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GroE[1] Assists Refolding of Recombinant Human Pro-Urokinase

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GroE, one of the molecular chaperones, facilitates correct protein folding both *in vitro* and *in vivo*. The refolding of recombinant human pro-urokinase, a protein with a high content of disulfide bonds, was used as a model system to illustrate the mechanism of action of GroE. Aggregation of this protein predominates during its *in vitro* refolding, as indicated by a strong, concentration-dependent increase in light scattering. The addition of GroE and Mg-ATP significantly increases the yield of the active protein. GroE specifically inhibits the aggregation reaction that competes with correct folding, as shown by a strong decrease in the intensity of light scattering. GroEL rapidly binds to unfolded or partially folded pro-urokinase molecules and thus protects them from the aggregation reaction. Interaction with GroES and ATP hydrolysis are required for the release of the polypeptide chain from GroEL and further acquisition of the completely folded, native conformation.

Key words: aggregation, chaperone, GroE, pro-UK, refolding.

Molecular chaperones, the cellular proteins that assist in the folding and assembly of oligomeric proteins, were described by Ellis and Hemmingsen (*1*) as preventing "improper interaction between potentially complementary surfaces and to disrupt any improper liaisons that may occur." These molecular chaperones, which belong to the group of heat-shock proteins, can interact with nonnative or partially folded polypeptide chains in an ATP-dependent manner (*2, 3*). One of the best-characterized molecular chaperones is GroEL from *Escherichia coli*, an abundant protein that is required for cell viability and the assembly of various other proteins (*4, 5*). GroEL exists as a complex of fourteen identical polypeptides (a 14-mer) of 549 residues each (subunit $M_r = 57,000$), whereas its partner, GroES, is a 7-mer of chains with 97 residues each (subunit $M_r = 10,000$) (*4-7*). Both proteins are encoded by the groE operon (*5, 8*). Similar molecules are found in mitochondria and chloroplasts (*9*). GroEL is a weak, potassium-dependent ATPase (*10*). Studies of the effects of GroE complex on the refolding and assembly of dimeric (*10, 11*) and monomeric (*12*) enzymes have demonstrated that GroEL binds the unfolded proteins tightly and releases them in a folded form, either assembled or able to become so, after hydrolysis of ATP and a physical interaction with GroES. It was proposed previously that GroEL is able to facilitate the correct folding of other proteins by preventing the formation of inactive aggregates (*11, 13*). This has been shown for the *in vitro* reconstitution of citrate synthase (CS), where GroEL and GroES together with Mg-ATP prevent the aggregation of partially folded intermediates by forming a transient complex (*11*).

For this study, we chose recombinant human pro-urokinase (pro-UK), a single-chain proenzyme of $M_r = 45,000$ with twelve disulfide bonds, as a model system to investigate the effect of GroE on its refolding *in vitro*. Pro-UK formed inclusion bodies when highly expressed in *E. coli*, and its *in vitro* folding has not been studied in detail (*14, 15*). We measured the reactivation and aggregation of pro-UK by means of enzymatic assays and light scattering, respectively, in both the absence and presence of the GroE complex. Our results show that GroE promotes the reactivation of pro-UK through effectively suppressing the aggregation of unfolded or partially folded molecules in an ATP-dependent manner.

MATERIALS AND METHODS

Materials—Plasmid pGroESL containing the *E. coli* groES and groEL genes was kindly provided by Professor Anthony A. Gatenby (Central Research and Development Department, Experimental Station, E.I. DuPont de Nemours, USA). The expression vector containing the human pro-UK gene and the *E. coli* strain were kindly provided by Professor Zichun Hua (Department of Biochemistry, Nanjing University, China).

Purification of GroEL and GroES—E. coli strain TG1 was transformed with plasmid pGroESL. The transformed cells were grown in LB broth (containing 25 μg/ml chloramphenical) at 37°C until A_{600} reached 0.6. IPTG was added to a final concentration of 40 μM and the culture was grown at 37°C for a total of 6 h. The cells were pelleted at 4,000 rpm for 30 min and then resuspended in a lysis buffer of 50

[1] GroE designates a complex of a 14-mer of GroEL with a 7-mer of GroES.

