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# Effect of temperature and denaturation conditions on protein folding assisted by GroEL-GroES chaperonin

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The goal of this study is to investigate effects of temperature and co-chaperonin requirement for *in vitro* protein refolding assisted by *E. coli* chaperone GroEL under permissive and nonpermissive temperature conditions. *In vitro* protein refolding of two denatured proteins was kinetically investigated under several conditions in the presence of GroEL. Effects of temperature and GroES-requirement on the process of prevention of protein aggregation and refolding of denatured protein were extensively monitored. We have found that *E. coli* GroEL chaperone system along with ATP is required for *in vitro* refolding of unfolded polypeptide under nonpermissive temperature of 37°C. However, under permissive condition spontaneous refolding can occur due to lower temperature, which can competes with chaperone-mediated protein refolding *via* GroEL chaperone system. Thus, GroEL seemed to divert spontaneous refolding pathway of unfolded polypeptide toward chaperone-assisted refolding pathway, which is more efficient protein refolding pathway.

**Key words** – Chaperone, GroEL/GroES, Protein aggregation, Protein refolding

## Introduction

Chaperone proteins are ubiquitous in bacteria, archaea, and eukarya and assist correct folding and assembling of other proteins in a ATP-dependent manner [11]. Molecular chaperones are characterized by their remarkable ability to recognize and bind nonnative proteins, which is often doomed to aggregate [17]. One of the best characterized chaperones is the GroEL/GroES chaperonin system from *Escherichia coli. In vivo*, the GroEL system is responsible for the folding of ~10 % of the polypeptide chains to their native structure [3,13]. Under stress condition, such as elevated temperature, the GroEL chaperonin maintains viability by stabilizing unfolding proteins or by keeping unfolding intermediates in a reactivable state, leading to a prevention of irreversible aggregation [7,9,14,15]. The fundamental mechanism of GroEL/GroES action involves encapsulation of a single molecule of nonnative protein in a cage-like structure, thereby allowing folding to occur protected from aggregation-prone environment [5,6].

Under thermal stress conditions, GroEL is capable of stabilizing its substrate proteins like rhodanese [16] and citrate synthase (CS) [2]. Other substrates are not stabilized, but they are bound by GroEL during heat inactivation and kept in a reactivable state [7,9,14,]. After shifting the conditions from an elevated temperature, under which spontaneous refolding is not possible (nonpermissive condition), to a lower temperature (permissive condition), the bound substrate could be efficiently refolded in the presence of ATP or GroES/ATP, or even without need of chaperonin in some cases. Experiments in which proteins are bound to GroEL and subsequently reactivated under permissive conditions are important to gain insights about *in vitro* properties of GroEL system. Folding intermediates are often formed and recognized by GroEL during the process of chemical or thermal denaturation. They contain a decreased number of tertiary contacts [19], partially or incompletely dissociated domains [10], and/or incorrectly formed disulfide bonds [4]. As a result, such intermediates tend to be highly hydrophobic, and consequently they can easily form large aggregates and precipitate.

Here, we used CS and rhodanese as chaperone substrates to investigate the activity of the GroEL system under different temperature conditions such as permissive and nonpermissive temperature. We have also investigated the effect of different protein denaturation conditions on GroEL activity toward substrate proteins during the time

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courses of aggregation. Of importance, we have observed that requirement of GroES for prevention of aggregation is influenced by the initial status (of being denatured or not) and identity of substrate proteins. Under permissive condition at low temperature, GroEL tends to recognize and bind CS folding intermediate, thereby impeding spontaneous refolding. On the contrary, under nonpermissive condition of high temperature (37°C), GroEL/GroES plus ATP is required for efficient refolding of unfolded CS. These results suggest that GroEL seems to divert spontaneous refolding pathway of unfolded polypeptide toward chaperonin-assisted refolding pathway, which is more efficient protein refolding pathway.

