# Individual subunits of bacterial luciferase are molten globules and interact with molecular chaperones

### (protein folding/protein assembly)

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**ABSTRACT** We have studied the assembly of a large heterodimeric protein, bacterial luciferase, by mixing purified subunits expressed separately in bacteria. The individual subunits $\alpha$ and $\beta$ contain much (66% and 50%, respectively) of the $\alpha$-helix content of the native heterodimer as measured by circular dichroism, yet the $\alpha$ subunit lacks observable tertiary structure as measured by NMR. These results are consistent with the $\alpha$ subunit existing in a molten globule or collapsed form prior to assembly. The molecular chaperone GroEL binds reversibly to both subunits prior to assembly. Since these observations were obtained under physiological conditions, we propose that the molten globule exists as a stable form during folding or assembly in the cell. Either the molten globule form of the subunits is an authentic folding intermediate or it is in rapid equilibrium with one. GroEL may function by facilitating assembly through stabilization of these incompletely folded subunits.

Heat shock proteins hsp70 and hsp60 have been termed molecular chaperones for their likely role in assisting protein folding or refolding in the cell. The hsp60 class of chaperones is thought to function at a later stage in the folding pathway than hsp70 on the basis of kinetic and binding data (1–4), though the mechanism of action is still largely unknown. Bacterial hsp60 (GroEL) with its cofactor GroES and ATP can increase recovery yields of proteins refolding *in vitro* by suppressing aggregation. The list of proteins assisted in this fashion includes the monomeric protein rhodanese (5–7) and the homodimeric proteins ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum* (8) and citrate synthase (9). Whether GroEL and GroES assist in the assembly of multimeric proteins or exclusively the folding of individual subunits is unknown largely due to the difficulty in separating these events during the renaturation of the homooligomeric proteins studied.

To study the role of chaperones in protein assembly we have used a heterodimeric protein, bacterial luciferase from *Vibrio harveyi*. Bacterial luciferase is composed of two related but nonidentical subunits: $\alpha$ luciferase (40 kDa) (10) and $\beta$ luciferase (36 kDa) (11) that lack any intra- or inter-disulfide bonds. The enzyme produces light in the presence of oxygen, reduced flavin mononucleotide, and a long-chain aldehyde. Although the catalytic site has been shown to reside entirely within the $\alpha$ subunit (12–14), no activity can be detected with either individual subunit (15, 16). The function of the $\beta$ subunit is unknown. Heterologous subunits allow for the separate expression of the individual subunits and therefore the separation of subunit folding from protein assembly. Assembly of luciferase can be measured by monitoring enzymatic activity with a sensitive luminescence assay (17).

Early folding studies by Friedland and Hastings (16) found that assembly was the rate-limiting step in luciferase renaturation. More recently, Baldwin and coworkers (18–21) have extensively studied the refolding of bacterial luciferase *in vitro* and have proposed that luciferase assembles from partially folded subunits. We demonstrate here that the $\alpha$ subunit is a molten globule when expressed in the absence of the $\beta$ subunit yet remains competent to assemble into the native heterodimer. We propose that the partially folded forms observed kinetically by Baldwin and coworkers (19, 20) are also in molten globule-like states.

## MATERIALS AND METHODS

**Materials.** [$^{15}$N]Ammonium chloride and [*methyl*-$^{13}$C]methionine were purchased from Isotec (Miamisburg, OH). The bicinchoninic acid (BCA) protein assay was from Pierce, isopropyl $\beta$-D-thiogalactopyranoside and dithiothreitol (DTT) were from Boehringer Mannheim, and fraction V bovine serum albumin (BSA) and all other reagents were from Sigma.

