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6. AUTHOR(S)
Miriam M. Ziegler and Thomas O. Baldwin

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Texas A&M Research Foundation
Box 3578
College Station, Tx 77843-3578

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13. ABSTRACT (Maximum 200 words)

This project was focused on determination of the three-dimensional structure of bacterial luciferase. The structure of the enzyme is of fundamental importance to the understanding of the catalytic mechanism and the mode of interaction of the enzyme with accessory proteins, and is essential to future plans to develop biosensor technologies. In the course of this project, numerous crystallization trials were carried out and conditions were refined that permitted high resolution data to be collected and interpreted. In collaboration with Dr. Ivan Rayment of the University of Wisconsin, data have been collected from native crystals and 3 derivatives at 2.8 Å. Higher resolution data are being collected at this time, and we fully expect to have a high resolution structure within the next few months, certainly by the end of the calendar year 1994. We have also developed crystallization protocols for several mutant luciferases. Structural analysis of the mutant luciferases should enable us to locate the active site in the three-dimensional structure of the wild-type enzyme, permit mechanistic interpretation of numerous experiments that have been reported over the past ca. 25 years, and assist us in designing the next generation of mutant enzymes to test hypotheses regarding the mechanism of light production by this intriguing and important enzyme.

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Bacterial Luciferase: Determination of the Structure by X-Ray Diffraction

FINAL REPORT

Office of Naval Research
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prepared by

Miriam M. Ziegler and Thomas O. Baldwin
Co-Principal Investigators

Department of Biochemistry and Biophysics
Texas A&M University
and the Texas Agricultural Experiment Station
College Station, TX 77843

Final Technical Report

This project was focused on determination of the structure of bacterial luciferase. The three-dimensional structure of bacterial luciferase is of fundamental importance to the long-term goals of the research program, understanding the catalytic mechanism and the mode of interaction of the enzyme with accessory proteins. In the course of this project, numerous crystallization trials were carried out and conditions were refined that permitted high resolution data to be collected and interpreted. In collaboration with Dr. Ivan Rayment of the University of Wisconsin, data have been collected from native crystals and 3 derivatives at 2.8 Å. Higher resolution data are being collected at this time, and we fully expect to have a high resolution structure within the next few months, certainly by the end of the calendar year 1994.

We have also developed crystallization protocols for several mutant luciferases. Structural analysis of the mutant luciferases should enable us to locate the active site in the three-dimensional structure of the wild-type enzyme, permit mechanistic interpretation of numerous experiments that have been reported over the past ca. 25 years, and assist us in designing the next generation of mutant enzymes to test hypotheses regarding the mechanism of light production by this intriguing and important enzyme.

In parallel with the determination of the three-dimensional structure of luciferase, we pursued two related lines of research:

1. We discovered and characterized different conformational forms of the β subunit, obtained by folding of the protein under different conditions (in the presence or absence of the α subunit, at different temperatures, etc.), including a β_2 species. In the case of the luciferase β subunit, we have clearly shown that the finally folded structure is determined by kinetic factors rather than by the stability of the finally folded product. We are currently working on crystallization of the β_2 homodimer that forms when the β subunit is expressed without α in *E. coli*. Comparison of the structure of β in the homodimer with β in the heterodimer will allow the first evaluation of the effects of protein-protein interactions on the structures of the individual subunits involved in the oligomer.
2. We performed preliminary characterizations of two types of mutant luciferases. One group of mutants comprises temperature-sensitive folding mutants with various substitutions at position 313 of the β subunit. The β313 mutations affect the rate of folding of the enzyme, but not the stability of the finally folded structure. For the other set of mutants, each of the wild-type Trp residues was replaced by Phe or Leu, in an effort to identify aromatic side chains potentially involved in stacking interactions with the flavin substrate. Two of the mutant enzymes show marked changes in catalytic parameters and in spectroscopic properties of bound FMN, and thus represent possible active site mutants.

In summary, the structural information resulting from this project will allow us to begin to define the roles of specific amino acid residues in substrate binding and catalysis and in protein-protein interactions.

Major equipment purchased under this contract: Turner Designs Luminometer, Model 20e, $5390; accessories for Beckman Optima XL-A ultracentrifuge, $4071.

Publications acknowledging the support of this contract:

- Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F. and Baldwin, T. O. (1993) Kinetics of refolding of urea-denatured bacterial luciferase demonstrates the existence of multiple intermediates on the folding pathway. *J. Biol. Chem.* **268**, 10760-10765.

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- Baldwin, T. O., Ziegler, M. M., Sinclair, J. F., Clark, A. C., Chaffotte, A.-F. and Goldberg, M. E. (1994) Mechanism of folding and assembly of heterodimeric bacterial luciferase. In Flavins and Flavoproteins 1993, eds. K. Yagi (Walter de Gruyter, Berlin) pp. (in press)

- Sinclair, J. F., Ziegler, M. M. and Baldwin, T. O. (1994) Kinetic partitioning of protein folding results in multiple native states of the β subunit of bacterial luciferase. *Nature Struct. Biol.* (in press, May issue).

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Refolding of Luciferase Subunits from Urea and Assembly of the Active Heterodimer

EVIDENCE FOR FOLDING INTERMEDIATES THAT PRECEDE AND FOLLOW THE DIMERIZATION STEP ON THE PATHWAY TO THE ACTIVE FORM OF THE ENZYME*

(Received for publication, October 22, 1992, and in revised form, December 21, 1992)

Miriam M. Ziegler‡§¶, Michel E. Goldberg§, Alain F. Chaffotte§¶, and Thomas O. Baldwin‡§‖****

*From the **Center for Macromolecular Design of the Institute of Biosciences and Technology and the ‡Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843 and the §Unité de Biochimie Cellulaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France*

Conditions have been established that allow reversible refolding of luciferase from 5 M urea. The kinetics of formation of the active enzyme showed a concentration-independent lag, suggesting the existence of intermediate structures on the pathway of refolding. The rate of approach to the final level of activity was strongly concentration-dependent at protein concentrations below 10 μg/ml, but at concentrations above about 20 μg/ml, the rate of approach to the final activity value did not change with concentration. The concentration dependence presumably reflects the second-order step yielding the heterodimeric structure. The finding that at concentrations above 20 μg/ml, the rate becomes insensitive to concentration suggests that under these conditions, some step subsequent to dimerization becomes rate-limiting.

When the refolding reaction was initiated by dilution out of 5 M urea at 50 μg/ml followed at various times by a secondary dilution to a final concentration of 5 μg/ml, it was found that the increase in activity continued at the rate characteristic of the higher protein concentration for a period of about 1–2 min following the dilution before slowing to the rate expected for the lower protein concentration. These observations indicate that there are inactive heterodimeric species that form from assembly of the individual subunits and that these species must undergo further folding to yield the active heterodimeric species.

At protein concentrations of 5–50 μg/ml, the final yield of active enzyme was about 65–85%, decreasing at higher and lower concentrations. At higher concentrations, aggregation probably accounts for the limit in recovery, whereas at lower concentrations, it appears that the reduced yield of activity is due to the competing process of the folding of one or both individual subunits into some form incompetent to interact with each other.

These experiments demonstrate the existence of slow steps in the refolding of luciferase subunits from urea and the formation of the active heterodimeric structure, both preceding and following the dimerization. Furthermore, the failure of protein at low concentrations to efficiently reassemble into the active heterodimer is consistent with the prior finding that luciferase subunits produced independently in *Escherichia coli* fold into conformations that cannot interact to form the active heterodimer upon mixing (Waddle, J. J., Johnston, T. C., and Baldwin, T. O. (1987) *Biochemistry* 26, 4917–4921).

Unraveling the mechanism of folding for any protein will require information about the structures of intermediates on the folding pathway and knowledge of the existence of parallel pathways. Most proteins are either composed of multiple subunits or exist as a single polypeptide with multiple folding domains that interact within the context of the covalent continuity of the peptide chain. The forces that maintain the assemblage of a multisubunit complex are noncovalent. Studies on small model systems have provided and continue to provide extremely valuable insight into the folding of individual domains, but it is unlikely that a general understanding of the folding of larger or multisubunit proteins will come exclusively through studies of folding of small peptides and proteins. Based on the classic studies of Anfinsen and his coworkers (Anfinsen, 1973) on a small protein, ribonuclease A, it is generally accepted that the final structure of a protein, or of a folding domain, is determined by the amino acid sequence. The existence of the same supersecondary structural motifs in unrelated proteins suggests that the same folding pattern may be determined by a great many amino acid sequences, *i.e.* that the folding code is highly redundant. However, an amino acid sequence that obediently forms an α helix in a specific protein may well refuse to assume a helical conformation when isolated from the context of the protein. Such findings lead one to suggest that perhaps with larger proteins consisting of multiple independent folding domains and/or multiple subunits, the native structures might be significantly altered as a result of interdomain or intersubunit contacts. That is, will a single subunit that folds in isolation reliably assume the same structure it would assume in the

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¶ Present address: Dept. of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

‖ Visiting Professor at Université Paris 7 during the period of these investigations. The visit to France was also supported by a Fulbright Award from the Commission Franco-Américaine d'Échanges Universitaires et Culturels and by Fogarty Senior International Fellowship 1 FO6 TW016066-01 from the National Institutes of Health, as well as by funds for a Faculty Development Leave from Texas A&M University. To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

context of interacting with the other subunits, or might it assume some alternative structure? Homopolymeric proteins do not provide an appropriate model system for the approach of this question, since it would not be possible to study directly the folding of individual subunits in the absence of oligomerization. A heterodimeric protein provides the simplest model system for the dissection of the processes of folding of the individual subunits and assembly into the dimer.

Bacterial luciferase is a heterodimeric enzyme composed of two homologous but nonidentical subunits (Friedland and Hastings, 1967a; Hastings *et al.*, 1969; Meighen *et al.*, 1970; Baldwin *et al.*, 1979; Cohn *et al.*, 1985; Johnston *et al.*, 1986). The enzyme has a single active center that is located primarily if not exclusively on the α subunit. Although the role of the β subunit remains a subject for debate, it is required for the high quantum yield reaction catalyzed by luciferase (see Ziegler and Baldwin (1981) and Baldwin and Ziegler (1992) for reviews). There are no intra- or interchain disulfide bonds in the enzyme (Tu *et al.*, 1977a). Luciferase catalyzes the reaction of $FMNH_2$,[1] O_2 and an aliphatic aldehyde to yield FMN and the carboxylic acid, and a photon of blue-green light (λ_{max} ~490 nm).

The genes encoding the α and β subunits, *luxAB*, have been cloned from *Vibrio harveyi* and expressed in *Escherichia coli* (Belas *et al.*, 1982; Baldwin *et al.*, 1984). Separation of the *luxA* gene and the *luxB* gene and expression of each from the *lac* promoter of pUC-derived plasmids allowed generation of significant levels of each subunit that had folded *in vivo* in the absence of the other (Waddle *et al.*, 1987). These separately produced α and β subunits each showed very low but authentic aldehyde- and flavin-dependent bioluminescence activity (Waddle and Baldwin, 1991; Sinclair *et al.*, 1993). Mixing of lysates containing the two subunits did not result in the expected formation of the much higher specific activity heterodimeric enzyme (Waddle *et al.*, 1987). However, if the subunits were first unfolded by the addition of urea, they were capable of recombining upon dilution of the urea. These observations led us to propose that in the normal folding of the luciferase subunits and assembly of the active heterodimer *in vivo*, the dimerization step occurs between either unfolded subunits or folding intermediates of the subunits, such that the active luciferase forms as the result of a kinetic trap. The individual subunits fold independently to form stable structures that are effectively unable to assemble. A minimal model describing our earlier results is presented diagrammatically in Fig. 1 (Waddle *et al.*, 1987).

The model presented in Fig. 1 makes certain predictions that are experimentally verifiable. First, at low concentrations of the individual subunits, the first-order off-pathway processes leading to the assembly-incompetent forms of the subunits would predominate, compromising the yield of the heterodimeric form of the enzyme; the yield of the heterodimer should increase at higher protein concentrations, since the rate of the second-order reaction would increase, whereas the competing first-order processes would not, leading to preferential partitioning of material into heterodimer formation. Second, if the luciferase subunits interact as partially folded intermediates following a slow folding step, the rate of formation of the active enzyme should show a concentration-independent lag due to initial folding steps of the individual subunits to the species competent to form heterodimer. Third, since the formation of the heterodimeric enzyme requires a second-order step, the rate of formation of the active enzyme should show a strong concentration dependence.

[1] The abbreviations used are: $FMNH_2$, reduced flavin mononucleotide; DTT, dithiothreitol; BSA, bovine serum albumin.

The experiments reported here were designed to test the above predictions, as well as to develop methods for the study *in vitro* of the folding of luciferase and its subunits. In these experiments, we monitor the formation of active luciferase following dilution from urea-containing solutions. Such measurements are greatly facilitated with bacterial luciferase due to the speed, simplicity, and sensitivity of the assay. Luciferase activity is measured in a single turnover assay by rapid injection of $FMNH_2$ into a vial containing enzyme, *n*-decyl aldehyde and O_2 dissolved in a buffer (Hastings *et al.*, 1978). The peak intensity of emitted light, which is achieved within 2 s of the time of injection, is proportional to the amount of active luciferase over many orders of magnitude (Hastings *et al.*, 1966). By monitoring the amount of active enzyme at various times following initiation of a refolding reaction, we have been able to begin to dissect the overall kinetic mechanism of the folding and assembly processes.

Prior work on the folding of luciferase from urea or guanidinium chloride suggested that the enzyme could be at least partially refolded following denaturation, but the extent of recovery varied significantly between the various reports (Friedland and Hastings, 1967a, 1967b; Hastings *et al.*, 1969; Gunsalus-Miguel *et al.*, 1972; Tu *et al.*, 1977b; Tu, 1978). We have previously reported the isolation of a series of mutants that we have designated temperature-sensitive folding mutants on the basis of the wild-type thermal stability of the folded proteins and the reduced ability of the proteins to fold at elevated temperatures (Sugihara and Baldwin, 1988). Further investigation of these mutants required the development of conditions that would reproducibly give high yields of active enzyme when the wild-type luciferase was refolded upon dilution out of denaturant. The experiments reported here describe simple and reproducible methods for the unfolding of luciferase in urea and the refolding of the active enzyme upon dilution of the urea. Furthermore, these experiments suggest the existence of multiple intermediates on the folding pathway leading to the active heterodimer. In a related series of experiments, we have 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 *et al.*, 1993). It appears likely that this species is one of the intermediates detected in the kinetic experiments reported here.

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 Schwarz-Mann. 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* (LE392) cells carrying the *V. harveyi luxAB* 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 $(mg/ml)^{-1} \cdot cm^{-1}$ (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 $FMNH_2$ photoreduced in a solution containing 2 mM EDTA (Hastings *et al.*, 1978).

Activity Recovery after Dilution of Luciferase from 5 M Urea into Buffer—Luciferase was denatured for 0.5–4.0 h in a 5 M urea buffer containing 50 mM phosphate, 1 mM EDTA, 1 mM DTT, pH 7.0, at 50 × the enzyme concentration desired for the refolding experiment. Refolding was initiated ("time 0") by a 1:50 dilution of the enzyme

from 5 M urea into renaturation buffer (50 mM phosphate, 0.2% BSA, 1 mM EDTA, 1 mM DTT, pH 7.0) at 18 °C. Addition of the enzyme to the buffer resulted in a final urea concentration of 0.1 M. In controls (native enzyme, never denatured), urea was added to the renaturation buffer to yield a final concentration of 0.1 M. Dilutions of enzyme out of urea were performed rapidly, with 20 μl of enzyme in 5 M urea buffer being added to 0.980 ml of renaturation buffer (or 60 μl of enzyme, 5 M urea to 2.94 ml of buffer) on a vortex mixer. We found that these conditions gave the most reproducible results, consistent with the observation of Goldberg *et al.* (1991) that rapid dilution from urea minimizes aggregation that may occur during slow mixing. The samples undergoing renaturation were maintained at 18 °C, and at intervals after initiation of refolding, aliquots (generally 10 μl) were withdrawn for assay. The time t was recorded as the time of dilution of the aliquot of renaturation mixture into 1.0 ml of assay buffer containing 15 μl of a sonicated suspension (0.01% v/v) of *n*-decyl aldehyde in H_2O; approximately 15 s elapsed between the recorded time (dilution into assay buffer) and the actual initiation of the assay by injection of $FMNH_2$.

RESULTS

The mechanism presented in Fig. 1 predicts two effects of protein concentration on the refolding reaction of bacterial luciferase. First, the rate of assembly of the heterodimer would be expected to show a second-order dependence on the concentration of the refolding subunits. Second, the expected yield of the heterodimer would be compromised at low protein concentrations by the competing first-order processes leading to α_x and/or β_x (see Fig. 1). To test these predictions, we investigated conditions for reversible unfolding of luciferase. For unfolding, we employed 5 M urea in 50 mM phosphate buffer, 1 mM EDTA. 1 mM DTT, pH 7.0, at 18–20 °C. Under these conditions, the unfolding reaction was complete within a few minutes, as shown by the ultraviolet circular dichroism spectrum in Fig. 2. The spectrum of the protein in 5 M urea did not change with time. For all subsequent experiments, the luciferase was unfolded in 5 M urea for at least 30 min prior to initiation of the refolding reaction.

Effect of Protein Concentration on the Final Recovery of Luciferase after Refolding from 5 M Urea—The optimal concentration of protein for reversible refolding was determined by investigation of the effect of concentration on the yield of active enzyme (Fig. 3). At low protein concentrations (<1 μg/ml), the control samples appeared to be unstable unless BSA was included in the renaturation buffer. BSA was included in the refolding buffers in the earlier studies on luciferase refolding, and we found that addition of BSA at 0.2% resulted in a dramatic stabilization of the activity of the controls at lower protein concentrations with no effect on the activity of controls or percent recovery of the refolded enzyme at higher protein concentrations (data not shown). We therefore included 0.2% BSA in the renaturation buffer for this experiment and all subsequent experiments. As predicted by the model in Fig. 1, the final yield of active enzyme was significantly reduced at low protein concentrations. Maximal yields of 75–90% were observed at 20–50 μg/ml, whereas the yield at 1 μg/ml was about 40%.