## Materials and Methods

### Expression and purification of *E. coli* GroEL/GroES

GroEL/GroES chaperonin proteins were prepared as described previously [20] with slight modifications. *E. coli* DH5α and BL21 (DE3) codon plus (Novagen, Inc., San Diego, CA) were used as the cloning and expression host cells, respectively. Briefly, GroEL/GroES were expressed and purified from *E. coli* (BL21) bearing the plasmid pGro11 under the control of *pzt1* promoter. The proteins were overexpressed with 20 ng/ml of tetracycline. The cell lysate obtained by sonication was loaded onto DEAE-cellulose (purchased from Sigma) column chromatography and the fractions containing GroEL and GroES were respectively collected. The pooled protein solution was concentrated using Amicon ultra-filtration device under nitrogen gas (Millipore, Bedford, MA, USA). Each concentrated protein fraction of GroEL and GroES was further purified by Sephadex G-150 gel-filtration column chromatography. The purity of recombinant protein was confirmed by SDS-PAGE. Protein concentration was determined using the following extinction coefficient: $E^{0.1\%}$ = 0.142 (at 276 nm) for GroES and $E^{0.1\%}$ = 0.173 (at 280 nm) for GroEL [20]. Hereafter, GroEL and GroES concentrations refer to 14-mer and 7-mer, respectively.

### Unfolding-Folding of rhodanese

Recombinant bovine rhodanese (thiosulfate:cyanide sulfur transferase, EC2.8.1.1) was purchased (from Sigma) and further purified as described previously [18] to remove ammonium sulfate in which the protein was suspended. Rhodanese concentrations were determined using a value of $E^{0.1\%}$ = 1.75 (at 280 nm) [1]. For chemical denaturation of the protein, 0.3 mg/ml (equivalent to 9 μM) rhodanese was denatured for at least 2 hrs at 25°C in the denaturation buffer (50 mM Tris/Cl, pH 7.8, plus 6 M Gunidinium chloride). For spontaneous folding, unfolded rhodaneses was diluted to 3.6 μg/ml in 50 mM Tris/Cl, pH 7.8, containing 50 mM sodium thiosulfate, 10 mM KCl, 10 mM $MgCl_2$, 0.2 M β-mercaptoethanol (rhodanese folding buffer) preincubated at the desired temperature. For GroEL-assisted refolding of the denatured rhodanese, the refolding mixture was incubated in the presence of GroEL (0.1 μM) with or without GroES (0.2 μM)/ATP (2 mM) at 20°C. For the case of thermal aggregation of rhodanese, the native rhodanese (3.6 μg/ml in rhodanese folding buffer) was incubated in the presence or absence of GroEL (0.1 μM) with other components at 50°C. The time course of thermal aggregation of rhodanese and aggregation during refolding of the chemically denatured rhodanese was monitored by the increase of light scattering at 500 nm, using an UV-visible spectrophotometer (Ultrospec 2100, Amersham Biosciences).

### Unfolding-Folding of citrate synthase

Mitochodrial CS from porcine heart (citrate synthase, EC 4.1.3.7) was obtained from Roche Molecular Biochemicals (Mannheim, Germany) and treated as described [21]. CS concentrations refer to monomer and measured as described [8]. For the case of thermal aggregation of CS, the native CS (0.15 μM in rhodanese refolding buffer) was incubated in the presence or absence of GroEL with other components at 43°C. For chemical denaturation of CS, the protein (15 μM) was treated and unfolded as described above. Chemically denatured CS was incubated in the presence or absence of GroEL (0.15 μM) at desired temperatures. Denatured CS was diluted to a final concentration of 0.15 μM into 50 mM Tris/Cl, pH 8.0, containing 10 mM KCl, 10 mM $MgCl_2$ (CS folding buffer) at 37°C. Light-scattering due to protein aggregation during thermal aggregation of CS and refolding of the chemically denatured CS was measured using spectrometry same as above.

### Thermal inactivation and reactivation of citrate synthase

For thermal inactivation of CS, native CS protein (0.15 μ

M) in the CS refolding buffer was initially incubated at 25°C in the presence or absence of GroEL (0.15 μM) and other components (0.3 μM GroES and 2 mM ATP), and the activity of CS was determined and set to 100 %. The inactivation reaction was started by placing the mixture in a 43°C water bath. Aliquots taken at the indicated time points were assayed to determine remaining CS activity as described [21].