**Expression and Purification of Luciferase Subunits.** Competent cells of *Escherichia coli* strain JM109(DE3) were transformed with ampicillin-resistance plasmids carrying the genes for either $\alpha$ luciferase (*luxA*), $\beta$ luciferase (*luxB*), or both, under control of a bacteriophage T7 promoter (22). The cells were grown to mid-logarithmic phase with ampicillin (100 $\mu$g/ml) and then induced with 1 mM isopropyl $\beta$-D-thiogalactopyranoside for either 3 hr at 37°C (*luxB* or *lux A* plus *luxB*) or 6 hr at 22°C (*luxA*). Cell pellets from 1 liter of mid-logarithmic culture were suspended in 40 ml of 200 mM sodium potassium phosphate/1 mM DTT, pH 7.0 (buffer A), and disrupted by passage through a French pressure cell. Breakage was >95%. Inclusion bodies and cell debris were separated from soluble proteins by sedimentation (15,000 × *g*, 5 min). The soluble form of $\alpha$ luciferase was purified from the supernatant of lysed cells by anion exchange chromatography and dialysis. Supernatants from extracts from bacteria expressing $\alpha$ luciferase grown at 25°C were dialyzed into 20 mM sodium potassium phosphate/1 mM DTT, pH 7.0. The protein was loaded onto Whatman DE-52 anion-exchange resin (2 mg/ml of resin) by a protocol similar to that used to purify the $\alpha$–$\beta$ dimer (23). The protein was eluted between 0.15 and 0.3 M phosphate. The active $\alpha$–$\beta$ luciferase dimer was purified by using a scheme from Holzman and Baldwin (24). Protein concentrations were determined by the BCA colorometric assay using BSA as a standard (25).

To renature $\alpha$ or $\beta$ luciferase, frozen inclusion bodies were first denatured with urea. Each frozen inclusion-body aliquot from 12 OD$_{600}$·ml of cells was dissolved in 200 $\mu$l of 8 M urea at room temperature by gentle periodic agitation over 10 min.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; D$_2$O, $^2$H$_2$O.
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Insoluble material was removed by centrifugation (12,000 × *g*, 10 min). The subunit was then allowed to fold by rapid 200-fold dilution of the supernatant from the clarified sample into buffer A at 4°C. The subunits were concentrated in a Centriprep 10 ultrafiltration unit (Amicon) and then in a Centricon 10 unit to the desired concentration and were clarified (12,000 × *g*, 5 min) before use.

α luciferase uniformly labeled with $^{15}N$ was produced by expressing the subunit in *E. coli* grown in M9 minimal medium with glucose and [$^{15}N$]ammonium chloride as a nitrogen source (26). α luciferase was isotopically labeled with $^{13}C$ exclusively in the methyl position of methionine by expressing the protein in a defined medium containing 19 unlabeled amino acids and [*methyl*-$^{13}C$]methionine (27). Isotopically labeled α luciferase was renatured from protein recovered from inclusion bodies as described above.

**Assembly of Luciferase.** The assembly reaction was initiated by mixing 5 μl of clarified α extract with 25 μl of clarified β extract in 1 ml of Buffer A with BSA (2 mg/ml) at 2°C. These volumes corresponded to roughly equimolar amount of the two subunits. Assembly was measured as the attainment of luciferase activity by the dithionite flash assay (17) with 25 μl of the renaturation reaction mixture.

To test assembly competence of the α luciferase subunit preparations, clarified extracts (20 μl) containing α luciferase were mixed with 80 μl of either 10 M urea or buffer A for 10 min at room temperature and then diluted 50-fold into buffer A containing a large excess (0.5 mg/ml) of the β luciferase subunit and BSA (2 mg/ml) at 22°C. The β subunit had been refolded from inclusion bodies as described above. After 1 hr the luciferase activity was measured. Samples were adjusted to contain identical final urea concentrations. Similar experiments were performed to test the assembly competence of the β subunit.

In the GroEL binding reaction, 60 nM GroEL (14-mer) was mixed with either 5 μl of clarified α luciferase or 25 μl of β luciferase extract in a total volume of 200 μl of buffer B (50 mM Tris/35 mM KCl/50 mM sodium phosphate/2 mM MgCl₂/1 mM EGTA/1 mM DTT, pH 7.5) for 5 min at 22°C and then centrifuged for 39 min in a Beckman TLA-100 rotor at 70,000 rpm at 22°C. Over 99% of GroEL was removed from the sample under these conditions. The top 150 μl of the sample was removed and then mixed with the complementary subunit (5 μl of α or 25 μl of β extract) in 1 ml of buffer A with BSA (2 mg/ml). Luciferase was used at 45 nM in a control experiment based on the estimate of α luciferase in bacterial extracts by gel electrophoretic analysis. GroEL was purified by velocity sedimentation from an overproducing strain grown at 32°C (7).