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Abbreviations: CS, citrate synthase; Hsp70, heat-shock protein of $M_r = 70,000$; IPTG, isopropyl-β-D-thiogalactoside; PB, sodium phosphate buffer; PMSF, phenylmethylsulfonylfluoride; pro-UK, pro-urokinase; rpm, rotations per minute; S2444, pyroglutamyl-glycyl-arginine-*p*-nitroanilide (Glp-Gly-Arg-NH-Np).

mM Tris/HCl, pH 8.0, and 10% (w/v) sucrose (4 ml/g of pellet), buffer A (0.1 M potassium phosphate, pH 7.0, 0.1 M ammonium sulfate, 5 mM 2-mercaptoethanol, 5 mM EDTA, and 10% glycerol) (3 ml/g of pellet), and PMSF (to a final concentration of 100 μM) was then added. Ultrasonic waves were used for cell lysis. After centrifugation at 15,000 rpm for 30 min, the supernatant was applied to a DE-52 cellulose column (equilibrated with buffer A). GroEL and GroES were found in the second peak together with other *E. coli* proteins on SDS-PAGE. The fraction containing GroEL and GroES was precipitated with ammonium sulfate (35-55% saturation). The sediment was dissolved in buffer B (50 mM Tris/HCl, pH 8.0, 5 mM 2-mercaptoethanol, 5 mM EDTA, 10% glycerol, 1 M KCl, and 0.5% Triton X-100) and then passed through a Sephacryl S-200 HR column (equilibrated with buffer B). GroEL was found in the first peak, but GroES in the second peak. After dialysis against buffer C (50 mM Tris/HCl, pH 8.0, 5 mM 2-mercaptoethanol, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5% Triton X-100), the fractions containing GroEL and GroES were further bound to a DEAE-Sepharose column (equilibrated with buffer C), respectively. Elution was achieved with a linear KCl gradient: 50 mM-400 mM for GroEL, 50 mM-1 M for GroES. The fractions containing GroEL and GroES were pooled, respectively, for future use.

Expression, Denaturation, and Reactivation of Recombinant Human Pro-urokinase—Transformed *E. coli* cells were grown in LB broth containing 20 μg/ml kanamycin and 100 μg/ml ampicillin at 37°C until A_{600} reached 0.6. IPTG was added to a final concentration of 200 μM and the cells were grown for a total of 5 h. The cells were harvested at 4,000 rpm for 20 min and then resuspended in buffer 1 (0.05 M PB, pH 7.5, and 0.3 M NaCl) (10 ml/g of pellet). Ultrasonic waves were used for cell lysis. After centrifugation at 2,000 rpm for 20 min, the paste was suspended in buffer 2 (0.05 M PB, pH 7.5, 2% Triton X-100, and 5 M urea) (10 ml/g of paste). Buffer 3 (0.05 M PB, pH 7.5, and 50% glycerol) (10 ml/g of paste) was then added slowly to the mixture. After centrifugation at 2,000 rpm for 20 min, the sediment was dissolved in buffer 4 (0.1 M PB, pH 7.5, 8 M urea and 50 mM 2-mercaptoethanol) and stirred for at least 12 h at 4°C. The entire mixture was centrifuged at 10,000 rpm for 20 min. The supernatant was dialyzed against buffer 5 (0.1 M PB, pH 7.5, 8 M urea, and 1 mM 2-mercaptoethanol) and then stored at 4°C. Renaturation was initiated by diluting the denatured pro-UK 100-fold in buffer 6 (2.5 M urea, 50 mM Tris/HCl, 5 mM EDTA, 0.01 M NaCl, 0.005% Tween-80, 0.25 mM GSH, 2.5 mM GSSG, 10 mM $MgCl_2$, and 10 mM KCl) at 4°C. The refolding solution was agitated vigorously to ensure rapid mixing during the addition of the unfolded protein. Spectroscopic measurement was started immediately after dilution.

Assaying of Enzymatic Activity of Pro-UK—(1) Pro-UK was assayed on a fibrin plate prepared as described by Ploug and Kjeldgaard (*16*). (2) Pro-UK was assayed using S2444 as a substrate according to the method described by Liu and Gurewich (*17*).

Protein Determination—Protein concentrations were measured as described by Bradford (*18*) with BSA as a reference.

