Contribution of Folding Steps Involving the Individual Subunits of Bacterial Luciferase to the Assembly of the Active Heterodimeric Enzyme*

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Bacterial luciferase is an αβ heterodimer with a single active center in which the reaction of reduced FMN, O₂, and an aliphatic aldehyde yields a photon of blue-green light. We have shown that refolding of the luciferase subunits from 5 M urea occurs via the intermediary of several species, one of which is an inactive heterodimeric structure, resulting from the dimerization of α and β, which isomerizes to the active αβ structure in a first-order reaction (Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F., and Baldwin, T. O. (1993) J. Biol. Chem. 268, 10760–10765). We have also demonstrated the existence of an inactive heterodimeric species that is well populated at equilibrium in the presence of 1.6–2.8 M urea (Clark, A. C., Sinclair, J. F., and Baldwin, T. O. (1993) J. Biol. Chem. 268, 10773–10779). We have separated the α and β subunits by ion exchange chromatography and investigated the effects on refolding of active luciferase of allowing the individual subunits to refold separately prior to mixing. These investigations show that the lag in formation of active luciferase is due to slow steps in folding of the individual subunits. The β subunit appears to fold faster than the α subunit, but folding of the β subunit also shows a distinct lag. When the α and β subunits were allowed to refold from urea for periods of several hours or more prior to mixing, the yield of active heterodimeric luciferase was compromised, which is consistent with the finding that individual subunits produced in vivo fold into structures incompetent to interact with each other to form the active heterodimer (Waddle, J. J., Johnston, T. C., and Baldwin, T. O. (1987) Biochemistry 26, 4917–4921). It appeared that the rate with which the β subunit assumed the heterodimerization-incompetent structure was faster than the rate with which the α subunit became heterodimerization-incompetent.

The observations support a model for folding and assembly of the subunits of luciferase in which the two subunits fold into assembly-competent structures that associate to form the heterodimer. In a slow competing process, the subunits undergo a conformational rearrangement to form stable structures incompetent to form heterodimers. It appears that the association of the luciferase subunits might constitute an example of one polypeptide modifying the folding pathway of another, a model that is consistent with the suggestion that the formation of the heterodimeric structure of luciferase is a kinetic trap on the folding pathway of the individual subunits (Sugihara, J., and Baldwin, T. O. (1988) Biochemistry 27, 2872–2880).

The luciferase from luminous marine bacteria catalyzes the bioluminescent oxidation of FMNH₂ and a long chain aliphatic aldehyde by molecular oxygen, producing FMN, the corresponding chain length fatty acid, and (presumably) H₂O, with the emission of blue-green light (see Baldwin and Ziegler (1992) for a recent review). The enzyme, an αβ dimer (Friedland and Hastings, 1967; Hastings et al., 1969), lacks disulfide bonds (Tu et al., 1977); the subunits are homologous, with 32% amino acid sequence identity between the α and β subunits from Vibrio harveyi (Cohn et al., 1985; Johnston et al., 1986). Although the high quantum yield reaction requires both subunits and appears to result from a single active center on the heterodimer, the separate α and β subunits expressed in Escherichia coli each shows very low but authentic bioluminescence activity in the absence of the other (Waddle and Baldwin, 1991; Sinclair et al., 1993). We have found that the individual luciferase subunits fold in vivo into stable structures that do not interact to form the active heterodimeric structure (Waddle et al., 1987). Furthermore, we have reported the existence of variant forms of the enzyme from V. harveyi that do not fold correctly at temperatures of 30 °C but that are stable at 30 °C once folded (Sugihara and Baldwin, 1988). These mutants appear to be very similar to the temperature-sensitive folding mutants of the phage P22 tail spike protein that have been described by King and co-workers (Goldenberg et al., 1982). The luciferase subunits interact during the folding process, but if they fail to form the heterodimer, the folding will proceed toward stable structures that do not interact to form the active heterodimer (Waddle et al., 1987). Conformational rearrangements in the subunits would be expected to yield to a conformation that is competent to form the heterodimer, the active structure that catalyzes the bioluminescent reaction.

