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Contribution of cotranslational folding to the rate of formation of native protein structure

(protein folding/translation/bacterial luciferase)

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ABSTRACT **To compare the process of protein folding in the cell with refolding following denaturation *in vitro*, we have investigated and compared the kinetics of renaturation of a full-length protein upon dilution from concentrated urea with the rate of folding in the course of biosynthesis. Formation of enzymatically active bacterial luciferase, an $\alpha\beta$ heterodimer, occurred 2 min after completion of β-subunit synthesis in an *Escherichia coli* cell-free system. Renaturation of urea-denatured β subunit, either in the presence of the cell-free protein synthesis system or in buffer solutions, proceeded more slowly. Cellular components present in the cell-free protein synthesis system slightly accelerated the rate of refolding of urea-unfolded β subunit. The results indicate that the luciferase β subunit begins the folding process cotranslationally and that cotranslational folding contributes to the rapid formation of the native structure in the cell.**

The classical approach to the study of protein folding has been to investigate refolding after transfer from conditions under which the protein exists primarily as the unfolded molecule to conditions under which the native structure predominates (1, 2). One of the central questions is how the patterns observed for refolding *in vitro* relate to protein folding within the living cell. There are two main differences between these processes. First, the nascent polypeptide may start to fold during biosynthesis on the ribosome (3–6). This process could lead to different intrapeptide contacts during folding from those that occur during refolding of the covalently intact but unfolded polypeptide. Second, there are multiple cellular components that are able to catalyze or assist the folding of newly synthesized polypeptides, such as peptidylprolyl isomerases, protein disulfide isomerase, and heat shock proteins (7, 8). Moreover, physicochemical conditions employed for protein renaturation *in vitro* often are very different from conditions *in vivo*.

Protein folding in the cell appears to be significantly faster than refolding of the denatured protein *in vitro*, especially for large oligomeric proteins. However, there are no examples for which the rate of biosynthetic folding has been determined for comparison with the refolding of the denatured polypeptide. The experiments reported here were designed to compare directly protein refolding with the process of protein folding during synthesis in a cell-free system in order to determine whether the protein synthesis mechanism and/or cellular components present in the cell-free system might contribute to the overall kinetics of native structure formation in the cell.

Bacterial luciferase, an $\alpha\beta$ heterodimer, was chosen for this study because of the extremely sensitive enzymatic assay, which permits monitoring the appearance of the native polypeptide synthesized in a cell-free expression system. Knowledge of kinetic (9, 10) and thermodynamic (11, 12) parameters for the refolding reaction has allowed us to establish experimental conditions with which to compare the refolding reaction with folding during biosynthesis.

MATERIALS AND METHODS

Plasmid Construction. Plasmid pTZ313 was constructed by ligation of a *ca.* 1200-base-pair *Ase* I–*Pvu* II fragment carrying the *luxB* gene from pTB7 (13) into the *Sma* I and *Pvu* II sites of the plasmid vector pTZ18R (Pharmacia). The resulting plasmid, which contained the *luxB* gene behind the phage T7 promoter, was linearized by *Ngo*MI and used for the production of mRNA. A variant of this plasmid with a *luxB* gene devoid of the TAA translation stop codon was constructed by site-directed mutagenesis (14). The stop codon was changed to TAT, encoding tyrosine, and a *Dra* I restriction site was introduced several bases downstream. The plasmid was linearized by *Dra* I for production of mRNA.

Transcription and Translation. The resultant linearized plasmids were used for the production of mRNAs with T7 polymerase (Promega) as described (15). The *Escherichia coli* cell-free expression system was prepared as described (16). L-[^{35}S]Methionine (1140 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was used as a radiolabeled marker. Translation was carried out at 29°C with 70 μg of the luciferase β-subunit mRNA per ml. The translation mixture was supplemented with native luciferase α subunit at a final concentration of 10 μg/ml. To monitor products of synthesis, 5-μl aliquots of the translation mixture were added to the sample buffer for SDS gel electrophoresis and subjected to electrophoretic analysis (17); the [^{35}S]methionine-labeled polypeptides were visualized by autoradiography. Incorporation of radioactivity into the full-length β subunit was quantitated as described below.