**Luciferase Structural Determination.** Circular dichroism (CD) spectra were measured at 4°C with a JASCO model J-600 spectropolorimeter. α and β luciferase subunits were prepared by renaturation as described above. Cell path lengths were 0.01 cm (α/β), 0.02 cm (α), and 0.05 cm (β). CD spectra were analyzed by the method of Hennessey and Johnson (28). The spectra were obtained at concentrations from 0.1 to 6 mg/ml.

α luciferase samples prepared for NMR analysis were concentrated to 6 mg/ml with a Centriprep 10 concentrator (Amicon) in buffer A with 5% (vol/vol) $^2H_2O$ (D₂O). Rapid buffer exchange into buffer A in 99% D₂O (final D₂O/H₂O ratio was ≈90%) was accomplished by spin desalting the 0.5-ml sample through a 5-ml Sephadex G-25 column equilibrated in buffer A in 99% D₂O. The pD of the exchange buffer is the uncorrected pH reading. The exchanged NMR spectrum was completed 4 min after the initiation of buffer exchange into D₂O. NMR spectra were obtained on a 500-MHz GE Omega spectrometer.

## RESULTS

By separate expression of the subunits in *E. coli*, each subunit of *V. harveyi* luciferase was given the opportunity to fold in the absence of the complementary subunit. Extracts from cells expressing α luciferase were mixed with extracts from cells expressing β luciferase, and the assembled enzyme was measured as the increase in activity in the light-producing enzyme assay (Fig. 1). The rate of assembly was slow but increased significantly at higher temperatures. This result is similar to that of Friedland and Hastings (16), who found that assembly was the rate-limiting step in luciferase renaturation. More recently, Baldwin and coworkers (18–20) have extensively studied the folding and assembly of luciferase and have proposed that luciferase assembles from partially folded subunits, since individual subunits become assembly-incompetent with time.

When the subunits of *V. harveyi* luciferase were expressed separately in *E. coli*, the proteins were found in the cytosol (soluble) and in inclusion bodies (insoluble), which can be separated by centrifugation. After bacterial growth at 22°C, the α subunit partitioned equally between the soluble and insoluble forms, whereas after growth at 37°C, >80% of the protein was insoluble. When the α and β subunits were renatured from inclusion bodies and then mixed together, they assembled with kinetics similar to those of subunits folded and soluble *in vivo* (which had not been put through a denaturation–renaturation cycle; data not shown). Subunits recovered from the inclusion bodies (α or β) were >95% pure. Refolded α luciferase and α luciferase solubly expressed in bacteria are competent for assembly. About 90% of luciferase activity can be recovered through renaturation from the urea-denatured dimer (15, 20). To test assembly competence, the soluble form of α luciferase was either added directly to a large molar excess of refolded β subunit (assembly reaction) or first denatured in 8 M urea prior to dilution into a similar solution containing the β subunit (refolding reaction). The assembly reaction yielded luciferase activity that was 105% of that yielded by the refolding reaction. By a similar experimental scheme, the assembly competence of the solubly expressed β subunit was determined to be 47%. While the α subunit remains active, some of the β subunit becomes inactivated during the 3-hr expression period in bacteria. This inactive form of the β subunit may be the β' form described by Waddle *et al.* (18). Subunits of luciferase have recently been purified which are assembly-incompetent but native-like (21). The relationship between these native-like subunits and the molten globule subunits studied here is unknown.
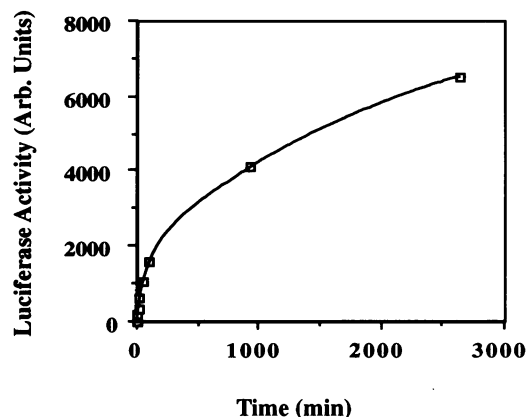


FIG. 1. Time course of the assembly at 2°C of bacterial luciferase from α and β subunits expressed as soluble proteins in bacteria. Assembly was monitored as appearance of enzymatic activity, shown here in arbitrary (Arb.) units.