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The abbreviations used are: FMNH₂, reduced flavin mononucleotide; DTT, dithiothreitol.
The native form of the luciferase enzyme constitutes a kinetic trap for the folding subunits (Waddell et al., 1987; Sugiha and Baldwin, 1988).

The heterodimeric quaternary structure of luciferase is obviously advantageous to the investigator with an interest in the detailed dissection of the folding pathway of a multimeric protein, since it permits distinction between (first-order) folding processes of the individual α and β subunits and the (second-order) step of assembly into the heterodimeric structure. The rapidity of the single-turnover assay (Hastings et al., 1978) permits the monitoring of the kinetics of formation of active enzyme from urea-unfolded luciferase upon dilution of the urea. The preceding paper demonstrated that at low protein concentrations, dimerization appears to be rate-limiting and that there are one or more isomerization steps between the initial dimeric complex and the final active enzyme (Ziegler et al., 1993). Upon dilution of an equimolar mixture of unfolded α and β subunits from 5 M urea into 0.1 M urea, a lag of 3.5-4 min was observed prior to the onset of recovery of active enzyme. This lag was essentially independent of the luciferase subunit concentration, suggesting that the lag was due to slow first-order steps preceding dimerization. At low protein concentrations, the yield of active heterodimer was compromised, apparently due to the competing first-order folding of one or the other, or both, of the subunits into the presumed assembly-incompetent form (Ziegler et al., 1993). Based on these observations, we have proposed the model presented in Fig. 1 (Ziegler et al., 1993).

This model makes certain predictions regarding the folding of the individual subunits and the assembly of the heterodimer. Specifically, if the lag in the formation of the active enzyme is due to first-order steps in the folding of the α and β subunits following dilution from urea, then allowing the individual subunits to refold for a short time prior to mixing should reduce or eliminate the lag. Furthermore, by allowing one subunit to refold from urea for a short time prior to mixing with the other unfolded subunit, it should be possible to determine whether the lag is due to slow steps on the refolding pathway for one subunit or the other, or both. The proposed dimerization process involving intermediates on the refolding pathway of the two subunits suggests that the rate of dimerization should depend on the concentration of both subunits. If a low concentration of one unfolded subunit was titrated with the other unfolded subunit, the rate of recovery of activity should depend on the concentration of the subunit in excess, regardless of which subunit is in excess. Finally, if the dimerization requires intermediate structures of subunits that dimerize in a kinetically controlled interaction but that also could independently into structures that do not interact with each other, then refolding of the subunits independently from urea should result in structures that are heterodimer assembly-incompetent. To investigate these possibilities, we have separated the luciferase subunits using chromatographic methods so that we could investigate the effects of varying the concentrations of the two subunits independently in the refolding mixture and varying the time of refolding prior to mixing of the subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—FMN was obtained from Fluka and was used without further purification. Bovine serum albumin (Fraction V powder) and n-decyl aldehyde were purchased from Sigma. Ultra-Pure urea was the product of Schirra-Mann. DEAE-Sephadex A-50 was a product of Pharmacia LKB Biotechnology Inc. All other chemicals were of the highest quality commercially available and were used without further purification.

Phosphate buffers were prepared by mixing the appropriate proportions of the monobasic and dibasic sodium or potassium salts to obtain the desired pH.

**Luciferase Purification and Assay**—E. coli (LB302) cells carrying the luxCDABE genes on a pUC9-derived plasmid, pLAV1, were grown, and the luciferase was purified as previously described (Baldwin et al., 1989), the purification method being a modification of that described by Hastings et al. (1978) for purification of the enzyme from the native organism, V. harveyi. Enzyme concentrations were determined by absorbance at 280 nm, using an extinction coefficient of 0.94 (mg/ml)⁻¹·cm⁻¹ (Gunsalus-Miguel et al., 1972). The enzyme was assayed (22°C) using a photomultiplier-photometer to detect the light emitted with n-decyl aldehyde as the substrate upon rapid injection of FMNH₂ photoreduced in a solution containing 2 mM EDTA (Hastings et al., 1978).

