Folding of Firefly (*Photinus pyralis*) Luciferase: Aggregation and Reactivation of Unfolding Intermediates[†]

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ABSTRACT: The guanidine-induced unfolding of firefly *(Photinus pyralis)* luciferase involves two inactive equilibrium intermediates and is freely reversible at low protein concentration and low temperature. However, reactivation is exceedingly slow so that the equilibrium is attained only after several days of incubation and reactivation yields decrease strongly with increasing protein concentration, suggesting that aggregation is a competing side reaction [Herbst et al. (1997) *J. Biol. Chem. 272*, 7099−7105]. We investigated the role of the equilibrium intermediates in the aggregation process using size-exclusion chromatography and dynamic light scattering to monitor their association state. Although the more unfolded intermediate aggregated much more rapidly, both intermediates associated irreversibly without a conformational change visible by fluorescence or circular dichroism, forming small oligomers which remained soluble in the presence of the denaturant. The association kinetics are compatible with a nucleated polymerization mechanism. Unfolding kinetics at 1 M denaturant indicated the presence of a further inactive intermediate capable to reactivate rapidly with kinetics similar to those observed for luciferase reactivation in the presence of cell extracts. The data suggest a kinetic trap in luciferase refolding that is accessible from both equilibrium intermediate conformations and is avoided in the presence of molecular chaperones.

Some small single-domain proteins have been shown to refold rapidly in vitro with the native state appearing in a simple direct path (*1*−*4*). But with the majority of model proteins that have been used to investigate in vitro refolding processes, a more complex behavior is observed. In early occurring refolding steps, partially structured intermediate states accumulate transiently and are subsequently converted into native molecules (*5*, *6*). Whether such populated intermediates generally play the role of unproductive, so-called "trapped" conformations, or represent essential states that must be passed by the polypeptide chain in a linear folding process, has remained an open question (*7*−*9*).

An outstanding case in this regard is the 61-kDa monomeric firefly luciferase, the light-producing enzyme from the North American beetle *Photinus pyralis*. The freely reversible unfolding and refolding of luciferase, induced by varying the concentration of guanidinium chloride (GdmCl),[1] has recently been reported (*10*). But compared to other proteins, the reactivation process was remarkably slow, requiring several days at 10 °C and many hours at higher temperatures. As apparent from analytical chromatography experiments, an enzymatically inactive long-lived kinetic intermediate was populated during refolding. A detailed characterization of denaturant-induced unfolding transitions, using different spectroscopic techniques, revealed the existence of at least two inactive equilibrium intermediates which were highly populated around 1 and 3 M GdmCl. A possible relationship between one of these and the kinetic intermediate has been discussed (*10*).

In addition to being slow, luciferase folding is complicated by competing reactions reducing the yield of refolding. Reactivation yields close to unity could only be achieved at low temperatures and very low protein concentrations. The overall recovery decreases drastically with increasing protein concentrations, reaching just a few percent at concentrations above 30 μM, as expected for a kinetic competition between an on-pathway reaction leading to the native state and nonproductive pathways leading to irreversible aggregation (*11*, *12*). Its aggregation sensitivity has made luciferase a popular model protein used to investigate the action and role of molecular chaperones, a class of folding-helper proteins, believed to function by suppressing protein aggregation (*13*−*18*).

Although little is known about the molecular mechanism of aggregate formation, this process is assumed to occur through contacts between hydrophobic regions, exposed in the unfolded or partially folded states of the polypeptide chain (*19*). These interchain contacts may be highly specific and correspond to nativelike interactions between secondary structure elements, subdomains or domains, normally used in chain folding (*20*−*22*). Accordingly, aggregation processes occurring in vivo, like inclusion body formation or amyloidosis, have been linked to the association of folding or unfolding intermediates (*23*, *24*).

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[1] Abbreviations: CD, circular dichroism; DTE, dithioerythritol; GdmCl, guanidinium chloride; SEC, size-exclusion chromatography.

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The present study investigates some molecular aspects of the aggregation process occurring during luciferase refolding in vitro. By characterizing the physical properties of distinct aggregation products formed at different starting conditions, and the kinetics of their formation, we gained insight into the role of the previously observed folding intermediates, as possible aggregation precursors, and by analyzing the kinetics of reactivation from different unfolding intermediates, we assessed the nature of the rate-limiting step of the extremely slow process. Our results may contribute to understanding the nature of slow folding reactions and may provide a basis for a more detailed analysis of the role of molecular chaperones in luciferase folding.

MATERIALS AND METHODS

Materials

Recombinant *P. pyralis* luciferase was purchased from Promega (Madison, WI) and diluted into 0.5 M Tris−acetate buffer, pH 7.5; aliquots of 20 μL were rapidly frozen in liquid nitrogen and stored at −70 °C at protein concentrations of 1 or 5 mg/mL. GdmCl was obtained from ICN/Schwarz-Mann (Cleveland, OH), and dithioerythritol was from Roth (Karlsruhe, Germany). Other chemicals were analysis-grade from Merck (Darmstadt, Germany), and solutions were made up from doubly distilled water.

Methods

Luminescence Assay. Luciferase activity was assayed using the GenGlow100-kit from BioOrbit (Turku, Finland). Unless indicated otherwise, 50 μL of luciferin reagent and 50 μL of ATP reagent, both preincubated at 25 °C, were mixed in a polystyrene tube (3.5 mL, 55 × 12 mm). The light reaction was started by addition of 10 μL of luciferase solution with 2−4 μg/mL luciferase and was recorded in a Berthold MiniLumat LB 9506 luminometer for 2 min. Correction for autoinactivation was performed as previously described (*10*).

Reactivation of GdmCl-Treated Luciferase. Luciferase was completely unfolded in phosphate buffer A (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM dithioerythritol) containing 5 M GdmCl as described previously (*10*), or partially unfolded in phosphate buffer A containing 1.0 M GdmCl at 5 °C at the indicated protein concentrations and for the given time periods. Unless indicated otherwise, dilution of the unfolded protein 100-fold with phosphate buffer A or phosphate buffer B (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM dithioerythritol, 40 mM GdmCl) initiated the refolding reaction at a final denaturant concentration of 50 mM GdmCl and a temperature of 5 °C. To follow the reactivation, 10 μL aliquots were taken from the renaturing samples at different times and assayed for luciferase activity.

The two control reactions shown in Figure 6 were performed as follows: Luciferase was completely unfolded for 40 min at 5 °C in phosphate buffer A containing 5 M GdmCl at a protein concentration of 370 μg/mL. One fraction of the unfolded protein was directly diluted in phosphate buffer A with a corresponding amount of GdmCl to final concentrations of 2 μg/mL luciferase and 27 mM denaturant (slow reactivation of completely unfolded luciferase). The other fraction was diluted in two steps (slow reactivation from the I_1 state): Before the final dilution step to 2 μg/mL luciferase and 27 mM GdmCl, the protein solution was first brought to 1 M GdmCl and 74 μg/mL luciferase and incubated for 5 min at 5 °C to populate the I_1 state.

Reactivation in Assay Buffer. Native luciferase was partially unfolded in phosphate buffer A containing 1.0 M GdmCl at 5 °C (see above) and at a luciferase concentration of 82 nM (5 μg/mL). After the given periods of time (20 s up to 4 h), unfolding was interrupted by quickly diluting 3 μL aliquots into a thermostated (25 °C) mixture of 30 μL of luciferin reagent, 30 μL of ATP reagent, and 3 μL of phosphate buffer A. In the case of the native control, the 3 μL of phosphate buffer A was replaced by 3 μL of GdmCl-balance-buffer, containing a corresponding amount of GdmCl to keep the overall GdmCl concentration in the assay mixture constant at 45 mM. The mixture was transferred into a thermostated (25 °C) fluorescence microcuvette (Hellma, Type 105.251 QS), and light emission was followed at 550 nm and a spectral bandwidth of 30 nm for 10 min using a Spex FluoroMax spectrofluorometer with its light source turned off.