Separation of the ribosome-bound luciferase β-subunit nascent chains was achieved by size-exclusion chromatography. Translation was carried out in a cell-free system (140 μl) supplemented with mRNA for β subunit without a stop codon in the presence of 10 μg of α subunit. The translation mixture was applied to an Ultrogel AcA34 (LKB) column (1 cm × 10 cm) equilibrated with buffer A (20 mM Hepes, pH 7.6/100 mM potassium acetate/10 mM magnesium acetate/1 mM dithiothreitol) and eluted with the same buffer. Fractions of 0.5 ml were collected. The column was calibrated with blue dextran (2000 kDa) and cytochrome *c* (12 kDa).

The proportion of the luciferase β subunit in the translation system supplemented with the mRNA lacking a stop codon that was ribosome-bound was determined by reaction with puromycin. Translation in 100 μl was carried out as above; the mixture was applied onto 0.5 ml of 10% sucrose prepared with buffer A. Centrifugation in a Beckman TLA 100.2 rotor was done at 90,000 rpm for 1 hr at 4°C. The pellet was dissolved in 100 μl of 20 mM Hepes buffer, pH 8.0/100 mM potassium

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acetate/10 mM magnesium acetate/1 mM dithiothreitol. Two 10-μl portions were taken, their volumes were adjusted to 50 μl with the same buffer, and 0.5 μg of α subunit was added. In one of them puromycin was added to 1 mM final concentration. Both samples were incubated 40 min at 29°C and then recentrifuged as discussed above. Pellets were dissolved in 50 μl of sample buffer for SDS gel electrophoresis. Aliquots from the translation mixture and subsequent fractions were subjected to electrophoretic analysis. Incorporation of radioactivity into the full-length β subunit was quantitated, and total incorporation in the corresponding fractions was calculated.

For quantitation of radioactivity incorporated into full-length β subunit, the gels with samples after electrophoretic separation were exposed in a β-particle imager, β-imager 1000 (BioSpace Instruments, Paris) or Betascope 603 (Betagen, Waltham, MA).

Luciferase β-Subunit Renaturation. Individual luciferase subunits were purified from the luciferase heterodimer as described (10). Renaturation was carried out either in the translation mixture without added mRNA or in the buffer 20 mM Hepes, pH 7.6/100 mM potassium acetate/1 mM magnesium acetate/1 mM dithiothreitol. Native α subunit (11) was added to a final concentration of 10 μg/ml. Renaturation was initiated by addition of denatured β subunit at a final concentration of 150 ng/ml.

Measurement of Bioluminescent Activity. Aliquots (5 μl) from the translation system or renaturation buffer were used for measurement of bioluminescence activity by the reduced flavin mononucleotide ($FMNH_2$) injection assay as described (18). Luciferase activity was measured with a Turner (Palo Alto, CA) TD-20e luminometer with one light unit corresponding to 3.22×10^5 quanta per sec based on a radioactive light standard as described (18).

Modeling of the Rate of β-Subunit Folding in a Cell-Free System. A mathematical simulation of the β-subunit folding and luciferase assembly reaction was performed based on the assumption that cotranslational folding does not occur. The kinetic profile for formation of active enzyme following dilution of unfolded β subunit into the cell-free system was approximated by a polynomial fit to the data presented in Fig. 3. It was assumed that the β subunit released from the ribosome would fold with the same kinetic profile. The rate of introduction of unfolded β subunit from ribosomes into solution was determined by the rate of production of the full-length protein as shown in Fig. 1 *Lower*. The increment of β subunit produced at each time point was then allowed in the simulation to fold and form active luciferase; the summation of the incremental additions was the predicted formation of luciferase based on the assumption of no cotranslational folding. In a second modeling experiment, further increase in the amount of full-length β subunit was blocked at 21 min to simulate the effect of a block of synthesis. Both radioactivity incorporation into the full-length β subunit and bioluminescence activity were normalized to the final values as 100%.