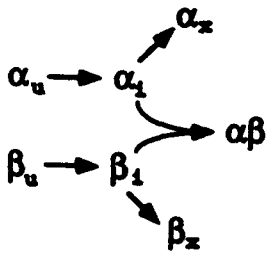

FIG. 1. **Initial model for folding and assembly of luciferase *in vivo* (adapted from Waddle *et al.* (1987)).** α_u and β_u are new partially or completely synthesized subunits, α_i and β_i represent partially folded intermediates, α_x and β_x represent folded conformations incompetent to form heterodimers, and $\alpha\beta$ is the active heterodimeric enzyme.

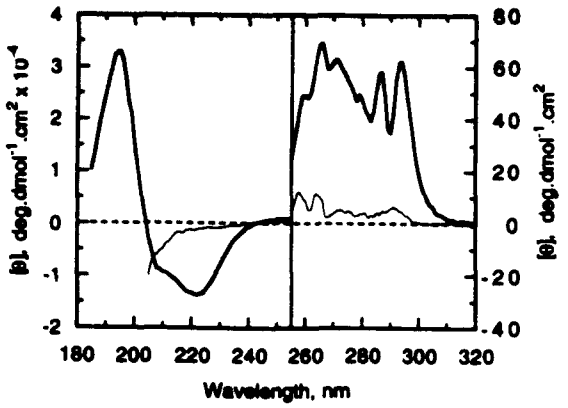

FIG. 2. **Ultraviolet circular dichroism spectrum of luciferase in buffer and in 5 M urea.** The CD spectrum of 25 μg/ml luciferase at 18 °C is shown under native conditions (*heavy line*; 50 mM phosphate, 1 mM EDTA, 1 mM DTT, 0.1 M urea) and after several minutes in the same buffer but 5 M in urea (*thin line*).

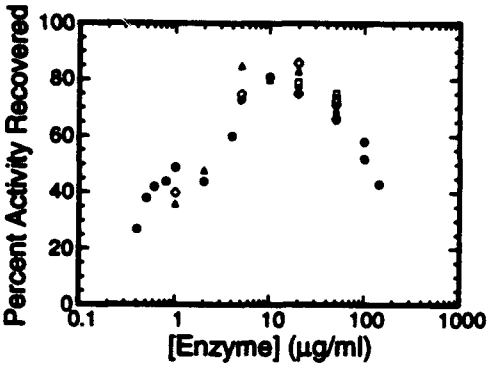

FIG. 3. **Effect of luciferase concentration on final yield of active enzyme following dilution from 5 M urea.** Luciferase at the concentration indicated was permitted to refold for 24 h at 18 °C after rapid 50-fold dilution from 5 M urea into renaturation buffer (50 mM phosphate, 0.2% BSA, 1 mM EDTA, 1 mM DTT, pH 7.0) (final concentration, 0.1 M urea). The different *symbols* represent the yields obtained in different experiments. Percent recovery is expressed relative to the activity of a native control sample at each concentration diluted into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

At protein concentrations above 50 μg/ml, the percent yield was compromised, presumably due to aggregation, a phenomenon that has been reported for other proteins (London *et al.*, 1974; Orsini and Goldberg, 1978; Zettlmeissl *et al.*, 1979; Mitraki *et al.*, 1987) and attributed to intermolecular interactions of folding intermediates (Goldberg and Zetina, 1980; Goldberg, 1985; Mitraki and King, 1989). We have not further investigated the cause for the reduced yield at higher protein concentrations, but we have limited the conditions of our experiments to protein concentrations of 50 μg/ml and below.

Effect of Protein Concentration on the Rate of Formation of Active Enzyme—The time course of formation of active enzyme following dilution from 5 M urea for a series of luciferase concentrations is presented in Fig. 4*A*, with earlier times expanded in *panels B* and *C*. These data demonstrate four aspects of the concentration dependence of the refolding process. First, the yield of active enzyme was reduced at both low and high protein concentrations (Figs. 4*A* and 3). Second, the refolding reaction showed a definite lag at early times that

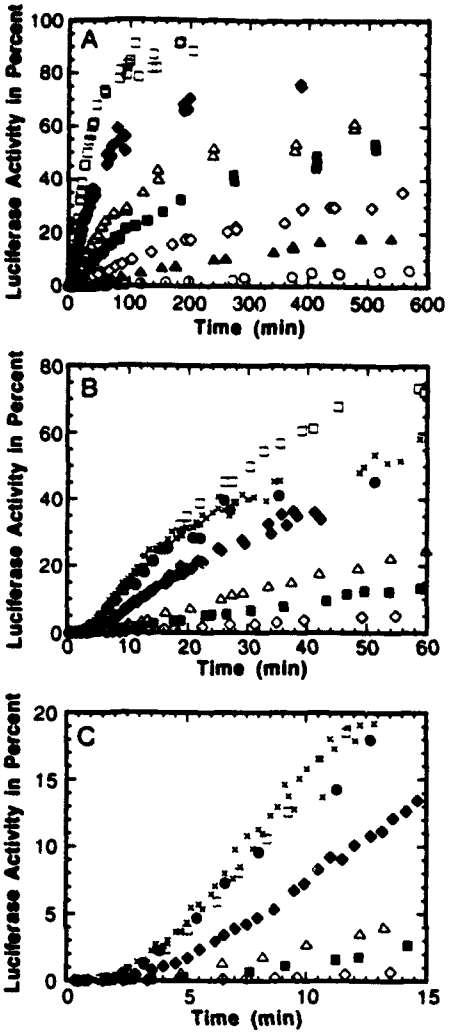

FIG. 4. **Effect of luciferase concentration on rate and extent of recovery of active enzyme.** The enzyme was denatured in 5 M urea, and after initiation of refolding by rapid 50-fold dilution of the enzyme into renaturation buffer, the time course of formation of active luciferase was monitored by removal of aliquots for assay (see "Experimental Procedures"). The complete time course is shown in *panel A*, the first 60 min are expanded in *panel B*, and the initial 15 min are expanded in *panel C*. Protein concentrations in the refolding mixtures were 0.2 (○), 0.4 (▲), 0.8 (◇), 2.0 (■), 4.0 (△), 10 (◆), 20 (□), 50 (×), and 100 (●) μg/ml. Percent recovery is expressed relative to the activity of a native control sample at each concentration diluted into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

was comparatively independent of concentration (Fig. 4, *B* and *C*), indicating the existence of folding intermediate(s) whose formation involved first-order processes, *i.e.* partial folding of the individual subunits prior to formation of the heterodimeric form required for high specific activity. Third, from low protein concentrations up to about 10 μg/ml, the rate of formation of the active form of the enzyme was strongly dependent on the concentration of the refolding subunits, as would be expected if the rate-determining step were a second-order process (interaction between the partially folded α and β subunits). Fourth, at concentrations of 20 μg/ml and above, the rate of refolding into the active form appeared to be concentration-independent. Interpretation of this observation was complicated by the fact that at higher concentrations, the initial rate (following the lag) was rapid, but the reaction appeared to terminate prematurely, compromising the final yield (Figs. 3 and 4).

The saturation in the rate of refolding at high protein concentrations was not predicted by the model presented in Fig. 1. One explanation for the observed saturation in rate at high protein concentrations is that at high concentrations some first-order process becomes rate-limiting. If the initial product of the subunit association reaction were inactive, requiring additional first-order folding steps to become active $\alpha\beta$, then the maximum observed rate of recovery of activity would be limited by the rate of the first-order process at high protein concentrations. Alternatively, the apparent saturation could be due to limiting of the observed rate by higher order competing processes such as aggregation that become significant only at the higher concentrations. To distinguish these possibilities, we performed refolding experiments at a concentration that gave the maximal rate (50 μg/ml) and, 6 min after initiation of the refolding reaction, diluted the protein 10-fold, conditions under which the rate should be much slower and strongly concentration-dependent (see Fig. 4). As shown in Fig. 5, upon dilution of the refolding mixture from 50 to 5 μg/ml, the rate did not decrease immediately to the rate expected for the lower concentration, but rather continued at the same (maximal) rate for 2–3 min before changing to the slower rate. Similar results (not shown) were obtained when secondary dilutions were performed 4 or 8 min after initiation of the refolding reaction. These results suggest that at the time of dilution, there exists a subpopulation of luciferase molecules that have already formed heterodimer, but have not yet become active.

DISCUSSION

Since the classic experiments of Anfinsen and his colleagues on ribonuclease (Anfinsen, 1973), it has been generally agreed that the information that dictates the folding of a polypeptide into its native, biologically active structure is resident in the sequence of amino acids that comprise the polypeptide, and thus the process of protein folding has been referred to as "the second translation of the genetic message" (Goldberg, 1985). It has been an article of faith among many investigators in the field of protein folding that the native structure of a protein is at a global energy minimum. However, our previously reported investigations of the folding *in vivo* of the

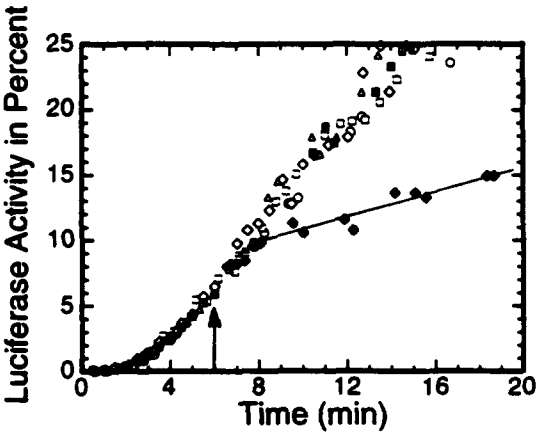

FIG. 5. **Secondary 10-fold dilution of luciferase during refolding.** Luciferase was diluted 50-fold from 2.5 mg/ml in 5 M urea to 50 μg/ml in renaturation buffer (0.1 M urea) at time 0, and after 6 min of refolding, an aliquot was diluted 10-fold into recovery buffer (again 0.1 M in urea) to yield 5 μg/ml luciferase. The time course of activity recovery in several replicate original samples (*open symbols*) and in the secondary dilution (◆) was monitored by removal of aliquots for assay. Activity is expressed as percent of a native control sample at 50 μg/ml in renaturation buffer, 0.1 M in urea; the activities in the diluted sample were multiplied by 10 to correct for the dilution.

subunits of luciferase led us to question the latter dogma (Waddle *et al.*, 1987; Sugihara and Baldwin, 1988). The separate luciferase subunits, α and β, appear to fold *in vivo* into structures that do not interact to form active luciferase when mixed *in vitro* unless they are first unfolded in urea-containing buffers. These observations led us to conclude that the active heterodimeric enzyme was not at a global energy minimum, but rather constituted a kinetic trap, and that if the subunits did not associate during folding, they ultimately achieved stable structures that were assembly-incompetent (Waddle *et al.*, 1987). The issue of whether the native structure of a protein is at a global energy minimum has been the subject of some controversy in recent years, and recent reviewers have been careful to point out that the folded structure must be the thermodynamically most stable state that is *kinetically accessible* (Goldberg, 1985; Kim and Baldwin, 1990; Jaenicke, 1991a, 1991b) and not necessarily at a global energy minimum.

Earlier examples of competing off-pathway folding processes in other systems, such as the tail spike protein of bacteriophage P22 (Mitraki and King, 1989) and denatured-reduced egg white lysozyme (Goldberg *et al.*, 1991), generally involved aggregation of intermediates. The luciferase subunits, however, did not aggregate but rather folded into soluble structures (Waddle *et al.*, 1987; Sugihara and Baldwin, 1988; Waddle and Baldwin, 1991). More recently, other examples of proteins with kinetically controlled folding processes (α-lytic protease and the serine protease inhibitors antithrombin and plasminogen activator inhibitor-1) have been reported (Baker *et al.*, 1992; Carrell *et al.*, 1991; Mottonen *et al.*, 1992).

The experiments reported here were designed to begin the process of dissecting the overall kinetic mechanism of the folding and assembly of the subunits of bacterial luciferase. The kinetic features of the refolding reaction that were predicted by the model advanced by Waddle *et al.* (1987) (Fig. 1) were confirmed in these experiments. First, at low protein concentrations, the yield of active heterodimeric enzyme was reduced, due to the alternative (off-pathway) first-order folding processes available to the individual subunits. Second, a marked, protein concentration-independent lag in recovery of activity was observed, suggestive of first-order folding steps for one or both subunits prior to assembly into the heterodimer. Further investigation of the cause of this lag has shown it to be due to slow steps in the folding of both the α and the β subunits prior to the step in which heterodimer is formed (Baldwin *et al.*, 1993). Third, the rate of formation of the active heterodimeric enzyme after the lag was strongly concentration-dependent, as expected for a second-order process. At the highest protein concentrations investigated (100–144 μg/ml), the reduced yield of active enzyme (Fig. 3) suggests that there may be aggregation occurring as well.

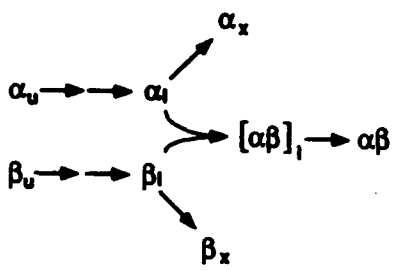

FIG. 6. **Revised model for folding of luciferase subunits, assembly into the heterodimer, and isomerization to the active enzyme.** α_u and β_u are unfolded subunits; α_i and β_i represent partially folded intermediates that are competent to interact, forming a heterodimer if both are present; α_x and β_x represent folded conformations incompetent to form heterodimers; $[\alpha\beta]_i$ represents inactive heterodimeric intermediate; and $\alpha\beta$ is the active heterodimer. Although there is presently no direct evidence for intermediate forms of the subunits between the fully unfolded forms and the species (α_i and β_i) competent to form heterodimer, the conversion is surely not a single-step process.

The results presented here also suggested a new feature of the refolding reaction not shown in the original model, the apparent saturation in the rate of recovery of active enzyme at higher protein concentrations (Fig. 4). Below 10 μg/ml, the rate of formation of active enzyme after the initial lag appeared to be determined by the second-order dimerization process; at 20 μg/ml and above, the rate appeared to be limited either by the first-order isomerization of inactive heterodimer to form active heterodimer or by competing higher order aggregation processes that would become significant only at higher concentrations, leading to an apparent limit to the rate of formation of active enzyme. The results of the secondary dilution experiment presented in Fig. 5 permitted us to postulate the intermediacy of an inactive heterodimeric species, $[\alpha\beta]_i$, which is converted to active enzyme by one or more (first-order) isomerization steps. By switching from conditions (50 μg/ml) under which the rate was presumably limited by the isomerization of the inactive heterodimer to conditions (5 μg/ml) under which the rate was limited by the second-order assembly step, we were able to monitor directly the conversion of the inactive heterodimer to the active heterodimer (Fig. 5). We have incorporated both first-order folding steps for the individual subunits and an inactive heterodimeric intermediate into a revised scheme for the pathway of folding and assembly of the luciferase subunits, presented in Fig. 6.

We have established conditions (18 °C, 50 mM phosphate at pH 7.0, protein concentrations of 15–50 μg/ml) under which luciferase can refold reproducibly to its active structure in high yield following rapid dilution out of 5 M urea. These methods should allow a more complete examination of the properties of the temperature-sensitive folding mutants described in our earlier report (Sugihara and Baldwin, 1988). Based on the results of the experiments reported here, we conclude the following.

1) Refolding of the α and β subunits of bacterial luciferase occurs by a multistep process involving intermediates both preceding and following assembly of the heterodimer.

2) The encounter complex between the two subunits, involving intermediates on the pathway of folding of the individual subunits, is inactive; formation of the active structure requires one or more subsequent isomerization steps.

3) At low protein concentrations, the yield of active heterodimer is compromised by competing first-order folding processes involving folding of one or both individual subunits into structures incompetent to form heterodimer, as predicted by Waddle *et al.* (1987).

These results support our earlier hypothesis that the formation of the active heterodimeric luciferase is a kinetically controlled process. Under conditions that limit the ability of the intermediate α_i and β_i structures to associate, the individual subunits appear to assume thermodynamically stable structures (α_x and/or β_x) incompetent to interact with each other, by processes that are experimentally irreversible on a time scale of days (Waddle, 1990; Sinclair *et al.*, 1993). This interpretation suggests that the biologically active heterodimeric structure of native luciferase may reside at a local energy minimum with a lifetime, determined by high activation energies of interconversion, that is meaningful on a biological time scale, rather than at the global energy minimum that would prevail on a geological time scale.

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

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Thomas O. Baldwin‡§¶‖, Miriam M. Ziegler§¶**, Alain F. Chaffotte‡¶**, and Michel E. Goldberg¶

From the ‡Center for Macromolecular Design of the Institute of Biosciences and Technology and the §Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843 and the ¶Unité de Biochimie Cellulaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

Bacterial luciferase is an $\alpha\beta$ heterodimer with a single active center in which the reaction of reduced FMN, O_2, 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 intermediacy of several species, one of which is an inactive heterodimeric structure, resulting from the dimerization of α and β, which isomerizes to the active $\alpha\beta$ 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 reformation 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. These 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 $FMNH_2$[1] and a long chain aliphatic aldehyde by molecular oxygen, producing FMN, the corresponding chain length fatty acid, and (presumably) H_2O, with the emission of blue-green light (see Baldwin and Ziegler (1992) for a recent review). The enzyme, an $\alpha\beta$ 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

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** Present address: Dept. of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

[1] The abbreviations used are: $FMNH_2$, reduced flavin mononucleotide; DTT, dithiothreitol.

alternative structures that do not interact. Furthermore, the final structures that the subunits assume do not appear to be in equilibrium with the structures that are capable of interacting to form heterodimer. That is, we have suggested that the native form of the luciferase enzyme constitutes a kinetic trap for the folding subunits (Waddle *et al.*, 1987; Sugihara 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 were 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 can fold 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.

