Equilibrium Intermediates in the Reversible Unfolding of Firefly (Photinus pyralis) Luciferase*

(Received for publication, July 31, 1996, and in revised form, January 6, 1997)

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Firefly luciferase has been used as a model protein to study cotranslational and chaperone-assisted protein folding. We found conditions for reversible unfolding of luciferase in the absence of cellular factors, and we characterized denaturant-induced equilibrium unfolding transitions and refolding kinetics of the enzyme. Luciferase unfolding induced by guanidinium chloride at 10 °C can be described as a four-state equilibrium with two inactive intermediates highly populated around 1 and 3 M denaturant. The transitions occur around 0.3, 1.7, and 3.8 M denaturant. The free energy of denaturation to the first inactive intermediate (ΔG°N=11 = 15 ± 3 kJ mol⁻¹) is small for a protein of 60 kDa. Fluorescence and circular dichroism spectra of the intermediates indicate that I₁ has a compact conformation, whereas aromatic side chains are highly exposed in the secondary structure. In the presence of a hydrophilic detergent, significant reactivation of luciferase is observed up to temperatures at which the native protein is unstable. Reactivation kinetics of luciferase are exceedingly slow and probably not limited by proline isomerization, as suggested by their independence from the time spent in the unfolded state.

The observation that some organisms are able to convert chemical energy into visible light has been fascinating researchers for many years. In Photinus pyralis, a beetle in the superfamily of Cantharididae, the 61-kDa monomeric protein firefly luciferase is responsible for the production of light. It is located in specialized peroxisomes in the abdominal lantern organ of the insect and was one of the first enzymes to be investigated in biochemical detail (1). Although subsequent research has led to a more detailed understanding of the light reaction and although the crystal structure of the enzyme has recently been solved, some aspects of its enzymatic mechanism still remain mysterious (2–4).

Luciferase catalyzes the Mg²⁺-ATP-dependent oxygenation of the heterocyclic component firefly luciferin, yielding ultimately an electronically excited oxyluciferin species. This excited state product then returns to the ground state emitting yellow-green light with an emission quantum yield close to 1. Luciferase activity could be detected almost immediately after release of the polypeptide from the ribosome (11). Moreover, when chemically denatured or heat-inactivated luciferase was diluted into reticulocyte lysate or wheat germ extract, the enzyme reactivated in minutes and with high yields of 60–80% when ATP was provided by a regenerating system (10–14). In contrast, reactivation of luciferase in buffer lacking cellular components was inefficient, and the enzyme was found to aggregate during refolding (9, 10). Reconstitution experiments with purified molecular chaperones identified DnaK, DnaJ, and GroE as candidates for the chaperoning system facilitating luciferase folding upon expression in Escherichia coli (9, 15).

On the other hand, it is generally held that the information required for correct folding is entirely encoded in the amino acid sequence of a protein (16), and proteins found to depend on molecular chaperones for efficient folding in vivo generally can be efficiently renatured if refolding conditions are carefully optimized (17). Such refolding experiments are a prerequisite for understanding critical steps in protein-folding pathways and to identify folding intermediates as candidate targets of chaperone action. The purpose of the present work was to find conditions for efficient refolding of P. pyralis luciferase in the absence of accessory proteins and to characterize the folding pathway of the enzyme. We found that firefly luciferase does indeed refold spontaneously and with high yield at low temperature and low protein concentration, conditions chosen to minimize aggregation as a competing side reaction. This allowed us to determine the thermodynamic stability of the enzyme from equilibrium unfolding transitions, to physically characterize two highly populated folding intermediates, and to follow the kinetics of luciferase refolding.