Aggregate Determination—Light scattering was measured with a Shimadzu RF-540 Spectrofluorophotometer with excitation and emission at 500 nm. The spectral band width was 2 nm for both excitation and emission. Data were collected to reflect the difference in the formation of pro-UK aggregates under various refolding circumstances rather than to determine the exact amount of aggregates.

RESULTS

The Yield of Reactivated Pro-UK Is Concentration- and pH-Dependent—It has been reported that recombinant human pro-UK accumulates as inclusion bodies in the cytoplasm when expressed in *E. coli*, and that the refolding of pro-UK from inclusion bodies is inefficient (*14, 15*). Since the yield of the active protein is dependent on the refolding conditions, we varied the pH and concentration in the refolding of pro-UK. Table I shows that the yield of the reactivated enzyme increases as the pH increases from 8.0 to 10.0. The results in Fig. 1 indicate that the reactivation yield is nearly zero when the protein concentration in the refolding mixture is more than 0.3 μM. The yield can be increased greatly if the final concentration is lowered. The decrease in the extent of reactivation at a very low concentration (below 0.06 μM) is probably due to unspecific adsorption to the surface of the glass cuvette, as indicated by Buchner *et al.* (*11*).

GroE Increases the Amount of Correctly Folded Pro-UK and Its Effect Is Dependent on Both pH and Concentration—The refolding of pro-UK was performed in the presence of GroE so as to investigate the effect of the GroE complex on protein refolding. The results in Fig. 2 demonstrate that the reactivation is strongly promoted in the presence of GroE and Mg-ATP in a concentration-dependent fashion. A 10-fold increase in the reactivation yield is observed with a 6-fold molar excess of GroE over pro-UK compared to renaturation in the absence of the chaperone.

In addition, when renaturation of pro-UK is carried out in the presence of a constant 6-fold molar excess of GroE and Mg-ATP, the yield of the active protein depends on both the pH (Table I) and pro-UK concentration (Fig. 1). The facilitation of pro-UK refolding by GroE is much more significant under pH 8.0 than under pH 9.0 or 10.0 (Table I).

Aggregation Competes with Folding of Pro-UK—Protein aggregation is frequently observed as a major nonproductive side-reaction of protein folding. As illustrated in Fig. 3, aggregation during the refolding of pro-UK does occur, which leads to a strong time- and concentration-dependent

TABLE I. **Influence of pH and GroE on the yields of active pro-UK.** Unfolded pro-UK was diluted 100-fold in the refolding buffer at 4°C. The concentration of pro-UK was 0.32 μM in the refolding mixture. The concentrations of the GroE complex were 1.92 μM. The concentration of ATP was 2 mM. Each refolding reaction was repeated four times. Pro-UK was assayed on a fibrin plate (*16*). The activity of the refolding pro-UK is given as a percentage relative to the theoretical result derived by assuming that all the expressed pro-UK is active (the activity of 1 mg pure pro-UK is 10^5 IU).

Refolding system	Reactivation yield (%)
pH 8.0 without GroE	2.9±0.18
pH 8.0 with GroE and Mg-ATP	27.8±0.36
pH 9.0 without GroE	8.0±0.22
pH 9.0 with GroE and Mg-ATP	16.1±0.24
pH 10.0 without GroE	11.9±0.27
pH 10.0 with GroE and Mg-ATP	21.1±0.14

increase in light scattering. The increase in light scattering slows down and shows a small amplitude when the protein concentration is lowered. In fact, the yield of correctly renatured molecules increases with decreasing initial protein concentration (Fig. 1). So an increase in the yield and a decrease in the extent of aggregation are correlated with each other. Aggregation proceeds rapidly after dilution of the unfolded protein under native conditions, suggesting that it occurs at a very early, transient stage of folding.

GroE Suppresses Aggregation but Has No Influence on Aggregates—To determine whether the increase in the yield of native pro-UK in the presence of GroE is due to suppression of aggregation, the kinetics of aggregation were monitored directly as light scattering. Although the refolding concentration of pro-UK may vary, the increase in light scattering always exhibits a much smaller amplitude in the presence of GroE compared to that in its absence (Fig. 4). At the same time, light scattering measurements with various GroE concentrations (Fig. 5) showed that GroE suppresses aggregation in the same concentration-dependent way as it increases the yield of active pro-UK (Fig. 2).