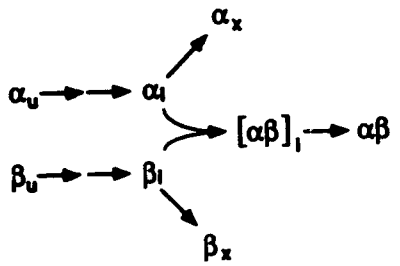

FIG. 1. **Model for folding of luciferase subunits, assembly into the heterodimer, and isomerization to the active enzyme.** α_u and β_u are unfolded subunits; α_i and β_i represent partially folded intermediates that are competent to interact, forming a heterodimer if both are present; α_x and β_x represent folded conformations incompetent to form heterodimers; $[\alpha\beta]_i$ represents inactive heterodimeric intermediate; and $\alpha\beta$ is the active heterodimer (from Ziegler *et al.*, 1993).

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 Schwarz-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* (LE392) cells carrying the *V. harveyi luxAB* 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 $(mg/ml)^{-1} \cdot cm^{-1}$ (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 $FMNH_2$ 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 (85 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 280 nm, using the extinction coefficients for denatured subunits determined by Waddle (1990), which are very similar to those determined by Gunsalus-Miguel *et al.* (1972): E for α = 1.23 $(mg/ml)^{-1} \cdot cm^{-1}$ and for β = 0.72 $(mg/ml)^{-1} \cdot cm^{-1}$.

Refolding 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 1:100 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 H_2O, followed approximately 12–15 s later by initiation of the assay by injection of $FMNH_2$. 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 various 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 Fig. 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 I), as predicted from the model presented in Fig. 1.

An additional observation apparent in Fig. 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

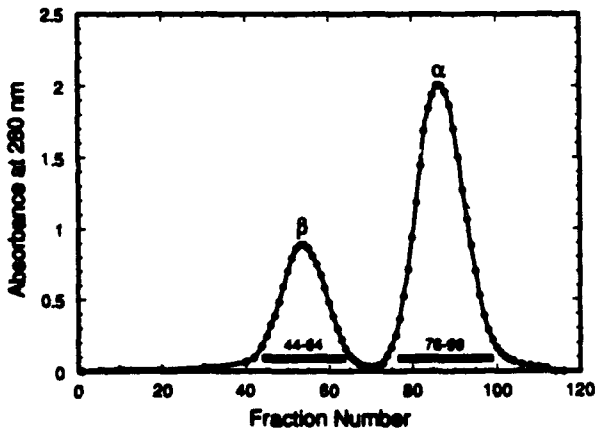

FIG. 2. **Separation of luciferase subunits by DEAE-Sephadex A-50 column chromatography in 5 M urea.** Luciferase purified from *E. coli* was denatured in 5 M urea, and the α and β subunits were resolved by a phosphate gradient in 5 M urea as described under "Experimental Procedures."

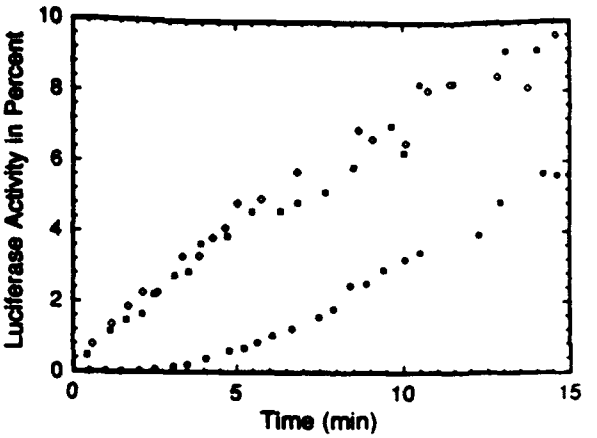

FIG. 3. **Time course of recovery of luciferase activity following refolding of the individual subunits prior to mixing.** The α and β subunits (each 1.0 mg/ml in 5 M urea) were permitted to refold separately after 1:50 dilution into renaturation buffer (see "Experimental Procedures"), for 30 min (■) or 60 min ( ) prior to mixing. The subunit concentration during the separate refolding was 20 μg/ml, and the final urea concentration in each refolding mixture was 0.1 M. At time 0 (30 or 60 min after dilution from 5 M urea), equal volumes of the solutions of the two refolding subunits were mixed, so that the final protein concentrations were 10 μg/ml α and 10 μg/ml β, or 20 μg/ml total, and aliquots were removed periodically for activity assay. In the control (●), equal volumes of the 1 mg/ml subunits in 5 M urea were mixed prior to dilution, and the mixture was diluted 1:50 (to 20 μg/ml final protein concentration) into renaturation buffer at time 0 to initiate refolding. Percent recovery is expressed relative to the activity of a native sample diluted to 20 μg/ml into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

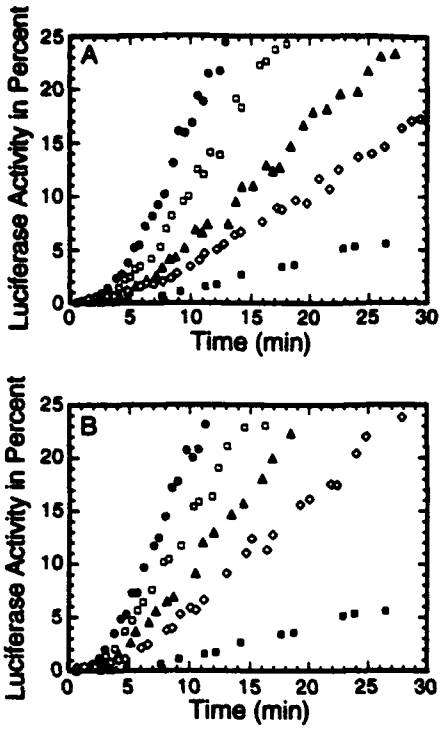

FIG. 4. **Time course of recovery of luciferase activity following dilution from 5 M urea in the presence of excess α subunit (*panel A*) or β subunit (*panel B*).** Luciferase (200 μg/ml) in 5 M urea was mixed with an equal volume of α subunit in 5 M urea or of β subunit in 5 M urea (or of 5 M urea buffer alone if no subunit was to be added), and at time 0, refolding was initiated by a 1:50 dilution of the mixture from 5 M urea into renaturation buffer (see "Experimental Procedures"). The final luciferase concentration in the refolding mixture was thus 2 μg/ml (1 μg/ml α and 1 μg/ml β) alone (■), 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

Relative yield of active luciferase after renaturation in the presence of excess α or β subunit

Luciferase sample	Relative yield*
2 μg/ml luciferase alone	1.0
+ 5 μg/ml α subunit	1.4
+ 10 μg/ml α subunit	1.3
+ 20 μg/ml α subunit	1.6
+ 36.6 μg/ml α subunit	1.5
+ 5 μg/ml β subunit	1.8
+ 10 μg/ml β subunit	1.7
+ 20 μg/ml β subunit	1.9
+ 38.2 μg/ml β subunit	1.9

* 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.

a reduction in the duration of the lag, suggesting that the α subunit refolds more slowly than does the β subunit.

The final prediction that we explored in these studies was the issue of the folding of the individual subunits into structures that do not interact with each other. The model shown in Fig. 1 is based on two observations. First, if the two subunits are synthesized independently in *E. coli*, one or the other or both fold into structures that do not assemble into active

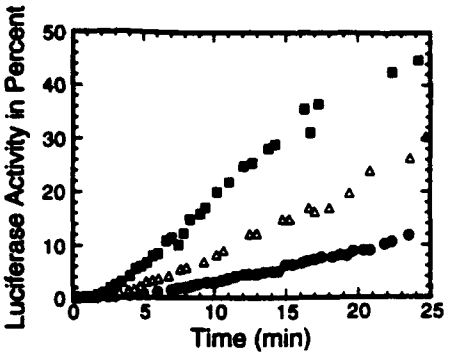

FIG. 5. **Time course of recovery of luciferase activity in the presence of excess α or β subunit that had been allowed to refold for 4 min prior to the addition of luciferase from 5 M urea.** The individual α or β subunit (2 mg/ml in 5 M urea) was diluted 1:100 into renaturation buffer. After "prefolding" of each subunit alone for 4 min at 18 °C, luciferase in 5 M urea was added (time 0) to each prefolding subunit solution such that the final luciferase concentration was 5 μg/ml in the presence of 20 μg/ml excess prefolded α (■) or β (△) subunit, and the final urea concentration was 0.1 M. In the control (●), luciferase was permitted to refold at 5 μg/ml after dilution from 5 M urea in the absence of prefolded subunits. Percent recovery is expressed relative to the activity of a native sample diluted to 5 μg/ml into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time. Final yields in this experiment (relative to the activity of the native sample at 5 μg/ml) were 73% for renatured luciferase alone and 77% for luciferase plus either subunit in excess.

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

TABLE II

Refolding both subunits separately prior to mixing

Effect on the final yield of active enzyme. The experiment was performed as described in the legend to Fig. 3, with the subunits being permitted to refold separately at 20 μg/ml for various periods of time prior to mixing. The final protein concentration following mixing was 20 μg/ml, 10 μg/ml of each subunit.

Duration of refolding prior to mixing	% yield[a]
0 min	50
30 min	50
60 min	45
90 min	40
167 min	40
21 h	6

[a] 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 at 20 μg/ml recovered about 80% of the control activity after 21 h at 18 °C (Ziegler *et al.*, 1993).

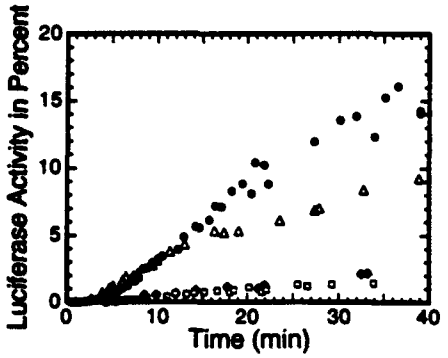

FIG. 6. **Time course of recovery of luciferase activity following refolding of one subunit for 24 h prior to dilution of the other subunit from 5 M urea.** The α and β subunits (each 1.0 mg/ml in 5 M urea) were permitted to refold separately upon 1:50 dilution into renaturation buffer (see "Experimental Procedures"), the urea concentration being 0.1 M, for 24 h prior to addition of the other subunit from 5 M urea. After 24 h of prefolding, 0.5 ml of the refolded 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 other subunit (in 5 M urea) was 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 unfolded β (Δ), or 10 μg/ml unfolded α and 10 μg/ml refolded β (□). 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 (◆) (as described in 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 μg/ml into the same renaturation buffer, 0.1 M in urea, and incubated for the same period of time.

α subunit is mixed with unfolded 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 heterodimer assembly-incompetent conformation when permitted to refold for 24 h in the absence of the β subunit.

TABLE III

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

Effect on final yield of active enzyme

Sample	% yield[a]
$\alpha_{urea} + \beta_{urea}$	50[b]
$\alpha_{refolded} + \beta_{urea}$	26[c]
$\alpha_{urea} + \beta_{refolded}$	5[c]
$\alpha_{refolded} + \beta_{refolded}$	6[d]

[a] 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).

[b] The subunits in 5 M urea were mixed prior to 1:50 dilution of the mixture into renaturation buffer.

[c] Either the α or the β subunit was permitted to refold for 24 h prior to the addition of the second subunit by dilution from 5 M urea, as described in the legend to Fig. 6.

[d] Both subunits were permitted to refold separately for 21 h prior to mixing, as in the experiment described in the legend to Fig. 3.

DISCUSSION

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 (α_u and β_u) and the heterodimer assembly-competent subunit species (α_i and β_i), which can either fold on to form the heterodimer assembly-incompetent species (α_x and β_x) or associate to form heterodimeric species, the inactive heterodimer ($[\alpha\beta]_i$), and the active heterodimer ($\alpha\beta$). 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,[2] but we observed a prolonged lag of 3–4 min between the time of dilution of the subunits from

[2] T. O. Baldwin, M. M. Ziegler, A. F. Chaffotte, and M. E. Goldberg, unpublished results.

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 (α_i and β_i). 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 α subunit to refold from urea for 4 min prior to mixing with unfolded β subunit, we observed a somewhat shorter lag (Fig. 5). The converse experiment, in which we allowed the β subunit to refold briefly prior to mixing with unfolded α 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 α subunit, but that although the β subunit appeared to fold faster than the α 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 α subunit with unfolded β subunit resulted in some reduction in yield of $\alpha\beta$ relative to the yield observed when unfolded α was mixed with unfolded β. This observation suggests that the α_i species may be very slowly converted to α_x, 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 β subunit appeared to convert to the β_x species slowly as well but significantly faster than the α 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 β subunit was allowed to refold for 24 h prior to mixing with either unfolded or refolded α, very little $\alpha\beta$ was formed, indicating that about 90% of the β subunit had been converted to the β_x species. These observations suggest that the half-time for the $\beta_i \rightarrow \beta_x$ 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 α subunit of *V. harveyi* (then called "MAV") luciferase to interact with β and only about a 32% decrease in the ability of the refolded β subunit to interact with α. Presumably, the apparent difference in the rates of the $\alpha_i \rightarrow \alpha_x$ and $\beta_i \rightarrow \beta_x$ 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 18 °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, α_x and β_x, 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; Ohta *et al.*, 1991) and the α-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 α-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 to an active conformation, but then slowly is converted into an inactive hyperstable species, apparently through the insertion of a stretch of polypeptide into a β sheet to yield a structure of enhanced stability (Carrell *et al.*, 1991; Mottonen *et al.*, 1992). The β 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 β subunit is similar to the proteases as well, in that it appears to be the interaction with the α subunit that converts it into the biologically active form.

We have separated the α and β 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 β is faster than the rate of refolding of α. 3) The rates of formation of the heterodimerization-incompetent species, α_x and β_x, are indeed quite slow, with half-times of hours. 4) The heterodimerization-incompetent species that form *in vivo* (observed by Waddle *et al.*(1987)) also form upon refolding *in vitro*.

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Folding of Bacterial Luciferase Involves a Non-native Heterodimeric Intermediate in Equilibrium with the Native Enzyme and the Unfolded Subunits*

(Received for publication, October 22, 1992, and in revised form, December 21, 1992)

A. Clay Clark‡, James F. Sinclair‡, and Thomas O. Baldwin‡§¶‖

From the §Center for Macromolecular Design of the Institute of Biosciences and Technology and the Departments of ‡Biochemistry and Biophysics and ¶Chemistry, Texas A&M University, College Station, Texas 77843

Bacterial luciferase is a heterodimeric enzyme that catalyzes the reaction of reduced FMN, O_2 and an aliphatic aldehyde to yield the carboxylic acid and an excited flavin that emits blue-green light upon return to ground state. The two subunits of the luciferase from *Vibrio harveyi*, α and β, have molecular weights of 40,108 and 36,349, respectively; the single active center resides primarily, if not exclusively, on the α subunit.

We have found that bacterial luciferase can be unfolded in urea-containing 50 mM phosphate buffer, pH 7.0, and refolded by dilution of the urea with final luciferase concentrations of 5–25 μg/ml. We have analyzed the urea-induced equilibrium unfolding of bacterial luciferase by monitoring changes in both the far ultraviolet circular dichroism (predominantly secondary structure) and intrinsic fluorescence emission (predominantly tertiary structure) resulting from incubation in various concentrations of urea at 18 °C for 18–24 h. Both spectral methods indicated a biphasic unfolding transition; the first phase was protein concentration-independent, whereas the second phase was protein concentration-dependent. Equilibrium unfolding curves showed an increase in fluorescence up to 2 M urea followed by a decrease in intensity and red shift of the emission maximum. The ratio of the fluorescence emission in the presence of 2 M urea relative to that in the absence of urea was greater when fluorescence was excited at 295 nm than at 280 nm. The fluorescence increase in the 0–2 M urea range corresponded to the first phase of the biphasic unfolding process. The urea-induced loss of luciferase enzymatic activity appeared to correspond to the first transition observed with the spectroscopic methods, and likewise to be protein concentration-independent. These observations suggested a three-state unfolding mechanism in which the native heterodimeric enzyme rearranges to an inactive heterodimeric species that is well populated, followed by dissociation and unfolding of the α and β subunits. The data were fit to a three-state mechanism using a nonlinear least squares method. At 18 °C in 50 mM phosphate, pH 7.0, the free energy change for the interconversion of the active heterodimer and the inactive heterodimeric species was estimated to be 4.52 ± 0.30 kcal/mol; the free energy change for the interconversion of the inactive heterodimer and the individual subunits was 19.7 ± 0.2 kcal/mol. These measurements demonstrate that the equilibrium unfolding of bacterial luciferase proceeds through a well populated inactive heterodimeric species that appears to be partially unfolded and are consistent with the observation (Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F., and Baldwin, T. O. (1993) *J. Biol. Chem.* 268, 10760–10765) of an inactive heterodimeric intermediate that forms from the individual subunits and precedes the active heterodimeric enzyme on the refolding pathway.

Extensive investigations of many small globular proteins have yielded detailed information concerning the thermodynamics and kinetics of folding (see Creighton (1990), Dill (1990), Kim and Baldwin (1990), and Jaenicke (1991), for recent reviews). Most proteins are only marginally stable, and in many cases, folding can be approximated by a two-state model in which only the native or the unfolded protein can be found in significant quantities (Pace *et al.*, 1991; Shortle *et al.*, 1990; Serrano *et al.*, 1990; Chen and Schellman, 1989; Pakula and Sauer, 1989; Bowie and Sauer, 1989). However, intermediates on the folding pathway have been detected for both small and large polypeptides (Fuchs *et al.*, 1991; Hughson *et al.*, 1991; Zetina and Goldberg, 1980; Hurle *et al.*, 1987; Touchette *et al.*, 1986; Tandon and Horowitz, 1986; Borden and Richards, 1990). If the long term goal of protein folding studies is the prediction of the tertiary structure of a protein from the primary sequence, then detailed information concerning the existence and structures of even transient intermediates is crucial and generally lacking. Models based mainly on studies of small globular proteins may not fully explain the folding and assembly of larger multidomain or multisubunit proteins.