EXPERIMENTAL PROCEDURES

Materials—Purified, lyophilized P. pyralis luciferase was obtained from Boehringer Mannheim, dissolved in storage buffer (0.5 M Tris acetate buffer, pH 7.5), and stored at −70 °C at protein concentrations of 1 or 5 mg/ml. The enzyme was found to be homogeneous by SDS gel electrophoresis and silver staining. Peptidylprolyl cis-trans-isomerases from the periplasm of E. coli (rotamase) and from rabbit cytoplasm (FKBP-52) were kindly provided by Dr. Elke Prohaska and Dr. Sushira Bose (Universität Regensburg). For reactivation experiments and equilibrium transitions, ultrapure Tween 20 from Sigma was used; for gel filtration experiments, it was protein-grade Tween 20 from Calbiochem. Dithioerythritol was purchased from Roth (Karlsruhe, Germany) and...
Luciferase Luminescence Assay—Unless indicated otherwise, luciferase activity was assayed using the GenGlow 100 kit from Bio-Orbit (Turku, Finland). A fluorescence microcuvette (Hellma, Type 105.251-001) was filled with 25 μl of luciferin reagent and 25 μl of ATP reagent, both preincubated at 25 °C as recommended by the supplier. For samples from equilibrium transitions induced by GdmCl, 2 μl of a corresponding GdmCl buffer solution were mixed into the assay to keep the overall GdmCl concentration constant at 55 mM. Addition of 3 μl of luciferase solution (0.2–18 μg/ml enzyme) started the light reaction. Light emission was followed at 550 nm at a spectral bandwidth of 25–40 nm for 2 min using a Spex FluoroMax or a Perkin-Elmer MFP-2A spectrofluorometer with switched-off light source. Because autoactivation causes a slight decrease in emission intensity with time (less than 5% over 2 min), the signals were corrected by extrapolation to zero time. The test was linear up to at least 18 μg/ml luciferase in the sample.

Spectroscopy—Fluorescence emission spectra were recorded in quartz cells (Hellma, Type 119.004F-QS) in a Spex FluoroMax spectrofluorometer equipped with a thermostatted cell holder. The excitation wavelength was set to 280 nm, and the spectral bandwidths were 4 and 8 nm for excitation and emission, respectively. With an increment of 0.05 nm, the emission time for each data point was 0.5 s. All spectra were corrected for fluorescence of buffers. Fluorescence of samples from folding equilibrium transitions was measured in 1-cm semimicro cells (Hellma, Type 104-QS) at the emission maximum of the native protein (334 nm) or at the maximum of the difference spectrum between native and unfolded luciferase (331 nm).

Circular dichroism was measured in calibrated fused silica semimicro fluorescence cells (path length, 4.2 mm; Hellma, Type 104F-QS) or in standard 1-mm silica cells (Hellma, Type 100-QS) in an Aviv 62A-DS spectropolarimeter at 10 °C at a spectral bandwidth of 1 nm unless indicated otherwise. For CD spectra, data points were taken at 0.2-nm intervals with integration over 1 s. Spectra were accumulated over four repeated scans, corrected for solvent background, and smoothed using polynomial interpolation. To follow conformational transitions, CD spectra were measured at 220 nm with integration over 180 s. Residue ellipticities were calculated using a mean residue weight of 110.5 calculated from the amino acid composition.

Reactivation of GdmCl-denatured Luciferase—Luciferase was denatured in buffer A (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM dithioerythritol) or buffer B (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM dithioerythritol, 0.2% v/v Tween 20) containing 5 mM GdmCl for 30 min at 10 °C. These conditions are sufficient to unfold the protein completely, as demonstrated by fluorescence and circular dichroism spectroscopy (see Fig. 2). Renaturation was initiated by 100-fold dilution into thermostatted buffer A or buffer B (10–40 °C, as indicated). To follow the reactivation course, the renaturation samples were further incubated at the given temperature by taking 3-μl aliquots at the indicated times and assaying bioluminescence activity. Reactivation experiments in the presence of pep tidylprolyl cis-trans-isomerase were done at 10 and 25 °C at a luciferase concentration of 60 nM with 120 mM FKBP52 or 1 μM rotamase, respectively.

Analytical Chromatography—Native luciferase and the folding intermediate were separated on a Superdex 200 HR 10/30 column (Pharmacia Biotech Inc.) at a flow rate of 30 ml/h and a temperature of 5 °C using buffer B as the running buffer. The enzyme was denatured, and renaturation was initiated at 10 °C in buffer B as described above. After varied times of refolding, 200-μl samples were taken and subjected to gel filtration chromatography. The fluorescence emission of the eluted protein was detected by a Merck/Hitachi F-1050 spectrofluorometer. Fractions were collected to be assayed for luciferase activity immediately after chromatography or after further incubation for 1 or 3 days at 10 °C. For comparison of peak areas with the time course of luciferase reactivation, elution profiles were fit to the sum of two Voigt peaks using PeakFit (Jandel Scientific).