Unassembled $\alpha$ luciferase was prepared from inclusion bodies by renaturation and concentration. ($\beta$ luciferase was more prone to inactivation from aggregation at the concentrations required for this study.) Sedimentation and gel filtration analysis suggested that the protein was a compact monomer with a sedimentation value of 3.5 S and a Stokes radius consistent with a globular protein of about 35 ± 3 kDa. Far-ultraviolet CD analysis of the $\alpha$ and $\beta$ subunits yielded spectra characteristic of proteins containing secondary structure. More concentrated samples of $\alpha$ luciferase (up to 6 mg/ml), including those prepared in $D_2O$ for NMR analysis, or higher temperatures (up to 25°C) gave similar results. $\alpha$, $\beta$, and native-assembled luciferase have spectra in the 190- to 230-nm range, consistent with approximately 20%, 15%, and 30% $\alpha$-helix, respectively (Fig. 2). However, the $\alpha$ subunit did not display a one-dimensional $^1H$ NMR spectrum indicative of a folded protein. Folded globular proteins typically show "upfield methyl" resonances above 1 ppm that result from the precise arrangement of aliphatic protons near $\pi$ electron systems such as aromatic rings (29). These are absent from the proton spectrum of $\alpha$ luciferase (data not shown). $\alpha$ luciferase is in a state with a relatively high secondary structure content but lacking any stable tertiary structure. This state has been termed a molten globule (30, 31) or a collapsed form (32) and is thought to be a folding intermediate.

The $\alpha$ subunit of luciferase has eight methionines, well distributed throughout the linear sequence of the molecule (10). The surrounding side chains will create differing environments for each methionine within a folded protein, which will translate into distinct NMR chemical shifts. For example, bacteriophage T4 lysozyme, a globular protein of 164 residues, contains five methionines, which can be labeled exclusively in the methyl position by expressing the protein in a defined medium containing 19 unlabeled amino acids and [*methyl*-$^{13}C$]methionine (23). The proton resonances of the methyl groups observed by $^{13}C$ editing of the proton NMR spectra are shown in Fig. 3A. The five resonance peaks from the folded bacteriophage T4 lysozyme are shown in the bottom spectrum. This chemical-shift dispersion of protons from the [$^{13}C$]methyl demonstrates the differing environments of methionines in a folded protein. Bacteria expressing $\alpha$ luciferase were grown in the same medium. The $^{13}C$-edited spectrum of the renatured $\alpha$ luciferase obtained from these cells is shown in the upper spectrum of Fig. 3A. Very little chemical shift dispersion can be detected among its eight methionines. The weak dispersion in chemical shift of the resonances in the 1.5- to 2.1-ppm region of the spectrum
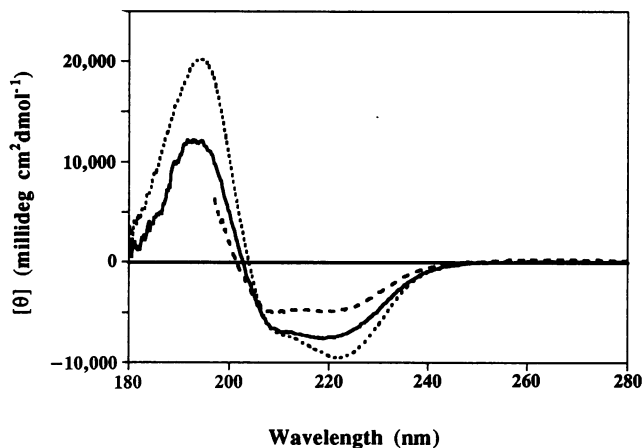






FIG. 2. Far-ultraviolet CD spectra of luciferase subunits. Protein samples were $\alpha$–$\beta$ dimer (2.0 mg/ml) ($\cdots$), $\alpha$ subunit (0.6 mg/ml) (——), and $\beta$ subunit (0.2 mg/ml) (– – – –).