For these and related reasons, we have begun to study the folding of bacterial luciferase, a heterodimer consisting of two dissimilar subunits, α and β, with molecular weights of 40,108 and 36,349, respectively, for the subunits of the enzyme from *Vibrio harveyi* (Cohn *et al.*, 1985; Johnston *et al.*, 1986). Bacterial luciferase is a flavin monooxygenase that yields a quantum of light as a product of the enzymatic reaction (for reviews of the system, see Ziegler and Baldwin (1981) and Baldwin and Ziegler (1992)). The overall reaction is $FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light$, where $FMNH_2$ is reduced flavin mononucleotide, RCHO is long chain aldehyde, typically *n*-decyl aldehyde, and RCOOH is

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‖ To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

the corresponding long chain fatty acid. Measuring the intensity of emitted light allows rapid and sensitive analysis of enzymatic activity over a wide range of protein concentrations. This assay has been used extensively to monitor production of active luciferase *in vivo* and *in vitro* (Baldwin and Ziegler, 1992). The luciferase assay is uniquely sensitive and well suited to such investigations.

The genes encoding the α and β subunits of bacterial luciferase from *V. harveyi* have been cloned and expressed in *E. coli* (Cohn *et al.*, 1983; Baldwin *et al.*, 1984; Belas *et al.*, 1982), and the amino acid sequences of the subunits are known (Cohn *et al.*, 1985; Johnston *et al.*, 1986). Since bacterial luciferase is a heterodimer and the genes encoding the α and β subunits (*luxA* and *luxB*, respectively) may be expressed together or individually, the products of folding of the individual subunits may be studied in the absence of formation of the heterodimer (Waddle *et al.*, 1987; Waddle and Baldwin, 1991; Sinclair *et al.*, 1993). The individual subunits and the heterodimer have been overexpressed and purified in large quantities (Baldwin *et al.*, 1989; Sinclair *et al.*, 1993), and the system is amenable to mutagenesis (Baldwin *et al.*, 1989; Sugihara and Baldwin, 1988; Chen and Baldwin, 1989; Chlumsky *et al.*, 1991). There is no posttranslational modification of the luciferase subunits, and the enzyme is not prone to aggregation (Baldwin and Ziegler, 1992). Because of these features, bacterial luciferase appears to be an ideal protein with which to study the basic properties of the folding and assembly of multisubunit enzymes.

By measuring the kinetics of the overall refolding reaction by which subunits diluted from 5 M urea refold and assemble into the active heterodimeric enzyme, we have shown that on a time scale of a few minutes, the individual subunits assume conformations that are competent for heterodimer formation (Ziegler *et al.*, 1993; Baldwin *et al.*, 1993). At low concentrations of subunits, heterodimer assembly appears to be rate-determining, whereas at higher protein concentrations, the rate-determining step is independent of protein concentration and appears to comprise a rearrangement of an inactive heterodimeric species into the active $\alpha\beta$ structure. The results of these studies confirmed our earlier proposal, based on luciferase subunit folding *in vivo* (Waddle *et al.*, 1987), that the luciferase subunits interact as partially folded intermediates to form the heterodimer; subunits that do not interact (or cannot interact due to their synthesis in different cells) fold into stable structures that cannot interact upon mixing, even with prolonged incubation.

In this paper, we describe the urea-induced unfolding of bacterial luciferase monitored at equilibrium using enzyme activity and spectroscopic probes that are sensitive to protein secondary and tertiary structure. Bacterial luciferase contains 8 tryptophanyl residues, 6 in the α subunit and 2 in the β subunit (Cohn *et al.*, 1985; Johnston *et al.*, 1986). We have monitored fluorescence emission with excitation either at 295 nm, which excites primarily tryptophanyl residues, or at 280 nm, which allows excitation of both tyrosinyl and tryptophanyl residues. We have also monitored circular dichroism at 222 nm and enzymatic activity over a range of protein concentration. The data presented demonstrate that the unfolding of bacterial luciferase occurs by a multistep process that includes as a minimum an inactive heterodimeric species as a folding intermediate that is well populated at equilibrium between 1.6 and 2.8 M urea.

EXPERIMENTAL PROCEDURES

Materials—Ultra-Pure urea was purchased from Schwarz/Mann. Dithiothreitol and Tween 20[1] were obtained from Boehringer Mannheim, and NaH_2PO_4 and K_2HPO_4 were from J. T. Baker. All other chemicals were reagent-grade. Bacterial luciferase was isolated from *E. coli* LE392 carrying the plasmid pLAV1 as described previously (Baldwin *et al.*, 1989) and was judged greater than 95% pure by SDS-polyacrylamide gel electrophoresis analysis (Laemmli, 1970).

Stock Solutions—Urea stock solutions (10 M), prepared as described previously (Pace *et al.*, 1989) in a bufter of 50 mM NaH_2PO_4/K_2HPO_4, pH 7.0 (±0.02), 1 mM dithiothreitol, and 0.005% Tween 20, were prepared daily for each experiment and filtered (0.22-μm pore) prior to use. The urea concentration of each stock solution was calculated by weight and by refractive index (Pace *et al.*, 1989), and solutions were used only if these two values differed by less than 1%. This buffer solution without urea is referred to below as "phosphate buffer."

Equilibrium Unfolding Curves—All equilibrium unfolding experiments in urea were performed as described by Pace *et al.* (1989). Briefly, stock protein solutions were prepared in phosphate buffer to be 10 times the desired final protein concentration. Phosphate buffer, urea from the 10 M stock solution, and 200 μl of stock protein solution to give a final volume of 2 ml were added to 2 ml siliconized Eppendorf tubes (National Scientific Supply). This yielded final urea concentrations of 0–6 M and the final protein concentrations indicated in the figure legends. Each sample was mixed by vortexing and incubated in a water bath at 18 °C for a minimum of 18 h.

For renaturation experiments, protein was denatured in 2 ml siliconized Eppendorf tubes that contained phosphate buffer and 10 M stock urea such that when the protein was added, the final urea concentration was 6 M and the protein concentration was 10 times the desired final concentration used in the experiment. After incubation for 1 h at 18 °C, 200 μl of denatured protein was added to tubes containing phosphate buffer and urea such that the final volume was 2 ml, and the final urea and protein concentrations were as indicated. Each sample was mixed by vortexing and was incubated for a minimum of 18 h at 18 °C.

Fluorescence emission at each denaturant concentration was measured using an SLM-Aminco 8000C spectrofluorometer; the signal was averaged for 50 s. All measurements were corrected for background signal. Circular dichroism was measured using a Jasco J600A spectropolarimeter using either a 5- or 10-mm cell. The CD signal was averaged for 60 s using a program created in the Microsoft Quickbasic environment by J. F. Sinclair. Both instruments were equipped with thermostatted cell holders, and temperature was held constant at 18 °C (±0.1 °C) using a circulating water bath.

Activity Measurements—Bioluminescence activity was measured using the $FMNH_2$ injection assay (Baldwin and Ziegler, 1992; Hastings *et al.*, 1978) and a Turner Designs TD-20e luminometer. Assay vials were maintained at 18 °C using a circulating water bath. Samples for activity measurements in urea-containing buffers were incubated in urea for a minimum of 18 h at 18 °C; a 1-ml aliquot was used for each assay, and a minimum of three assays were performed at each urea concentration.

Data Analysis—Experimental data, collected as described above, could not be reconciled with a simple two-state mechanism. We therefore developed a three-state treatment with which all of the data were satisfactorily analyzed. In developing this mechanism, several assumptions were made. First, we assumed a single heterodimeric intermediate in equilibrium with the native enzyme and the unfolded subunits. That is, we assumed the three-state mechanism

$$[\alpha\beta]_N \overset{K_1}{\rightleftarrows} [\alpha\beta]_I \overset{K_2}{\rightleftarrows} \alpha + \beta \qquad \text{(Eq. 1)}$$

in which the protein is assumed to be in either the native heterodimeric state (N), a non-native dimeric state (I), or in the unfolded monomeric state ($\alpha + \beta$). If we set the molar concentration of the native heterodimer $[\alpha\beta]_N = [N]_T$ when all the protein is native, we may define the mole fraction of each species as

$$f_N = \frac{[N]}{[N]_T} \qquad \text{(Eq. 2)}$$

$$f_I = \frac{[I]}{[N]_T} \qquad \text{(Eq. 3)}$$

$$f_S = \frac{[\alpha]}{[N]_T} = \frac{[\beta]}{[N]_T} = f_\alpha = f_\beta \qquad \text{(Eq. 4)}$$

where f_N = mole fraction in the native state. f_I = mole fraction in the

[1] The abbreviation used is: Tween 20, poly(oxyethylene)$_{20}$-sorbitan monolaurate.

intermediate heterodimeric state, and f_S = mole fraction in the subunit state. Note that

$$f_N + f_I + f_S = 1 \quad \text{(Eq. 5)}$$

The equilibrium constants, K_1 and K_2, are then related to the mole fraction of each species present and the total concentration of protein $[N]_T$ by Equations 6 and 7.

$$K_1 = \frac{f_I}{f_N} \quad \text{(Eq. 6)}$$

and

$$K_2 = \frac{[N]_T f_S^2}{f_I} \quad \text{(Eq. 7)}$$

where f_N, f_I, and f_S represent the fraction of the protein that is in each form at equilibrium. Note that when $[\alpha] = [N]_T = [\beta]$, $f_S = 1$.

By rearranging and combining Equations 5 and 6, the following is obtained.

$$f_I = \frac{K_1(1 - f_S)}{(1 + K_1)} \quad \text{(Eq. 8)}$$

Using Equations 5–8, it is possible to solve for the mole fraction of each species present at equilibrium in terms of the total protein concentration, N_T, and the two equilibrium constants, K_1 and K_2. Substituting Equation 8 into Equation 7 and solving for f_S yields Equation 9.

$$f_S = \frac{-K_1K_2 + \sqrt{(K_1K_2)^2 + 4[N]_T(1 + K_1)(K_1K_2)}}{2[N]_T(1 + K_1)} \quad \text{(Eq. 9)}$$

Substituting Equation 9 into Equation 8 yields the following equation.

$$f_I = K_1 \left| \frac{2[N]_T(1 + K_1) + K_1K_2 - \sqrt{(K_1K_2)^2 + 4[N]_T(1 + K_1)(K_1K_2)}}{2[N]_T(1 + K_1)^2} \right| \quad \text{(Eq. 10)}$$

Finally, substituting Equation 10 into Equation 6 yields Equation 11.

$$f_N = \frac{2[N]_T(1 + K_1) + K_1K_2 - \sqrt{(K_1K_2)^2 + 4[N]_T(1 + K_1)(K_1K_2)}}{2[N]_T(1 + K_1)^2} \quad \text{(Eq. 11)}$$

From Equations 6, 7, and 9–11 and the relationship

$$\Delta G = -RT \ln(K_{eq}) \quad \text{(Eq. 12)}$$

where R is the gas constant and T is the temperature in K, one may calculate the equilibrium constant and the value of ΔG at each urea concentration. We assumed the free energy change for each step in the reaction to be linearly dependent on denaturant concentration as described previously (Thomson *et al.*, 1989) (Equations 13 and 14).

$$\Delta G_1 = \Delta G_1^{H_2O} - m_1[\text{denaturant}] \quad \text{(Eq. 13)}$$

and

$$\Delta G_2 = \Delta G_2^{H_2O} - m_2[\text{denaturant}] \quad \text{(Eq. 14)}$$

where $\Delta G_1^{H_2O}$ and $\Delta G_2^{H_2O}$ are the free energy changes in the absence of denaturant corresponding to steps K_1 and K_2, respectively, and m_1 and m_2 are the cooperativity indices associated with each step. The amplitude of the spectroscopic signal determined at each urea concentration was assumed to be a linear combination of the fractional contribution from each species (Equation 15).

$$Y = Y_N f_N + Y_I f_I + Y_S f_S \quad \text{(Eq. 15)}$$

where Y_N, Y_I, and Y_S are the amplitudes of the signals for the respective species. Note that no distinction was made for the signal from the α and the β subunit. The amplitudes associated with the native and unfolded forms of the protein were assumed to be linearly dependent on urea concentration such that

$$Y_N = Y_{N'} + m_3[\text{urea}] \quad \text{(Eq. 16)}$$

and

$$Y_S = Y_{S'} + m_4[\text{urea}] \quad \text{(Eq. 17)}$$

where $Y_{N'}$ and $Y_{S'}$ are the amplitudes of the signals in the absence of urea for the native and unfolded species, respectively, and m_3 and m_4 are the slopes that describe the dependence of the amplitudes for native and unfolded protein, respectively, on urea concentration.

Nonlinear least squares fitting of the data to these equations employed a Macintosh version of Nonlin (Robelko Software, Carbondale, IL; see Johnson and Frasier (1985)) to determine the eight unknown parameters, $\Delta G_1^{H_2O}$, $\Delta G_2^{H_2O}$, m_1, m_2, m_3, m_4, Y_I, and Y_S, from each unfolding curve. The value of $Y_{N'}$, determined in the absence of urea, was not allowed to vary during the fitting process. Nonlinear least squares fits of measurements of enzyme activity in the presence of urea were done using a simple two-state transition model (Santoro and Bolen, 1988) with the program Kaleidagraph (Synergy Software, Reading, PA).

Error Analysis—Equation 15 (and associated definitions) and the average values of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ from Table I were used to calculate a "perfect" data set. To determine if these parameters, extracted by fitting experimental data to Equation 15, represented a global minimum in the fitting procedure, random error was introduced into the calculated data, and the initial estimates of the four parameters were varied in separate experiments. The data could withstand approximately 15% error without divergence from the original values of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ during the fitting procedure. Likewise, the initial estimates of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ were varied individually and in groups of two or more. These parameters could be varied by about ±7% and the fitting routine still converged on the values of the parameters used to calculate the perfect data. These two tests demonstrated that the average values of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ in Table I represent global minima in the nonlinear least squares fit of the experimental data to Equation 15. To estimate the error in these parameters, error was introduced into the calculated data and the initial estimates of the four parameters simultaneously. Random error of 3, 5, and 7% was introduced into the calculated data set; the residuals of the data in Figs. 2 and 3 fit to Equation 15 showed about 4% variance (not shown). The initial estimates of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ were again varied up to ±10%. Evaluation of the variance in the values of m_1, m_2, $\Delta G_1^{H_2O}$, and $\Delta G_2^{H_2O}$ that were determined from the various initial estimates of these parameters and the error-containing calculated data set suggested that the error in these parameters presented in Table I is less than 6%.

RESULTS

Denaturation of Luciferase—The spectral changes associated with the denaturation of luciferase were studied by fluorescence and circular dichroism. As shown in Fig. 1, native luciferase had a fluorescence maximum at about 330 nm that was decreased and red-shifted to approximately 345 nm upon treatment with 6 M urea. At intermediate urea concentrations, the fluorescence emission spectra were not intermediate between the native spectrum and that in 6 M urea. As shown in Fig. 1*B*, the emission intensity increased in 2 M urea when excitation was at 295 nm, whereas emission increased only slightly when excitation was at 280 nm (Fig. 1*A*). In the range of 0–2 M urea, the fluorescence intensity increase was not accompanied by a large red shift. Above ~2 M urea, the fluorescence intensity decreased and the spectral maximum shifted to longer wavelengths. The maximum difference in fluorescence between the native and denatured protein occurred at about 324 nm, so in subsequent experiments in which fluorescence emission was to be monitored, the emission wavelength was 324 nm.

The circular dichroism spectrum of native luciferase in the far UV is suggestive of a high α-helical content (Cohn *et al.*, 1985; Johnston *et al.*, 1986; Ziegler *et al.*, 1993), and the near-UV spectrum is indicative of defined packing of the aromatic side chains (Ziegler *et al.*, 1993). Upon denaturation, both far-UV and near-UV signals show a large change due to loss of both secondary structure and tertiary structure (Ziegler *et al.*, 1993). The fluorescence and CD spectral data indicate that luciferase was largely unfolded in the presence of 6 M urea, 50 mM phosphate, pH 7.0. Protein unfolded in 6 M urea was shown to be at equilibrium by monitoring fluorescence emission (excitation, 280 nm) at 324 nm and CD at 222 nm for 2 h (not shown). Circular dichroism and fluorescence measure-

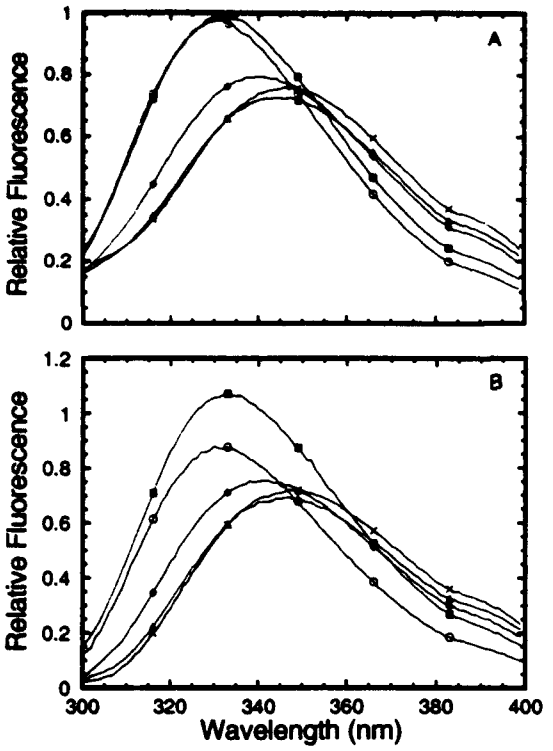

FIG. 1. **Fluorescence emission spectra of bacterial luciferase in different concentrations of urea.** *A*, excitation at 280 nm. *B*, excitation at 295 nm. Luciferase samples (25 μg/ml) were incubated for 18 h at urea concentrations as follows: 0 M (○), 2 M (■), 3 M (◇), 4 M (▲), and 6 M (×).