**Purification of Luciferase Subunits**—The α and β subunits of luciferase purified from recombinant E. coli (Baldwin et al., 1989) were resolved by chromatography on DEAE-Sephadex in 5 M urea as previously described (Tu, 1978). The enzyme to be applied to the column (65 mg) was dialyzed at 4°C versus 40 mM phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0, immediately before application to the column, enough solid urea was added to make the sample 5 M in urea. The sample was then applied to a 2.5 × 42-cm column previously equilibrated in 40 mM phosphate, 1 mM EDTA, 1 mM DTT, 5 M urea, pH 7.0, at 4°C and eluted with a linear gradient consisting of 400 ml of the equilibration buffer and 400 ml of the same buffer 120 mM in phosphate.

The pooled subunits (in 5 M urea) were concentrated by ultrafiltration using CentriPrep-10 centrifugal concentrators (Amicon), dialyzed against 5 M urea, 50 mM phosphate, 1 mM EDTA, 1 mM DTT, pH 7.0, and stored at −20°C. Subunit concentrations in 5 M urea were determined by absorbance at 290 nm, using the extinction coefficients for deactuated subunits determined by Wadde (1990), which are very similar to those determined by Gunsalus-Miguel et al. (1972): E for α = 1.22 (mg/ml)⁻¹·cm⁻¹ and for β = 0.72 (mg/ml)⁻¹·cm⁻¹.

**Refrolding of Luciferase and of Individual Subunits from 5 M Urea**—When refolding of luciferase alone was to be followed, the enzyme in 5 M urea (50 mM phosphate, 1 mM EDTA, 1 mM DTT, pH 7.0) was diluted (defining time 0) 1:100 into renaturation buffer (50 mM phosphate, 0.2% bovine serum albumin, 1 mM EDTA, 1 mM DTT, pH 7.0), which was also 0.05 M in urea so that the final urea concentration after enzyme addition would be 0.1 M in the sample during refolding. When individual subunits alone were to be refolded, the same procedure was followed. When luciferase was to be refolded in the presence of added subunit, the enzyme and the subunit (both in 5 M urea) were premixed, and the mixture was diluted 1:50 at time 0 into renaturation buffer. When a subunit was to be “prefolded” for a given length of time prior to addition of luciferase, the subunit in 5 M urea was diluted 1:60 into renaturation buffer (so the urea concentration during prefolding was 0.05 M), and the luciferase refolding was subsequently initiated by 1:100 dilution (time 0) of the enzyme in 5 M urea into the solution of prefolded subunit in renaturation buffer (final urea concentration, 0.1 M). All dilutions of enzyme or
subunit from 5 M urea into buffer were carried out by rapid addition to the buffer on a vortex mixer. Activity recovery was monitored by withdrawal of 10-μl aliquots of the renaturation mixture and dilution at time t into 1.0 ml of assay buffer containing 15 μl of a sonicated suspension (0.01% v/v) of n-decyl aldehyde in H2O, followed approximately 12–15 s later by initiation of the assay by injection of FMNH2. In experiments in which luciferase was renatured in the presence of added excess α or β subunit, or in which a subunit was "prefolded" prior to mixing with the other subunit or with luciferase, the activities of the individual subunits were monitored in separate control samples and the activity attributable to the free subunit was subtracted from the activity obtained in the final mixture. This correction was significant only for the α subunit and only at early times or high concentrations.

RESULTS

Upon dilution of unfolded luciferase subunits in 5 M urea into buffer with a final urea concentration of 0.1 M, a lag of about 4 min is observed prior to the recovery of bioluminescence activity (Ziegler et al., 1993). The rate of recovery following the lag is strongly concentration-dependent below 10 μg/ml, whereas the duration of the lag is comparatively concentration-independent (Ziegler et al., 1993). This observation suggested that the lag might be due to slow (first-order) steps in the folding of either α or β (or both) preceding the dimerization step. Experiments to test this possibility required pure isolated subunits. For this purpose, we have separated the subunits of luciferase using DEAE-Sephadex column chromatography in buffers containing 5 M urea (Fig. 2). The resolution afforded by this method was excellent, but nonetheless, to avoid contamination of one subunit with the other, we were conservative in the pooling of fractions.