RESULTS AND DISCUSSION

The biologically active form of *Vibrio harveyi* luciferase is an αβ heterodimer. Unfolded β subunit can fold and assemble with the native α subunit to form the active enzyme, while folded β subunit will not interact with folded α subunit (10, 11). Therefore, we have added native α subunit to the cell-free protein synthesis system used to produce β subunit and monitored the rate at which the native αβ enzyme was formed in the reaction. The concentration of α subunit used in the study was sufficiently high that the second-order α-β subunit association reaction was faster than the folding of the β subunit, so that folding of the nascent chain was rate-limiting.

Expression *in vitro* employed an *E. coli* cell-free system supplemented with mRNA encoding luciferase β subunit. Purified native α subunit was added at a final concentration of 10 μg/ml prior to initiation of translation. Translation reactions were maintained at 29°C, since higher temperatures promoted formation of inactive subunits (data not shown). The same temperature dependence of luciferase folding and assembly *in vivo* and refolding *in vitro* has been observed (19). The time dependence of β-subunit synthesis and appearance of enzymatically active αβ dimer are shown in Fig. 1. Full-length β subunit is the predominant product in the translation system *in vitro* (Fig. 1 *Upper*). To quantitate β-subunit synthesis, incorporation of [^{35}S]methionine in the polypeptide was determined by use of a β-particle imager. The results, together with the kinetics of appearance of luciferase activity, are shown in Fig. 1 *Lower*. The kinetics of the two processes exhibit similar patterns, although there is a 3- to 4-min lag between completion of synthesis of the entire β subunit and appearance of active αβ heterodimer. This lag may reflect not only folding/association events following release of the β subunit but also termination and release of the completed polypeptide from the ribosome (20, 21).