To determine whether GroEL has any influence on already-formed aggregates, renaturation was initiated without a chaperone, and GroE was then added at different stages of refolding. Figure 6 shows that further aggregation will be inhibited once GroE becomes available in the refolding solution, but previously aggregated pro-UK molecules can not be dissolved and subsequently regain their activity since the intensity of light scattering does not decrease after the addition of GroE.

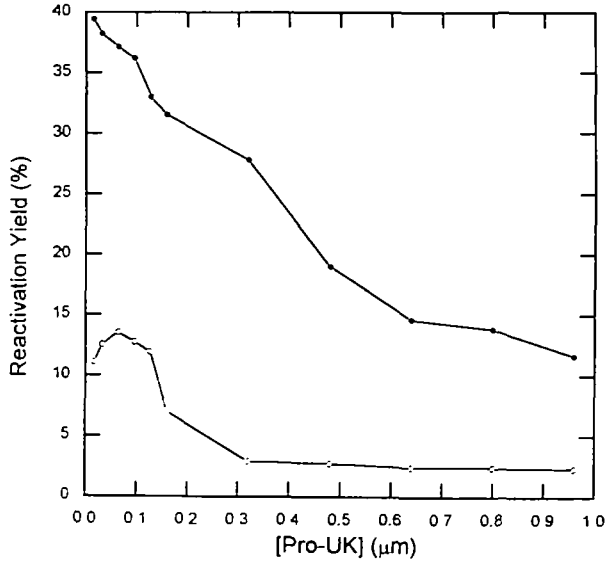

Fig. 1. **Concentration dependence of the yield of reactivated pro-UK in the presence (●) and absence (○) of GroE at 4°C.** Pro-UK was completely denatured at various protein concentrations. Refolding was initiated by 100-fold dilution of the unfolded protein in the refolding buffer at pH 8.0 to the indicated pro-UK concentrations. A 6-fold molar excess of GroE over pro-UK was added to the refolding solution for the reactions in the presence of GroE. The reactivation yield was measured on a fibrin plate after 24 h refolding. Each refolding reaction was repeated four times. The activity of the refolding pro-UK is given as a percentage relative to the theoretical result derived by assuming that all the expressed pro-UK is active (the activity of 1 mg pure pro-UK is 10^5 IU).

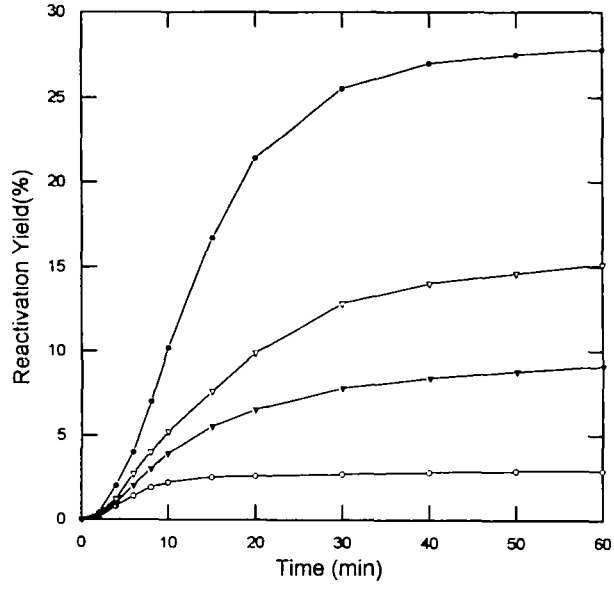

Fig. 2. **Time course of reactivation of pro-UK in the presence of 1.92 μM (●), 0.64 μM (▽), and 0.16 μM (▼) GroE, and in the absence of GroE (○).** Unfolded pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentration of pro-UK in the refolding solution was 0.32 μM. Activity assays using S2444 were performed after various times of refolding. Each refolding reaction was repeated four times. The activity of the refolding pro-UK is given as a percentage relative to the theoretical result derived by assuming that all the expressed pro-UK is active (the activity of 1 mg pure pro-UK is 10^5 IU).