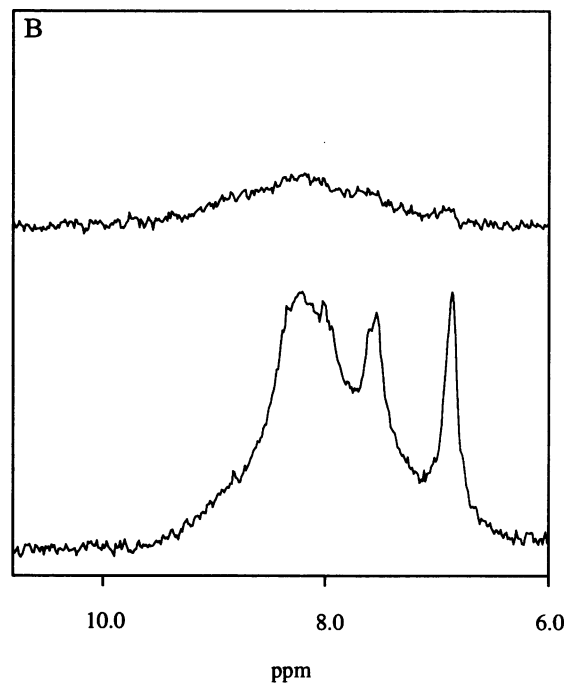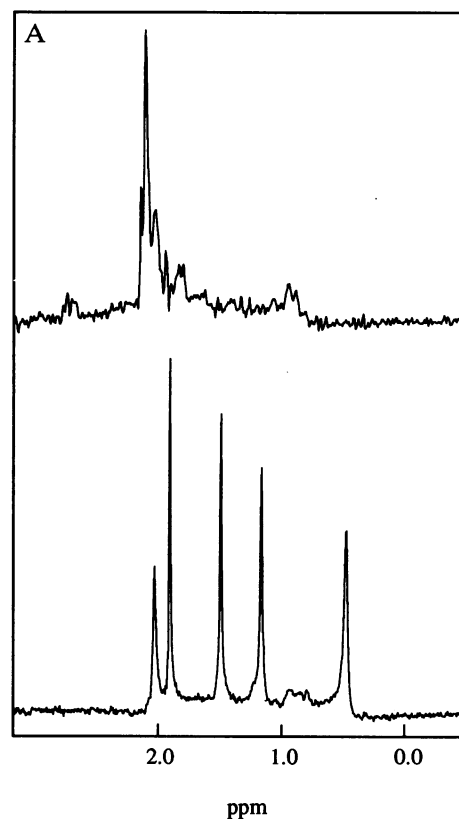FIG. 3. NMR analysis of $\alpha$ luciferase. ($A$) $^{13}C$-edited $^1H$ NMR spectra of methionine methyl protons of renatured $\alpha$ luciferase and T4 lysozyme. Upper spectrum, 0.15 mM renatured $\alpha$ luciferase in buffer A with 5% $D_2O$ at 20°C; lower spectrum, 0.25 mM bacteriophage T4 lysozyme in 20 mM phosphate, pH 7.0/10 mM KCl with 5% $D_2O$ at 20°C (22). The signal resonances at ≈0.8 ppm arise from the presence of nuclei at natural abundance and low-level scrambling of the [$^{13}C$]methyl groups. ($B$) $^{15}N$-edited proton NMR spectra at 22°C of the amide region of $\alpha$ luciferase uniformly labeled with $^{15}N$. Spectra were taken before (lower spectrum) and after (upper spectrum) exchange from buffer A in $H_2O$ to buffer A in $D_2O$.

possibly could be an indication of some local tertiary structure. Upon full unfolding of a protein such as T4 lysozyme, the chemical-shift dispersion is lost as the methionines become exposed to solvent. The single resonance peak of T4 lysozyme after denaturation coincides with that of unassembled $\alpha$ luciferase (data not shown), confirming the general lack of tertiary structure.