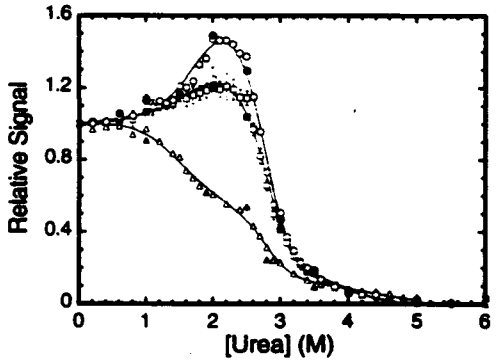

FIG. 2. **Noncoincidence of equilibrium unfolding data.** Urea-induced unfolding of luciferase (25 μg/ml) was measured by CD at 222 nm (△) and by fluorescence emission at 324 nm with excitation at either 280 nm (□) or 295 nm (○). *Closed symbols* represent renatured protein to show reversibility. *Error bars* show the standard deviation from four unfolding curves. For clarity, *error bars* are not shown for the other data sets. *Solid lines* represent curve fits as described under "Experimental Procedures."

ments were made about 30 s after introduction of the protein into the denaturant; it appeared that the unfolding process was complete within the time required to manually mix the sample and introduce it into the spectropolarimeter.

Equilibrium Unfolding of Luciferase—The unfolding of luciferase at equilibrium was investigated by monitoring circular dichroism at 222 nm and intrinsic fluorescence emission at 324 nm. Fig. 2 shows typical unfolding curves for luciferase. With excitation either at 280 or 295 nm, there was an increase in fluorescence between 0 and 2 M urea (see also Fig. 1). Excitation at 295 nm gave a higher relative signal change than did excitation at 280 nm (approximately 50%, as compared with approximately 20% with excitation at 280 nm at this protein concentration). Fig. 2 also shows the corresponding equilibrium unfolding curve monitored by changes in CD at 222 nm. The reduction in secondary structure appeared to be biphasic. The first phase of unfolding, between 0 and 2 M urea, corresponded to the fluorescence increase shown in Fig. 2. The fluorescence emission spectrum of the luciferase in 2 M urea suggested that the tryptophanyl residues remained buried (see Fig. 1). These results are consistent with the existence of stable intermediates in equilibrium with the native and unfolded forms. The *error bars* in Fig. 2 show the standard deviation obtained from four denaturation curves at 25 μg/ml luciferase performed on separate days, demonstrating that the data were highly reproducible.

Effect of Tween 20 on the Unfolding Transition—In order to obtain maximum refolding of luciferase under all conditions tested, a small amount (0.005%) of Tween 20 was included in the buffer and urea solutions (see "Experimental Procedures"). In reconstitution experiments (not shown) in which protein denatured in 6 M urea was diluted 1:50 into phosphate buffer and allowed to refold, greater than 85% activity recovery was obtained in the protein concentration range of 5–25 μg/ml when Tween 20 was included in the phosphate buffer. In analogous experiments without Tween 20, only 25 μg/ml protein gave greater than 85% activity recovery. There was a dramatic decrease in activity recovery both below and above the protein concentration range of 5–25 μg/ml, even in the presence of Tween 20. At higher protein concentrations, the reduced yield has been attributed to aggregation, whereas at lower concentrations, the dimerization process appears to be compromised by the competing first-order folding of the subunits into dimerization-incompetent structures (Ziegler *et al.*, 1993; Baldwin *et al.*, 1993). The protein concentration range of 5–25 μg/ml, within which the activity recovery is optimal, was used in the experiments reported here. The effect of Tween 20 on the equilibrium unfolding of luciferase was minimal (data not shown). There was a larger effect on reversibility at lower protein concentrations than at higher concentrations as measured by activity recovery, suggesting that the detergent may act primarily to prevent protein from sticking to the incubation tubes. Because there was little apparent effect on protein stability and because of enhanced reversibility at the lower protein concentrations, we used 0.005% Tween 20 in the buffer and urea solutions.

Effect of Luciferase Concentration on the Unfolding Transition—Because bacterial luciferase is a heterodimer, it was of interest to determine the concentration dependence of the equilibrium unfolding process. As shown in Fig. 3, *A* and *B*, as the luciferase concentration increased, there was both a shift in the position of the midpoint of the second transition to higher denaturant concentration and an increase in relative fluorescence signal between 2 and 3 M urea. Measurement of relative CD at 222 nm (Fig. 3*C*) showed that the first transition was independent of protein concentration, whereas the second transition was apparent only at higher protein concentrations. Since luciferase is heterodimeric in the absence of urea, the insensitivity of the spectroscopic signals to changes in protein concentration in the 0–2 M urea region suggests that the changes in the protein that occur upon equilibration with urea-containing buffer in this concentration range do not involve subunit dissociation. In contrast, the spectroscopic signals showed a strong protein concentration dependence at urea concentrations greater than 2 M, suggesting that the spectroscopic changes reflect, at least in part, subunit dissociation. The results shown in Fig. 3 suggested the 3-state mechanism

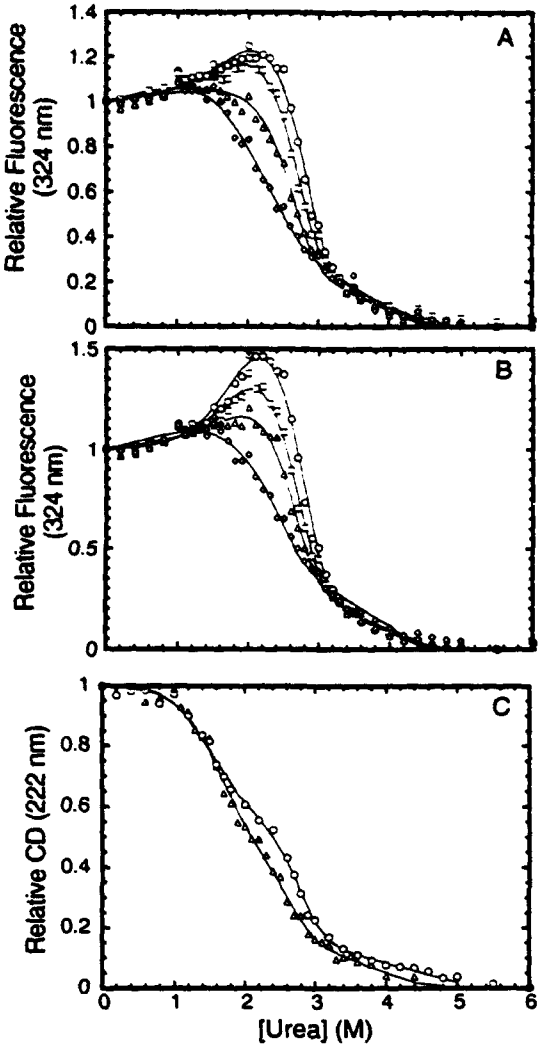

FIG. 3. **Dependence of equilibrium unfolding of bacterial luciferase on protein concentration.** For *panels A* and *B*, unfolding was monitored by fluorescence emission at 324 nm with excitation at either 280 nm (*panel A*) or 295 nm (*panel B*), at protein concentrations of 5 μg/ml (◇), 10 μg/ml (△), 15 μg/ml (□), and 25 μg/ml (○). For *panel C*, unfolding was monitored by CD at 222 nm at protein concentrations of 10 μg/ml (△) and 25 μg/ml (○). *Solid lines* represent curve fits as described under "Experimental Procedures." Parameters for these fits are given in Table I.

$$[\alpha\beta]_N \overset{K_1}{\rightleftharpoons} [\alpha\beta]_I \overset{K_2}{\rightleftharpoons} \alpha + \beta \qquad \text{(Eq. 18)}$$

The free energy changes and the cooperativity indices associated with each step were analyzed as described (see "Experimental Procedures") and are summarized in Table I. These data showed that $\Delta G_1^{H_2O}$ is relatively constant over the protein concentration range tested. The free energy change and cooperativity index associated with the first step in unfolding were 4.5 ± 0.3 kcal/mol and 2.38 ± 0.21 kcal/mol/M, respectively, based on measurements of the circular dichroism and fluorescence emission spectra in 11 experiments. The free energy change and cooperativity index associated with the second step were also relatively constant over the protein concentration range studied. $\Delta G_2^{H_2O}$ and m_2, which encompass the dissociation of the apparent intermediate into the two subunits, showed an average free energy change and cooperativity index of 19.7 ± 0.2 kcal/mol and 3.99 ± 0.04 kcal/mol/M, respectively, from the 11 experiments. The data in Table I confirm that bacterial luciferase is a very stable protein, with a total free energy change for subunit unfolding and dissociation of approximately 24 kcal/mol. Furthermore, the relatively high m values suggest that the two steps apparent in these data are strongly cooperative.

Loss of Enzymatic Activity upon Unfolding—In addition to the spectroscopic probes discussed above, we used enzymatic activity to monitor the denaturation of bacterial luciferase. Samples were prepared the same way as those used for spectroscopic measurements, as described under "Experimental Procedures," at the same protein concentrations. The data from these experiments are summarized in Table II. The midpoint of the denaturation profiles occurred at about 1.6 M urea and was independent of the protein concentration. The values of $\Delta G_2^{H_2O}$ and m, determined by fitting the data to a two-state (native *versus* denatured) mechanism, were 4.4 ± 0.8 kcal/mol and 2.78 ± 0.8 kcal/mol/M, respectively, in reasonable agreement with the same parameters determined from spectroscopic measurements (Table I).

DISCUSSION

A detailed understanding of protein folding will require not only information about the structures of the unfolded and native species and the rates of their interconversion, but also about intermediates on the folding pathway and possible parallel pathways. Much of the debate today regarding protein folding centers on the question of the structures of intermediates relative to that of the native protein. Although a great deal has been learned about protein folding from investigations of small globular proteins or peptides, it is unlikely that a general understanding of protein folding will come exclusively from investigations of small model systems. Most proteins are composed either of multiple subunits or of multiple folding domains that interact in part through the covalent continuity of the peptide chain. The interactions between subunits of a multisubunit protein are exclusively noncovalent (with the exception of disulfide bonds), but otherwise, for many proteins, interdomain interactions and intersubunit interactions appear to be similar (Wetlaufer, 1981).

Wetlaufer (1981) proposed a simplifying assumption that reduces the folding problem for large multidomain proteins to a series of folding problems involving the individual domains, many of which are small and globular. This model of folding suggests that the final step of folding involves intercalation of the side chains of the interdomain contact residues. More recently, Ptitsyn *et al.* (1990) has suggested that as a protein folds, it passes through a series of intermediates arriving at a structure resembling in fold the structure of the native protein, except that the amino acid side chains are not properly intercalated, either between or within individual domains. This proposed structure, known as the molten globule on the basis of the fluidity of its structure and its globular shape, slowly converts to the native structure as the final arrangements of the amino acid side chains are achieved. It appears likely that a molten globule-like structure might exist as an intermediate on the folding pathway of many proteins (Ptitsyn *et al.*, 1990; Kuwajima, 1989); this fact and the similarity of the molten globule to the native structure suggest that little will be learned about the pathways of folding by its study. Rather, it would appear that detailed investigation of transient intermediates that occur during the folding of a protein might yield more valuable information about the pathway(s) of folding. To date, the best structural information regarding folding intermediates comes from studies of bovine pancreatic trypsin inhibitor with which intermediates have been trapped as a result of the formation of disulfide bonds during refolding (Creighton, 1978; Weissman and Kim, 1991).

TABLE I

Thermodynamic parameters obtained from equilibrium denaturation of bacterial luciferase

The data were analyzed as described under "Data Analysis."

[Protein]	Signal*	$\Delta G_1^{H_2O}$	$\Delta G_2^{H_2O}$	m_1	m_2
μg/ml		kcal/mol	kcal/mol	kcal/mol/M	kcal/mol/M
25	Fl. (280 nm)	4.62	19.9	2.40	3.97
25	Fl. (295 nm)	4.67	19.8	2.18	4.00
25	CD (222 nm)	3.86	20.1	2.63	4.08
15	Fl. (280 nm)	4.63	19.7	2.20	3.98
15	Fl. (295 nm)	4.64	19.7	2.21	3.99
15	CD (222 nm)	4.07	19.4	2.74	3.94
10	Fl. (280 nm)	4.72	19.7	2.31	3.98
10	Fl. (295 nm)	4.71	19.7	2.20	3.97
10	CD (222 nm)	4.33	19.7	2.69	3.96
5	Fl. (280 nm)	4.69	19.7	2.30	4.04
5	Fl. (295 nm)	4.73	19.6	2.32	4.04
Average		4.52 ± 0.30	19.7 ± 0.2	2.38 ± 0.21	3.99 ± 0.04

* Fl., fluorescence; CD, circular dichroism. The excitation wavelength is shown in parentheses; the emission wavelength for all fluorescence experiments was 324 nm.

TABLE II

Thermodynamic paremeters obtained from measurements of loss of enzymatic activity

[Protein]	ΔG^{H_2O}	m	$\text{urea}_{1/2}$
μg/ml	kcal/mol	kcal/mol/M	M
25	3.70 ± 0.74	2.35 ± 0.39	1.6
15	4.10 ± 0.36	2.28 ± 0.24	1.8
5	5.30 ± 1.15	3.71 ± 0.85	1.4
Average	4.4 ± 0.8	2.78 ± 0.8	1.6

TABLE III

Effect of Tween 20 on stability of bacterial luciferase

Signal*	Tween 20	$\Delta G_1^{H_2O}$	$\Delta G_2^{H_2O}$	m_1	m_2
		kcal/mol	kcal/mol	kcal/mol/M	kcal/mol/M
Fl. (280 nm)	+	4.62	19.9	2.40	3.97
Fl. (280 nm)	−	6.26	17.4	2.17	3.44
Fl. (295 nm)	+	4.67	19.8	2.18	4.00
Fl. (295 nm)	−	4.67	19.7	2.19	4.00
CD (222 nm)	+	3.86	20.1	2.63	4.08
CD (222 nm)	−	4.15	20.5	2.53	4.39

* Fl., fluorescence; CD, circular dichroism. Excitation wavelength is shown in parentheses; the emission wavelength for each fluorescence experiment was 324 nm.

Without the stabilizing influence of the covalent disulfide bonds, it is exceedingly difficult to investigate the structure of a transient intermediate on the folding pathway of a protein.

Investigation of the kinetics of refolding of bacterial luciferase following dilution from solutions of 5 M urea demonstrated the existence of multiple transient intermediates on the folding pathway. Of particular interest was the finding that the initial heterodimeric structure that formed from interaction between partially folded α and β subunit species was not active, but on a time scale of many seconds, it was converted into the active heterodimer (Ziegler *et al.*, 1993). Bacterial luciferase is largely unfolded in urea-containing buffers of 5 M and above (Fig. 1; Ziegler *et al.*, 1993; Baldwin *et al.*, 1993). We have established conditions by which the protein may be diluted from the urea to refold into the biologically active form. The reversibility of the unfolding process was enhanced slightly by the addition of 0.005% Tween 20 to the buffers; this detergent, chosen from a series of nonionic detergents on the basis of experimental performance, had very little effect on the apparent stability of the folded state. A comparison of the thermodynamic parameters for the unfolding reaction determined in the presence and absence of the detergent is presented in Table III. Although there appeared to be slight changes in the stability of the protein in the presence of Tween 20, the primary effect was to give more reproducible results, especially in the intermediate urea concentration range, ~2–3 M.

The measurements of the rates of formation of active luciferase following dilution from urea used bovine serum albumin rather than Tween 20 to enhance recovery (Ziegler *et al.*, 1993; Baldwin *et al.*, 1993). To avoid spectral interference, the experiments reported here employed Tween 20 rather than bovine serum albumin. It appeared that the effects of bovine serum albumin and Tween 20 were similar.

The equilibrium unfolding processes monitored by intrinsic fluorescence and far-UV circular dichroism appeared to be biphasic. The increase in fluorescence in the 0–2 M urea range suggested the existence of an intermediate that had a higher fluorescence quantum yield than the native heterodimer. The fluorescence emission spectrum of this apparent intermediate was not strongly red-shifted, suggesting that the tryptophanyl residues remained largely buried (Figs. 1 and 2). At 2 M urea, the circular dichroism in the peptide region (222 nm) had decreased about 30%, suggesting partial unfolding. Interpretation of this observation is complicated by the contributions of aromatic amino acid residues to the CD signal in this region of the spectrum. It is therefore not possible without further experimentation to interpret the decrease in CD signal at 222 nm in terms of α-helical content of the protein.

The first transition (0–2 M urea) appeared to be independent of protein concentration, suggesting that structural changes were limited to isomerization of heterodimer. Above 2 M urea, there was a strong concentration dependence, suggesting that subunit dissociation occurred in this transition (Fig. 3). The data were fit to a three-state model in which the first step involved isomerization of the native heterodimer to a nonnative heterodimer and the second step involved dissociation of the nonnative heterodimer to yield subunits. In our treatment, we assumed the α and β subunits to be identical. The results of this treatment (Table I) were highly consistent, both between spectral methods and with different protein concentrations. By using the average values from the experimental data for $\Delta G_1^{H_2O}$ (4.52 kcal/mol), $\Delta G_2^{H_2O}$ (19.7 kcal/mol), m_1 (2.38 kcal/mol/M), and m_2 (3.99 kcal/mol/M), we could calculate the equilibrium distribution of the three species, native enzyme, intermediate, and subunits, at each urea concentration. Results of these calculations are shown in Fig. 4. Data were calculated for three different protein concentra-

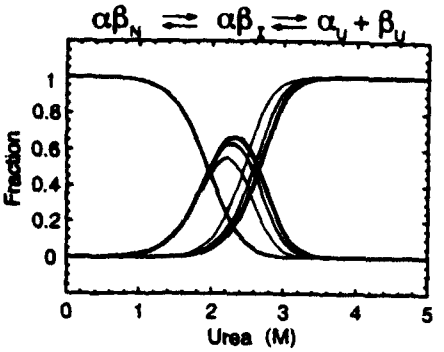

FIG. 4. **Theoretical calculation of the fraction of each species as a function of urea concentration in the unfolding process.** The fractions of native, intermediate, and unfolded protein were calculated as a function of urea concentration for protein concentrations of 5 (*thin line*), 10 (*medium line*), and 25 μg/ml (*heavy line*). $\alpha\beta_N$ refers to the native form of luciferase, $[\alpha\beta]_I$ refers to a dimeric intermediate, and α_u and β_u refer to the individual unfolded subunits.

tions, 25, 10, and 5 μg/ml. The fraction of protein in the intermediate form is highest for the highest protein concentration, about 65% at 2.3 M urea and 25 μg/ml total protein.