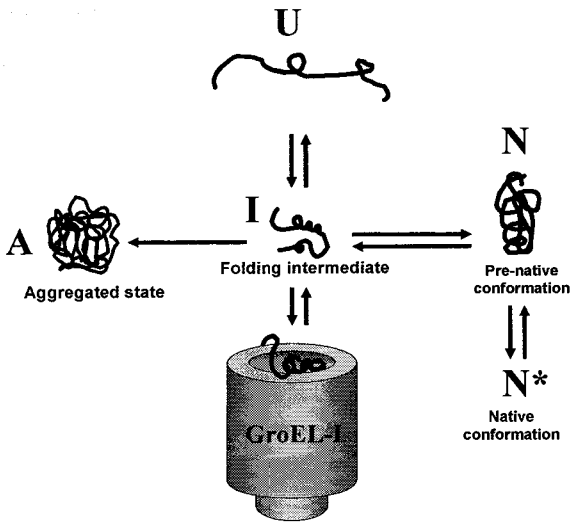
Chemically denatured CS was reactivated in the presence and absence of GroEL (0.15 μM) and other components (0.3 μM GroES and 2 mM ATP) at two different temperatures (20°C and 37°C). Reactivation of denatured CS (15 μM) was initiated by diluting CS 100-fold into the CS refolding buffer under vigorous stirring. The renaturation sample was kept at the desired temperature. At the each time points indicated, aliquots were withdrawn and the activity was determined by assaying CS activity. The activity of 0.15 μM native CS was set to 100 %.

## Results and Discussion

GroEL and GroES proteins expressed in *E. coli* were respectively purified as described in "Materials and Methods". Figure 1 shows a SDS-PAGE of the purified GroEL and GroES with each purification step. The purified GroEL was appeared by single band of 57 kDa, which corresponds to a molecular weight of GroEL monomer, and GroES protein was identified as 10 kDa (monomer) single band in SDS-PAGE.

A model has been proposed for folding-unfolding process of the substrate proteins. In the present work, we use this model presented in Scheme 1 to interpret our data. According to this model, a folding intermediate (**I**) forms when refolding is initiated for denatured proteins (**U**). This folding intermediate (**I**) partitions to two states of the protein, one for aggregated state (**A**) and the other for pre-native state (**N**). The form **N** is capable of folding to the complete native structure (**N***), which is fully active protein. In this model, GroEL can bind the folding intermediate (**I**), and the **GroEL-I** protein complex inhibits the intra-protein processes that would lead to inactive species (**A**).

Figure 2A shows the effect of GroES requirement on the prevention of the chemically denatured rhodanese by monitoring light scattering using spectrometry. At 20°C, time-dependent protein aggregation extensively starts by placing the denatured rhodanese into dilution in the folding




Scheme 1.

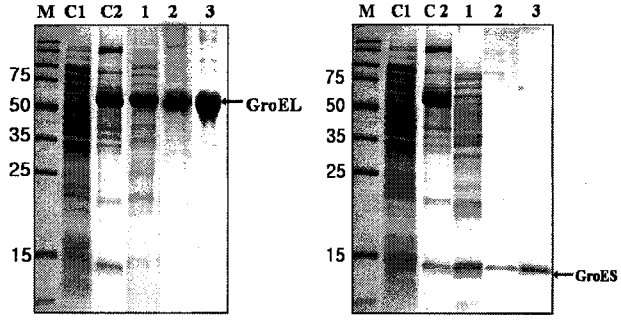



Fig. 1. Purification of recombinant GroEL/GroES. Lane M, molecular mass marker; lane C1, crude cell extract without induction; lane C2, crude cell extract with induction; lane 1, DEAE-cellulose peak fractions; lane 2, Protein fractions after ultrafiltration; lane 3, Protein fractions after gel-filtration chromatography. The gel was stained with Coomassie brilliant blue.

buffer. This protein aggregation process is significantly inhibited by 60 % in the presence of GroEL. This result suggests that GroEL protects the aggregation-prone unfolded state of the chemically denatured rhodanese from aggregation. In the model, this case can be described by as following; the step **I** → **A** can be inhibited by partition of **I** → **GroEL-I** by GroEL. Prevention of protein aggregation was further intensified in the presence of whole GroEL system including GroES and ATP. Thus, denatured rhodanese is readily captured by GroEL system and protected from converting to the aggregated state. We have also tested whether GroEL is capable of suppressing thermal aggregation of native rhodanese at high temperature (50°C). As shown in Fig. 2B, elevated temperature results in rapid increase of light scattering of rhodanese due to extensive protein