Hydrogen–deuterium exchange experiments have been used to determine the tertiary structural content within regions of small proteins (33, 34). The formation of stable hydrogen bonds and/or the burial of amides within the protein causes the peptide backbone amide protons to be more resistant to exchange with solvent than those exposed when the protein is unfolded. Selected amide protons in the interior of highly stable folded proteins such as T4 lysozyme exchange up to $10^{10}$ more slowly than those of the unfolded state, whereas amide protons on molten globules are protected to an intermediate degree, with protection factors $10^2$ to $10^3$ over unfolded proteins (35–37). A hydrogen–deuterium exchange experiment performed with $^{15}$N-labeled $\alpha$ luciferase is shown in Fig. 3B. The lower spectrum represents all the amide protons present in buffered $H_2O$. Again this spectrum shows very little chemical-shift dispersion. This can be seen quite clearly in the resonances arising from primary amides seen at 6.9 and 7.5 ppm. This is characteristic of a protein with little or no tertiary structure. After the solvent was switched to buffered $D_2O$, the signal rapidly decreased (top figure) to 15% of the original signal intensity within 4 min, correlating to a protection factor of $10^3$ or less. Following this initial drop, the signal remained relatively constant for several hours. In folded globular proteins 30–50% of the amide protons are well protected. Since the $^{15}$N sample after exchange contained $\approx$10% residual $H_2O$, and contaminating proteins in the $^{15}$N-labeled $\alpha$ luciferase preparation can account for some of the signal following exchange, we conservatively estimate that <10% of the amide protons of $\alpha$ luciferase were protected from exchange to any degree. Our estimate of a hydrogen–deuterium protection factor of <$10^3$ for $\alpha$ luciferase is consistent with previously determined values for molten globules (36). From this and the previously described experiments we conclude that the $\alpha$ subunit of luciferase has little if any stable tertiary structure prior to assembly.

We tested whether molecular chaperones could recognize the preassembly forms of luciferase. Addition of a slight molar excess of bacterial hsp60 (GroEL) completely inhibited the assembly. However, this inhibition was abolished upon addition of the cofactor GroES and ATP to the reaction mixture (Fig. 4A). Both subunits were able to bind to the chaperone. Extracts could be depleted of $\alpha$ or $\beta$ subunit by sedimenting the GroEL-bound subunits (Fig. 4B). Sedimenting GroEL in solution with native luciferase did not remove the dimer from solution or inhibit the luciferase reaction. Nor did the order of addition or the subunit chosen for sedimentation have any effect on assembly. These results provide additional evidence that the $\alpha$ and $\beta$ subunits were in a similar partially folded conformation.

A larger portion of the $\alpha$ subunit could be expressed in a soluble form at lower growth temperatures. This soluble form possessed structural features similar to those of the subunit renatured from inclusion bodies. The $^1$H NMR spectra of the purified soluble $\alpha$ subunit and the renatured form are virtually identical. Both spectra contain broad resonance lines and are devoid of any upfield methyl signals (data not shown). Like the renatured form, the soluble form contains a high helix content as determined by far-ultraviolet CD analysis. Moreover, neither form of $\alpha$ possesses any enzymatic activity alone, but the two forms are equally capable of assembling with $\beta$ into the active enzyme. Therefore, $\alpha$ luciferase subunits, whether expressed in a soluble form *in vivo* or
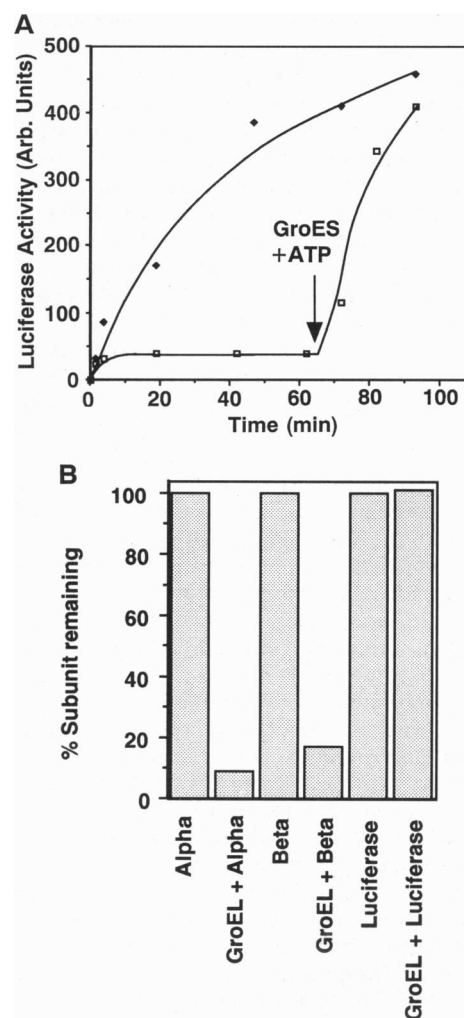