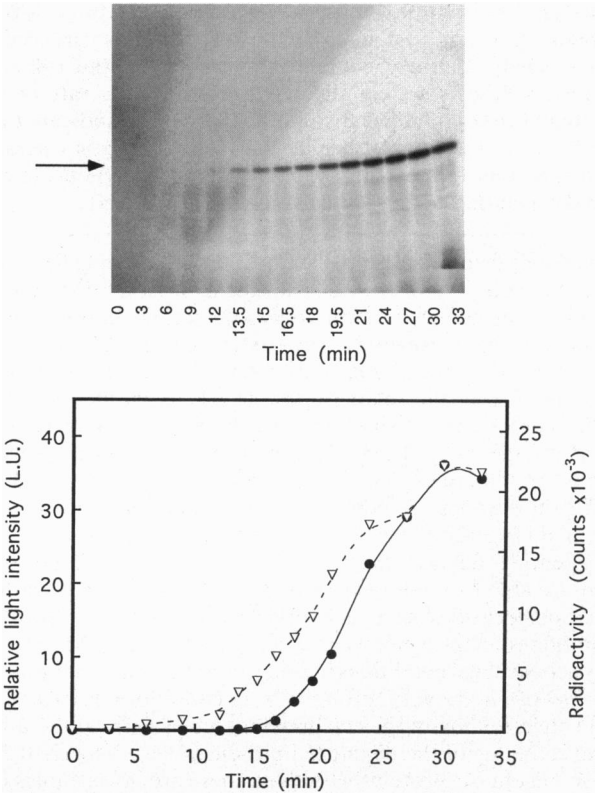

FIG. 1. Time course of luciferase β-subunit synthesis in an *E. coli* cell-free system and appearance of luciferase αβ heterodimer enzymatic activity. (*Upper*) Kinetics of full-length β-subunit synthesis as revealed by gel electrophoresis followed by autoradiography. (*Lower*) Quantitative analysis of β-subunit synthesis and appearance of the enzymatic activity. Translation was carried out in a total volume of 180 μl with the luciferase β-subunit mRNA in presence of [^{35}S]methionine and supplemented with native luciferase α subunit. At each indicated time point, two 5-μl aliquots of the translation mixture were removed simultaneously. One was used for measurement of bioluminescence activity (●), and the other aliquot was subjected to electrophoretic analysis to monitor polypeptide synthesis. The same gel was used for quantitation of radioactivity incorporation on a β-imager. The total number of counts in each sample corresponds to the radioactivity detected for polypeptides with the molecular weight of full-length β subunit (▽). The experimental error of the measurements was ±7%. Specific enzymatic activity of luciferase obtained with synthesized β subunit was $1.6 \times 10^{13} \pm 0.4 \times 10^{13}$ quanta per sec per mg. L.U., light unit(s).

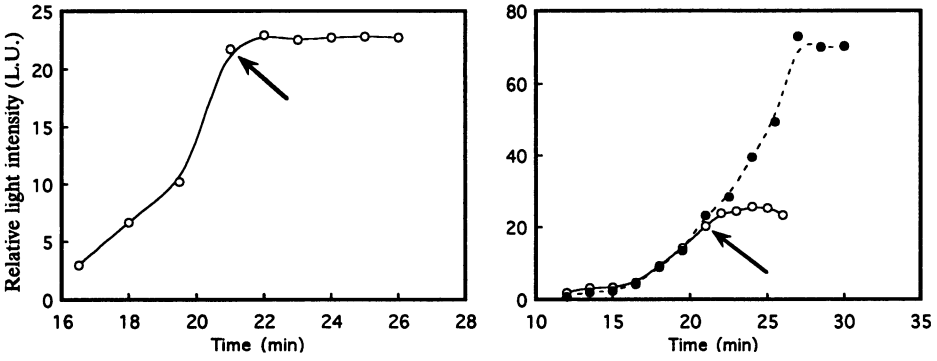

FIG. 2. Effect of blocking translation on the time course of appearance of luciferase activity in an *E. coli* translation system. (*Left*) The [^{35}S]methionine translation mixture for β-subunit synthesis supplemented with α subunit was prepared in a total volume of 180 μl with luciferase β-subunit mRNA and was subdivided into two portions which were incubated simultaneously. In one portion, $MgCl_2$ was added after 21 min of incubation to block further elongation (time indicated by arrow). At each time point, two 5-μl aliquots were removed simultaneously, one for electrophoretic analysis (data not shown) and the other for determination of the enzymatic activity. Active enzyme from the control (without block) was determined at 30 min, and light intensity was found to be 91 light units (L.U.). This level was greater than that observed in the experiment shown in Fig. 1, probably because a different batch of mRNA was employed. (*Right*) Cell-free translation with unlabeled methionine (final concentration, 50 μM). The translation mixture (total volume, 180 μl) was divided into two portions and kept at 4°C. Translation was carried out with the addition of $MgCl_2$ at 21 min to block elongation in one portion (time indicated by arrow) (○) and without the translational block in the other portion (●). The enzymatic activity of 5-μl aliquots was measured.

To obtain some understanding of the rate of folding and assembly of the β subunit following release from the ribosome, we have used a different experimental approach. By increasing the Mg^{2+} concentration, translation was blocked; this treatment has been shown to "freeze" nascent polypeptides on ribosomes and completely block further synthesis (6, 22). Any further increase in enzymatic activity following the block in translation should reflect only folding/association events of the β subunit that had already been released into the reaction mixture. A translation block was applied at 21 min, which was in the linear range of increase in enzymatic activity in the cell-free system. Quantitative analysis of the translation products confirmed that no further synthesis occurred after the Mg^{2+} concentration was increased. Within 1 min of application of the translation block, the luciferase activity reached a plateau at 27 light units (Fig. 2 *Left*), while the control reaction without the block reached 91 light units at 30 min (not shown). In other translation mixtures, unlabeled methionine (instead of [^{35}S]methionine) at a concentration identical to that of other amino acids was added. The results were the same: after application of the block of translation, the activity reached a plateau of 28 light units within 2 min, while without the block, the activity reached 74 light units at 30 min (Fig. 2 *Right*). The duration of the lag between cessation of synthesis of the β subunit and cessation of formation of active luciferase was 2 min or less, while the delay in appearance of luciferase following completion of the full-length β subunit, shown in Fig. 1, was 3–4 min. In the experiment presented in Fig. 1, the delay may be due both to termination and release of the full-length protein and subsequent folding, while in the experiment presented in Fig. 2, we believe that the delay is due only to folding.