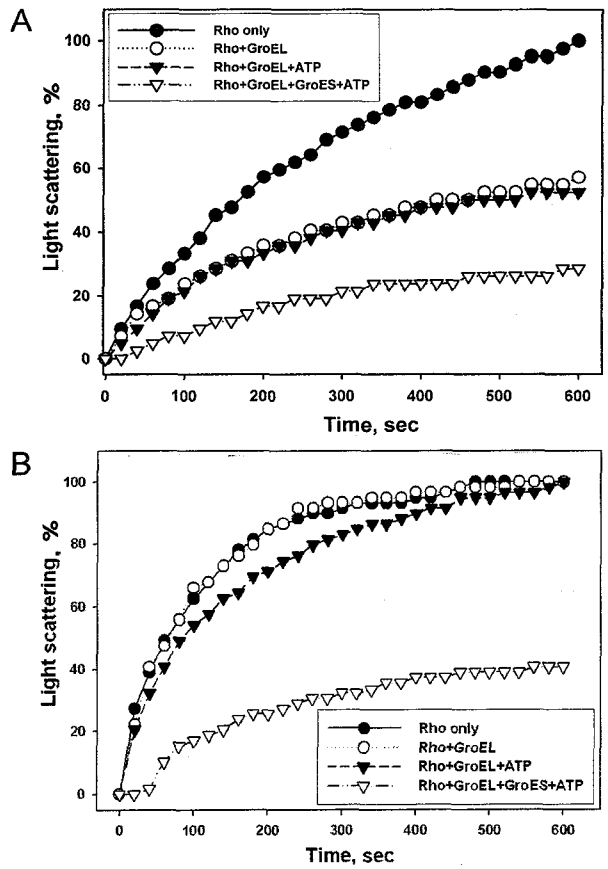



Fig. 2. (A) GroEL/ES lessened aggregation of chemically denatured rhodanese (20°C). Rhodanese protein (Rho) was chemically denatured and diluted by 100-fold into the renaturation buffer (+/- GroEL/ES) at 20°C. Time-dependent protein aggregation was monitored by absorbance increase at 500 nm due to light scattering. (B) Both GroEL and GroES is required for prevention of thermal aggregation of rhodanese at elevated temperature. Same experiment starting with the native rhodanese (0.10 μM) was performed as (A) except at the elevated temperature (50°C).

aggregation. Rhodanese aggregation could not be prevented by including GroEL alone in the mixture. However, complete GroEL system including GroES and ATP efficiently lessened progress of the thermal aggregation by 60 % (Fig. 2B). As compared with the result shown in Fig. 2A, it indicates that only the GroEL/GroES can recognize denaturing rhodanese intermediate, which can be distinguished from the unfolding intermediate originated from the unfolded state (U). In contrast, in the aggregation of chemically denatured rhodanses, GroEL alone was capable of recognizing the folding intermediate.

Similar experiments were carried out using other substrate protein, citrate synthase (CS). CS is well suited, because unfolding and refolding can be easily monitored. The use of CS as a substrate protein for GroEL allows us to address whether chaperonin suppresses aggregation, interacts with folding intermediate, and reactivates the denatured substrate protein. CS is a commercially available, dimeric, mitochondrial protein, composed of two identical subunits (49 kDa each). In the absence of any substrates, CS is readily inactivated on incubation at higher temperatures with a midpoint of transition at 48°C [23]. CS loses its activity after major structural changes during denaturation. At high temperature (37°C and 43°C), either chemically denatured or native CS was readily aggregated (Fig. 3). This is based on unspecific hydrophobic interactions between unfolding intermediates and results in the formation of high molecular weight particles. GroEL