Analytical Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium runs were performed at 2 °C in a Beckman Instruments Model E analytical ultracentrifuge in an AN-D rotor at 60,000 and 12,000 rpm, respectively. The sample contained 0.2 mg/ml luciferase in storage buffer, and the sedimentation data were corrected to water and 20 °C. For the determination of the molecular mass from sedimentation equilibrium profiles, a partial specific volume of 0.748 ml/g, as calculated from the amino acid composition, was used (18).

RESULTS

In an initial series of experiments, we tested whether efficient reactivation of unfolded firefly luciferase is possible in the absence of molecular chaperones. P. pyralis luciferase was diluted into denaturant solution to a final concentration of 5 mM GdmCl and kept for 30 min at 10 °C. Similar conditions have been employed previously (10) and are sufficient to completely unfold the protein (cf. Fig. 2 below). To prevent off pathway aggregation, refolding was attempted at low protein concentration (2–5 μg/ml) and low temperature (10 °C). Samples of unfolded luciferase were rapidly diluted 100-fold into cold phosphate buffer without additional ingredients and kept at 10 °C for several days. Within the first 24 h, refolding samples exhibited 50 ± 5% of the luminescence activity of native controls, for which no change in activity with time was observed under these conditions. Upon further incubation for 2 days, the activity of the refolding samples increased to 65 ± 5% of the controls. Addition of 1 mM dithioerythritol did not affect these results.

A further increase in reactivation yield was achieved by addition of a hydrophilic detergent. In the presence of 0.2% (v/v) Tween 20 and 1 mM dithioerythritol (to suppress peroxide formation; see Ref. 19) samples refolded for 24 h or 3 days exhibited luminescence activities of 65 ± 5 and 80 ± 5% of those observed for native controls, respectively. The activities of the controls were also higher after incubation in the presence of the additives (20–50% compared with samples incubated in buffer alone), indicating that the detergent may act by preventing adsorption of the enzyme to vessel walls, as observed with other proteins (20). In our hands, the addition of Tween 20 into the enzyme assay did not have a stimulatory effect, as reported previously (21).

Equilibrium Unfolding of Firefly Luciferase—Equilibrium transitions induced by GdmCl and measured as changes in enzymatic activity and fluorescence in the absence of detergent at low protein concentration are depicted in Fig. 1, A and B. Because GdmCl has an inhibitory effect on the enzymatic activity of P. pyralis luciferase (data not shown), corresponding amounts of GdmCl were added to the luminescence assay buffer in all experiments, keeping the overall GdmCl concentration in the luminescence assay constant at 55 mM.

After 3 days at 10 °C further change in luminescence activity (Fig. 1A) or intrinsic protein fluorescence (Fig. 1B) were observed, and the midpoints of unfolding and refolding transitions were essentially coincident. Although the enzymatic activity was lost in a single cooperative transition with a midpoint around 350 mM GdmCl, the denaturant-induced fluorescence change was clearly biphasic. In a first transition, the fluorescence intensity at the emission maximum of the native protein (334 nm) was reduced by ~35% with little change in the position of the emission maximum. This transition coincided with the inactivation of the enzyme. Beyond 1.2 μM GdmCl, a further reduction in fluorescence intensity was observed, with a transition midpoint around 1.7 μM GdmCl, and the emission maximum shifted to 351 nm. The presence of a pronounced plateau region around 1 μM GdmCl indicated a highly populated equilibrium unfolding intermediate.

The unfolding transition, as observed by fluorescence, was not affected by changes in protein concentration (Fig. 1C). The transition measured at 3.7 μg/ml enzyme was superimposable onto that determined at 50 μg/ml. In contrast, renaturation yields were clearly dependent on luciferase concentration (Fig.
Reactivation yields, measured relative to controls of native enzyme diluted to and incubated at the same protein concentration, approached 100% below 2 \( \mu \text{g/ml} \) but decreased to less then 40% at 10-fold higher protein concentration. To a first approximation, the concentration dependence could be described by a second-order aggregation reaction competing with a unimolecular folding process (22), as represented by the solid line in Fig. 1D.