Analytical Treatment of the Kinetic Mechanism. To formulate an analytical solution for the refolding reaction described in Scheme 2, two processes were taken into account: the reactivation of I_{fast} and the inactivation process of native luciferase. These processes can be described as a system of two coupled differential equations:

$$\frac{dI_{fast}(t)}{dt} = -k_{fast}I_{fast}(t) \quad \text{(I)}$$

$$\frac{dN(t)}{dt} = k_{fast}I_{fast}(t) - k_i N(t) \quad \text{(II)}$$

Equation I is a homogeneous equation with the general solution $I_{fast}(t) = I_{fast}(0)e^{-k_{fast}t}$. Introducing this term into eq II yields the nonhomogeneous linear differential equation:

$$\frac{dN(t)}{dt} + k_i N(t) = k_{fast}I_{fast}(0)e^{-k_{fast}t}$$

which can generally be solved as the sum of a special solution of the inhomogeneous equation

$$N^i(t) = \frac{k_{fast}}{k_i - k_{fast}}I_{fast}(0)$$

and the general solution of the homogeneous equation $N^h(t) = N^h(0)e^{-k_i t}$.

With $N(0) = N_0$, the general solution of eq II can be written as

$$N(t) = N_0 e^{-k_i t} - \frac{k_{fast}}{k_i - k_{fast}}I_{fast}(0)e^{-k_i t} + \frac{k_{fast}}{k_i - k_{fast}}I_{fast}(0)e^{-k_{fast}t} \quad \text{(III)}$$

The data depicted in Figure 7 were fitted to eq III using Sigma Plot (Jandel Scientific) for the two possible cases $N(0) = 0$ (Figure 7B) or $N(0) \neq 0$ (Figure 7A). The latter case,

which assumes that a background of not yet denatured luciferase was still present in the denaturation samples, is indistinguishable from the solution obtained for a system of three coupled differential equations which would account for one further intermediate reactivating in the dead time of mixing.

Analytical Size-Exclusion Chromatography. All high-resolution gel filtration chromatography experiments were performed on a Superdex 200 HR 10/30 column (Pharmacia Biotech Inc.) at 5 °C and at a flow rate of 30 mL/h. For each run, a 200 μL sample of the corresponding luciferase solution was applied to the column. Unless indicated otherwise, the final luciferase concentration in these samples was 10 μg/mL, to ensure that further aggregation occurring during the column run was negligible. The eluting protein was detected by its fluorescence emission using a Merck/Hitachi F-1000 spectrofluorometer, with excitation and emission wavelengths set to 280 and 331 nm, respectively. To determine peak areas, elution profiles were fit to the sum of asymmetric double sigmoidal peaks using PeakFit (Jandel Scientific).

To follow aggregation kinetics, sets of identically composed protein samples were prepared by dilution of luciferase in phosphate buffer C (100 mM potassium phosphate, pH 7.8, 1 mM EDTA) containing GdmCl (final concentrations of luciferase and denaturant as indicated). Each sample was incubated at the temperature indicated for the given period of time, followed by dilution with running buffer to a final luciferase concentration of 10 μg/mL if necessary (see above) and immediate chromatography. The running buffer was phosphate buffer C containing the same concentration of GdmCl as present in the samples.

To check if the aggregation phenomenon was due to disulfide bonding, several protein samples were treated as described above, but in the presence of 1 mM DTE in all buffers including the running buffer. As a further control, it was investigated whether aggregates can be redissolved in 5 M GdmCl: 640 μg/mL luciferase was incubated in phosphate buffer C with 1.0 or 3.0 M GdmCl, respectively, at 5 °C for 4 days. These conditions are sufficient to aggregate the majority of the luciferase monomers present in the solution (see Figure 3). Then, both solutions were brought to 5 M GdmCl by adding a corresponding amount of phosphate buffer C with 8.3 M GdmCl, incubated for another 2 h at 5 °C, diluted 100-fold into running buffer (phosphate buffer C with 1.0 M GdmCl), and applied to the column.

A possible reversibility of the observed association process was excluded in a two-step procedure: 100 μg/mL luciferase was incubated in phosphate buffer C with 1.0 M GdmCl for 2 h at 5 °C. A 200 μL sample was directly applied to the chromatography column, and the eluted protein was collected in small fractions. One fraction representing the earlier eluting, aggregated luciferase was incubated for another 3 h at 5 °C and then reapplied to the column. The observed elution position was unchanged.

Dynamic Light Scattering. The instrumentation used to measure static and dynamic light scattering, refractive indices, and solvent viscosities and the procedures for data acquisition and analysis have been described in detail (*25*). The investigations were done at a fixed scattering angle of 90°, and at the wavelength 514.5 nm of an argon laser Lexel 3500 operating at 1 W output power. The homodyne time-autocorrelation functions of the scattering intensity, $G^2(\tau)$, were calculated by a 90-channel multibit multiple-τ correlator. The initial time interval for data accumulation was 16.8 s. This time interval defines the attainable time resolution of the light scattering data. The experimentally observed slow changes allowed a further averaging over several time intervals in order to improve the signal-to-noise ratio. By using the program CONTIN (*26*), we have derived distribution functions of the translational diffusion coefficient D from $G^2(\tau)$. The diffusion coefficients D were converted into Stokes radii R_S via the Stokes−Einstein equation: $R_S = k_B T/6\pi\eta_0 D$, where k_B is Boltzmann's constant, T is the temperature in Kelvin, and η_0 is the solvent viscosity. To equilibrate luciferase with phosphate buffer A, the protein was passed over a column of Sephadex G50 (20 × 1 cm) at 5 °C. The protein concentration in the pooled fractions was determined by UV absorption using a specific absorbance of 0.75 cm^2 mg^{-1} at 278 nm. For the light scattering measurements, the protein was diluted in phosphate buffer A or in the same buffer containing 1 M GdmCl, and the solution was transferred into a 100 μL micro flow-through cell (Hellma type 176.752-QS) through a membrane filter (pore size 100 nm) at 5 °C.

For the native protein, scattering intensities and diffusion coefficients were measured at four protein concentrations between 0.1 and 0.5 mg/mL, and the apparent molecular mass, calculated from the scattering intensity using a refractive index increment of $\Delta n/\Delta c = 0.19$ mL/g, and the diffusion coefficient were extrapolated to zero protein concentration. To follow the kinetics of aggregation at 1 M denaturant by dynamic light scattering, autocorrelation functions were calculated at 16.8 s time intervals and stored for post-experiment analysis. In the calculation of the apparent molecular mass from the static scattering intensity data in this solvent, a refractive index increment of 0.17 mL/g was used.

Fluorescence and CD Spectroscopy. Native or completely denatured luciferase was quickly diluted into phosphate buffer C containing corresponding amounts of GdmCl to achieve overall GdmCl concentrations of 0, 1, or 5 M, respectively. The final luciferase concentration in all samples was 100 μg/mL. Resulting changes in the fluorescence intensity and circular dichroism were recorded as a function of time over 10 h at a temperature of 5 °C. All spectroscopic signals were corrected for buffer background.

Fluorescence intensity was measured in a fluorescence microcuvette (Hellma, Type 105.251-QS) using a Spex FluoroMax spectrofluorometer equipped with a thermostated cell holder. Excitation and emission wavelengths were 280 and 331 nm, and the spectral bandwidths were 2 and 4 nm for excitation and emission, respectively. To avoid a loss of fluorescence signal caused by photobleach, a time increment of 200 s, during which the shutter was closed, separated the data collection steps of 2 s for each data point.

The change in circular dichroism signal was recorded in standard 1-mm silica cells (Hellma, Type 100-QS) in an Aviv 62A-DS spectropolarimeter at 5 °C and a spectral bandwidth of 1 nm. In analogy to the fluorescence experiment, data points were taken every 200 s with an integration time of 30 s and the shutter was closed between data acquisition periods. Residue ellipticities were calculated using a mean

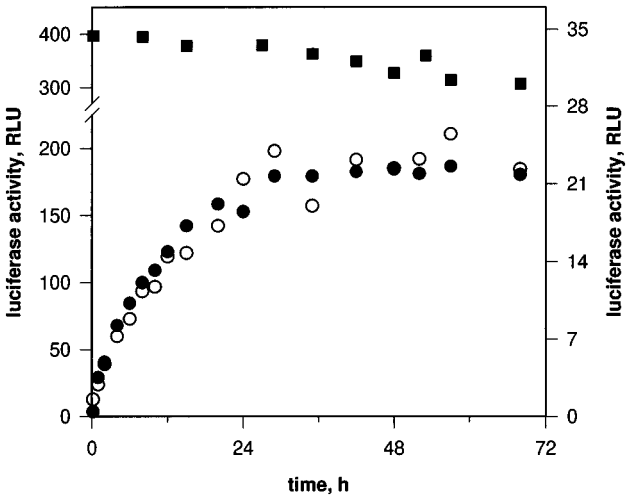


FIGURE 1: Kinetics of reactivation from completely and partially unfolded states. Luciferase was denatured in 5 M GdmCl (●, left ordinate) or 1 M GdmCl (○, right ordinate) at 5 °C and a protein concentration of 370 μg/mL for 120 min and diluted 100-fold into buffer A or B, respectively, to initiate refolding at 50 mM GdmCl. After varied times of refolding, 10 μL samples were assayed for enzymatic activity. (■) Native luciferase incubated in buffer A containing 50 mM GdmCl.

residue weight of 110.5 calculated from the amino acid composition.

