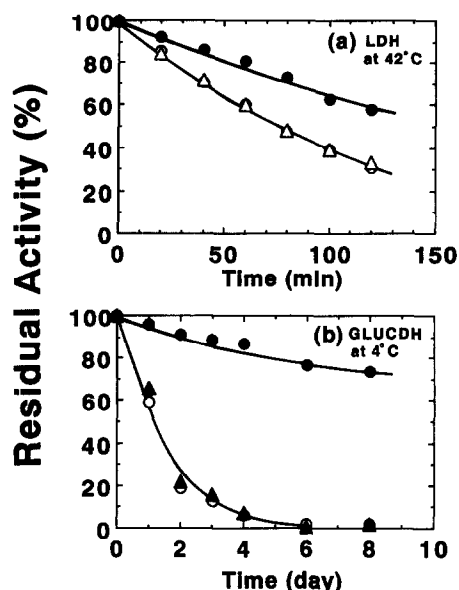


Fig. 2. Protection of LDH from heat inactivation at 42°C (a) and of GLUCDH at 4°C (b). (●), in the presence of GroEL + GroES (5-fold molar excess relative to protomer) and 2 mM ADP; (△), 30 $\mu\text{g}/\text{ml}$ dextran; (▲), 0.7 mg/ml bovine serum albumin; (○), none.

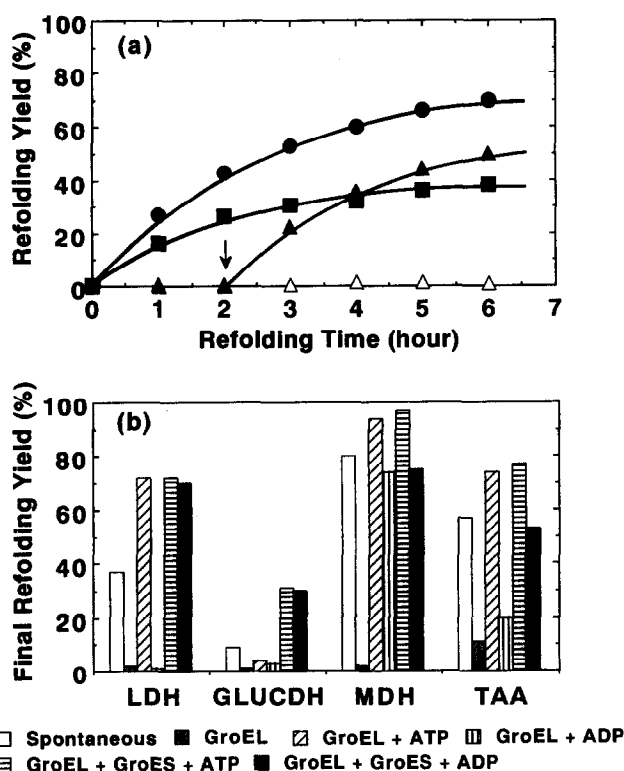


Fig. 1. GroE-facilitated protein refolding characteristics at 25°C of LDH (a) and final refolding yields of various enzymes (b). The refolding time when the refolding activity reached plateau were: 7 h (LDH), 24 h (GLUCDH), 30 min (MDH), 2 h (TAA). (●), LDH (4 $\mu\text{g}/\text{ml}$) + GroEL and GroES (5-fold molar excess relative to LDH protomer) + 2 mM ADP; (■), spontaneous refolding; (△), LDH + GroEL and GroES; (▲), 2 mM ADP or ATP was added to (△) at the time shown by the arrow.

ing intermediates of all of these enzymes. Upon adding ATP to the GroEL-intermediate complex solution, all enzyme activities except GLUCDH were restored efficiently (Fig. 1b). The refolding of GLUCDH required GroES even in the presence of ATP. This refolding characteristic displayed by GLUCDH is the same as those of ribulose biphosphate carboxylase [13,14], rhodanese [15,16], and ornithine transcarbamylase [17]. In the presence of GroES and GroEL, the activities of all the enzymes were efficiently recovered in the presence of ADP as well as ATP. This result was consistent with the refolding of enolase as reported previously [9]. Taken together this result and the previous results regarding tryptophanase [8] and enolase [9], strongly support the idea that GroE (GroEL and GroES) and ADP are also capable of facilitating the folding of many proteins regardless of various differences in structural characteristics, cellular location, or origin.