The loss of secondary structure during unfolding at 50 \( \mu \text{g/ml} \) was followed by circular dichroism (Fig. 1E). Close to half of the CD signal amplitude at 220 nm was lost in a cooperative transition coincident with inactivation between 0 and 1 \( \mu \text{M GdmCl} \). Surprisingly, only a minor change paralleled the second phase of unfolding around 1.7 \( \mu \text{M GdmCl} \), whereas more than one-third of the CD change occurred in a highly cooperative transition with a midpoint at 3.8 \( \mu \text{M denaturant} \). The change in secondary structure around 3.8 \( \mu \text{M GdmCl} \) was fully reversible (Fig. 1F) and rapid. When samples of native and completely unfolded luciferase were diluted to denaturant concentrations between 3 and 5 \( \mu \text{M GdmCl} \), equilibrium ellipticities were attained in the dead time of manual mixing (data not shown). Thus, two equilibrium intermediates with varying contents of secondary and tertiary structure are populated during the reversible unfolding of luciferase.

Characterization of the Equilibrium Intermediates—Fluorescence emission and CD spectra of the intermediates in comparison with native and completely unfolded luciferase are presented in Fig. 2. Both types of spectra indicated that the enzyme was completely unfolded at 5.0 \( \mu \text{M GdmCl} \). The CD signal around 220 nm was 180 ± 600 degree\( \cdot \text{cm}^2\cdot\text{dmol}^{-1} \), proving that the protein had lost its secondary structure. Maximum fluorescence emission occurred at 351 nm, near the emission maximum of solvent-exposed tryptophan, and a distinct peak around 305 nm indicated spatial separation of tyrosine and tryptophan fluorophores. Very similar fluorescence spectra were observed in the plateau region of the CD transition around 3 \( \mu \text{M GdmCl} \). In contrast, the fluorescence spectrum of the first inactive intermediate was similar to that of native luciferase, with an emission maximum at 334 nm, differing mainly in fluorescence intensity. The data indicate that the intermediate populated around 1 \( \mu \text{M GdmCl} \) is compact enough to largely shield its aromatic side chains from the solvent. In
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FIG. 2. Spectroscopic characterization of the unfolding intermediates.
Fluorescence emission spectra (A, 3.7 μg/ml) and circular dichroism (B, 50 μg/ml) of samples from the plateau regions of the unfolding transitions depicted in Fig. 1, C and E, in comparison with those of native and completely unfolded luciferase. Fluorescence spectra were invariant between 3 and 6 M GdmCl: the spectrum shown was taken at 4.5 M denaturant. CD spectra taken at 0.8 and 1.2 M GdmCl were essentially identical to the spectrum at 1 M denaturant. The spectra indicated by heavy lines in B were measured at 4.2 nm, and the native spectrum indicated by a thin line was taken at 1 nm path length.

contrast, the intermediate observed around 3 M GdmCl, although retaining a large part of the secondary structure, does not significantly protect the fluorophores.

Quantitative Analysis of Luciferase Unfolding—It is apparent from the results presented above that luciferase unfolding is not completely reversible at elevated protein concentrations, i.e., under conditions in which a full set of spectroscopic and activity data can be acquired that might be subjected to global fitting procedures. Under certain conditions, however, individual inactivation and unfolding transitions could be sufficiently well separated to allow a quantitative analysis.

As pointed out above, the reversibility of the inactivation transition could be improved by addition of Tween 20. Unfolding and refolding transitions measured by fluorescence and bioluminescence after incubation in the presence of Tween 20 were very similar to those observed in buffer alone (Fig. 3, A and B), although the activation yields were higher and the midpoints of unfolding and refolding transitions coincided more precisely. In the presence of Tween 20, the inactivation transition, as well as the first phase of the fluorescence transition, were somewhat more cooperative than in the absence of the detergent. This suggests an interaction of the unfolding intermediate with the detergent molecules. Since Tween 20 concentrations far above the critical micelle concentration were used, Tween 20 micelles might be the interacting species. The reactivation was superimposable with the inactivation transition when the data were normalized to the final yield obtained at very low denaturant concentrations. It was well separated from the second phase of the unfolding transition by a pronounced plateau in the fluorescence signal around 1 M GdmCl (Fig. 3B). On the basis of these observations, a thermodynamic analysis of the inactivation transition appeared justified.