The loss of enzymatic activity in urea occurred in a single transition that was protein concentration-independent and corresponded to the first transition observed in the spectroscopic measurements (Table II). These results demonstrate that the heterodimeric intermediate that has the enhanced fluorescence is largely or completely inactive in the bioluminescence reaction. This interpretation is consistent with the finding from kinetic measurements of an inactive heterodimeric intermediate on the refolding pathway (Ziegler *et al.*, 1993). The results presented here confirm the existence of this heterodimeric intermediate and suggest that the intermediate is sufficiently stable to allow detailed spectroscopic analysis.

We have established conditions that allow the investigation of the conformational stability of the bacterial luciferase heterodimer. In the course of these studies, we have demonstrated that the inactive heterodimeric intermediate that isomerizes to the active form of the enzyme, demonstrated in the kinetic experiments of Ziegler *et al.* (1993), is well populated at equilibrium. In addition, our experiments show the following. 1) The unfolding of bacterial luciferase at 18 °C, in 50 mM phosphate, pH 7.0, has been fit to a three-state model with a free energy change for the first step of 4.52 kcal/mol and, for the second step, of 19.7 kcal/mol. 2) The first step in the unfolding reaction involves isomerization of the native heterodimer to yield an inactive heterodimer that is spectroscopically distinguishable from the native enzyme. 3) The inactive heterodimer appears to be prone to aggregation. However, at low protein concentrations, the unfolding transitions appear to be fully reversible.

These observations explain in quantitative terms the failure to observe free luciferase subunits in equilibrium with the heterodimer under nondenaturing conditions (Hastings *et al.*, 1966). Furthermore, the folding mechanism supported by these data is entirely consistent with a model of luciferase assembly in which the interaction between partially folded subunits leads to a final native conformation in which the α and β subunits are intertwined, rather than interacting through a clearly discernible interface between compactly folded subunits (Waddle *et al.*, 1987; Sugihara and Baldwin 1988; Ziegler *et al.*, 1993; Baldwin *et al.*, 1993).

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Purified Native Subunits of Bacterial Luciferase Are Active in the Bioluminescence Reaction but Fail To Assemble into the $\alpha\beta$ Structure†

James F. Sinclair, Jenny J. Waddle,‡ E. Florence Waddill, and Thomas O. Baldwin*

Center for Macromolecular Design and the Departments of Biochemistry and Biophysics and of Chemistry, Texas A&M University, College Station, Texas 77843-2128

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ABSTRACT: We have expressed the α and β subunits of bacterial luciferase, encoded by *luxA* and *luxB*, from separate plasmids in *Escherichia coli* and developed an efficient purification scheme that yields many milligrams of protein of greater than 90% homogeneity. Earlier experiments showed that subunits synthesized separately assume conformations that do not assemble into the active luciferase heterodimer without prior denaturation. This observation led to the proposal that formation of the luciferase heterodimer involved interactions between intermediate conformations on the folding pathway of one or both of the subunits [Waddle, J. J., Johnston, T. C., & Baldwin, T. O. (1987) *Biochemistry 26*, 4917–4921]. Both of the subunits catalyze reduced flavin- and aldehyde-dependent bioluminescence reactions that are similar to that of the heterodimer in terms of reduced flavin binding affinity, aldehyde binding and inhibition, and kinetics of the overall bioluminescence reaction, but at an efficiency of about 5×10^{-6} that of the heterodimer. Spectrophotometric analyses suggest that the structures of the individual subunits are similar to, but *not* identical to, the structures of the subunits in the heterodimer. Mixing of the two subunits under nondenaturing conditions did not lead to formation of the high specific activity heterodimer, even after prolonged incubation. Likewise, treatment of a stoichiometric mixture of the individual subunits with 5 M urea followed by 50-fold dilution of the urea did not yield the active heterodimer under the same conditions that yield high levels of active enzyme following denaturation of the native heterodimer [Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F., & Baldwin, T. O. (1993) *J. Biol. Chem. 268*, 10760–10765]. However, refolding of the α and β subunits together from 5 M urea following unfolding with 5 M guanidine HCl resulted in formation of the high specific activity $\alpha\beta$ species, suggesting that the native isolated α and/or β species is resistant to unfolding by 5 M urea. The results indicate that formation of the heterodimer *in vivo* must occur by interaction of transient subunit species that are distinct from the stable forms of the subunits that we have purified from cell extracts.

Bacterial luciferase is a heterodimeric ($\alpha\beta$) enzyme with a single active center residing primarily if not exclusively on the α subunit [see Ziegler and Baldwin (1981) and Baldwin and Ziegler (1992) for reviews of the system]. The α and β subunits of the enzyme from *Vibrio harveyi* are 355 amino acid residues (Cohn et al., 1985) and 324 amino acid residues (Johnston et al., 1986) in length, respectively. The two subunits are clearly homologous; 80% of the residues in β are either identical to or chemically similar to the corresponding residue in the α subunit. The shorter length of the β subunit results from an apparent deletion of residues 258–286 relative to the α subunit (Baldwin & Ziegler, 1992).

Luciferase catalyzes the bioluminescent reaction of $FMNH_2$, O_2, and an aliphatic aldehyde to yield FMN, the carboxylic acid, and blue-green light with a quantum yield of about 0.1. The stoichiometry of the reaction requires 1 mol of $FMNH_2$ (Becvar & Hastings, 1975) and 1 mol of aldehyde (Holzman & Baldwin, 1983) per mole of the heterodimer. The preponderance of the evidence from mutant enzyme analysis and chemical modification studies [discussed by Baldwin and Ziegler (1992)] indicates that the single active center is associated primarily with the α subunit.

Waddle et al. (1987) have shown that expression of the individual luciferase α and β subunits from recombinant plasmids in *Escherichia coli* results in accumulation of large amounts of subunit in cell lysates, demonstrating that the individual subunits fold *in vivo* into structures that are stable and soluble within the cell. However, mixing of lysates containing large amounts of the individual subunits did not yield the highly active $\alpha\beta$ species. Unfolding of the proteins with 8 M urea and refolding together by dilution of the urea led to excellent recovery of the active heterodimer. On the basis of these observations, Waddle et al. (1987) suggested that *in vivo* the subunits must interact as partially folded species and that the final steps of folding must occur within the heterodimeric species. They also suggested that the individual subunits must be able to fold into stable structures that are beyond and not in equilibrium with the subunit species that are capable of interaction to form $\alpha\beta$. These observations raised the possibility that formation of the biologically active heterodimer might constitute a kinetic trap, since the "completely" folded individual subunits do not recombine upon mixing, even with prolonged incubation.

It was thought for many years that the individual subunits of luciferase lacked bioluminescence activity. While subunits refolded individually from urea-containing buffers following chromatographic separation do exhibit low activities, it was concluded that the activity was the result of failure of the chromatographic systems employed to completely separate

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* Author to whom correspondence should be addressed at the Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843. Email: Baldwin@BIGRAF.TAMU.EDU.

‡ Present address: Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802.

the two subunits (Friedland & Hastings, 1967a,b; Tu, 1978). Recently, Waddle and Baldwin (1991) reported that *both* of the luciferase subunits catalyze a low-efficiency bioluminescence reaction. This observation was not expected. If the active center is confined to the α subunit, then the observation of bioluminescence activity from the β subunit would be difficult to understand. In this regard, it is interesting to note that there is some indication from NMR studies (Vervoort et al., 1986) that more than one flavin can bind to luciferase, though the second molecule is bound very weakly, perhaps "nonspecifically". The homology between the subunits suggests a similar three-dimensional structure and the potential for a residual active center on the β subunit. The apparent deletion of a region of about 28 residues from the β subunit could account for lack of a fully functional active center on the β subunit (Baldwin & Ziegler, 1992).

The initial report of the catalytic activity of both individual subunits (Waddle & Baldwin, 1991) was based on studies of individual subunits produced within *E. coli* cells carrying plasmids that encode only a single luciferase subunit, either α or β. Since the luciferase subunits were produced from a recombinant plasmid in *E. coli*, there was no possibility of residual cross-contamination of one subunit with the other, as would occur with subunits separated chromatographically (Friedland & Hastings, 1967a,b; Tu, 1978). The activity measurements of Waddle and Baldwin (1991) were made with partially purified subunits.

The purpose of the experiments presented in this paper was to develop a highly efficient method to purify the individual subunits from the cellular constituents of *E. coli*, and using the highly purified subunits, to investigate the low bioluminescence activity of the subunits and to begin to develop a better understanding of the structures of the folded individual subunits. We have determined the binding affinities of the subunits for the substrates, $FMNH_2$ and aldehyde, and the kinetics and quantum efficiencies of the reactions catalyzed by the subunits relative to the heterodimer. The physical properties of the separate subunits indicate that they exist as well-defined globular structures that are similar to but distinct from the structures of the subunits as they exist in the heterodimer. As was found with partially purified subunits, the pure α and β subunits do not recombine under native conditions to form the high specific activity $\alpha\beta$. Furthermore, the subunits incubated together in 5 M urea did not associate to form the highly active heterodimer, indicating that they did not unfold in 5 M urea. The same conditions have been shown to lead to complete (or nearly so) unfolding of the subunits of the heterodimer (Ziegler et al., 1993). It appeared that unfolding of the individual subunits required 5 M guanidine-HCl, after which dilution from denaturant resulted in association of the two subunits to form the high specific activity heterodimeric luciferase.

EXPERIMENTAL PROCEDURES

Materials. DEAE Sephadex A-50 was purchased from Sigma, Ultrogel AcA 54 from IBF Biotechnics, dithiothreitol from Boehringer Mannheim Biochemicals, EDTA from Research Organics, *n*-octanal from Sigma, *n*-decanal and *n*-dodecanal from Aldrich, FMN from Calbiochem, and UltraPure urea from Schwartz-Mann. All inorganic salts were purchased from Baker or Fisher and were of the highest purity grade available.

Bacterial Growth and Cell Lysis. E. coli strain LE392, an r_k^-, m_k^- strain derived from ED8654 (Maniatis et al., 1982), was chosen for its ability to overexpress cloned structural genes (Baldwin et al., 1989). Plasmid pJH2, described previously (Waddle et al., 1987), carries *luxA* encoding the luciferase α subunit from *V. harveyi* under control of the *lac* promoter in pUC9. Plasmid pJH5 (Waddle et al., 1987) carries *luxB* encoding the luciferase β subunit from *V. harveyi* under the control of the *lac* promoter in a pUC9 derivative that carries a kanamycin resistance marker. The *luxA* and *luxB* genes were derived from the primary clone screened from a genomic bank (Baldwin et al., 1984). The media used were LB supplemented with carbenicillin (100 μg/mL) for LE392/pJH2 and with kanamycin sulfate (100 μg/mL) for LE392/pJH5.

Waddle and Baldwin (1991) noted that growth of *E. coli* cultures at 30 °C or above resulted in production of luciferase subunits in the insoluble fraction of the cell lysates, whereas at 25 °C the majority of the subunit was in the soluble fraction for both subunits. Similar results were observed in the current study. Single colonies from overnight growth at 25 °C on LB agar plates were picked and used to inoculate 5 mL of LB medium. Cultures were allowed to grow at 25 °C with aeration (250 rpm) for approximately 6 h. The 5-mL liquid culture was used to inoculate 50 mL of medium and allowed to grow at 25 °C with aeration for 7 h. This culture was used to inoculate 1.5 L of medium which was then grown at 25 °C for 24 h. Cells were harvested when the OD[1] at 600 nm reached about 4.2.

Purification Procedures. Cells were harvested by centrifugation at 6370*g* for 15 min at 10 °C. The cell pellet was resuspended in 72 mL (minimum volume required) of buffer consisting of 0.2 M phosphate, 0.5 mM DTT, and 1 mM EDTA, pH 7.0 for the α subunit, or pH 6.2 for the β subunit, and lysed in an SLM/Aminco French pressure cell with 1000 psi applied to the drive. The cells and cell lysate were kept on ice throughout the procedure. Cell debris was removed by centrifugation at 27200*g* for 20 min at 4 °C. The cell lysate containing the β subunit was treated with ammonium sulfate, and the protein precipitating between 40% and 75% saturation at 4 °C was collected by centrifugation at 27200*g* for 15 min at 4 °C. The precipitated protein was resuspended in 0.2 M phosphate and 0.5 mM DTT, pH 6.2, and dialyzed overnight against the same buffer (three changes of 1 L each). The lysate containing the α subunit was not treated with ammonium sulfate. Unless otherwise stated, all steps in the α subunit purification were carried out at pH 7.0, while the β subunit purification was performed at pH 6.2.

DEAE Sephadex A-50 was equilibrated in 0.2 M phosphate buffer and used to prepare a column with a bed volume of 412 mL (5-cm diameter). The dialyzed protein was applied to the column and allowed to equilibrate with the resin for 30 min, after which it was eluted from the column at a flow rate of 150 mL/h with a linear gradient between 750 mL of 0.2 M phosphate and 750 mL of 0.6 M phosphate, both with 0.5 mM DTT and 1 mM EDTA (1500 mL total, pH 7.0 for α and pH 6.2 for β). Column fractions (20 mL) were monitored for bioluminescence activity, and protein concentration was estimated by measuring the absorbance at 280 nm. Fractions were selected for pooling based on bioluminescence activity and the results of polyacrylamide gel electrophoresis in the presence of SDS. Pooled fractions were concentrated in an Amicon ultrafiltration cell (PM30 membrane) and then dialyzed against 0.2 M phosphate, 0.5 mM DTT, and 1 mM EDTA (pH 7.0 for α and 6.2 for β). The samples were applied

[1] OD, optical density; BSA, bovine serum albumin; DTT, dithiothreitol; CD, circular dichroism; SDS, sodium dodecyl sulfate.

to a second DEAE Sephadex A-50 column (same dimensions as the first column) and eluted with the same gradient as the first column, but with a flow rate of about 45 mL/h. Fractions with the highest specific activity were pooled, concentrated, and dialyzed against 0.2 M phosphate buffer. Concentrated β subunit was applied to an Ultrogel AcA 54 column (2.5 cm × 90 cm) and eluted at about 15 mL/h with 0.1 M phosphate, 0.5 mM DTT, and 1 mM EDTA, pH 7.0. Fractions of 3 mL were collected, activity and protein concentration were measured, and component proteins were analyzed by SDS gel electrophoresis. Fractions containing the highest purity subunit were pooled, concentrated, and stored frozen. All chromatographic procedures were carried out at 0–4 °C.

Determination of Molar Extinction Coefficients. The molar extinction coefficients of the luciferase heterodimer, α subunit, and β subunit were determined by the method of Edelhoch (1967). Highly purified protein samples ($A_{280} \sim 1.5$–2.5) were dialyzed overnight against 50 mM phosphate, pH 7.0, at 4 °C. Following centrifugation at 15000 rpm for 2 min in an Eppendorf microcentrifuge, absorbance spectra between 250 and 450 nm were recorded against a baseline of the buffer that had been used in the dialysis. The spectra confirmed that the centrifugation had removed any light scattering aggregated materials.

Protein samples were diluted 1:4 into 8 M guanidinium chloride in 50 mM phosphate, pH 6.5, to yield samples in 6 M guanidinium chloride. The absorbance at 280 nm of each sample was measured. Equivalent native samples were prepared by 1:4 dilution of the protein stock into 50 mM phosphate, pH 7.0, and the absorbances at 280 nm were determined. Protein concentrations in 6 M guanidinium chloride were determined from the extinction coefficients (Edelhoch, 1967) of *N*-acetyl-L-tryptophanamide and glycyltyrosinylglycine and the tryptophanyl and tyrosinyl content of the α and β subunits (Cohn et al., 1985; Johnston et al., 1986). Spectral measurements were taken with a Hewlett-Packard model 8452A spectrophotometer at 24 °C.

Measurement of Bioluminescence Activity. Bioluminescence activity was determined by the flavin injection method (Hastings et al., 1978) in which the enzyme is incubated with the aldehyde substrate in an aerobic buffer solution over a photomultiplier tube. The reaction was initiated by the rapid injection of 1 mL of $FMNH_2$ prepared by catalytic reduction. Light emission was detected by a Turner Designs model TD-20e luminometer with a sensitivity of 3.66×10^5 quanta·s^{-1}·(light unit)$^{-1}$. Data were recorded by means of a Macintosh computer and Superscope software (GWI, Cambridge, MA). The data were fit using the model developed by Abu-Soud et al. (1992) with the program Kinsim (Barshop et al., 1983). Different chain length aldehyde substrates were prepared by sonication in water to obtain a 0.01% v/v suspension. Assays were performed at room temperature (~24 °C) in 1 mL of 50 mM phosphate, pH 7.0, 0.2% BSA with 10 μL of the aldehyde suspension.

Aldehyde Inhibition. Suspensions of *n*-decanal were prepared by sonication in water for a 0.01% v/v suspension (Holzman & Baldwin, 1983). Fresh aldehyde was prepared every hour to avoid potential interference from oxidation. Assays were performed in the same manner as described above, but without BSA. Peak light intensity for each reaction was measured with a Turner luminometer. Multiple assays were performed at each aldehyde concentration.