To test the possibility that the lag in recovery of activity was due to folding steps that precede dimerization, we allowed the individual subunits to refold separately for varying periods of time prior to mixing (Fig. 3). Upon mixing of subunits that had been allowed to refold separately for 5 min or longer, recovery of luciferase activity was observed without a lag, demonstrating that the lag was in fact due to refolding steps that preceded dimerization.

The active form of bacterial luciferase is the heterodimer; formation of the heterodimer on the folding pathway would require a second-order process that should be apparent in the concentration dependence of the rate of dimer formation. We have shown that the rate of formation of active luciferase is strongly concentration-dependent at concentrations below 10 μg/ml (Ziegler et al., 1993). Above 10 μg/ml, the rate of formation of the active enzyme appears to saturate, suggesting that some other process becomes rate-limiting (see Fig. 1). At low concentrations (2 μg/ml and below) a marked reduction in yield of active enzyme is observed that appears to be due to competing first-order folding steps involving the individual subunits that lead to stable structures that are incompetent to form heterodimer (Ziegler et al., 1993). This model (Fig. 1) predicts that if the concentration of one subunit were held constant at 1 μg/ml, and the concentration of the other varied from 1 μg/ml to above 20 μg/ml, the rate of formation of the active enzyme should increase with the concentration of the subunit in excess and that the excess subunit should rescue the limiting subunit from undergoing the competing first-order folding reaction. The results of this experiment are presented in Figs. 4, A and B. Addition of an excess of either subunit to a limiting concentration of the other resulted in a concentration-dependent increase in the rate of formation of active enzyme after the lag (Fig. 4) and also an increase in the yield of active enzyme (Table 1), as predicted from the model presented in Fig. 1.

An additional observation apparent in Figs. 4, A and B, was that with high concentrations of either the α or the β subunit present, there was still a lag preceding formation of the active heterodimer. This result suggested that the lag might be due to slow steps in the folding of both subunits. However, to better approach this question, we allowed one subunit to refold from urea for 4 min prior to mixing with the other unfolded subunit. By so doing, we hoped to obtain some idea of the relative rates (for α versus β) of the presumed first-order steps that precede the dimerization step in the assembly of active luciferase. The results are presented in Fig. 5. In both cases, the lag was still apparent, indicating the existence of slow steps in the refolding of both subunits. However, addition of excess prefolded α subunit to unfolded β subunit resulted in

![Fig. 2. Separation of luciferase subunits by DEAE-Sephadex A-50 column chromatography in 5 M urea. Luciferase purified from E. coli was denatured in 5 M urea, and the α and β subunits were resolved by a phosphate gradient in 5 M urea as described under "Experimental Procedures."](image-url)
the issue of the folding of individual subunits into structures that do not assemble into active.

The final luciferase concentration in the refolding mixture was thus 2 µg/ml (1 µg/ml α and 1 µg/ml β) alone (A), or 2 µg/ml plus additional subunits as follows. A, α subunit at 5 µg/ml (●), 10 µg/ml (▲), 20 µg/ml (◇), or 36.6 µg/ml (○); B, β subunit at 5 µg/ml (●), 10 µg/ml (▲), 20 µg/ml (◇), or 38.2 µg/ml (○). Percent recovery is expressed relative to the activity of a native sample diluted to 2 µg/ml into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