The inactivation data were fit to a N ↔ I2 two-state model (Fig. 3A, solid lines) because sedimentation velocity and sedimentation equilibrium ultracentrifugation experiments indicated the native enzyme to be monomeric (s20,w = 3.9 S, molecular mass = 60,600 Da). The analysis yielded a denaturation free energy of ∆G°N=I1 = 15 ± 3 kJ mol⁻¹ in the absence of denaturant and a denaturant dependence of m = 4.5 ± 1 kJ mol⁻¹ M⁻¹. Because no reliable CD data could be obtained under conditions in which the first two phases of the transition (N → I1 → I2) were found to be highly reversible, i.e., at nanomolar protein concentrations and/or in the presence of Tween 20, a rigorous quantitative description of the complete four-state unfolding transition is not possible at present.

Time Course of Luciferase Reactivation—The equilibrium experiments described had already indicated that luciferase reactivation occurs very slowly at 10 °C. Therefore, we undertook a more detailed analysis of reactivation kinetics at various temperatures. Results are illustrated in Fig. 4. Because the time course of reactivation did not follow monophasic first-order kinetics, no attempt at calculating rate constants was made. The time of half-maximal reactivation at 10 °C was 4 h. It was independent of protein concentration between 0.2 and 4 μg/ml. With increasing temperature, it decreased to 2.5 h at 15 °C and to 1.5 h at 20 °C. Above 20 °C, the activity of the native control decreased significantly during the experiment, and the activity of the refolding sample went through a maximum before decreasing at the rate observed for the control. When reactivation was calculated relative to the activity of the control sample at equivalent times, the time of half-maximal reactivation was close to 50 min at 30 °C. At temperatures above 15 °C, the effect of Tween 20 on refolding yields was more pronounced; however, omitting the detergent did not accelerate the reactivation reactions.

Slow folding of monomeric proteins is frequently caused by the rate-limiting isomerization of peptide bonds preceding proline residues. Although such peptide bonds are in a defined conformation, either cis or trans, in native proteins, an equilibrium of 10–40% cis and 60–80% trans conformers is attained for any given Xaa-Pro peptide bond in the unfolded state. Because this equilibrium is reached slowly, a test for proline isomerization as a rate-limiting folding reaction comprises rapid unfolding of a protein at high denaturant concentration in a time that is short compared with the half-time of proline isomerization in the unfolded state (around 5 min at 10°C; compare Ref. 23) followed by immediate dilution into refolding buffer (24). Thus, firefly luciferase was diluted into buffer containing 5 M GdmCl at 10°C. Under these conditions, complete unfolding of the enzyme occurs in the dead time of mixing (data not shown). Renaturation, initiated within 10 s by a second dilution into refolding buffer, occurred at the same rate as it did in a sample diluted after 5 h of incubation in unfolding buffer (Fig. 4A). Accordingly, the presence of stoichiometric amounts of proline isomerases from the E. coli periplasm or from the eucaryotic cytosol did not accelerate
concentration during refolding was 2 for the effect of proline isomerization on refolding kinetics. The protein luciferin, pH 7.8.

Transitions. Both the I2 and the I1 intermediates thus appear to partly coincide with the slow reactivation of unfolded luciferase.