FIG. 4. Interaction of GroEL with luciferase subunits. (A) GroEL inhibition of luciferase assembly. Bacterial extracts containing $\alpha$ and $\beta$ luciferase were mixed in 50 mM Tris/35 mM KCl, 2 mM MgCl$_2$/50 mM sodium phosphate, pH 7.5, at 22°C with GroEL (0.6 mg/ml) with ($\blacklozenge$) or without ($\square$) GroES (0.6 mg/ml) and 1 mM ATP. At the time indicated by the arrow GroES (0.6 mg/ml) and 1 mM ATP were added to the sample that lacked GroES and ATP. (B) Binding of luciferase subunits to GroEL. Controls samples run without GroEL are defined as 100% assembly. The assembly was allowed to proceed at room temperature for 30 min and then measured by the light assay given above. Within 30 min the assembly reaction was >50% complete at this temperature.

denatured and renatured *in vitro*, are in a similar molten globule state under physiological conditions.

## DISCUSSION

The use of a heterodimeric protein has allowed us to separate folding of monomers from the assembly of the active dimer. Previous studies with small protein factors (6 kDa) have demonstrated structural changes associated with assembly (38, 39). For the example demonstrated here, the subunits of a large globular protein remain only partially folded until assembly occurs. Put differently, the subunit association seems to drive the final folding of the enzyme. The folding and stability that occur from the assembly event may also ensure the unidirectional movement across intracellular membranes.

Molten globules can be formed from some monomeric proteins under denaturing conditions such as low pH (35, 37) or removal of the prosequence prior to folding (40). $\alpha$

luciferase lacks a prosequence, and the conditions used in our study were close to physiological (200 mM sodium potassium phosphate, pH 7.0/1 mM DTT at 22°C). Therefore, we conclude that the α subunit exists as a molten globule when expressed in bacteria and can be isolated stably in this form. Moreover, since α luciferase is expressed prior to β luciferase on a multicistronic message in luminescent bacteria (41), α luciferase may persist in the cell in a free state for a relatively long time prior to assembly. This evidence strongly suggests that the molten globule state either exists and is an authentic folding/assembly intermediate in the cell or exists in rapid equilibrium with a folding intermediate.

A current model of hsp60 action proposes that a folding protein undergoes conformational changes on the surface of hsp60 (GroEL) in an ATP- and GroES-dependent manner. During this process the protein passes through a molten globule state (6). The form of the folding polypeptide that can be first recognized by GroEL is not known. From binding studies this form has been suggested to be partially folded (4, 6, 8). We have shown here that GroEL can bind to a subunit of a protein which at equilibrium is in a molten globule state. Whether GroEL can recognize other, less folded forms or will convert the molten globule into different forms has yet to be tested. It also remains a possibility that GroEL recognizes the intersubunit face region of the subunits prior to assembly.

Some *in vitro* studies demonstrating that GroEL facilitates refolding have used homooligomeric enzymes, such as citrate synthase (9) and ribulose-1,5-bisphosphate carboxylase (8), that could not distinguish between folding and assembly events. We found that the molecular chaperone GroEL inhibited assembly of bacterial luciferase, possibly by binding to both the α and β subunits in their preassembly molten globule form. Upon addition of the cofactor GroES and ATP, this inhibition was reversed (Fig. 4). The chaperones GroEL and GroES may assist assembly by preventing aggregation of this partially folded intermediate. GroEL can prevent aggregation of both α and β luciferase *in vitro* (data not shown). Our study suggests a way in which the hsp60 class of chaperones may function in the cell. Oligomeric enzymes assembling from molten globule subunits would have a greater propensity for aggregation due to this relatively long-lived folding intermediate and would have a commensurate requirement for the molecular chaperone hsp60 to prevent this aggregation.

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