RESULTS

Upon chemical denaturation with guanidinium chloride (GdmCl), luciferase unfolds in a four-state equilibrium with two inactive intermediates, I_1 and I_2, populated around 1 and 3 M denaturant (*10*). Enzyme that has been incubated at a final concentration of 5 M GdmCl for several minutes at 5 °C is totally unfolded and can be renatured by rapid dilution into cold phosphate buffer in a slow reaction with a half-time of $t_{1/2} = 10$ h. A similarly performed reactivation experiment starting with the partially unfolded equilibrium intermediate I_1, populated in buffer with 1 M GdmCl at a final protein concentration of 370 μg/mL for 120 min at 5 °C, is shown in Figure 1. Evidently, the reactivation was equally slow, suggesting that the rate-limiting steps were identical for both kinetics. Compared to the renaturation from the fully unfolded state, however, the reactivation yield was drastically reduced to less than 20%. Moreover, when starting with enzyme partially denatured at 3 M GdmCl, the obtained reactivation yields were even smaller (data not shown). This suggested that irreversible aggregation occurred either during the dilution step or in the presence of the corresponding amounts of denaturant, in its extent related to the prevailing intermediate.

Qualitative Analysis of the Aggregation Processes. Techniques used to analyze aggregation pathways comprise nondenaturing electrophoresis and light scattering (*22*, *27*). We decided to use analytical size-exclusion chromatography (SEC) for the majority of our studies, because it is suitable for a wide range of protein concentrations. Its resolution approaches that of native gel electrophoresis, but it is relatively rapid and compatible with the presence of GdmCl. Moreover, individual fractions can easily be collected and reanalyzed after further incubation steps.

Initially, we investigated how the hydrodynamic properties of monomeric firefly luciferase changed during GdmCl-induced unfolding along the four-state equilibrium transition. To assess the influence of GdmCl on the separation characteristics of the column material, the column was calibrated at 0 and 1 M GdmCl, using different stable proteins which were native under both conditions. Their elution positions in the presence and absence of denaturant were identified, and no significant differences could be detected. Luciferase was diluted to a final concentration of 10 μg/mL into buffer, containing 0−5 M GdmCl at 5 °C. Immediately after dilution, the samples were applied to a high-resolution gel-filtration column, equilibrated with the same buffer. Fluorescence emission was used to detect the eluted protein (Figure 2A). As expected for a more and more unfolding protein, an increase in the amount of GdmCl in the buffer resulted in an earlier elution of the enzyme, indicating an enlarging Stokes radius. The detected change in the elution position reflected the course of the unfolding transition monitored by CD spectroscopy with two characteristic kinks at 1 and 3 M GdmCl (Figure 2B, ref *10*).

At two GdmCl concentrations, 2.5 and 3.0 M, a small shoulder eluting before the main peak was observed, suggesting an association process. A more detailed qualitative investigation revealed that a longer term incubation of luciferase in the presence of intermediate amounts of GdmCl was accompanied by a considerable change in the elution profile. As an example, the elution profiles obtained from luciferase incubated for different periods of time at a final GdmCl concentration of 1 M are depicted in Figure 2C,D. The incubation temperatures were 5 and 30 °C, respectively, and the protein concentration was 10 μg/mL. Luciferase that was subjected to the column immediately after its dilution eluted as a single peak, but bimodal elution patterns were observed after further incubation. A second, substantially earlier eluting peak continually increased with progressive incubation time, while the amplitude of the originally observed peak decreased simultaneously. Upon incubation for several hours or days, the earlier eluting peak broadened, and several distinct shoulders became visible at its front side. Thus, the overall peak pattern shifted from that of a single homogeneous molecule to that of a mixture of different species with increased Stokes radii. Addition of 1 mM DTE to all buffer solutions including the running buffer had no influence on the observed peak patterns, indicating that the expanded particles did not emerge from disulfide formation.

A bimodal distribution of elution volumes in SEC patterns can be observed for slow transitions occurring between compact and less compact states during the unfolding of a protein (*28*). To distinguish between this possibility and an irreversible self-association, we performed a two-step experiment: Luciferase was partially unfolded in 1.0 M GdmCl for 2 h, so that the earlier eluting species was populated. The two species were separated by gel-filtration chromatography, collected in small fractions, and further incubated for 3 h at 5 °C to allow reequilibration in case of a slow transition process. Then, one sample representing the earlier eluting species was reapplied to the SEC column. Only a single peak was detected, eluting at the same volume as it had been collected before. Moreover, treatment with 5 M GdmCl, an amount sufficient to completely unfold luciferase (*10*), followed by dilution back into buffer with 1 M GdmCl converted the enlarged state back to its more