As pointed out above, fully unfolded luciferase was observed to be in rapid equilibrium with the I2 conformation at 3–5 M GdmCl. Similarly, a rapid fluorescence change in the dead time of manual mixing was observed when the unfolded enzyme was diluted to denaturant concentrations between 1 and 3 M and the final amplitudes corresponded to those observed in the equilibrium transitions. Both the I2 and the I1 intermediates thus appear to slowly reactivate with the fully unfolded state. In contrast, slow reactivation with half-times of many hours was observed when luciferase reactivation was initiated after denaturing the enzyme overnight at 1 M GdmCl. Furthermore, a fluorescence increase of around 20% of the final signal was observed to coincide with the slow reactivation of unfolded luciferase. Taken together, the observations suggest that the conformation of luciferase preceding the rate-limiting step in the reactivation pathway may be related to the I1 equilibrium intermediate. For a direct comparison with the equilibrium unfolding intermediates, the putative kinetic intermediate accumulating during refolding at low denaturant concentration would have to be characterized further by fluorescence and CD spectroscopy. This was precluded by the low protein concentrations necessary for efficient refolding and by the strong tendency of luciferase to adsorb to vessel walls during refolding in fused silica cells. However, it was possible to characterize some properties of the kinetic intermediate by analytical gel filtration chromatography (Fig. 5).

In a series of experiments, equal amounts of luciferase, which had been allowed to renature for different time periods at 10 °C as described before, were applied to a high-resolution gel filtration column. Fluorescence emission was used to detect the eluted protein. Native luciferase eluted from the column after 31.3 min, corresponding to an apparent molecular mass of 53 kDa when the column was calibrated with globular proteins. As is apparent from the elution profiles depicted in Fig. 5A, two forms of luciferase were present in the refolding samples in varying proportions. In the absence of detergent, only the native protein fraction was eluted as a distinct peak, increasing with longer renaturation time (data not shown). The remainder of the protein was lost by adsorption to the column material. Addition of Tween 20 to the running buffer efficiently suppressed this interaction with the column matrix. Two protein peaks were now resolved in the elution profile, one eluting at 28.9 min and a second peak at 31.3 min coeluting with native luciferase. The peak at 28.9 min continually decreased and finally disappeared with progressive renaturation, whereas the peak corresponding to the native enzyme increased simultaneously. During refolding, the time course of formation of the kinetic intermediate by analytical gel filtration chromatography (Fig. 5).

The Kinetic Intermediate of Luciferase Refolding—As is apparent from the elution profiles depicted in Fig. 5A, two forms of luciferase were present in the refolding samples in varying proportions. In the absence of detergent, only the native protein fraction was eluted as a distinct peak, increasing with longer renaturation time (data not shown). The remainder of the protein was lost by adsorption to the column material. Addition of Tween 20 to the running buffer efficiently suppressed this interaction with the column matrix. Two protein peaks were now resolved in the elution profile, one eluting at 28.9 min and a second peak at 31.3 min coeluting with native luciferase. The peak at 28.9 min continually decreased and finally disappeared with progressive renaturation, whereas the peak corresponding to the native enzyme increased simultaneously. During refolding, the time course of formation of the native peak coincided closely with the appearance of enzymatic activity (compare Figs. 5A and 4A). To verify the precursor-product relationship, fractions of the two peak regions observed with the sample renatured for 10 min were collected and assayed for luciferase activity (Fig. 5B). When the fractions were assayed immediately after chromatography, only a very low amount of enzymatically active luciferase could be observed. Its elution position around 31 min indicated that it corresponded to the fraction of native protein formed from the intermediate during chromatography. However, when the fractions were assayed after further incubation for 1 or 3 days at 10 °C, the earlier eluting protein species became enzymatically active, proving that it represented a precursor of the native protein (Fig. 5B). The elution position of the intermediate corresponds to a globular protein of molecular mass 104 kDa. The 25% enlarged Stokes radius of the intermediate might indicate that
FIG. 5. Analysis of luciferase refolding by size-exclusion chromatography. A, samples of luciferase (60 nM) refolding at 10 °C for the times indicated and samples of a native control were subjected to size-exclusion chromatography, and the eluting protein was detected by its fluorescence. The solid lines represent the fits used to integrate the elution profiles for comparison with refolding kinetics. B, fractions from the chromatographic separation of a sample refolded for 10 min (●, ○, ■) and of a native control (□) were assayed for luciferase activity immediately after chromatography (●, ○) and after further incubation in buffer B for 24 (□) and 72 h (■) at 10 °C.

it is a partially folded form, highly expanded compared with the native enzyme, or that it may be caused by an interaction with Tween 20 micelles.