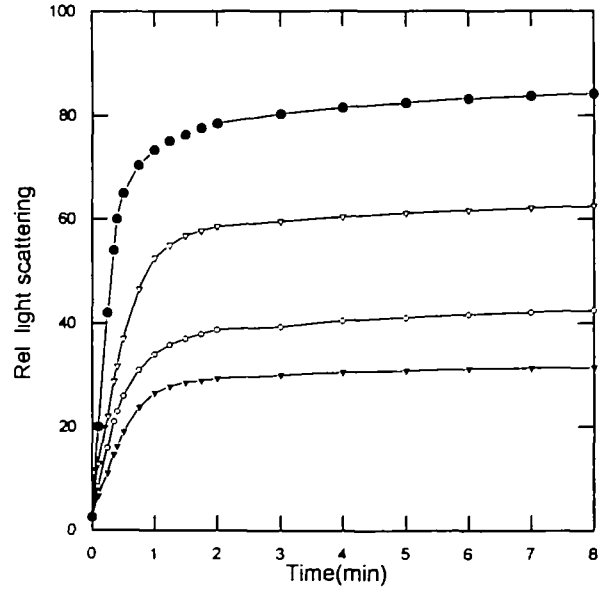

Fig. 3. **Concentration dependence of the increase in light scattering in the time course of refolding of pro-UK.** Completely denatured pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentrations of pro-UK in the refolding solution were 0.32 μM (●), 0.16 μM (▽), 0.128 μM (○), and 0.096 μM (▼). Light scattering was measured at 500 nm. Each refolding reaction was repeated four times.

Only GroEL Can Suppress Aggregation—Components in the refolding mixture of pro-UK were varied in order to probe the function of GroE. The light scattering-time curves in Fig. 7 demonstrate that GroEL alone is sufficient to prevent the aggregation of pro-UK during refolding. The effect of GroEL is even more pronounced in the absence of Mg-ATP than in its presence.

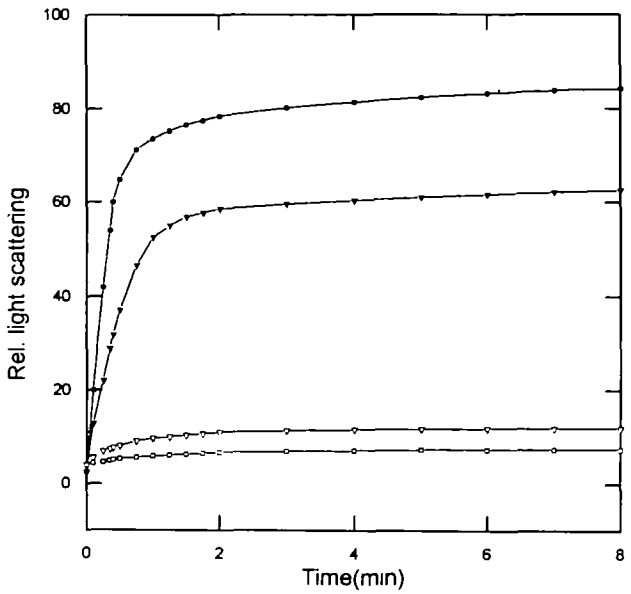

Fig. 4. **The effect of GroE on the intensity of light scattering in the time course of refolding of pro-UK: 0.32 μM Pro-UK in the absence (●) and presence (▽) of GroE and Mg-ATP, and 0.16 μM pro-UK in the absence (▼) and presence (□) of GroE and Mg-ATP.** Completely denatured pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentrations of the GroE complex and ATP were 1.92 μM and 2 mM, respectively. Each refolding reaction was repeated four times.