Table I

<table>
<thead>
<tr>
<th>Luciferase sample</th>
<th>Relative yield$^a$</th>
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</thead>
<tbody>
<tr>
<td>2 µg/ml luciferase alone</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 5 µg/ml α subunit</td>
<td>1.4</td>
</tr>
<tr>
<td>+ 10 µg/ml α subunit</td>
<td>1.3</td>
</tr>
<tr>
<td>+ 20 µg/ml α subunit</td>
<td>1.6</td>
</tr>
<tr>
<td>+ 36.6 µg/ml α subunit</td>
<td>1.5</td>
</tr>
<tr>
<td>+ 5 µg/ml β subunit</td>
<td>1.8</td>
</tr>
<tr>
<td>+ 10 µg/ml β subunit</td>
<td>1.7</td>
</tr>
<tr>
<td>+ 20 µg/ml β subunit</td>
<td>1.9</td>
</tr>
<tr>
<td>+ 35.2 µg/ml β subunit</td>
<td>1.9</td>
</tr>
</tbody>
</table>

$^a$Yields are given relative to that of 2 µg/ml luciferase alone diluted 1:50 from 5 M urea into renaturation buffer after 21 h at 18°C, from the experiment described in the legend to Fig. 4. The recovery from the 2 µg/ml sample with no added subunits was itself about 45% of a control that had not been denatured.

luciferase upon mixing (Waddle et al., 1987). Second, the yield of active heterodimeric enzyme that forms during refolding from urea is compromised at low concentrations, indicating a competing folding process involving the independent subunits (Ziegler et al., 1993). Neither of these results demonstrated over what time course the conversion of the subunits to the assembly-incompetent form was occurring, or which of the two subunits is responsible.

To address the question of the time course of the competing off-pathway folding of the individual subunits, we permitted the subunits to refold separately for various periods of time up to 21 h and then mixed them and followed the time course and final yield of active heterodimer recovery. The early time courses of two such experiments, involving prior refolding of both subunits for 30 and 60 min, were shown in Fig. 3, and the effect on the lag in activity recovery is discussed above. The final yields of active enzyme recovered in those two experiments, as well as following prefolding for longer periods of time, are shown in Table II. Permitting both of the subunits to refold separately for up to about 3 h prior to mixing had relatively little effect on the final recovery of active enzyme, but the yield obtained in the experiment with 21 h of prefolding was much lower, suggesting that the off-pathway conversion of the species competent to form heterodimer to the assembly-incompetent form must be slow, with a first-order rate constant in the range of 0.06-0.12 h$^{-1}$ at 18°C.

To address the question of which of the two subunits was being converted to a stable, heterodimer assembly-incompetent form, we allowed each to refold for 24 h prior to mixing. Upon mixing of the separately refolded subunits, very low activity that formed at a very slow rate was observed (Fig. 6 and Table III). Likewise, dilution of unfolded α subunit into a solution containing folded β subunit resulted in a very low yield of active enzyme, indicating that most of the β subunit had refolded over the 24-h period into an assembly-incompetent form. Dilution of unfolded β subunit into a solution of folded α subunit, however, resulted in rapid recovery of activity with a shorter initial lag than was observed when unfolded.
Luciferase Subunit Folding and Kinetic Control of Assembly

Table II

Refolding both subunits separately prior to mixing

<table>
<thead>
<tr>
<th>Duration of refolding prior to mixing</th>
<th>% yield$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>50</td>
</tr>
<tr>
<td>30 min</td>
<td>50</td>
</tr>
<tr>
<td>60 min</td>
<td>45</td>
</tr>
<tr>
<td>90 min</td>
<td>40</td>
</tr>
<tr>
<td>167 min</td>
<td>40</td>
</tr>
<tr>
<td>21 h</td>
<td>6</td>
</tr>
</tbody>
</table>

$^*$Final activities were determined 24 h after mixing of the subunits. Yields are given relative to a control consisting of 20 μg/ml luciferase alone that had never been denatured. For reference, luciferase diluted 1:50 from 5 M urea into renaturation buffer (final concentration, 10 μg/ml of each subunit) was permitted to refold separately for 21 h at 18°C (Ziegler et al., 1993).