DISCUSSION

In vitro renaturation of firefly luciferase in the absence of chaperone proteins has repeatedly been described as a very inefficient process blocked by the rapid aggregation of non-native protein chains (10, 12, 15). Our results, however, demonstrate that the enzyme is able to efficiently renature in the absence of cellular components. The conditions of efficient luciferase refolding, i.e., low protein concentration and low temperature, are those found to favor the refolding of other popular chaperone substrates such as citrate synthase, ribulose-bisphosphate carboxylase/oxygenase, malate dehydrogenase, and rhodanese (25, 26). Luciferase refolding yields its fluorescence. The solid lines represent the fits used to integrate the elution profiles for comparison with refolding kinetics. B, fractions from the chromatographic separation of a sample refolded for 10 min (●, ○, ■) and of a native control (□) were assayed for luciferase activity immediately after chromatography (●, ○) and after further incubation in buffer B for 24 (□) and 72 h (■) at 10 °C.

Nevertheless, we found no evidence for a role of proline isomerization in the slow refolding process due to the much faster refolding of small proteins lacking disulfide bonds. Luciferase contains 29 proline residues and is therefore proline-rich (27). Proline isomerization is most often found to be the rate-limiting step in the refolding of small proteins lacking disulfide bonds. Luciferase refolding in cell extracts is superimposed upon thermal inactivation processes in which the native protein as well as folding intermediates could be involved. Even at low temperatures, at least two exponentials are necessary to describe the refolding process, a faster first phase in the minutes time range and a very slow second phase taking hours. Slow equilibrium transitions in unfolded protein chains, such as the cis-trans isomerization of Xaa-Pro peptide bonds, create a mixture of species (24). Proline isomerization is most often found to be the rate-limiting step in the in vitro refolding of small proteins lacking disulfide bonds. Luciferase refolding explains in part why firefly luciferase refolding in the absence of chaperones has not become apparent in earlier experiments.

The time course of luciferase refolding does not follow first-order kinetics. At temperatures above 20 °C, renaturation is superimposed upon thermal inactivation processes in which the native protein as well as folding intermediates could be involved. Even at low temperatures, at least two exponentials are necessary to describe the refolding kinetics, a faster first phase in the minutes time range and a very slow second phase taking hours. Complex kinetics in the formation of native molecules during protein refolding are often caused by the coexistence of fast folding and slow folding species in the unfolded state. Slow equilibrium reactions in unfolded protein chains, such as the cis-trans isomerization of Xaa-Pro peptide bonds, create a mixture of species (24). Proline isomerization is most often found to be the rate-limiting step in the in vitro refolding of small proteins lacking disulfide bonds. Luciferase refolding in cell extracts is superimposed upon thermal inactivation processes in which the native protein as well as folding intermediates could be involved. Even at low temperatures, at least two exponentials are necessary to describe the refolding kinetics, a faster first phase in the minutes time range and a very slow second phase taking hours. Complex kinetics in the formation of native molecules during protein refolding are often caused by the coexistence of fast folding and slow folding species in the unfolded state. Slow equilibrium reactions in unfolded protein chains, such as the cis-trans isomerization of Xaa-Pro peptide bonds, create a mixture of species (24). Proline isomerization is most often found to be the rate-limiting step in the in vitro refolding of small proteins lacking disulfide bonds. Luciferase refolding explains in part why firefly luciferase refolding in the absence of chaperones has not become apparent in earlier experiments.

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activity are compatible with a four-state model according to
\[ N \rightleftharpoons I_1 \rightleftharpoons I_2 \rightleftharpoons U, \]
where \( N \) is native, enzymatically active luciferase, \( I_1 \) and \( I_2 \) are partially unfolded, inactive intermediates, and \( U \) is the completely unfolded polypeptide. The free energy of denaturation (\( \Delta G^o_{N\rightarrow U} \)) of around 15 kJ mol\(^{-1}\) at 10 °C is small for a protein of 61 kDa. A significant fraction (around 0.2%) of the enzyme is denatured at equilibrium even at 10 °C and in the presence of the stabilizing phosphate ion. This is compatible with the observation that the enzyme is inactivated when it is incubated with the chaperonin GroEL. Although a rigorous thermodynamic characterization of the complete four-state transition has not been possible so far, it is obvious that the three unfolding steps add up to a thermodynamic stability relative to the completely unfolded state of roughly 50 kJ mol\(^{-1}\). Thus, firefly luciferase is not an unusually unstable structure; rather, the presence of stable folding intermediates destabilizes the native state.