As the refolding yields of LDH and GLUCDH were higher in the presence of GroE and ADP compared to their respective spontaneous refolding yields, it may be said that ADP may play an important role in the function of GroEL, although only in the presence of GroES. This result also strongly suggests that the binding of nucleotide to GroEL, and the subsequent conformational changes which are triggered, are very important in the expression of chaperonin function, as reported previously [9]. Recently, it has also been reported that the binding of GroES to GroEL induces a specific GroEL conformational change [18].

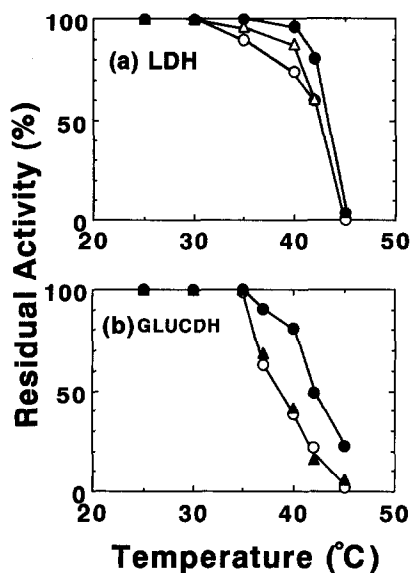


Fig. 3. Stability of LDH (a) and GLUCDH (b) against short-time heat treatment. Incubation times were 60 min for LDH and 30 min for GLUCDH. Symbols are the same as in Fig. 2.

3.2. Enhancement of heat stability of LDH and GLUCDH in the presence of GroEL, GroES, and ADP

In order to study whether GroE/ADP is also effective in protecting enzymes from heat inactivation in solution, we measured the enzyme activities of various proteins in the presence of GroE and ADP at various temperatures. LDH and GLUCDH were used in these experiments as the MDH used in our studies was obtained from thermophilic sources and TAA has been determined to be especially resistant to heat inactivation (data not shown). All other conditions were the same for the refolding experiments. As shown in Fig. 2a, the enzyme activities were preserved for a longer time at 42°C when GroE/ADP was present in the solution. Very surprisingly, as shown in Fig. 2b, GLUCDH activity was protected even at 4°C. Also, both enzymes were protected by GroE/ADP from heat inactivation at various temperatures (at 45, 42 and 40°C for LDH, and at 45, 42, 25 and 4°C for GLUCDH) more effectively than by the addition of an equivalent molar concentration of dextran or bovine serum albumin. The effects of ATP and ADP were almost the same for both enzymes. Fig. 3 shows the protecting effects (60 min-incubation for LDH and 30-min incubation for GLUCDH) of GroE/ADP at varied temperatures. GroE/ADP protects enzyme activity also during short-time heat treatment. It should be noted that the GroEL, GroES, and ADP system would be useful for protecting enzymes from heat inactivation in solution in an industrial sense, as ADP is not hydrolyzed by GroEL. All these results suggest that GroE and ADP quite adequately perform as a chaperonin with regard to many proteins.

3.3. Mechanism of chaperonin GroE function

As shown above, GroE/ADP(or ATP) not only facilitates the refolding of enzymes but also protects them from heat inactivation. These two GroE-related mechanisms, i.e. a mechanism by which GroE effectively refolds a protein from the completely unfolded state and a mechanism by which GroE protects native protein structure from heat inactivation, seem apparently different but they might be uniformly expressed as shown in Scheme 1:



where, N, and U represent native and unfolded molecules, I is refolding and thermal unfolding intermediates, and X is an irreversibly formed molecule such as an aggregate. GroEL binds to both the refolding intermediate and the thermally unfolding intermediate (I) that are prone to the irreversible step in the same manner ($I \rightarrow X$), thereby segregating and stabilizing them ($\text{GroE} \cdot I$). In the presence of nucleotide, the intermediate states are dissociated from GroEL and refold to the native conformation (N). If the nucleotide is ADP, GroES is necessary for the reaction, and if the nucleotide is ATP, GroES is not (except for GLUCDH) [9]. A similar mechanism regarding protection of rhodanese from heat inactivation was postulated by Mendoza et al. [19].

Most proteins are shown to be fluctuating to a large extent in solution; for instance, the variable and constant fragments of an immunoglobulin light chain were shown to undergo a global unfolding transition ($N \rightleftharpoons U$) even under physiological conditions [20, 21]. The results obtained in this study, in particular the protective effects of GroE/ADP at temperatures as low as 4°C, suggest that LDH and GLUCDH also seem to undergo a transition which produces aggregation-prone intermediates in solution at physiological conditions. To clarify this point, the characteristics of the refolding and unfolding intermediates must be elucidated in more detail.

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