To directly compare the rate of β-subunit renaturation under the same conditions as were used for translation of the mRNA, urea-denatured β subunit was added to the translation mixture supplemented with α subunit but without mRNA. The β subunit was used at approximately the same concentration as was obtained in the translation system *in vitro* (as quantitated by [^{35}S]methionine incorporation in the translation experiments). The appearance of luciferase activity was monitored as a function of time (Fig. 3). Following a lag of about 3 min, enzyme activity increased rapidly, reaching about 50% of the final value in about 9 min. The same experiment was repeated by using a buffer resembling that of the translation mixture supplemented with α subunit but without the cellular constituents contained in the cell-free translation system (Fig. 3). In this case, after a comparable lag, enzyme activity increased more slowly, reaching 50% of the final value in about 13 min. Different concentrations of Mg^{2+} have been found to have no effect on the rate of formation or yield of luciferase (data not shown). A possible explanation of the effect of components of the cell-free system on the rate of refolding is the action of chaperones (7, 8). At subunit concentrations of 10–50 μg/ml, excellent yields of active enzyme are obtained without the aid of chaperones (9, 10). While β-subunit renaturation in the cell-free system is faster than in the buffer, it is still much slower than folding of β subunit during synthesis.

The most obvious interpretation of the results of the experiments described above is that the β subunit, when it is released from the ribosome, has attained appreciable structure so that the time required to complete the folding process is significantly less than is required for the denatured polypeptide to refold under identical condition. We have modeled the folding/assembly reaction based on the following assumptions: (*i*) synthesis of the β subunit occurs at the rate shown by the data

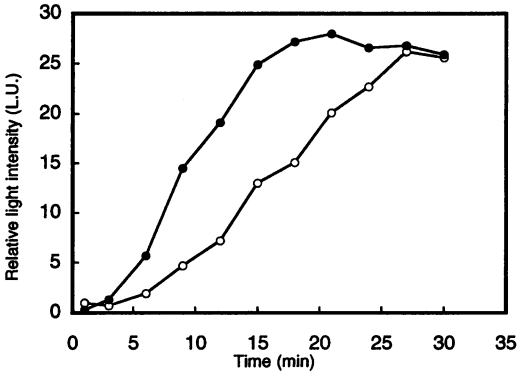

FIG. 3. Renaturation of full-length luciferase β subunit. Renaturation in the translation mixture was initiated by addition of urea-denatured β subunit to 180 μl of the translation mixture with added α subunit but without added mRNA; 5-μl aliquots were removed for measurement of enzymatic activity (●). Renaturation in buffer was initiated by addition of urea-denatured β subunit to 1 ml of buffer supplemented with α subunit; 5-μl aliquots were removed to measure enzymatic activity (○). Both renaturation mixtures contained the same final concentration of urea-denatured β subunit (0.15 μg/ml). L.U., light unit(s).

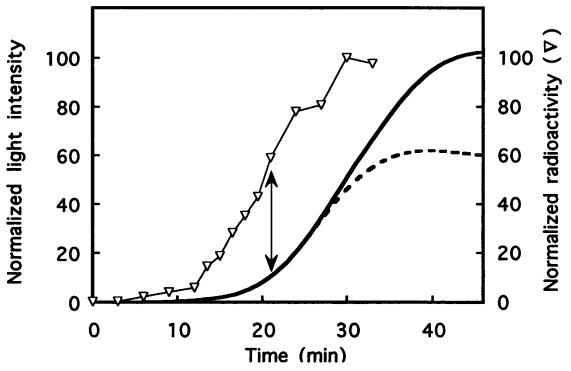

FIG. 4. Mathematical modeling of the β-subunit folding and luciferase assembly reaction. ▽, Time course for production of full-length β subunit (data from Fig. 1 *Lower*); ▬, simulated time course for formation of active luciferase from the synthesized β subunit [calculated by assuming that the β subunit (released in an unfolded state) folded and assembled into active enzyme at the same rate as denatured β subunit refolded under identical conditions (Fig. 3)]; ---, simulated time course for formation of active luciferase following a block in synthesis at 21 min. Further details are given in the text.