Surfactants have been used in a number of cases to increase refolding yields of proteins (29–31). In the case of carbonic anhydrase II, the addition of different surfactants to the renaturation buffer not only affects refolding yields at high protein concentration but also accelerates or slows down renaturation (32). Tween 20 is a relatively hydrophilic detergent with a large, bulky polyoxyethyleneorbisorbit head group. It is widely used as a non-denaturing detergent, and this, as well as related detergents, were used previously as additives in the enzymatic assay for firefly luciferase (21, 33). It clearly increases luciferase renaturation yields, but it does not significantly affect the rate of luciferase refolding. Although it hardly shifts the midpoints of the two phases of the guanidine-induced equilibrium transition, the cooperativity of the \( N \rightleftharpoons I_1 \) transition, measured as inactivation or by fluorescence, is somewhat higher in its presence. This indicates a weak stabilizing interaction of Tween 20 with the equilibrium unfolding intermediate.

For a further analysis of the kinetic intermediate of refolding by gel filtration chromatography, the addition of Tween 20 to the elution buffer was inevitable because the detergent efficiently suppressed the adsorption of the non-native protein species to the column material. In its presence, an enzymatically inactive kinetic folding intermediate was quantitatively regained from the column. It eluted notably earlier than native luciferase at a position typical for a globular protein of about twice its molecular mass. A number of observations indicate that the kinetic intermediate identified by gel filtration chromatography is related to the equilibrium unfolding intermediate \( I_1 \): (i), reactivation from the equilibrium intermediate and from the completely unfolded protein are equally slow; (ii), the conversion of the kinetic intermediate to the native protein coincides with a fluorescence increase; and (iii), CD and fluorescence changes across the second and third transitions at 1.7 and 3.8 M GdmCl occur rapidly. Since the equilibrium transitions indicated an interaction of \( I_1 \) with the detergent, a possible explanation of its elution position is complex formation between the intermediate and a Tween 20 micelle. However, an enlarged Stokes radius should be expected for the intermediate on the basis of the reduced far-ultraviolet CD and fluorescence signals. The spectra suggest that about half the native secondary structure is retained in the intermediate \( I_1 \) and that the aromatic side chains are still in a moderately hydrophobic environment. In contrast, the aromatic fluorophores are highly exposed in the intermediate \( I_2 \) populated around 3 M GdmCl. Because both intermediates unfold and fold rapidly and their unfolding transitions are only moderately cooperative, they would be compatible with the definitions of collapsed, “molten globule” and “pre-molten globule” conformations (34).

Alternative explanations may be suggested by the crystal structure of \( P. pyralis \) luciferase that has recently been reported (3). Firefly luciferase folds into two compact domains connected by a short linker; a large N-terminal domain comprising residues 4–436 and a smaller C-terminal domain formed from amino acids 440–544. Since the active site of the enzyme is presumed to be located between the two domains, an intermediate with one of the two domains unfolded, although retaining large parts of the secondary and tertiary structure, would be enzymatically inactive. Moreover, the two tryptophan residues are located close to the linker region. The biphasic change in the fluorescence signal could therefore represent successive denaturation of the two domains, and the large CD amplitude of \( I_2 \) might correspond to a subdomain of the large and highly complex N-terminal domain. We are currently trying to distinguish between the two possibilities using a fragmentation approach.

In any case, the observed equilibrium and kinetic intermediates in the folding of firefly luciferase constitute possible targets for the action of molecular chaperones. Because luciferase folding in the cell and in cellular extracts occurs orders of magnitude faster than in our refolding experiments, cellular folding helpers must either prevent the formation of the kinetically trapped species or catalyze its conversion to the native enzyme.

Acknowledgments—We thank Rainer Jaenicke for performing the ultracentrifugation experiments and Tatjana Tannenberg for gel filtration experiments with partially unfolded luciferase.

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Equilibrium Intermediates in the Reversible Unfolding of Firefly (Photinus pyralis) Luciferase
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doi: 10.1074/jbc.272.11.7099

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