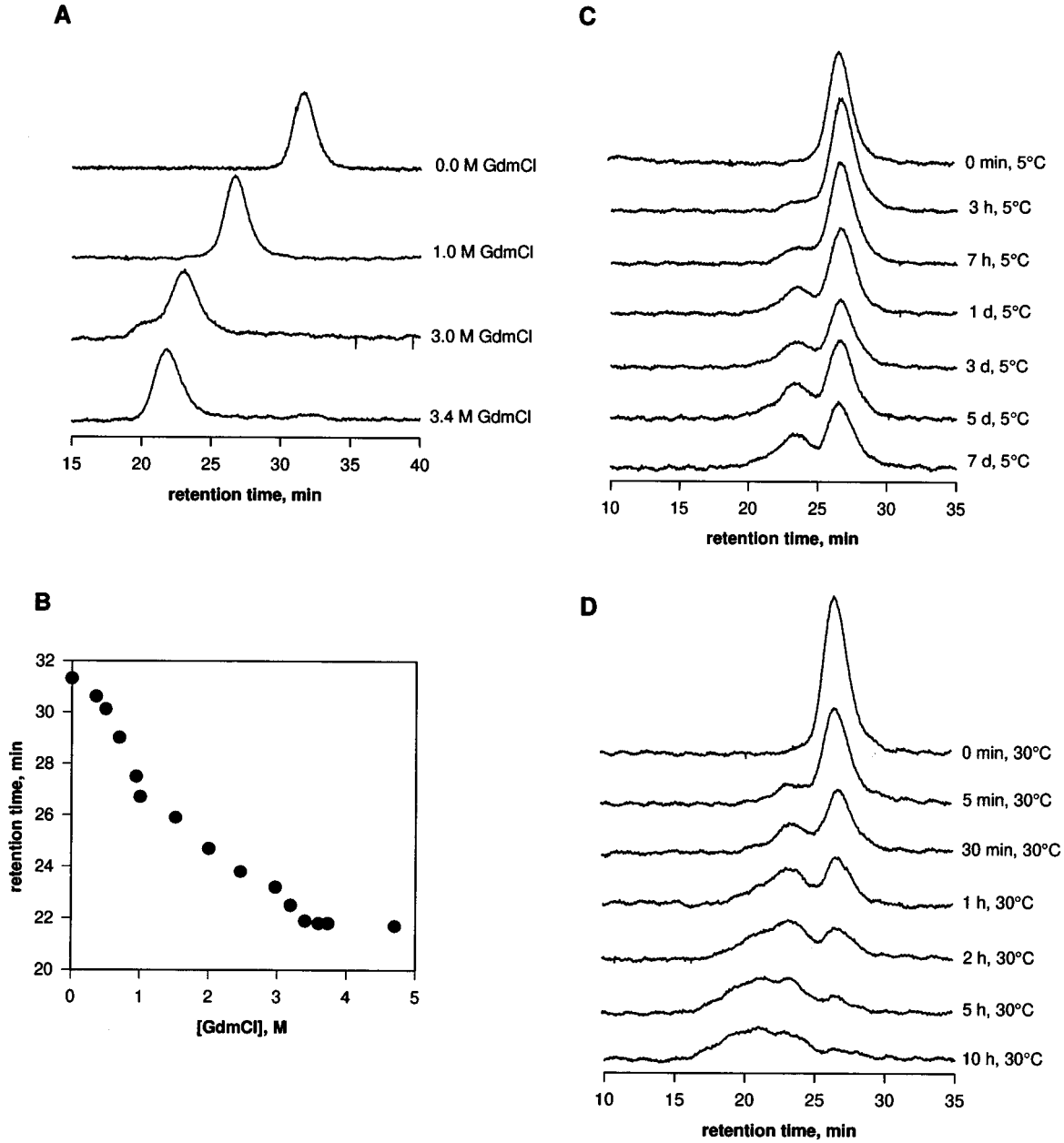


FIGURE 2: Analysis of luciferase unfolding and aggregation by size-exclusion chromatography. (A and B) Changes in hydrodynamic volume in the unfolding transition. Luciferase was diluted into buffer containing the indicated concentration of GdmCl to a final protein concentration of 10 μg/mL at 5 °C. Immediately after dilution, each sample was applied to a size-exclusion column equilibrated in the same buffer, and the eluted protein was detected by its fluorescence. (B) The determined retention time was plotted versus the guanidine concentration in the elution buffer. (C and D) Analysis of luciferase aggregation. Luciferase was diluted to a final concentration of 10 μg/mL in buffer with 1 M GdmCl thermostated at 5 °C (C) or 30 °C (D). The samples were further incubated at the corresponding temperature for the indicated period of time and then analyzed by size-exclusion chromatography at 5 °C and in the presence of 1 M GdmCl.

compact initial form. We therefore concluded that the change in the elution profile was due to a noncovalent self-association reaction, which was not reversible upon dilution on the time scale of the experiment.

Parameters Affecting the Aggregation Reaction. The self-association process could be observed at GdmCl concentrations between 1.0 and 3.3 M, different incubation temperatures, and different protein concentrations, albeit with vastly varying kinetics. Surprisingly, the process reached a state of apparent standstill after a certain incubation time which was significantly different for each corresponding starting condition, i.e., a combination of denaturant concentration, incubation temperature, and initial protein concentration in the sample.

Kinetics of aggregate formation measured by gel-filtration (SEC) at various temperatures and amounts of luciferase in solution are shown in Figure 3A,B. The analysis was done at a final GdmCl concentration of 1.0 M in the incubation and running buffer. This corresponds to the first plateau region of the equilibrium transition (*10*), where a partially unfolded, inactive intermediate is populated. In analyzing the temperature dependence, the final luciferase concentration in the samples was constant at 10 μg/mL; in measurements of the protein concentration dependence, the incubation temperature was constant at 5 °C. To stop the association reaction prior to chromatographic analysis, all the analytical column runs were done at 5 °C and a final luciferase concentration of 10 μg/mL, i.e., under conditions where the

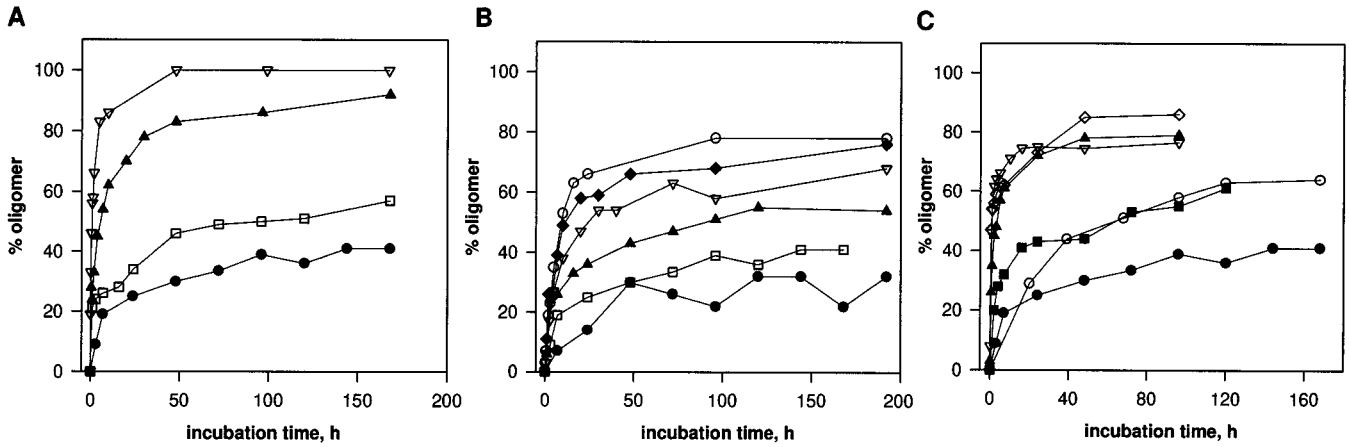


FIGURE 3: Influence of temperature and protein and denaturant concentrations on luciferase aggregation. (A) Temperature dependence. Samples of luciferase were diluted to 10 μg/mL in buffer containing 1 M GdmCl at 5 °C (●), 10 °C (□), 20 °C (▲), and 30 °C (▽), incubated for the given periods of time, and then analyzed by size-exclusion chromatography at 5 °C. (B) Protein concentration dependence. Samples of luciferase were diluted to a final protein concentration of 5 μg/mL (●), 10 μg/mL (□), 20 μg/mL (▲), 40 μg/mL (▽), 70 μg/mL (◆), and 100 μg/mL (○) in buffer with 1 M GdmCl thermostated to 5 °C. The samples were incubated for the given periods of time, rapidly diluted to a protein concentration of 10 μg/mL, and immediately applied to the size-exclusion column. (C) Denaturant dependence. Samples of luciferase were diluted to 10 μg/mL in buffers containing 1 M (●), 1.5 M (■), 2.0 M (▲), 2.5 M (▽) 3.0 M (◇), and 3.2 M (○) GdmCl thermostated at 5 °C, incubated for the given periods of time, and then analyzed by size-exclusion chromatography. In all cases (A−C), the sum of the peak areas was the same for all samples representing one aggregation time course, indicating that the protein subjected to the column was completely regained. Therefore, the ratio of the peak areas was used to calculate the amount of enzyme aggregated.

aggregation reaction is slow compared to the length of the run. Samples with higher protein concentration were rapidly diluted and immediately afterward applied to the column. As reported for other proteins, the aggregate formation significantly speeded up when increasing either the temperature or the protein concentration in solution. The kinetic course of the reaction was not that of a simple bimolecular reaction. After an initial progressive phase, the association process reached a certain degree of saturation at 20−100% of the total amount of protein aggregated, depending on the individual starting condition. Further aggregation appeared to continue only slowly (Figure 3A,B).

As already mentioned, the aggregation reaction could be observed at GdmCl concentrations between 1.0 and 3.3 M, suggesting that it corresponded to the association of the partially unfolded intermediates populated in this denaturant range. To explore their role as possible aggregation precursors, we further investigated how a change of the amount of GdmCl in the incubation buffer affected the speed and extent of the process. The protein samples were incubated at 5 °C and a final luciferase concentration of 10 μg/mL, and aggregate formation was followed using SEC. To avoid a disturbance of the system, the gel-filtration runs were carried out at the same denaturant concentrations as present in the samples. As apparent from Figure 3C, the aggregation reaction became faster with increasing amount of denaturant, reached a maximum between 2.5 and 3.0 M GdmCl, and slowed again at higher GdmCl concentrations. At GdmCl concentrations higher than 3.3 M, aggregate formation could no longer be observed, probably due to the solubilizing effect of the denaturant (*29*). The observed GdmCl dependence suggests that the more unfolded equilibrium intermediate of luciferase present around 3 M GdmCl associated significantly faster and to a higher extent than the more compact one. Again, the aggregation reaction did not go to completion but saturated at levels which were dependent on the GdmCl concentration in the buffer.