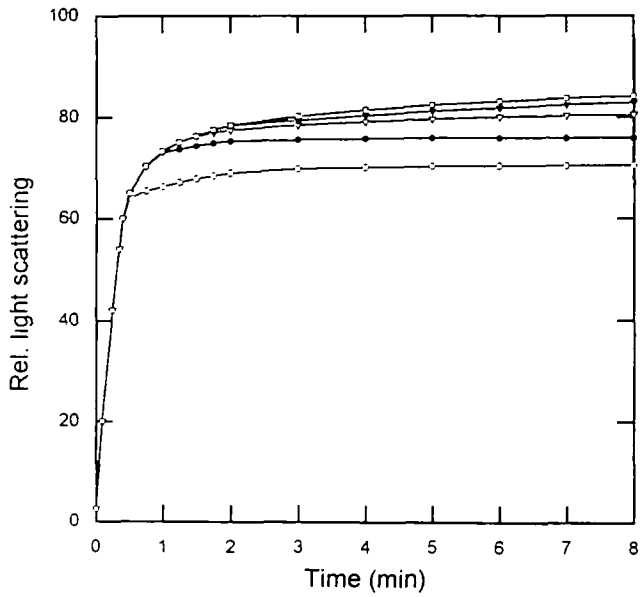

Fig. 6. **Effect of GroE added after various times of refolding of pro-UK.** 1.92 μM GroE and 2 mM ATP were added 30 s (○), 60 s (●), 90 s (▽), and 120 s (▼) after initiation of refolding. Light scattering in the time course of reactivation in the absence of GroE (□) is shown for reference. Denatured pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentration of pro-UK in the refolding step was 0.32 μM. Each refolding reaction was repeated four times.

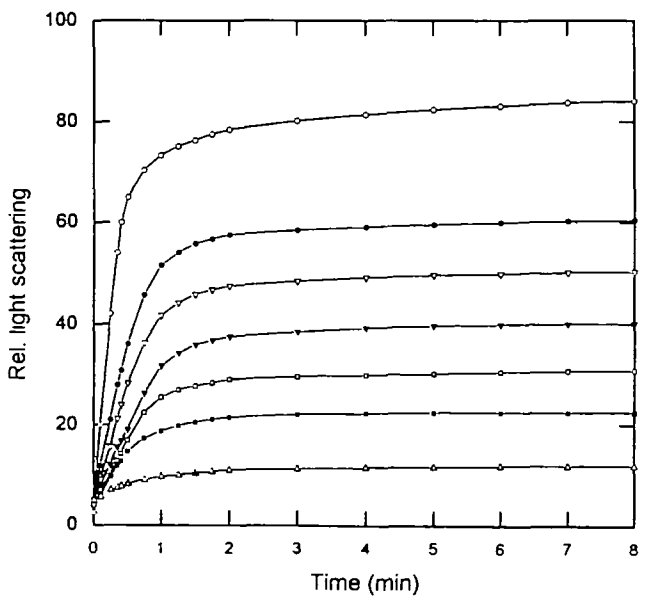

Fig. 5. **Light scattering in the time course of refolding of pro-UK in the presence of 1.92 μM (△), 0.96 μM (■), 0.64 μM (□), 0.32 μM (▼), 0.16 μM (▽), and 0.08 μM (●) GroE, and in the absence of GroE (○).** Completely denatured pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentration of pro-UK in the refolding step was 0.32 μM. Each refolding reaction was repeated four times.

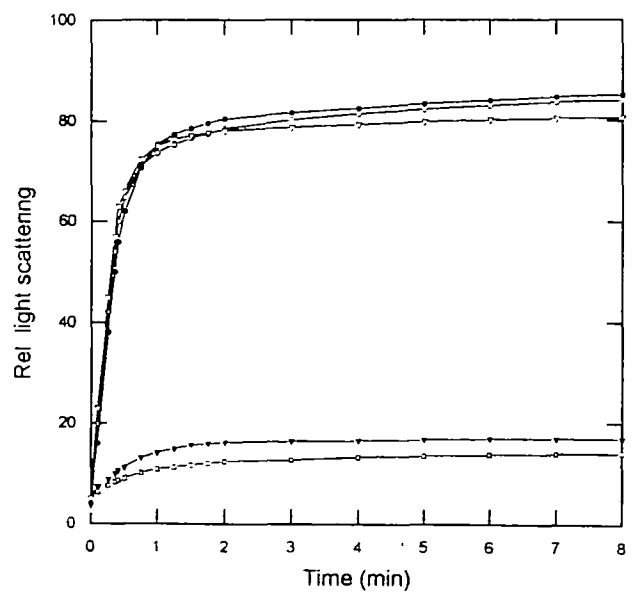

Fig. 7. **Light scattering of pro-UK refolding in the presence of 1.6 mg/ml BSA (●), 1.92 μM GroES (▽) and 1.92 μM GroEL in the presence (▼) and absence (□) of 2 mM ATP, and refolding in the absence of additional protein (○).** Denatured pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentration of pro-UK in the refolding step was 0.32 μM. Each refolding reaction was repeated four times.