Effect on final yield of active enzyme

Table III

Refolding the α and/or β subunit separately for 24 h prior to mixing with the other subunit

<table>
<thead>
<tr>
<th>Sample</th>
<th>% yield $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>αωure + βωure</td>
<td>50$^*$</td>
</tr>
<tr>
<td>αωrefolded + βrefolded</td>
<td>26$^*$</td>
</tr>
<tr>
<td>αωure + βrefolded</td>
<td>5$^*$</td>
</tr>
<tr>
<td>αrefolded + βrefolded</td>
<td>6$^*$</td>
</tr>
</tbody>
</table>

$^*$Final protein concentrations were all 20 μg/ml, 10 μg/ml of each subunit. Yields are given relative to a control consisting of 20 μg/ml luciferase alone that had never been denatured. For reference, luciferase diluted 1:50 from 5 M urea into renaturation buffer at 20 μg/ml recovered about 80% of the control activity after 21 h at 18°C (Ziegler et al., 1993).

The experiments reported here were undertaken in order to develop an understanding of the overall kinetic pathway for the folding and assembly of the luciferase subunits. Because of the nature of the experiments, we deemed it unreasonable to attempt to extract specific rate constants for individual steps or combinations of steps. Rather, we elected to utilize the approach described in this and the preceding publication (Ziegler et al., 1993) to investigate the overall folding reaction. Knowledge of the overall folding mechanism should allow us to design spectroscopic methods by which we may monitor the rates of conversion of one identifiable intermediate into the next. Our results allow qualitative estimates to be made of the rates of interconversion of folding intermediates.

We have demonstrated the existence of a series of intermediates involved in the folding and assembly of the luciferase subunits. These include the unfolded subunits (αω and βω) and the heterodimer assembly-competent subunit species (αω and βω), which can either fold on to form the heterodimer assembly-incompetent species (αω and βω) or associate to form heterodimeric species, the inactive heterodimer ([αβ]ω), and the active heterodimer ([αβ]ω). Knowledge of these intermediates and their apparent interconversions allowed us to formulate a minimal model for the kinetic mechanism for the folding of the luciferase subunits and the assembly of the active heterodimer (Fig. 1) that is consistent with the results of studies carried out at equilibrium (Clark et al., 1993).

The results reported here demonstrate the existence of one or more comparatively slow steps between the unfolded subunits and the assembly-competent form of the subunits. Only a few proteins have been investigated by circular dichroism spectroscopy on the stopped-flow time scale, but for such proteins a clear generalization can be made. The majority of the secondary structure of a protein forms within much less than 1 s following dilution from a denaturant solution (Kuwajima et al., 1991; Chaffotte et al., 1992). The same is true for the luciferase subunits, but we observed a prolonged lag of 3–4 min between the time of dilution of the subunits from heterodimer assembly-incompetent conformation when permitted to refold for 24 h in the absence of the β subunit.

Discussion

Fig. 6. Time course of recovery of luciferase activity following refolding of one subunit for 24 h prior to dilution of the other subunit from 5 M urea. The α and β subunits (each 1.0 M, 5 M urea) were permitted to refold separately upon 1:50 dilution into renaturation buffer (see "Experimental Procedures"), the subunits being mixed at 37°C. The urea concentration being 0.1 M, for 24 h prior to dilution of the other subunit from 5 M urea. After 24 h of prefolding, 6.5 mg/ml of each subunit was diluted with an equal volume of renaturation buffer (so that the final concentration of refolded subunit was 10 μg/ml) and at time 0, the subunits in 5 M urea were diluted 1:100 into the solution of the refolded subunit, so that the final urea concentration was again 0.1 M and the final protein concentrations were 10 μg/ml refolded α and 10 μg/ml refolded β (Δ), or 10 μg/ml unfolded α and 10 μg/ml refolded β (C). For reference, subunits that had each been permitted to refold separately for 21 h were mixed in equal volumes (final concentrations, 10 μg/ml of each subunit) at time 0 ( ). (Fig. 3 for shorter times of refolding). In the control ( ), the subunits in 5 M urea were mixed prior to dilution and diluted together 1:50 into renaturation buffer (final concentrations, 10 μg/ml of each subunit) to initiate refolding at time 0. Percent recovery is expressed relative to the activity of a native sample diluted to 20 mg/ml into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