Aggregation Measured Directly by Dynamic Light Scattering. Although our gel filtration analysis did establish the irreversibility of the process, it provided only limited information about the state of association of luciferase folding intermediates prior to the rate-determining step of the irreversible aggregation reaction. This was because of the inevitable dilution connected with the chromatographic analysis. Thus, we attempted to follow the aggregation reaction directly by light scattering. Luciferase was diluted into phosphate buffer containing 1 M GdmCl at 5 °C so that the protein concentration was 0.1 mg/mL, and the solution was rapidly introduced into the observation cell of a laser light scattering apparatus through a 0.1 μm membrane filter. The changes in the scattering intensity (static light scattering) and its fluctuations with time (dynamic light scattering) were followed over a period of 8 h (Figure 4). This corresponds to the time period required for the irreversible aggregation reaction to come close to completion under these conditions (cf. Figure 3B). In a reference experiment, native luciferase was analyzed at varied concentrations in buffer lacking the denaturant. The resulting Stokes radius of 3.27 ± 0.06 nm and molecular mass of 64 ± 6 kDa, which is in excellent agreement with the value calculated from the amino acid composition, confirm the monomeric state of native luciferase, as determined previously by analytical ultracentrifugation (*10*). The initial values for the average molecular mass calculated from the scattering intensity (61 kDa) and the Stokes radius calculated from the measured diffusion coefficient (4.3 nm), both determined immediately after dilution into the buffer containing 1 M GdmCl, are consistent with an expanded monomer, indicating that the single peak eluting from the gel filtration column accurately represented the association state of the applied sample. During the incubation at 1 M GdmCl for 8 h, the scattering intensity increased 2.5-fold, and the Stokes radius increased 1.5-fold. Both values are consistent with the average state of association being approximately trimeric (*30*). The Stokes radius

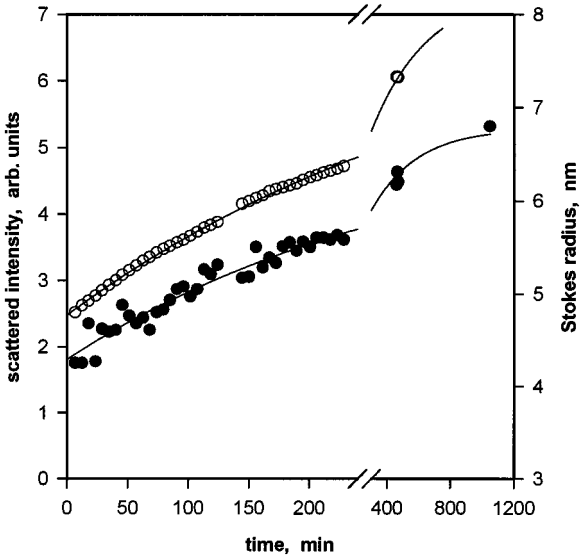


FIGURE 4: Aggregation observed by dynamic light scattering. Luciferase was diluted into buffer containing GdmCl to final concentrations of 100 μg/mL protein and 0.95 M denaturant, the sample was rapidly introduced into a laser light scattering apparatus through a membrane filter, and the changes in the scattered light intensity (●, left ordinate) and in the hydrodynamic radius (○, right ordinate) were followed over time. Each data point represents an average over 20 (originally measured) time intervals of 16.8 s (cf. Materials and Methods). The solid lines are only meant to guide the eye of the reader.

determined 18−20 h after dilution was 6.8 nm, corresponding to a 1.6-fold increase from the initial value in 1 M GdmCl and indicating that the aggregation process approached an end point. The time course of the changes in the scattering intensity and in the Stokes radius was in good agreement with the gel-filtration results.

Aggregation versus Slow Refolding. Luciferase, that was applied to the chromatography column immediately after its dilution into GdmCl buffer, eluted as a single peak at a position typical for the chosen GdmCl concentration. This was irrespective of whether the diluted protein had been in the native or in the completely unfolded state before the dilution, indicating that the reversible un- and refolding equilibrium (*10*) was attained rapidly and prior to aggregate formation. Similarly, both the CD and fluorescence signals reached their equilibrium values in the dead time of manual mixing, when completely unfolded luciferase was partially refolded to the intermediate state I_1 by dilution to 1 M GdmCl. No significant change in spectroscopic signals was detected during the aggregation process. In contrast, the partial unfolding of native luciferase in buffer containing 1 M GdmCl occurred in two phases. Only a part of the native signal was lost in the dead time. The remaining signal difference was observed over several hours and followed a single-exponential decay. Eventually, the same amplitude as in the refolding experiment was attained. Although it occurred slowly, the spectroscopic signal change was much faster than aggregate formation under the same conditions (Figure 3), suggesting that it represented a further so far unnoticed unfolding reaction, which was not associated with a significant change in the hydrodynamic properties and was, therefore, not visible in the SEC experiments. The rate constants determined for the changes in fluorescence and CD signals were identical at 1.7 ± 0.4 h^{-1}. Both changes are likely to represent the same process (Figure 5A,B).

The involvement of a conformational change preceding the formation of the monomeric equilibrium intermediate at 1 M GdmCl was confirmed by the results of reactivation experiments. When luciferase was partially unfolded in 1 M GdmCl for at least 90 min, i.e., the enzyme was converted to the I_1 equilibrium intermediate, subsequent reactivation was as slow as reactivation from the completely unfolded state (Figure 1) (*10*). However, if the time of incubation in 1 M GdmCl was reduced to less than 30 min, before the enzyme was diluted into renaturation buffer, a rapid phase appeared in the reactivation kinetics (Figure 6), and an increase of enzymatic activity during the bioluminescence assay was observed with samples taken early during the reactivation reaction. These observations suggested the presence of a luciferase denaturation intermediate that was inactive but competent for rapid reactivation. To assess its presence in more quantitative terms and with better kinetic resolution, we analyzed the time course of luciferase reactivation in the bioluminescence assay (Figure 7). For that purpose, native luciferase was inactivated in GdmCl buffer at a final denaturant concentration of 1 M. The protein concentration was 5 μg/mL, and the sample was incubated at 5 °C to ensure that aggregate formation was negligible during the experiment. After different periods of time, aliquots of the sample were diluted 30-fold into activity−assay solution thermostated at 25 °C. This step interrupted the unfolding reaction and initialized a reactivation process which was monitored as an increase of luminescence emission during the measurement. As apparent from the reactivation courses depicted in Figure 7, much of the inactivated enzyme reactivated rapidly compared to the slow reactivation of I_1 which was not detectable in the assay time. The proportion of fast reactivating enzyme decreased with increasing incubation time in 1 M GdmCl, confirming that at least one kinetic intermediate was transiently present during the unfolding reaction.

Kinetic Analysis of Luciferase Reactivation from 1 M GdmCl. To explain the observations described above, one has to consider at least one fast reactivating intermediate that is slowly converting to the very slowly reactivating equilibrium intermediate I_1 during denaturation at 1 M GdmCl:

Scheme 1

$$N \xrightarrow{k_{fast}} I_{fast} \xrightarrow{k_{slow}} I_1$$

The unfolding of native luciferase to form I_{fast} at 1 M GdmCl occurs within the dead time of mixing, and I_{fast} renatures rapidly upon dilution back to native conditions. The formation of the slowly refolding intermediate I_1 is likely responsible for the slow phase of the spectroscopic signal change visible in the unfolding. For the kinetic analysis of the reactivation measurements that were performed in assay buffer (Figure 7), a further process had to be considered: In the presence of the substrates firefly luciferin and ATP, luciferase undergoes a product inhibition reaction in which the active enzyme is successively removed from the system (*31*−*33*), resulting in a permanent loss of luminescence signal during the measurement (Figure 7). Under the given conditions, this inactivation followed a single exponential with a rate constant of $k_i = 0.038 \pm 0.003$ min^{-1} and was independent from the initial luciferase concentration at least