$FMNH_2$ Binding Affinities. The $FMNH_2$ binding affinities of the α and β subunits were determined by the dithionite assay method of Meighen and Hastings (1971) and compared with that of the heterodimer. Dithionite solutions were prepared as described by Tu and Hastings (1975). Enzyme was mixed with 1 mL of FMN containing 50 mM phosphate and 1 mM DTT. The flavin was reduced and molecular oxygen removed by addition of 4 μL of a 30 mg/mL solution of dithionite in water. The reaction was initiated by injection of 1 mL of 0.01% aldehyde containing 50 mM phosphate, pH 7.0, 1 mM DTT, and dissolved O_2. The peak light intensity was measured using a Turner luminometer; assays were performed in triplicate.

Spectroscopic Properties of the α and β Subunits. Circular dichroism spectra in the near- and far-UV of the individual subunits and of the heterodimer were recorded with a Jobin-Yvon CD-6 spectropolarimeter in the laboratory of Prof. Michel Goldberg of the Pasteur Institute. Protein samples in 25 mM phosphate, 1 mM EDTA, and 0.1 mM DTT, pH 7.0, were maintained at 18 °C while spectra were being recorded. The cuvettes used had a 1-cm path length for the 250–320-nm region and a 0.02-cm path length for the 185–255-nm region. Far- and near-UV spectra were recorded with a band path of 2 nm, a time constant of 5 s, and a step of 1 nm. The concentrations for the far-UV CD spectra were 4.38, 9.60, and 3.00 μM for α subunit, β subunit, and luciferase, respectively. Near-UV CD spectra were recorded with protein samples of 17.3, 37.8, and 11.3 μM for α subunit, β subunit, and luciferase, respectively. Spectra were first normalized on the basis of molar concentration of polypeptide and converted to mean residue ellipticity on the basis of the total number of amino acid residues per subunit (355 for α and 324 for β). Buffer baselines were recorded under identical conditions and subtracted from the spectra of the proteins. Fluorescence emission spectra were determined with an SLM 8000C spectrofluorometer with excitation at 280 nm. The concentration of all three samples was 1.0 μM.

Subunit Assembly. The individual subunits (15.2 μM) were incubated in 5 M guanidine-HCl or 5 M urea, both in 50 mM phosphate and 0.5 mM DTT, pH 7.0, for 30 min and then dialyzed against the same buffer with 5 M urea for 4 h at 18 °C. The refolding reaction was initiated by 50-fold dilution to 0.304 μM (~23 μg/mL) in 50 mM phosphate buffer, pH 7.0, at 18 °C and a final urea concentration of 0.1 M. The heterodimer was denatured in 5 M urea and renatured under the same conditions as the individual subunits, using methods described by Ziegler et al. (1993) and Baldwin et al. (1993). A fourth solution, with both α and β subunits, each at 15.2 μM in 50 mM phosphate buffer, was diluted to 0.304 μM in phosphate buffer at 18 °C and 0.1 M urea. The heterodimer in 50 mM phosphate buffer was diluted to 0.304 μM in phosphate buffer and 0.1 M urea at 18 °C. The appearance of activity in these solutions at 18 °C was monitored with a Turner luminometer over a period of several days.

RESULTS

Purification. We have found that very high levels of luciferase accumulate in *E. coli* strain LE392 transformed with a pUC9 plasmid encoding both subunits. For this reason, we chose to use LE392 carrying the *luxA* or *luxB* gene for overexpression of the individual subunits. Growth of these strains in LB medium at 20, 30, and 37 °C was monitored. The cell density (OD_{600}) giving the highest accumulation of α subunit as determined from Coomassie blue staining of SDS gels was 2.1 for growth at 20 °C, 1.0 for growth at 30 °C, and 0.8 for growth at 37 °C. Estimates were made of the fraction of the α subunit that was produced in soluble form by comparing the intensity of staining of the α subunit band

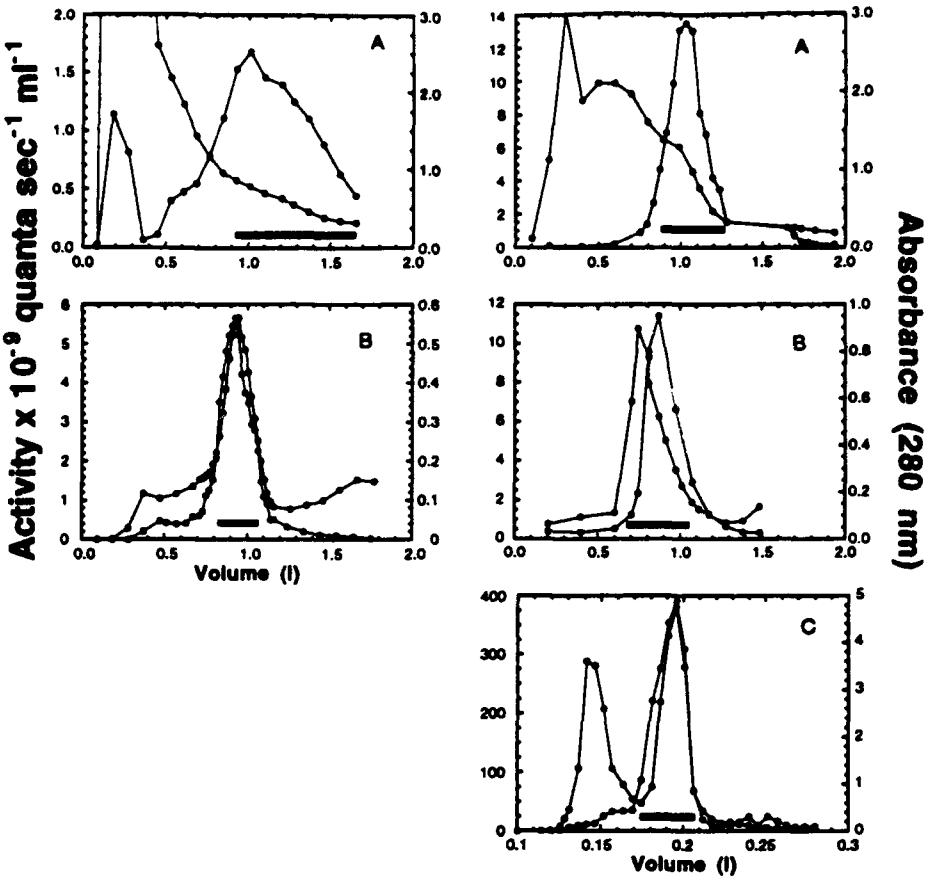

FIGURE 1: Column elution profiles for the α (left panel) and β (right panel) subunits. Bioluminescence activity (open symbols) and absorbance at 280 nm (closed symbols) are plotted against the elution volume for each column. (Left panel A) First DEAE-A50 column; (left panel B) second DEAE A-50 column; (right panel A) first DEAE A-50 column; (right panel B) second DEAE-A50 column; (right panel C) Ultrogel AcA 54 column. Horizontal bars indicate fractions which were pooled from each column. Activity in quanta·s^{-1}·mg^{-1} was determined with *n*-decanal as described under Experimental Procedures.

Table I: Purification of the α and β Subunits[a]

	total protein (mg)		total act. $\times 10^{-10}$ (quanta/s)		sp act. $\times 10^{-8}$ [quanta/(s·mg)]		% yield[b]	
purification step	α	β	α	β	α	β	α	β
crude lysate	12060	18443	97.4	7.1	0.8	0.04	100	100
first A-50	162	360	73.6	5	45.4	1.6	75	81
second A-50	108	262	190.0	6.3	176.0	2.6	194	97
Ultrogel AcA 54[c]		50		6.0		10.8		84

[a] Cultures of *E. coli* LE392 carrying plasmids pJH2 (α subunit) or pJH5 (β subunit), grown as described under Experimental Procedures, were the source of the crude lysates from which the subunits were purified. [b] The percent yield was calculated in each case relative to the total activity of the crude lysate. [c] The purification of the α subunit was complete after two DEAE A-50 columns, while an additional Ultrogel AcA 54 column was required for the final purification of the β subunit.

before and after centrifugation. At least 75% of the α subunit was insoluble in cells grown at 37 or 30 °C, while 75–90% of the subunit was soluble in cells grown at 20 °C. The β subunit behaved in a similar fashion, indicating that cell growth at 20 °C allowed the greatest accumulation of both subunits in soluble form into stationary phase. Under these conditions more than 90% of both subunits remained soluble after centrifugation at 27200*g* for 30 min at 5 °C. A growth temperature of 25 °C was chosen for routine work since the growth rate was much faster than at 20 °C, and production of soluble protein was at an acceptable level.

After 24 h of growth at 25 °C, cells from 6 L of culture were harvested, lysed, and treated with ammonium sulfate as described under Experimental Procedures. The dialyzed sample was applied to a DEAE Sephadex A-50 column and eluted as described. Both subunits eluted from the columns as single peaks at the end of the gradients, after the majority of the contaminating protein. In the case of the α subunit, additional 0.6 M phosphate buffer was added after the gradient to complete elution of the subunit. Chromatography of each subunit on a second DEAE Sephadex A-50 column yielded α subunit that was greater than 95% pure, as shown by SDS–PAGE gels, while the β subunit preparation retained one major and several minor contaminating bands. Chromatography of the β subunit preparation on an Ultrogel AcA 54 column yielded β subunit that was greater than 95% pure. The elution profiles of the various chromatographic steps are presented in Figure 1, and a summary of the purification is given in Table I. The luciferase heterodimer was purified as described by Gunsalus-Miguel et al. (1972) and modified by Baldwin et al. (1989).

Determination of Extinction Coefficients. Using the known amino acid composition of the α and β subunits (Cohn et al., 1985; Johnston et al., 1986), the extinction coefficients were determined for the individual subunits and for the heterodimer using the method of Edelhoch (1967). The values determined

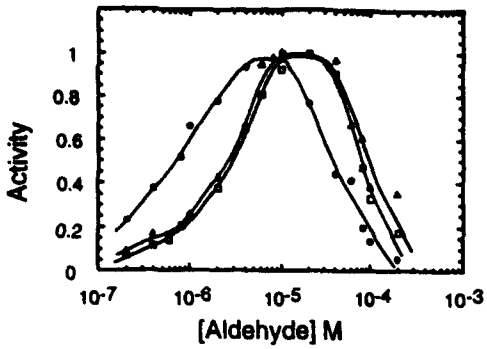

FIGURE 2: Effect of *n*-decanal concentration on bioluminescence activity. Peak light intensity was determined by the flavin injection assay in the presence of the indicated concentration of *n*-decanal. These values were then normalized to the same scale and plotted. The relative activities are shown for the luciferase heterodimer (circles), α subunit (squares), and β subunit (triangles). The protein concentrations used in these assays were 0.817 nM for luciferase, 0.394 μM for the α subunit, and 0.768 μM for the β subunit. The solid line is a smoothed curve drawn through the data points.

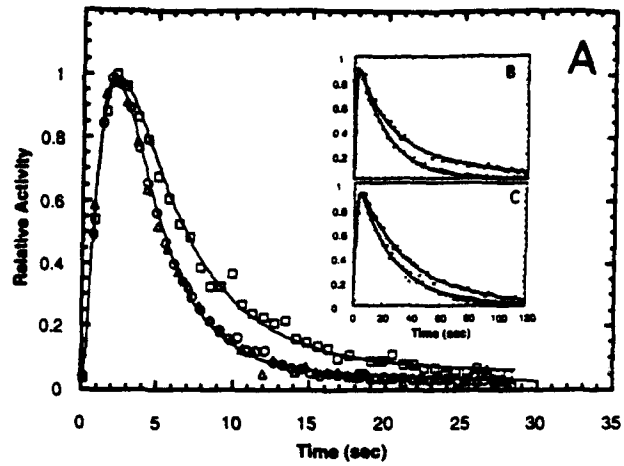

FIGURE 3: Time course of the bioluminescence reaction catalyzed by the heterodimer (circles), α subunit (triangles), and β subunit (squares). (Panel A) Light production using *n*-decanal as substrate. (Panel B) Light production using *n*-octanal as substrate. (Panel C) Light production using *n*-dodecanal as substrate. The solid lines represent simulated time courses based on the kinetic mechanism of the heterodimer proposed by Abu-Soud et al. (1992). The protein concentrations were the same as for the experiment depicted in Figure 2. The first-order decay rates used in the simulation are presented in Table II.

were 1.13 $(mg/mL)^{-1}\cdot cm^{-1}$, 1.41 $(mg/mL)^{-1}\cdot cm^{-1}$ and 0.71 $(mg/mL)^{-1}\cdot cm^{-1}$ for luciferase, α subunit, and β subunit, respectively. These correspond to molar extinction coefficients of 8.69×10^4 M^{-1} cm^{-1}, 5.64×10^4 M^{-1} cm^{-1}, and 2.59×10^4 M^{-1} cm^{-1}, respectively. Previously determined values for the extinction coefficient of luciferase are 0.94 $(mg/mL)^{-1}\cdot cm^{-1}$ (Gunsalus-Miguel et al., 1972) and 1.2 $(mg/mL)^{-1}\cdot cm^{-1}$ [see Tu et al. (1977)].

Comparison of the Bioluminescence Activity of Individual Subunits with that of Luciferase. Bacterial luciferase from *V. harveyi* is inhibited by high concentrations of the aldehyde substrate (Holzman & Baldwin, 1983). A recent detailed investigation of the kinetic mechanism of the enzyme suggests that the inhibition is due to formation of a dead-end enzyme–aldehyde complex; the decrease in activity appears to result from failure of this complex to bind $FMNH_2$, with $FMNH_2$ being removed from the reaction by the competing nonenzymatic reaction with O_2 (Abu-Soud et al., 1992, 1993). The bioluminescence activity of the α and β subunits was likewise inhibited by high concentrations of aldehyde (Figure 2). The highest bioluminescence activity occurred at 10 μM *n*-decanal for the heterodimer and at 20 μM *n*-decanal for both the α and β subunits.

Upon injection of $FMNH_2$ into a solution of enzyme, aldehyde, and O_2, there is a rapid rise in light intensity to a peak which is proportional to the amount of enzyme under conditions of saturating substrates. In this assay format, $FMNH_2$ that does not bind to the enzyme is rapidly removed by nonenzymatic reaction with O_2 such that turnover is not possible (Hastings & Gibson, 1963). The peak light intensity is followed by an exponential decay, thought to represent the decay of an enzyme-bound flavin 4a hydroperoxide–aldehyde complex to yield the excited state [see Baldwin and Ziegler (1992) for a discussion of the reaction]. The α subunit exhibited a first-order decay of light intensity that superimposed upon that of the heterodimer, while the β subunit displayed a slower decay rate than the heterodimer for all three aldehyde chain lengths tested (see Figure 3). The first-order rate constants are presented in Table II.

The binding of $FMNH_2$ to the individual subunits was monitored by an activity assay. Protein was incubated with various concentrations of $FMNH_2$ under anaerobic conditions (sodium dithionite), and the bioluminescence reaction was initiated by rapid injection of aldehyde and dissolved O_2. In this assay, it is assumed that the initial maximum light intensity following injection is proportional to the concentration of enzyme-bound flavin at the time of injection of O_2 and aldehyde (*Meighen & Hastings, 1971*). The data from such experiments are presented in Figure 4. The values of K_m for the complex of $FMNH_2$ with the heterodimer and with the individual subunits were determined from a nonlinear least-squares fit of a hyperbolic plot of light intensity versus $FMNH_2$ concentration using the Michaelis–Menten equation. These parameters, 0.44, 0.18, and 0.60 μM for the dimer and α and β subunits, respectively, are summarized in Table II.

Table II: Bioluminescence Decay Rate Constants and K_m for Reduced Flavin

	decay rate constants			
	n-octanal (s^{-1})	*n*-decanal (s^{-1})	*n*-dodecanal (s^{-1})	$K_m[FMNH_2]$ (μM)
heterodimer	0.050	0.30	0.040	0.44
α subunit	0.050	0.30	0.040	0.18
β subunit	0.033	0.21	0.028	0.60

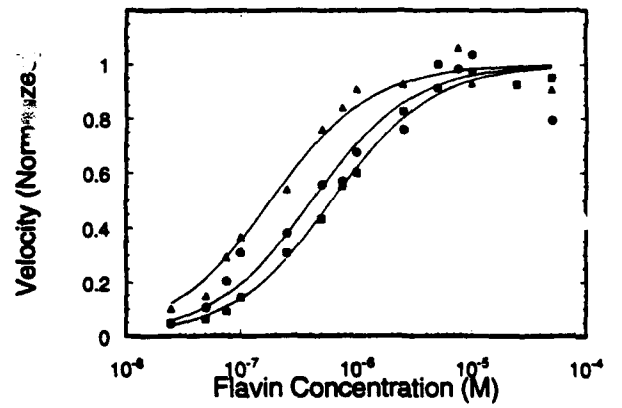

FIGURE 4: Interaction of luciferase and the α and β subunits with $FMNH_2$. Bioluminescence activity is plotted versus $FMNH_2$ concentration for the heterodimer (circles), the α subunit (triangles), and the β subunit (squares). The solid lines are the best fits of the data to the Michaelis–Menten equation. The protein concentrations were the same as for the experiment depicted in Figure 2.[2] The values of K_m determined from these data are presented in Table II.