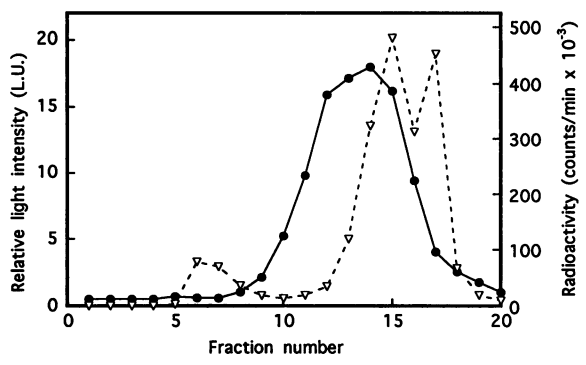

FIG. 5. Testing for the possibility of formation of enzymatically active αβ luciferase heterodimer by interaction of the nascent β subunit with free α subunit. Translation was carried out in a cell-free system supplemented with mRNA for β subunit without a stop codon in the presence of α subunit. The translation mixture was applied to a molecular sieve column (Ultrogel AcA34) to separate the ribosomal fraction from soluble proteins. Total radioactivity in 10-μl aliquots (▽) and enzymatic activity in 100-μl aliquots (●) were determined for each fraction.

presented in Fig. 1 *Lower*; (*ii*) the β subunit begins to fold only after release from the ribosome into the medium; (*iii*) the rate of folding of β subunit after release into the medium is the same as that of β subunit diluted from denaturant into the cell-free system (Fig. 3). The simulation was conducted by assuming that unfolded β subunit was introduced into the refolding reaction at the rate with which full-length β subunit was synthesized and that the unfolded polypeptides folded at the same rate as did unfolded β subunit introduced from denaturant.

As may be seen from the results of the modeling (Fig. 4), if the β subunit were released from the ribosome into the solution as an unfolded polypeptide, the native structure would form much more slowly than was observed experimentally. The delay between synthesis of the full-length polypeptide and appearance of the activity would have been about 10 min. To simulate the kinetics of appearance of luciferase activity following the Mg^{2+}-induced block of synthesis, the amount of β subunit synthesized prior to the 21-min time point, the time at which the block was applied, was held constant. The β subunit that had been synthesized prior to the block but folded after the block of synthesis would result in a large increase in the final amount of active enzyme (in contrast to Fig. 3); the plateau should not occur until about 17 min (see Fig. 4). Thus, the results expected based on the assumption of posttranslational folding of full-length β subunit are in clear contradiction to the experimentally observed rate of formation of active luciferase in the course of β-subunit synthesis.

It has been shown in a number of studies that the ribosome shelters 20–30 C-terminal amino acid residues during protein synthesis. Even though previous studies have implicated the C-terminal region of the β subunit in the heterodimerization process (19), we decided to test whether the β subunit may form a structure during synthesis with which the α subunit can combine to form an active complex prior to completion of synthesis and release from the ribosome. Luciferase β-subunit mRNA devoid of a stop codon was translated to produce a stable ternary complex of polypeptide–mRNA–ribosome. As in the above experiments, the cell-free system was supplemented with α subunit. The same level of polypeptide synthesis was obtained as for the mRNA with an in-frame stop codon. However, luciferase activity in the translation system was at the background level. Upon size-exclusion chromatography of the translation system, the radioactivity appeared in two peaks—the void volume, where extremely large complexes and particles including ribosomes are expected to be eluted, and the total accessible volume, where free [^{35}S]methionine is eluted (Fig. 5). Electrophoretic analysis of chromatographic fractions revealed that the β subunit was eluted predominantly in the void volume (not shown). There was no luciferase activity in these fractions, and no activity appeared after incubation of ribosomal complexes with additional α subunit. Enzymatic activity in the zone of free proteins is probably due to the α subunit alone (11) and perhaps to a small amount of luciferase (αβ) resulting from release of β subunit. By treatment with puromycin, which causes the release of nascent polypeptides from ribosomes, it has been shown that the β subunit produced from mRNA lacking a stop codon indeed was predominantly