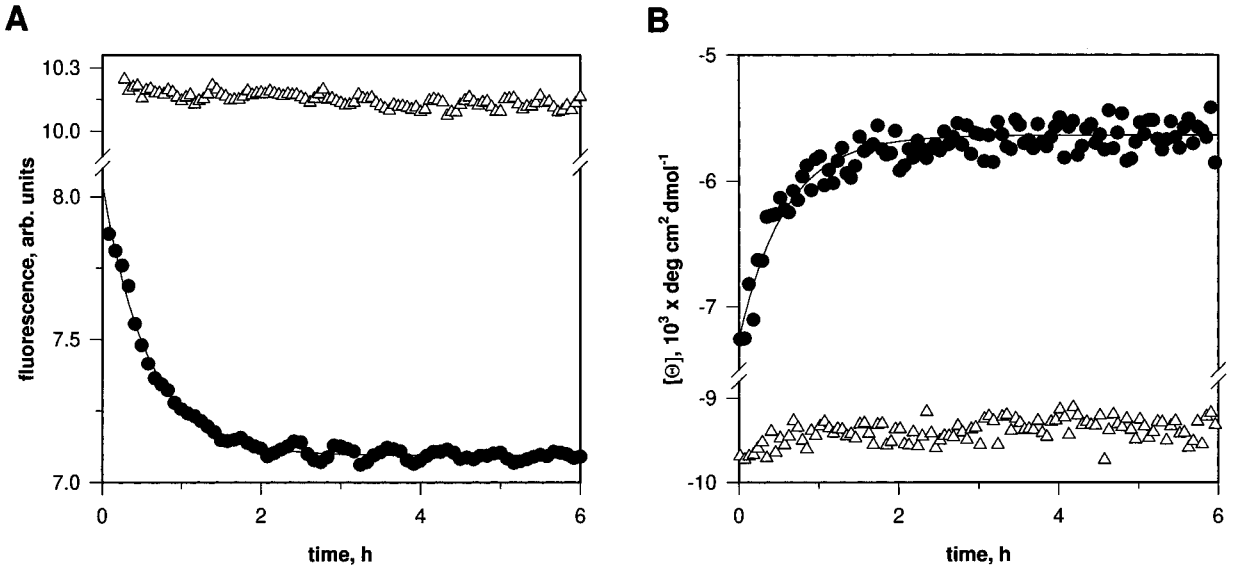


FIGURE 5: Slow step in luciferase denaturation. Native luciferase was manually mixed into buffer C with 1 M GdmCl (●) or without denaturant (△) at 5 °C, so that the protein concentration was 100 μg/mL in all samples. The resulting changes in the fluorescence intensity at 331 nm (A) and the circular dichroism at 220 nm (B) were recorded as a function of time. The solid lines represent single exponentials with a rate constant of $k = 1.7 \pm 0.4$ h^{-1} as obtained by nonlinear regression. When unfolded luciferase was diluted from 5 to 1 M GdmCl, constant fluorescence (7.0 ± 0.2 arbitrary units) and CD (−5800 ± 200 deg cm^2 dmol^{-1}) signals were observed after completion of the mixing period.

up to 5.5 μg/mL luciferase in the sample. Because the slow reactivation of the I_1 species was insignificant during the short time interval of the measurement, it was not taken into account for the kinetic analysis of a single reactivation time course. The mechanism minimally necessary to describe the measured reactivation kinetics was therefore:

Scheme 2

$$I_{fast} \xrightarrow{k_{fast}} N \xrightarrow{k_i} N_{inactivated}$$

This simple consecutive model served as a basis for nonlinear least-squares fits to the experimental data depicted in Figure 7. The fits approximated the data reasonably well, although systematic deviations were observed (Figure 7B). Introducing an initial signal $N_0 \neq 0$ at the onset of reactivation into the model could increase the quality of the fit significantly. The presence of an initial activity could be due to two different reasons. Either the enzyme had not been completely inactivated, when the unfolding was interrupted, or part of luciferase reactivated in an even faster reaction during the first 30 s not resolved in the measurement. The spectroscopic results described above make the latter case more likely, but distinguishing between the two possibilities would have required rapid mixing techniques. Although introducing a second exponential at the folding did improve the fits, the data measured with a dead time of 30 s did not reliably determine amplitudes and rate constants for 2 folding phases. Thus, we kept the model as simple as possible and did not allow for inhomogeneity in I_{fast}.

The simplified mechanism described above resulted in two sets of experimental parameters. The concentration of I_1, the slowly reactivating intermediate accumulating during the incubation in 1 M GdmCl, was calculated from the difference in the final signal (corrected for inactivation) between the native sample and the sample of the reactivated enzyme for each reactivation reaction (Figure 7). The increase in I_1

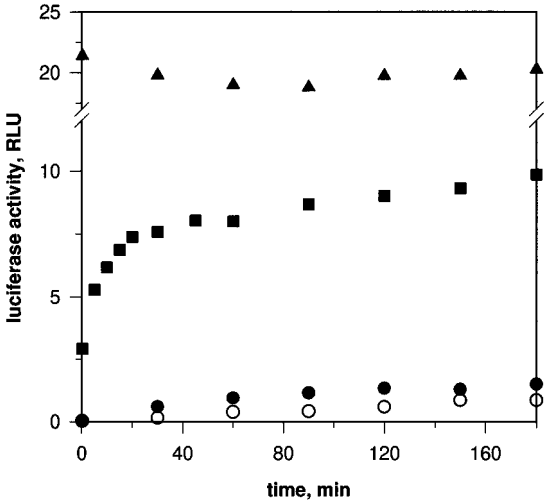


FIGURE 6: Reactivation of luciferase after short denaturation in 1 M GdmCl. Luciferase (74 μg/mL) was incubated in buffer A with 1 M GdmCl for 20 min at 5 °C and then rapidly diluted with buffer A lacking denaturant to a final protein concentration of 2 μg/mL and a GdmCl concentration of 27 mM. After the given time intervals, 10 μL aliquots were withdrawn and immediately assayed for luciferase activity (■). Initial bioluminescence activities are shown as determined by extrapolation to the start of the assay. Both the slow reactivation of completely unfolded luciferase (●) as well as of luciferase in the I_1 state (○), diluted to the same final conditions, are shown as controls (cf. Materials and Methods). The native signal (▲) remained stable during the short incubation at 5 °C.

[equivalent to the decrease in $(I_{fast} + N_0)$] during the period of incubation in 1 M GdmCl yielded a single exponential with a rate constant of 2.5 ± 0.5 h^{-1} (Figure 8), which agrees quite well with the rate constant of 1.7 ± 0.4 h^{-1} determined for the slow phase of the spectroscopic signal change. The second set of parameters was a set of rate constants k_{fast}. They were not identical for the individual reactivation reactions, as one would expect from the folding model, but systematically decreased from 0.89 ± 0.01 min^{-1} determined

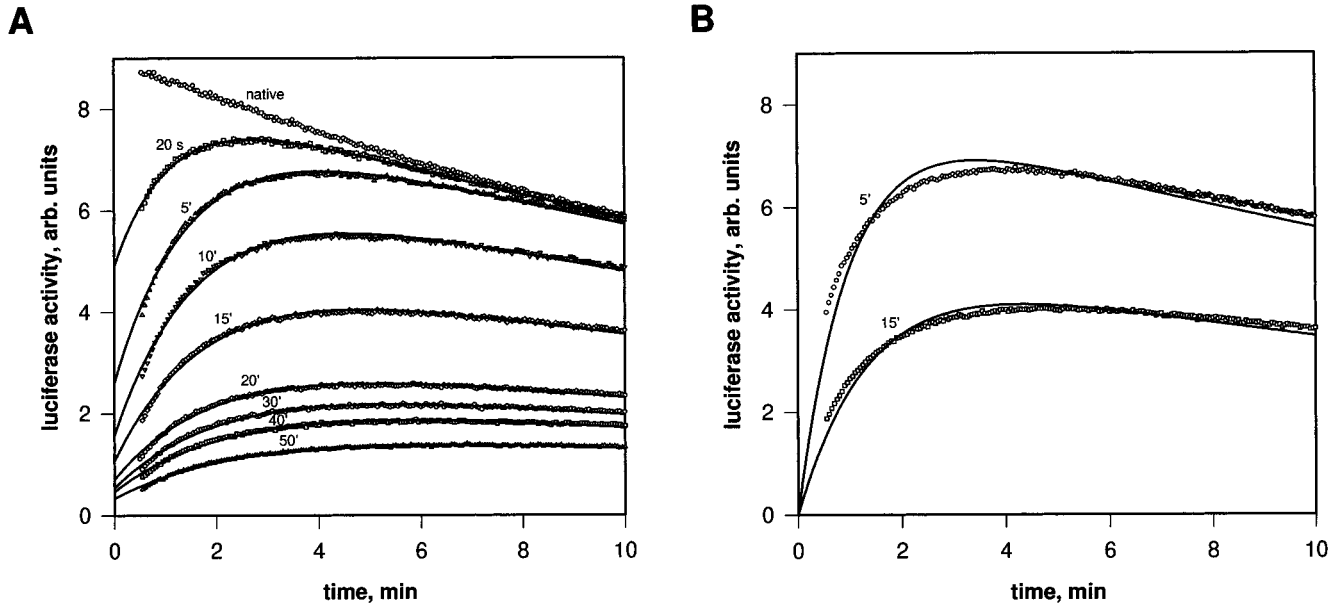


FIGURE 7: Reactivation of partially unfolded luciferase during the bioluminescence assay. Luciferase was denatured in 1 M GdmCl for the periods of time indicated. To interrupt the unfolding process, samples of 3 μL were mixed into activity assay buffer thermostated to 25 °C and transferred to a measuring cell, and the resulting reactivation was monitored as the increase in light emission. The solid lines represent nonlinear least-squares fits to the observed reactivation time courses, performed with Sigma Plot (Jandel Scientifics), on the basis of the reactivation model depicted in Scheme 2. In (B), the amount of native luciferase at $t = 0$ min was set to zero; in (A), an initial activity N_0 was allowed (cf. Materials and Methods).