α subunit is permitted to refold independently in the absence of β subunit, indicating that a substantial fraction of the 24 h-folded conformation of the α subunit is able to interact with β subunit as β refolds from urea. The shorter lag is consistent with the observation that the β subunit folded faster than did the α subunit, such that the folding of α determined the length of the lag when both subunits were diluted from urea at the same time (see Fig. 5). However, the sample in which the α subunit was permitted to fold for 24 h prior to the addition of unfolded β eventually recovered only half of the activity of the sample in which the two subunits refolded together from the outset (Table III), suggesting that some fraction of α may also be assuming a

$^*$O. Baldwin, M. M. Ziegler, A. F. Chaffotte, and M. E. Goldberg, unpublished results.
Luciferase Subunit Folding and Kinetic Control of Assembly

urea and the onset of accumulation of active enzyme (Ziegler et al., 1993). Thus, there must already be considerable structure present in the assembly-competent species ($\alpha$ and $\beta$). Although supplementation of luciferase during refolding with a large excess of either individual subunit indicated that the rate of recovery of the active heterodimer was a function of the concentration of the subunit in excess (Fig. 4), the lag appeared to be nearly independent of the concentration of the subunit in excess. This observation, and the observation that the lag was independent of the concentration of the refolding subunits maintained at a stoichiometry of 1:1 (Ziegler et al., 1993), suggested that the lag was due to a slow (first-order) step or steps in the folding of both subunits, since the lag persisted regardless of which subunit was in excess. The suggestion that the lag was due to slow steps preceding dimer assembly was confirmed by allowing the two subunits to refold for various periods of time prior to mixing (Fig. 3). By this method, we eliminated the lag, demonstrating that the lag was due to the delay in the formation of the assembly-competent forms of one or both subunits.

In all of our experiments, we found only a single way to alter the duration of the lag in the formation of active luciferase without completely eliminating it. By allowing the $\alpha$ subunit to refold from urea for 4 min prior to mixing with unfolded $\beta$ subunit, we observed a somewhat shorter lag (Fig. 5). The converse experiment, in which we allowed the $\beta$ subunit to refold briefly prior to mixing with unfolded $\alpha$ subunit, resulted in a lag of nearly the same duration as if both subunits were diluted together from urea simultaneously. This experiment demonstrated that the lag is determined primarily by the rate of folding of the $\alpha$ subunit, but that although the $\beta$ subunit appeared to fold faster than the $\alpha$ subunit, there was not a major difference in the rates.

The final conclusion that we may draw from these experiments relates to the failure of folded subunits to assemble into the active enzyme, as originally reported by Waddle et al. (1987) for subunits folded in vivo. We found that if the individual subunits were permitted to refold from urea for 21 h prior to mixing, little active enzyme was formed (Table II). To determine if both folded subunits were heterodimer assembly-incompetent, we mixed one folded subunit with the other unfolded subunit and monitored both the rate of formation and the yield of active enzyme. Mixing of refolded $\alpha$ subunit with unfolded $\beta$ subunit resulted in some reduction in yield of $\alpha\beta$ relative to the yield observed when unfolded $\alpha$ was mixed with unfolded $\beta$. This observation suggests that the $\alpha_1$ species may be very slowly converted to $\alpha_\alpha$, with a half-time for the conversion of the order of 24 h at 18°C in 0.1 M urea and 50 mM phosphate, pH 7.0. The $\beta$ subunit appeared to convert to the $\beta_\beta$ species slowly as well but significantly faster than the $\alpha$ subunit, with a half-time between 6 and 12 h. When both subunits were allowed to refold for periods of 60–90 min prior to mixing, the rates of formation and yield of active enzyme were not seriously compromised. However, when the $\beta$ subunit was allowed to refold for 24 h prior to mixing with either unfolded or refolded $\alpha$, very little $\alpha\beta$ was formed, indicating that about 90% of the $\beta$ subunit had been converted to the $\beta_\beta$ species. These observations suggest that the half-time for the $\beta \rightarrow \beta_\beta$ conversion is less than 12 h but greater than 3 h.