TABLE II. **Influence of various components on the yields of active pro-UK.** Unfolded pro-UK was diluted 100-fold in the refolding buffer at pH 8.0 and 4°C. The concentration of pro-UK was 0.32 μM in the refolding mixture. Each refolding reaction was repeated four times. Pro-UK was assayed on a fibrin plate (*16*). The activity of the refolding pro-UK is given as a percentage relative to the theoretical result derived by assuming that all the expressed pro-UK is active (the activity of 1 mg pure pro-UK is 10^5 IU).

Added components	Reactivation yield (%)
None	2.9±0.18
2 mM ATP	3.3±0.14
2 mM ATP+1.6 mg/ml BSA	3.0±0.23
1.92 μM GroEL	2.7±0.17
1.92 μM GroEL+2 mM ATP	10.1±0.21
1.92 μM GroES+2 mM ATP	3.0±0.19
1.92 μM GroEL+1.92 μM GroES+2 mM ATP	27.8±0.36
1.92 μM GroEL, addition of 1.92 μM GroES and 2 mM ATP after 45 min	27.7±0.26
1.92 μM GroEL+1.92 μM GroES, addition of 2 mM ATP after 45 min	28.1±0.29

As shown in Table II, while GroEL alone is able to significantly suppress aggregation, it does not exert any effect on the reconstitution of pro-UK. This suggests that GroEL and unfolded or partially folded pro-UK may form a stable complex without Mg-ATP and GroES. GroES has no influence on reactivation. The same holds true when ATP alone is added. Nevertheless, the addition of GroEL (in the absence of GroES) as well as ATP leads to a certain increase in the yield of native pro-UK. Although GroES is not necessary for the increase in the reactivation yield, the presence of GroES, together with GroEL and Mg-ATP, leads to the maximum yield of the active enzyme. Similar effects were observed when GroEL was added first, and GroES and Mg-ATP were added after 45 min or GroEL and GroES were added first and Mg-ATP was added later.

It has been reported that BSA facilitates the reactivation of several proteins in an unspecific way (*19*). However, the addition of BSA to the refolding solution of Pro-UK neither suppressed aggregation (Fig. 7) nor promoted reactivation (Table II).

The foregoing results are similar to the results regarding the refolding of CS (*11*). Unfolded or partially folded forms of pro-UK are recognized by GroEL specifically, and stable binding between them inhibits aggregation as well as further folding. Interaction with GroES and Mg-ATP is presumably required for the release of the polypeptide chain from GroEL. On the other hand, GroE does not interact with native pro-UK. This indicates that GroE can only recognize the nonnative conformation of unfolded molecules or folding intermediates, and does not affect the conformation of correctly folded pro-UK.

DISCUSSION

Human pro-UK is a 411 amino acid glycoprotein and contains up to 12 disulfide bonds. The molecule consists of three structural domains, an EGF-like domain (9-44aa), a kringle domain (45-133aa), and a serine-protease domain (134-411aa) (*20*). Folding of pro-UK occurs through the fast formation of the secondary structure, and subsequent slow, rate-limiting steps involving the formation of the tertiary structure and disulfide bonds, domain pairing, *etc.* *In vitro* refolding is inefficient because of kinetic competition between aggregation and rapid steps in the correct folding pathway. Aggregation has previously been considered as a major determinant for the decrease in the reactivation yield during protein refolding *in vitro* (*21*). Folding is a first-order reaction, while aggregation is a second or higher order reaction. Therefore, aggregation is favored by an increased protein concentration. The strong differences in rate between renaturation (Fig. 2) and aggregation (Fig. 3) indicate that aggregation occurs well before the rate-limiting steps of protein folding. In unfolded or partially folded polypeptides, buried hydrophobic residues inaccessible in native molecules may be exposed to the hydrophilic environment. Nonnative molecules are prone to aggregate through nonproductive association among those exposed hydrophobic surfaces. Consequently, aggregation is mainly in competition with the rapid formation of a critical intermediate that has already been protected against such off-pathway reactions and may enter the slow folding steps.