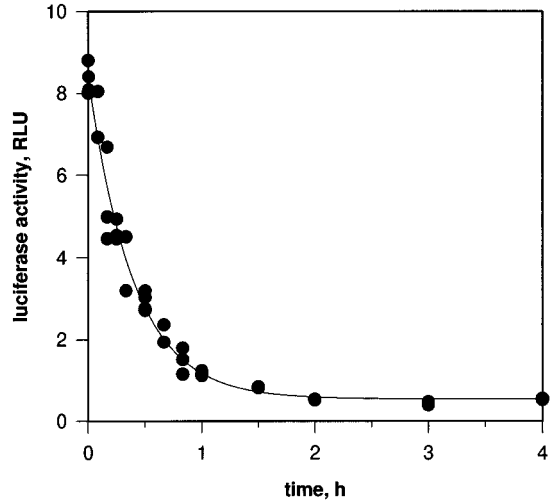


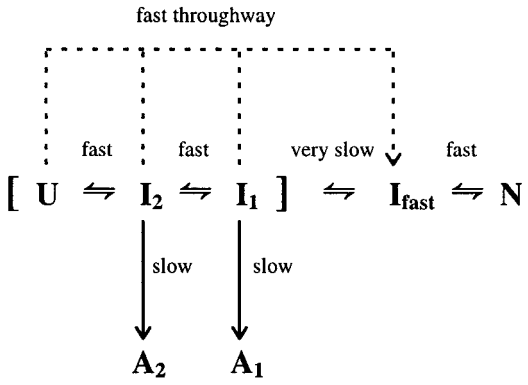
FIGURE 8: Loss of fast reactivating luciferase during denaturation in 1 M GdmCl. The fraction of luciferase that was not able to reactivate within a 20 min renaturation span after denaturation in 1 M GdmCl for variable periods of time was determined from the experimental data depicted in Figure 7. The data plotted as the final signal amplitude corrected for inactivation as a function of denaturation time measure the sum of the I_{fast} and N_0 fractions in the analysis depicted in Figure 7A. The solid line is a single exponential with the rate constant of 2.5 h^{-1} fit to the data by nonlinear regression.

for luciferase incubated for about 20 s in denaturant solution, to less than 0.23 ± 0.02 min^{-1} found for enzyme that had been inactivated for longer than 90 min. This suggests, together with the systematic deviations from a single exponential (cf. above), that the I_{fast} species is not homogeneous but represents a set of partially unfolded, interconverting molecules. A possible origin for heterogeneity in denatured states is *cis*−*trans* isomerization at peptidyl−prolyl bonds, which often becomes rate-limiting for the refolding of small model proteins (*34*). Luciferase contains 29 proline residues equally distributed along the polypeptide chain, some of which could isomerize in a partially unfolded state of the enzyme and therefore give rise to the observed slowdown of the reactivation process.

DISCUSSION

Together with the results of our previous study on the guanidine-induced equilibrium unfolding (*10*), the observations presented above may be condensed into the following model (Scheme 3) for the folding of *Photinus pyralis* luciferase:

Scheme 3



In Scheme III, I_1 and I_2 represent the equilibrium unfolding intermediates highly populated around 1 and 3 M denaturant, respectively. In contrast, I_{fast} has been observed only transiently as a kinetic intermediate of the denaturation at 1 M GdmCl, able to revert rapidly to the native state upon dilution of the denaturant. I_1 and I_2 significantly differ in their spectroscopic as well as their hydrodynamic properties. Circular dichroism and fluorescence spectra indicate that I_1 retains most of the secondary structure and at least partially

shields the two tryptophan residues of luciferase from the solvent, whereas the aromatic chromophores are highly solvent-exposed in I_2 despite a significant amount of secondary structure present in this intermediate. The two intermediates are formed rapidly upon dilution of completely unfolded luciferase (U) into buffer containing 1 or 3 M GdmCl, respectively, as apparent from changes occurring within the dead time of manual mixing in both the Stokes radius and the spectroscopic signals of the protein. This indicates that the completely unfolded protein and the intermediates I_1 and I_2 are in rapid equilibrium which is shifted to the more unfolded forms with increasing amounts of denaturant. In Scheme 3, the rapid equilibrium is depicted in a linear way [$U \rightleftharpoons I_2 \rightleftharpoons I_1$]. This is not meant to imply that I_1 and I_2 are obligatory folding intermediates, that would have to be passed during reactivation of the enzyme in a linear folding pathway. Therefore, the rapidly equilibrating species have been put in brackets.

A rapid equilibrium between the three species I_1, I_2, and U is further supported by similar reactivation kinetics observed upon dilution from 1, 3, and 5 M GdmCl, respectively. In all three cases, reactivation upon dilution into native buffer at low temperatures occurred unusually slowly and did not follow first-order kinetics. This suggests that the protein adopts the same long-lived conformation (or ensemble of conformations) probably functioning as a kinetic trap under native conditions, irrespective of whether it was in the I_1, I_2, or U state before its dilution. In gel-filtration experiments, performed in the presence of 0.2% (v/v) Tween 20 in buffer lacking denaturant, the kinetic folding intermediate described previously (*10*) could quantitatively be regained from the column, if I_1 was diluted into native buffer instead of completely unfolded luciferase (data not shown). Its elution position lies about halfway between that of I_1 observed in the presence of 1 M GdmCl and that of the native protein in buffer.

In contrast, luciferase that had been denatured for a short period of time at 1 M GdmCl reactivated within minutes (I_{fast}) and eluted at the position of the native protein upon injection onto the gel-filtration column. The observed half-time of reactivation from I_{fast} measured in the bioluminescence assay mixture at 25 °C increased from less than 1 min to more than 3 min with increasing incubation time at 1 M denaturant. This suggests that I_{fast} does not correspond to a single conformation, but represents an ensemble of interconverting faster folding and slower folding molecules. Proline isomerization has been identified as the cause of such a mixture of species in other protein systems (*34*). It may very well play a role here, although 28 of the 29 prolines in firefly luciferase are in the *trans* conformation (1 is localized in a flexible loop region) and proline isomerization is not responsible for the exceedingly slow reactivation of the completely unfolded enzyme (*10*). As the proline residues are equally distributed along the polypeptide chain, some of them could be flexible enough to isomerize, even in the incompletely unfolded protein. A detailed investigation of the role of proline isomerization in the reactivation from I_{fast} is hampered by the transient nature of the intermediate species precluding the use of double-jump techniques and by its compactness expected to prevent access of proline isomerases (*34*).

Luciferase in the I_{fast} conformation is enzymatically inactive. As shown by rapid gel filtration and by dynamic light scattering, conversion of I_{fast} to I_1 is not accompanied by a significant change in the Stokes radius and both the fast and the slowly reactivating intermediates are expanded relative to the native state. During denaturation in 1 M GdmCl, large fractions of the changes in the fluorescence and CD signals occur in the dead time; i.e., the spectroscopic signals for I_{fast} are more similar to those of I_1 than to those of native luciferase. All this indicates that both intermediates are structurally related and that the slow conversion of I_{fast} to I_1 is not a global unfolding transition. Instead, it may correspond to some local denaturation event releasing a small part of the large amino-terminal domain of luciferase and allowing it to engage in some non-native interactions that are stable in the absence of denaturant and have to be broken for the enzyme to fold into the native, enzymatically active conformation. The precise nature of this kinetic trap in luciferase folding may be approached by fragmentation experiments, by studies with luciferase mutants, and by comparative investigation of the folding of other luciferases and of structurally related enzymes, like the major subunit of gramicidin S synthetase (*35*).