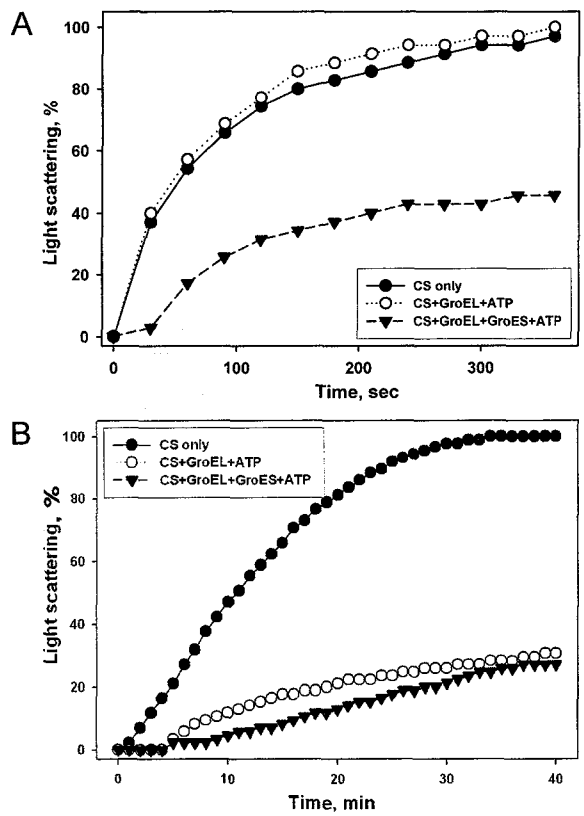



Fig. 3. (A) GroEL/ES lessened aggregation of chemically denatured CS. CS protein was chemically denatured and diluted by 100-fold into the renaturation buffer (+/- GroEL/ES) at 37°C. Time-dependent protein aggregation was monitored by absorbance increase at 500 nm due to light scattering. (B) GroEL/ES lessened thermal aggregation of CS. Native CS protein was incubated at 43°C. Time-dependent protein aggregation was monitored by absorbance increase at 500 nm due to light scattering.

requires co-chaperonin GroES for efficient prevention of aggregation of the chemically denatured CS (Fig. 3A). In contrast, as shown in Fig. 3B, the presence of stoichiometric amounts of GroEL leads to a suppression of thermal aggregation because GroEL forms apparently stable complexes with the unfolding intermediates (**I → GroEL-I** in Scheme 1). Thus, depending on whether unfolding rhodanese comes from either native state (N) or denatured state (U), GroEL seems to recognize different unfolding intermediates.

Citrate synthase readily inactivates on incubation at 43°C. This inactivation process was monitored by measuring the remaining activity of the enzyme during the incubation at elevated temperatures. Within minutes after start of the incubation at 43°C, the activity decreases and after 20 min no active CS can be detected (Fig. 4). Stoichiometric concentrations of GroEL did not influence the inactivation process. Inactive folding intermediates are bound readily by GroEL and are no longer in equilibrium with the native state. In the model, the folding pathway of **I → GroEL-I** is more favored without partition to **I → N**. It therefore seems that GroEL inactivates CS, a phenomenon that has been also observed in the case of various other substrate proteins with which GroEL is stably associating [12,22]. However, the extent and kinetics of thermal inactivation of CS was lessened with the complete GroEL system including GroES and ATP. Thus, it indicates that the unfolding intermediate of CS was recognized and protected from inactivation by the GroEL/GroES complex.

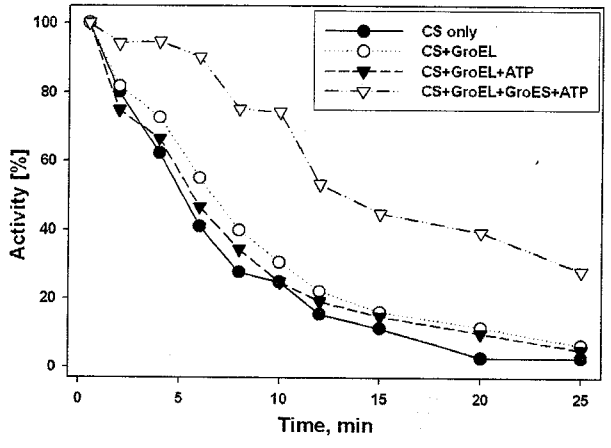



Fig. 4. Inactivation kinetics of CS at 43°C in the presence of GroEL/ES. Inactivation of CS (0.15 μM monomer in the folding buffer) was measured in the refolding buffer in the absence of chaperone or in the presence of GroEL +/- GroES.

In order to investigate how the GroEL system responds to denatured CS at each environment of permissive and nonpermissive condition, we have monitored reactivation kinetics of chemically denatured CS at two different temperature conditions, such as 37°C (nonpermissive condition) and 20°C (permissive condition). Reactivation of chemically denatured CS was strongly influenced by the folding conditions like temperature. As shown in Fig. 5A, the presence of both GroES and ATP is required to allow efficient folding of the denatured CS under nonpermissive condition, where the spontaneous refolding of denatured CS is not possible. Under permissive folding conditions (low temperature, 20°C) for the spontaneous folding of CS, the presence of GroEL alone was not sufficient for