Pro-UK is an alkaline protein. Its isoelectric point is 9.05-9.20. As indicated in Table I, renaturation of pro-UK improves under higher pH. The addition of GroE and Mg-ATP promotes the reactivation of pro-UK and this effect depends on pH. A 10-fold increase in the reactivation yield in the presence of GroE can be achieved at pH 8.0, but only a 2-fold increase at pH 9.0 or 10.0 (Table I). GroEL functions in the acidic environment of *E. coli* and is susceptible to a basic solution. As a result, the assistance by GroEL weakens with increasing pH.

The presence of GroE during refolding suppresses aggregation and concomitantly increases the amount of native pro-UK. It has been confirmed that GroEL specifically interacts with unfolded or partially folded molecules (*11, 22-25*). Once the correct native state has been reached, GroEL is no longer able to interact with pro-UK molecules. This suggests that GroEL recognizes those structural elements that only exist in unfolded or partially folded forms. Since hydrophobic areas have also been observed on the surface of the GroEL molecule (*22, 26*), the above-mentioned elements may be those hydrophobic residues exposed only at the surface of the nonnative polypeptide chain. Therefore, GroEL blocks aggregation by binding to unfolded molecules or folding intermediates of pro-UK through hydrophobic interaction.

While GroEL alone is sufficient to inhibit aggregation, the maximum recovery of pro-UK activity can only be achieved in the presence of GroEL, GroES, and Mg-ATP, which indicates an ordered sequence of events. Firstly, GroEL binds to unfolded pro-UK or folding intermediates, GroES and Mg-ATP not being required. This binding blocks both aggregation and further folding steps. Secondly, pro-UK must be released from the GroEL·pro-UK complex in order to complete with slow steps of folding and to acquire its native conformation. ATP hydrolysis is essential for the release, while the presence of GroES is not. Our results regarding the interplay between GroEL, GroES, and Mg-ATP correspond to the findings of Goloubinoff *et al.* (*27*).

Our conclusion is also consistent with that of Buchner *et al.* Their research was on the refolding and aggregation of CS (*11*). For both CS and pro-UK, aggregation is strongly suppressed by GroE and their reactivation yields reach

nearly 30% after the addition of GroE. However, the refolding of these two proteins is quite different. CS is a homodimer (subunit $M_r = 50,000$) with three disulfide bonds, while pro-UK is a monomeric protein with 12 disulfide bonds. The enzyme structure of CS can be classified as essentially all α-helix, and these helices of the two subunits are packed together tightly to give a globular molecule (*28*). The formation of α-helices and subunit assembly are probably the main slow steps during protein refolding. As to pro-UK, proper pairing between sulfhydryl groups is the key point of the refolding process. That the yields of the two proteins are the same suggests that GroE inhibits the aggregation reaction, and thus may have a certain effect on the achievement of correct disulfide bonds.

DnaK, a Hsp70 homologue in *E. coli*, could dissolve aggregates of heat-inactivated *E. coli* RNA polymerase and subsequently allow reactivation in the presence of ATP *in vitro* (*29*). In contrast, GroE cannot redissolve aggregated pro-UK. Similarly, the activity of native pro-UK is also not influenced by GroE. This confirms that GroE is only active during early stages of folding, when unfolded or partially folded pro-UK molecules are present in high amounts.

The rate of refolding of pro-UK is not increased after the addition of GroE. The mechanism for GroE is different from that for protein disulfide isomerase (PDI) (*30*) and peptidyl prolyl *cis-trans* isomerase (PPI) (*31, 32*). GroE binds to nonnative molecules and thus inhibits side reactions, while PDI and PPI accelerate the rate-limiting steps of the folding reaction, and thereby prevent aggregation. Besides, GroE is able to facilitate protein refolding, but it cannot guide the correct folding and assembly by selecting the correct folding pathway. It is the information encoded by the amino acid sequence that determines protein folding (*33*).

In summary, GroE increases the yield of reactivated pro-UK by binding to unfolded polypeptides or folding intermediates, thus preventing unproductive aggregation that may occur. The role of chaperones such as GroE is to protect folding molecules from off-pathway reactions. Recently molecular chaperones in the eukaryotic cytosol were shown to interact differently with chemically denatured proteins and their newly translated counterparts (*34*). Therefore, whether the proposed mode of action for GroE during protein refolding *in vitro* operates in the *de novo* folding of nascent polypeptide chains *in vivo* remains to be determined.

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