Table 1. Probing the ribosome-bound state of the nascent β subunit by puromycin

	Translation mixture		Ribosomal fraction			
			With puromycin		Without puromycin	
	Total	Pellet	Total	Pellet	Total	Pellet
Radioactivity, counts × 10^{-6}	7.5	5.4	0.54	0.115	0.54	0.50
β subunit, % of total	100	72	100	21	100	92

Translation was carried out in a cell-free system (in 100 μl) with the mRNA lacking a stop codon in the presence of α subunit. The translation mixture was subjected to centrifugation through 10% sucrose. The pellet was dissolved in 100 μl of the buffer; two 10-μl aliquots were removed, their volume was adjusted to 50 μl with the same buffer, α subunit was added, and the mixtures were incubated with or without puromycin. After recentrifugation under the same conditions, the pellets were dissolved in SDS buffer. After electrophoretic separation, incorporation of radioactivity into full-length β subunit was quantitated.

ribosome-bound (see Table 1). Large complexes and particles from a translation mixture were pelleted by centrifugation. This fraction contained the majority of the full-length β subunit. Addition of puromycin caused a dramatic decrease of high molecular weight β subunit, thus directly confirming that the polypeptide existed as a peptidyl-tRNA in the ribosome. The polypeptide released by puromycin formed enzymatically active luciferase in the presence of α subunit; 42 light units were obtained from an amount of the puromycin-treated ribosomal fraction that was derived from 5 μl of the original translation mixture. From these experiments, the possibility of an inactive complex between α subunit and the nascent β subunit cannot be excluded. These results demonstrate that the final folding step of the β subunit, that is, formation of the enzymatically active αβ heterodimer, can occur only after release of the β subunit from the ribosome.

Based on the results presented in this study, we conclude that the luciferase β subunit folds cotranslationally. Clearly, the polypeptide cannot fold prior to synthesis. The C-terminal region of the β subunit is produced about 10–11 min after initiation of translation. The time required for folding of at least this portion of the β subunit, known to be involved in heterodimerization, is less than the time required to refold the covalently intact but unfolded β subunit upon dilution from urea. Biosynthetic folding of luciferase, including αβ heterodimer association, is completed in less than 2 min after the β subunit is released from the ribosome. The apparent explanation for this observation is that cotranslational folding contributes to the rate of formation of native protein structure in the cell-free system and therefore, we believe, within the living cell. While components of cell extracts (chaperones, peptidylprolyl isomerases, etc.) play important roles in protein folding, they cannot be *per se* a decisive factor determining the fast rate of biosynthetic folding, at least for this protein. Whatever the interactions in the biosynthetic folding of the β subunit, folding of the polypeptide during its synthesis on the ribosome is a prerequisite condition for the fast acquisition of native structure.

These data suggest that the fully unfolded form of a complete polypeptide does not exist within the living cell. This is in clear contrast with the starting point of refolding experiments *in vitro*. Questions regarding possible differences in the pathway(s) of polypeptide refolding and biosynthetic folding remain open. In the case of luciferase, it appears that the overall folding mechanism is similar whether the β subunit is produced from ribosomes or supplied unfolded from urea. That is, the β subunit folds into a heterodimerization-competent monomeric structure that combines with the α subunit to form, ultimately, the active luciferase heterodimer. Vectorial appearance of the nascent polypeptide from the N-terminal to the C-terminal end may affect, at least, the temporal organization of folding events within the β-subunit monomer.

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