Gunsalus-Miguel et al. (1972) have reported a similar experiment (permitting the individual subunits to refold for 48 h, whereas our maximum refolding time prior to mixing was 24 h), with qualitatively similar but quantitatively different results. These authors allowed the individual subunits to refold at 4°C and found no loss in the ability of the refolded $\alpha$ subunit of V. harveyi (then called "MAV") luciferase to interact with $\beta$ and only about a 32% decrease in the ability of the refolded $\beta$ subunit to interact with $\alpha$. Presumably, the apparent difference in the rates of $\alpha_\alpha \rightarrow \alpha_\beta$ and $\beta_\beta \rightarrow \beta_\alpha$ conversions between our present results and those of Gunsalus-Miguel et al. (1972) is due to the temperature difference; the reactions appear to be quite slow at 15°C (present results) and to occur even more slowly at 4°C (Gunsalus-Miguel et al., 1972).

The structures of the heterodimer assembly-incompetent forms of the luciferase subunits, $\alpha_1$ and $\beta_2$, are of great interest but beyond the scope of the experiments reported here. We have investigated the circular dichroism spectra of the separately folded subunits and find that both have well defined spectra in both the far ultraviolet and the near ultraviolet, indicating that they have folded into well defined structures with the aromatic side chains packed into chiral environments (data not shown). A detailed investigation and interpretation of these observations will require much additional experimentation.

Our results suggest that the folding of the luciferase is similar in certain respects to the folding of proteases such as subtilisin (Zhu et al., 1989; Otta et al., 1991) and the $\alpha$-lytic protease (Silen and Agard, 1989; Baker et al., 1992), as well as the serpin plasminogen activator inhibitor (Carrell et al., 1991; Mottonen et al., 1992). The correct folding of the proteases requires interaction with the propolypeptide, either in cis or in trans (Zhu et al., 1989; Silen and Agard, 1989; Silen et al., 1989). These proteases appear to fold to a stable but inactive conformation, requiring interaction with the propolypeptide to be converted to the active conformation. For the $\alpha$-lytic protease, the activation barrier between the two conformations has been estimated to be 27 kcal/mol (Baker et al., 1992). A similar process has been reported for plasminogen activator inhibitor-1, which folds into an active conformation, but then slowly is converted into an inactive hyperstable species, apparently through the insertion of a stretch of polypeptide into a $\beta$ sheet to yield a structure of enhanced stability (Carrell et al., 1991; Mottonen et al., 1992). The $\beta$ subunit of bacterial luciferase appears to be similar to the serpin in that it folds into an assembly-competent species that slowly converts into an assembly-incompetent form. The $\beta_\beta$ subunit is similar to the proteases as well, in that it appears to be the interaction with the $\alpha$ subunit that converts it into the biologically active form.

We have separated the $\alpha$ and $\beta$ subunits of bacterial luciferase by column chromatography in 5 M urea so that we could study the effects of varying the concentrations of each in refolding experiments in which we measured the recovery of bioluminescence activity. The results of our experiments demonstrate the following. 1) The lag in recovery of activity, described in the experiments of Ziegler et al. (1993) is due to first-order steps in the refolding of both subunits prior to formation of the dimerization competent species. 2) The rate of refolding of $\beta$ is faster than the rate of refolding of $\alpha$. 3) The rates of formation of the heterodimerization-incompetent species, $\alpha_1$ and $\beta_2$, are indeed quite slow, with half-times of hours. 4) The heterodimerization-incompetent species that form in vitro (observed by Waddle et al. (1987)) also form upon refolding in vitro.

REFERENCES
Luciferase Subunit Folding and Kinetic Control of Assembly


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