The question of how luciferase avoids this kinetic trap during its in vivo folding still remains open. Results obtained from in vitro translation experiments suggest luciferase to become active very rapidly after its release from the ribosome (*15*, *36*). On the other hand, an involvement of molecular chaperones is supported by the observation that chemically denatured luciferase reactivated within minutes after dilution into reticulozyte lysate or wheat germ extract (*13*, *15*, *36*; Ruth Herbst and Robert Seckler, unpublished observation). Thus, luciferase must be able to reach the native conformation rapidly and efficiently, avoiding the kinetic traps using a folding throughway, as indicated in Scheme 3.

Besides being related to a kinetic trap, both intermediates, I_1 and I_2, are aggregation precursors. Longer term incubation of luciferase in buffers containing intermediate amounts of GdmCl results in irreversible self-association of the protein. The range of denaturant concentrations, where aggregation is observed, i.e., between 1 and 3.3 M GdmCl, corresponds to the conditions where the intermediate states I_1 and I_2 are highly populated. Aggregation was maximal between 2.5 and 3.0 M GdmCl. At first sight, this could be interpreted as an indication that only I_2 is the associating species. Because I_1 rapidly equilibrates with I_2, as discussed above, the continuous removal of I_2 would result in a slow aggregation even at denaturant concentrations where I_1 is stable. Two further observations, however, suggest otherwise: Neither aggregation observed at 1 M GdmCl nor aggregation at 3 M GdmCl were going along with changes in spectroscopic signals. On the other hand, the dilution of partly aggregated I_1 from 1 M GdmCl into buffer containing 3 M denaturant was accompanied by a fluorescence decrease, dependent in its rate and amplitude on the fraction of the protein aggregated before dilution (data not shown). Therefore, it appears to be more likely that both intermediates, I_1 and I_2, are sensitive to form aggregates, independently of each other. According to their different spectroscopic properties, I_1 and I_2 aggregates retain a different, characteristic portion of secondary structure, which is directly related to the structure of their immediate precursors, I_1 and I_2. As

suggested by the fluorescence change observed upon dilution into 3 M GdmCl, I_1 aggregates are redissolved when transferred into higher concentrations of denaturant. This is consistent with the view that aggregation is not an unspecific process, but occurs at least to some extent via specific interactions, involving distinct regions or segments of secondary structure which are exposed in the partially folded intermediate precursor (*20*−*22*). Accordingly, the aggregation maximum at 2.5−3.0 M GdmCl then reflects the differences in the association sensitivity of I_1 and I_2. Compared to I_1, I_2 aggregates faster and to a higher extent. Our results on luciferase differ significantly from observations on the denaturant dependence of the solubility of some small, single-domain proteins. In a careful study on apomyoglobin (*37*), Fink and co-workers observed a minimum of protein solubility at urea concentrations roughly corresponding to the midpoint of the cooperative unfolding transition. They showed convincingly that this solubility minimum can be explained without resorting to unfolding intermediates, merely by the differential effects of urea on the solubility of the native and the unfolded protein, thus confirming previous theoretical predictions (*38*).

Aggregation upon dilution of an unfolded protein into renaturation buffer has been described as a fast reaction of higher than second order (*11*, *12*, *39*). This fast aggregation process is one of the major problems in the in vitro refolding of large proteins, drastically limiting the overall reactivation yield especially at high protein concentrations and increased temperatures. The underlying kinetic competition between first-order folding and second-order aggregation (*12*) has been found to provide a quantitative description of the decrease in luciferase refolding yields with increasing protein concentration (*10*). Compared to the aggregation of lysozyme (*11*) or lactate dehydrogenase (*39*), the irreversible self-association of luciferase folding intermediates occurred very slowly. This, however, does not mean that the reactions described above are unrelated to the aggregation process limiting luciferase refolding, because the association experiments in this report have intentionally been performed under conditions suppressing hydrophobic interactions (low temperature, low protein concentration), to achieve a good kinetic resolution of the individual events. Compared to this, high local concentrations of protein emerge transiently during dispersion of a concentrated protein solution in the renaturation buffer, leading to rapid aggregation (*11*). The presence of strong denaturing agents such as GdmCl may stabilize aggregation-prone folding intermediates, as observed here (*20*, *40*). On the other hand, it is known to increase the solubility of a protein by destabilizing aggregates and thus slows down aggregation (*41*).

As expected for higher order reactions (*39*), the association process of I_1 and I_2 was accelerated with increasing temperature and protein concentration. Surprisingly, however, the reaction reached an apparent standstill after a certain amount of protein had aggregated. The incubation conditions not only affected the speed of the process, but the overall extent of aggregation varied systematically with increasing temperature and protein and denaturant concentrations. Related observations have been made in the case of the dimeric bacterial ribulose-1,5-bis-phosphate carboxylase (Rubisco; *42*). In that case, the rapid aggregation of a partially folded monomeric intermediate comes to a sudden halt, when the concentration of the intermediate has dropped below a "critical aggregation concentration" which is strongly dependent on the temperature, but essentially independent of the total protein concentration. Qualitatively, the concentration dependence of aggregate formation from the luciferase unfolding intermediate I_1 at 1 M GdmCl and 5 °C resembles the Rubisco observations, although the concentration of I_1 remaining in solution does increase from ∼70 nM to ∼300 nM with an increase in the total protein concentration between 80 nM and 1.7 μM. Critical aggregation concentrations are observed in the reversible formation of detergent micelles (*43*) and helical protein polymers (*44*), and are directly related to the free energy of subunit assembly onto a polymerization nucleus. The association reaction observed here, however, can only be reversed by increasing the denaturant concentration. Thus, kinetics rather than thermodynamics have to be considered.

The concentration dependence of the aggregation rate observed, with the rate slowing down dramatically below a critical concentration, is characteristic for a reaction order significantly higher than 2 and is consistent with assembly by a nucleated polymerization mechanism involving the reversible formation of small oligomers nucleating the irreversible aggregation reaction (*45*). A nucleated polymerization mechanism has been suggested for the irreversible protein aggregation connected with prion diseases (*46*, *47*) and has been found to explain the observations on the in vitro aggregation of transthyretin (*24*) and bacteriophage P22 tailspike protein (*22*). Moreover, reversible formation of small oligomers at early stages of irreversible aggregation processes has been observed experimentally with several proteins (*27*, *48*, *49*). From the gel filtration and light scattering data described above, we do not have evidence for a large fraction of luciferase folding intermediates being reversibly associated at early stages of the aggregation process occurring at 1 M GdmCl. Dynamic light scattering indicated the large majority of the material to be monomeric at early time points after the dilution into the denaturant and any material eluting before the monomer from the gel filtration column proved to be irreversibly aggregated when rechromatographed without prior dissociation by 5 M GdmCl. Both methods, however, would hardly detect a few percent of reversibly associated species.

The irreversible polymerization of luciferase folding intermediates does not lead to the formation of large, insoluble aggregates under the experimental conditions we employed, but rather yields dimers, trimers, and small multimers which remain in solution. This reflects the solubilizing effect of the denaturant present, as similar observations have been made with other proteins, when aggregation was studied in the presence of GdmCl or urea (*22*, *27*). In the absence of solubilizing denaturants, the proteins form large, insoluble aggregates, when productive folding is prevented (*50*, *51*). At GdmCl concentrations above 3 M, luciferase aggregation rates strongly decrease, and above 3.3 M denaturant, the aggregation reaction is blocked completely and a freely reversible unfolding transition is observed for the I_2 intermediate (*10*).

In summary, the in vitro folding of firefly luciferase is limited by a kinetic trap that dramatically slows down the formation of native molecules and leads to accumulation of partly folded intermediates prone to aggregation. Our results

provide a basis for the study of the mechanism by which molecular chaperones improve the folding efficiency of the enzyme and demonstrate the importance of analyzing folding kinetics as well as final yields when firefly luciferase is used as a model protein in studies of in vitro and in vivo protein folding processes.

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