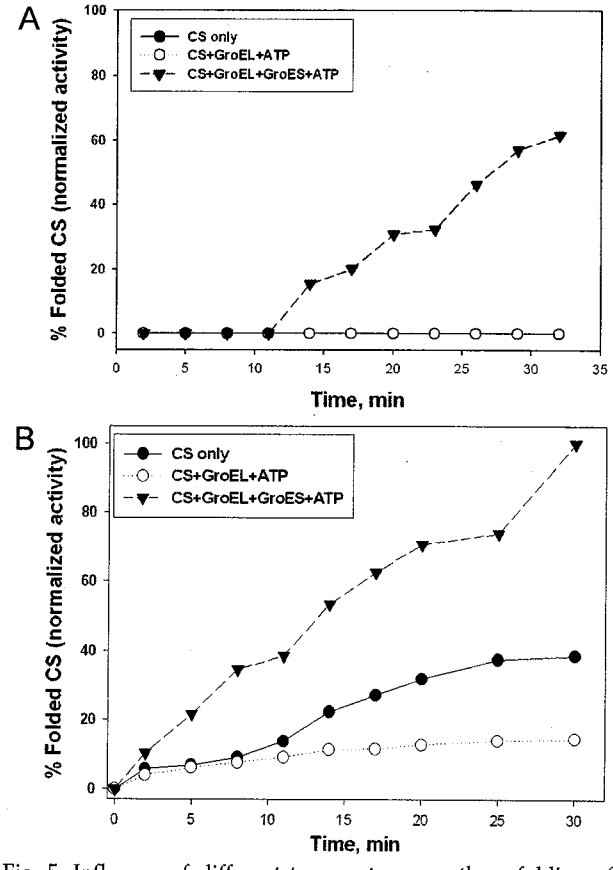



Fig. 5. Influence of different temperatures on the refolding of chemically denatured CS. (A) Chemically denatured CS protein was not able to spontaneously refold itself at 37°C (nonpermissive condition). Protein refolding occurred in the presence of GroEL/ES. (B) Chemically denatured CS protein was spontaneously renatured at low temperature (20°C, permissive condition). Protein reactivation was inhibited by incubation with GroEL alone, but this inhibitory effect was reversed by incubation with GroEL/ES.

reactivation of CS (Fig. 5B). In this case, the folding kinetics is slower than in the absence of the chaperone owing to rebinding to GroEL. This phenomenon can be explained by the model, in which partition of folding intermediate (**I**) to the native species (**N**) and subsequent active species (**N***) was favored by the GroEL/GroES complex. However, under permissive condition without GroES and ATP, GroEL readily binds to the folding intermediate and forms a stable complex, not releasing the folding intermediate into the native state. From our data, we suggest that under permissive condition spontaneous refolding can occur due to lower temperature, which can competes with chaperone-mediated protein refolding *via* GroEL chaperone system. Therefore, at the permissive temperature GroEL seemed to divert spontaneous refolding pathway of unfolded polypeptide toward chaperone-assisted refolding pathway, which is more efficient protein refolding pathway. In contrast, under nonpermissive condition unfolded protein could not be refolded without GroEL function.

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## 초록 : GroEL-GroES 샤페로닌에 의한 단백질 접힘에 있어서 온도와 변성조건의 영향

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이 연구의 목적은 대장균 분자 샤페론 GroEL의 시험관 내 단백질 접힘에 있어서 반응온도의 영향과 보조샤페론의 필요 여부를 자발적 재접힘이 가능한 온도와 그렇지 않은 온도조건에서 조사하는 것이다. 여러 조건하에서 GroEL에 의한 두 가지 기질 단백질의 재접힘을 반응속도론적으로 조사하기 위하여 GroEL에 의한 단백질 침전생성억제와 변성된 단백질의 재접힘을 광범위하게 조사하였다. 자발적 재접힘이 가능하지 않은 37°C에서는 ATP와 완전한 GroEL 시스템이 변성된 폴리펩티드의 재접힘을 위하여 필요하다는 것을 확인하였다. 하지만, 자발적 재접힘이 가능한 낮은 온도에서는 자발적 재접힘과 샤페론 의존적 단백질 재접힘이 서로 경쟁하는 것을 알 수 있었다. 따라서 GroEL은 변성된 폴리펩티드의 자발적 접힘 경로를 더 효율적인 단백질 재접힘 경로인 샤페론 의존적 단백질 재접힘 경로로 유도하는 것으로 보인다.