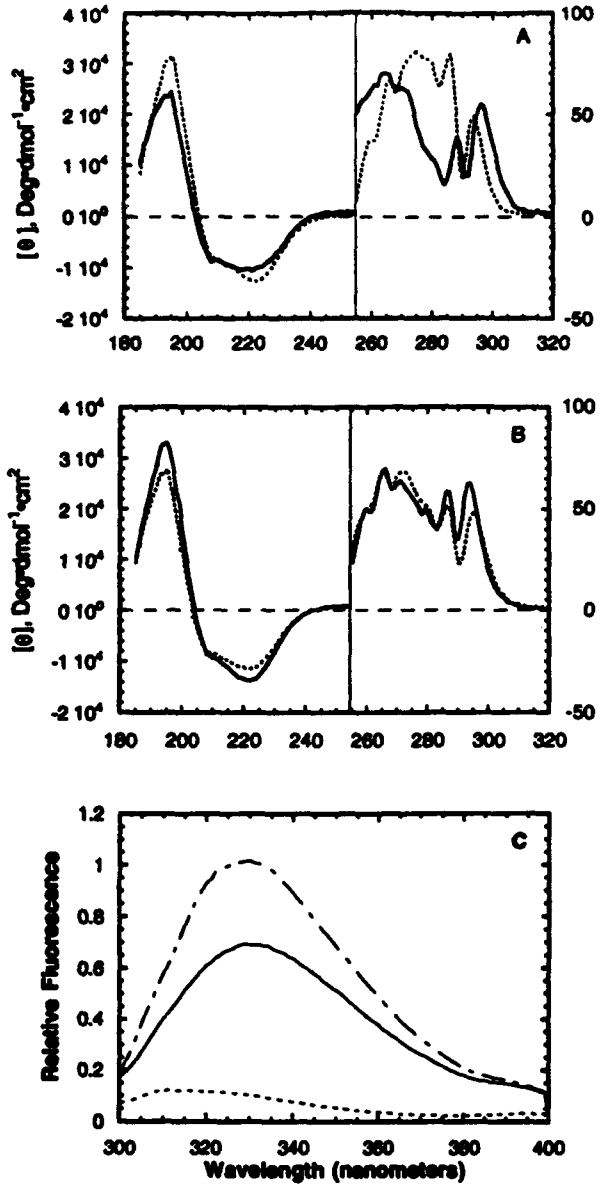

FIGURE 5: Circular dichroism and fluorescence emission spectra of luciferase, α subunit, and β subunit. (Panel A) Comparison of the circular dichroism spectra of equimolar concentrations of α subunit (—) and β subunit (- - -). (Panel B) Comparison of the sum of the spectra in panel A (- - -) to the circular dichroism spectrum of luciferase (—). (Panel C) Comparison of the fluorescence emission spectra (excitation at 280 nm) of equimolar concentrations of luciferase (—), α subunit (– – –), and β subunit (- - -). Spectra were recorded as described under Experimental Procedures.

Spectral Properties of the α and β subunits. Figure 5a shows the circular dichroism spectra for the individual subunits, and Figure 5b shows a comparison of the sum of the spectra in Figure 5a with the spectrum of the native heterodimer. The sum of the spectra for the subunits was similar to, but not equal to, the spectrum of the dimer in the far-UV, indicating either that there was some secondary structure content that was unique to the heterodimer or that some aromatic residue(s), which also contribute(s) to the far-UV CD spectrum, was in a different environment in the free subunit(s) than in the heterodimer. Differences between the sum of the near-UV CD spectra of the individual subunits and the spectrum of the native heterodimer suggest that several of the aromatic residues of the individual subunits reside in different environments from that which exists in the heterodimer. The fluorescence emission spectrum (Figure 5c) of the α subunit had a greater amplitude than that of the heterodimer, while the spectrum of the β subunit was very weak, due in part to the low content of tryptophan in the β subunit and an apparent quenching of the fluorescence in the folded state (Clark et al., 1993).

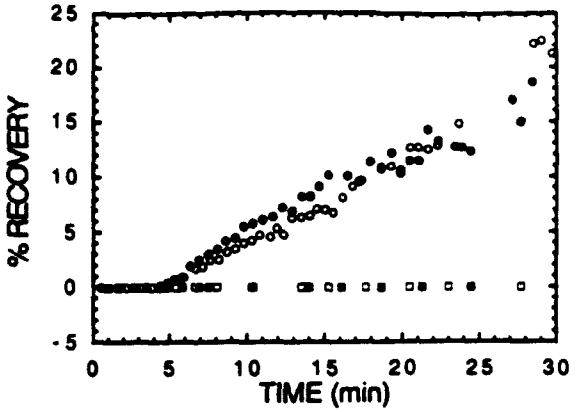

FIGURE 6: Time course of formation of the luciferase heterodimer following dilution of subunits from various solutions of denaturant at 18 °C. In the first experiment, equimolar amounts of α and β subunit were mixed in 5 M urea and diluted 50-fold to 0.1 M urea, and the activity was monitored as a function of time (closed squares). In the second experiment, luciferase was added to 5 M urea at the same concentration as the subunits in the first experiment and diluted 50-fold, and the luciferase activity was monitored (closed circles). In the third experiment, α and β subunits were treated with 5 M guanidine HCl, dialyzed into 5 M urea, and diluted 50-fold, and luciferase activity was monitored (open circles). Two control experiments were also performed. In the first, luciferase incubated in 0.1 M urea was used to indicate the stability of the folded protein under these conditions, and the activity from this experiment was the basis for the percent recovery for the other four experiments. The second control showed the activity of the mixed α and β subunits in the presence of 0.1 M urea (open squares).

Assembly of the α and β Subunits to Form Luciferase. Waddle et al. (1987) showed that individual α and β subunits produced in *E. coli* would not combine to form the active heterodimer unless first unfolded with 8 M urea. Baldwin et al. (1993) have suggested that this behavior is due to folding of the β subunit into an alternative conformation that does not interact with refolding α subunit. To better understand these observations, we have repeated the refolding experiments described by Ziegler et al. (1993) except that we used the individual α and β subunits for refolding rather than the heterodimer. Figure 6 shows that no bioluminescence activity was observed from mixtures of native α and β subunits or from mixtures of subunits that had been incubated in 5 M urea prior to dilution into the refolding buffer. Since Waddle et al. (1987) had obtained complementation with impure subunits unfolded in 8 M urea, we wished to employ stronger conditions than 5 M urea. Subunits first treated with 5 M guanidine-HCl were dialyzed into 5 M urea and then refolded in phosphate buffer under the same conditions as the first two experiments. For comparison under these conditions, heterodimer that had been unfolded in 5 M urea was refolded in phosphate buffer, and, as a control, heterodimer that had never been unfolded was incubated under the same conditions as the refolding samples. The mixture of native subunits and the subunits that had been treated with 5 M urea showed

[2] Because the concentrations of the individual subunits in this experiment were $\sim K_d$, we also plotted the data correcting for the concentration of enzyme-bound flavin. The K_d values obtained by this rigorous treatment of the data were within 20% of the values in Table II, indicating that subpopulations of the individual subunits bind the substrate $FMNH_2$ (see Discussion).

essentially no increase in activity over the time tested, while the subunits that had been unfolded in guanidine-HCl and subsequently transferred to 5 M urea and allowed to refold together showed essentially the same rate of refolding and yield of $\alpha\beta$ as the heterodimer unfolded in 5 M urea and allowed to refold at the same concentration.

DISCUSSION

The luciferase from *V. harveyi* is a remarkably soluble enzyme; the procedures that we have developed for overexpression of the enzyme in *E. coli* yield cells in which luciferase comprises over 50% of the soluble protein (Baldwin et al., 1989), so each subunit of luciferase comprises over 25% of the soluble protein. The same methods that result in accumulation of high levels of luciferase also yield high levels of the individual subunits, but there are several notable differences. First, the individual subunits appear to be less soluble than the heterodimer, especially when cells are grown at higher temperatures. Second, while the accumulation of the individual subunits appears to be similar to the level of accumulation of luciferase, the yield of subunit from the purification scheme is significantly less than from the purification of luciferase (Table I; Baldwin et al., 1986, 1989; Hastings et al., 1978). Purification of the luciferase subunits was facilitated both by the overexpression and by the fact that the subunits appear to be more acidic than the majority of the proteins in lysates of *E. coli* (Waddle et al., 1987). The purification that we have employed relied upon monitoring the activity of the subunits. We cannot rule out the possibility that the subunits fold into multiple stable conformations that are not in rapid equilibrium and that not all of these conformations are active. If this were the case, our purification protocol might resolve active from inactive conformers, thereby resulting in a lower than expected yield of protein. In this regard, it is interesting that the specific activity of the purified β subunit varies from one preparation to the next by up to 4-fold, while the specific activity of the α subunit preparations appears to be relatively constant (data not shown). Furthermore, the total bioluminescence activity of α subunit preparations increased significantly during the purification (note the 194% yield of α subunit activity in Table I), suggestive of removal of an inhibitor or conversion from an inactive to an active conformation. At this time, we have no explanation for the variability of the specific activity of purified β subunit.

Expressing the individual luciferase subunits in different cultures permitted purification of each subunit without contact with the other, thereby eliminating the possibility of trace contamination of one subunit with the other. By resolving the α and β subunits genetically, it has been possible to study each subunit in the absence of the other and to demonstrate that both subunits express flavin- and aldehyde-dependent bioluminescence activity. Both subunits were inhibited by high concentrations of aldehyde, as was the heterodimer. Like the heterodimer, both subunits had a K_m for the protein–$FMNH_2$ complex of about 0.5 μM. For all aldehyde chain lengths tested, the decay of bioluminescence emission from the α subunit was the same as for the heterodimer, whereas the decay of light for the β subunit was slightly slower. These experiments suggest that the active sites formed by the separate subunits are similar to that of the heterodimer. While the active site of the heterodimer has been shown to reside primarily on the α subunit (Cline & Hastings, 1972; Meighen et al., 1971a,b; Baldwin & Ziegler, 1992), the observation of authentic catalytic activity from the isolated β subunit demonstrates that the β subunit must also have a similar site. Whether this site on β is utilized in the heterodimer is a question that is open to debate (Baldwin & Ziegler, 1992). It is interesting to note that Vervoort et al. (1986), using NMR methods, have found two flavin binding sites per $\alpha\beta$, one of high affinity which appears to be the active site and one of lower affinity. It should be noted that we cannot, from the experiments presented here, distinguish between a low specific activity from all molecules of subunit or a higher specific activity from a smaller fraction of the total molecules. That is, the possibility definitely remains that the activity from the individual subunits results from a small subpopulation of each subunit.

As an initial step in characterization of the structures of the individual subunits and the structures of the subunits in combination as they form luciferase, the intrinsic fluorescence and the circular dichroism of the individual subunits were compared with the spectra of the heterodimer. If the environment of the eight tryptophanyl residues [6 in α (Cohn et al., 1985) and 2 in β (Johnston et al., 1986)] and the tyrosinyl residues were the same in the subunits as in the heterodimer, the arithmetic sum of the spectra of the subunits would be expected to yield the spectrum of the heterodimer. However, this is clearly not the case: the fluorescence emission intensity of the α subunit is substantially greater than that of the heterodimer (Figure 5c). The urea-induced unfolding of luciferase monitored by intrinsic fluorescence under equilibrium conditions has demonstrated the existence of a heterodimeric intermediate that is well-populated at equilibrium (Clark et al., 1993). This nonnative heterodimeric species has a higher fluorescence than the native heterodimer, suggesting that the fluorescence of the tryptophanyl residues in the native structure is partially quenched. In fact, the fluorescence per tryptophanyl residue in the native heterodimer is only about 30% of the fluorescence of BSA at equivalent concentrations of tryptophanyl residues (Waddle, 1990). The wavelength of maximum emission is the same for the intermediate as for the native heterodimer, indicating that the tryptophanyl residues in the intermediate have not contacted water but are still buried in the hydrophobic regions of the protein (Clark et al., 1993). Likewise, the spectral properties of the free α subunit suggest that the tryptophanyl residues are buried and that interaction with the β subunit to form the $\alpha\beta$ structure must result in substantial quenching of the intrinsic fluorescence, suggesting that the structure of the free α subunit more closely approximates that of the α subunit in the intermediate heterodimer than that of the α subunit in the native heterodimer. The β subunit has only two tryptophanyl residues, compared with 6 for the α subunit, but its fluorescence intensity is about 8-fold below that of the α subunit (Waddle, 1990).

The sum of the near-UV circular dichroism spectra of the α and β subunits is very close to the spectrum of the heterodimer. The differences are, however, significant and consistent with the observed enhanced fluorescence of the α subunit relative to that of the heterodimer. Such experiments require a precise determination of protein concentration; confidence in the sum of spectra is limited by the confidence in the concentrations of the three samples, α subunit, β subunit, and luciferase. In this case, however, there is not only a slight difference in the amplitude, which might be due to errors in concentration determination, but there are also shifts in peak wavelength in the region of the spectrum where tryptophanyl residues absorb (Figure 5b). In the far-UV, likewise, the spectra sum to yield a spectrum that is nearly the same as that of the heterodimer, but not identical. These results suggest

that the structures of the two subunits as they fold independently are very nearly the same as the structures of the subunits in the luciferase. The fluorescence and near-UV spectral probes sample the regions of the protein in the vicinity of the aromatic residues, while the far-UV samples both the aromatic residue environments and the secondary structure assumed by the peptide backbone.

Investigation of the effect of protein concentration on the rate of recovery of the active heterodimeric luciferase following dilution from 5 M urea demonstrated several features of the refolding of the enzyme (Ziegler et al., 1993; Baldwin et al., 1993). At low protein concentrations, both the rate of recovery and the yield of recovery were reduced. The rate of recovery was low due to the second-order requirement for formation of the high specific activity heterodimer. The reduced yield of active enzyme at lower protein concentrations was attributed to a competing first-order "off-pathway" folding of one or both of the individual subunits that was significant only at low concentrations when the second-order heterodimer assembly step was slow. At higher protein concentrations (≥20 μg/mL) the rate of formation of active luciferase appeared to saturate, which together with other evidence suggested the existence of a first-order (isomerization) step subsequent to the second-order heterodimerization step, the first-order step becoming rate determining when the assembly step is fast (Ziegler et al., 1993).

When the subunits separated by chromatography in 5 M urea were refolded separately and then mixed, there was only a very slight increase in activity, consistent with the conclusion that the activation barrier between the dimerization competent species and the dimerization incompetent species is very large and that there is little interconversion between the two species (Baldwin et al., 1993). It was thus not surprising that mixing of the native recombinant α and β subunits (Figure 6) did not lead to formation of the high specific activity heterodimeric luciferase. What was surprising was the observation that treatment of the individual native subunits with 5 M urea, mixing, and 50-fold dilution from the urea did not lead to formation of the active form of luciferase. The conditions employed were the same as in the refolding experiments described above, which yielded ≥80% active heterodimer (Ziegler et al., 1993; Baldwin et al., 1993). To obtain efficient refolding of the α and β subunits to form the $\alpha\beta$ structure, a stronger denaturant, 5 M guanidine HCl, was required. After denaturation in 5 M guanidine HCl followed by dialysis against buffer containing 5 M urea, the mixed subunits diluted 50-fold from the urea refolded to form luciferase in good yield. This result suggests that while heterodimeric luciferase placed into 5 M urea unfolds completely and rapidly (Ziegler et al., 1993), the native subunits treated with 5 M urea do not unfold sufficiently to interact upon dilution of the denaturant. However, treatment with 5 M guanidine-HCl apparently unfolded the subunits sufficiently to allow assembly of the heterodimer upon dilution; the unfolded subunits remained unfolded in 5 M urea during the dialysis step. Earlier experiments involving refolding of individual subunits from urea indicated that the β subunit assumes a conformation that does not interact with folding or refolded α subunit, while refolded α subunit can assemble with refolding β subunit (Baldwin et al., 1993). These experiments suggested that the β subunit might be resistant to unfolding by 5 M urea. These results are consistent with the earlier suggestion that formation of the active heterodimeric luciferase appears to comprise a kinetic trap and that the individual subunits have available alternative folding pathways that yield stable structures (Sugihara & Baldwin, 1988). The alternative subunit structures are similar but not identical to the subunits in luciferase, and, most interestingly, they are much more resistant to unfolding in urea than is the native luciferase. Kinetic control of protein folding processes has been suggested for other systems as well (Baker et al., 1992; Carrell et al., 1991; Mottonen et al., 1992), and Goldberg (1985) has pointed out that kinetic control might be expected when kinetic intermediates are detected.

ACKNOWLEDGMENT

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STUDIES ON THE KINETIC MECHANISM OF THE BACTERIAL LUCIFERASE-CATALYZED REACTION

W.A. Francisco[2], H.M. Abu-Soud[2,3], F.M. Raushel[1,2] and T.O. Baldwin[1,2]
[1]*Center for Macromolecular Design and Departments of* [1]*Biochemistry & Biophysics and* [2]*Chemistry, Texas A&M University, College Station, TX 77843-2128, U.S.A.*
[3]*Present Address, The Cleveland Clinic Foundation, 1 Clinic Center, 9500 Euclid Ave., Cleveland, OH 44195-5001*

INTRODUCTION

Bacterial luciferase catalyzes the reaction of $FMNH_2$ with molecular oxygen and an aliphatic aldehyde to yield FMN, the carboxylic acid and a photon of blue-green light. The kinetic mechanism of the reaction is complex; there are three substrates and multiple products. The order of binding and the rates of interconversion of the various intermediates have only recently become understood (1,2).

The overall reaction was defined earlier by studies using stopped flow methods (3). More recent studies have defined a consistent set of rate constants that allow simulation of the entire progress of this reaction (1,4). These results are critically important in interpretation of the effects of mutations within the active center of the enzyme on the reaction. Cline and Hastings (5) generated a large set of mutants of the enzyme, and characterized those mutants by steady-state methods. Chemical modification experiments demonstrated the existence of a highly reactive thiol in or near the active center at position α106 (2). An in-depth understanding of the role of the reactive thiol and of the residues modified in the Cline mutants will require a thorough investigation of the kinetic mechanisms of mutant enzymes. We have constructed a mutant αC106V and shown that the mutant enzyme is active, but that it binds $FMNH_2$ only weakly (6). Xi et al (7), using the same αC106V mutant luciferase, showed that the rate of formation of FMN was much faster with this enzyme than in the absence of the enzyme and concluded that the mutation resulted in a mechanistic shift of the enzyme from a flavin monooxygenase to a flavin oxidase. We have investigated this enzyme in detail and have found that the mutant enzyme forms the same 4a-peroxydihydroflavin intermediate that is formed by the wild type enzyme (4,8). The reason that FMN is formed so rapidly by this mutant enzyme is that the peroxyflavin intermediate breaks down much more rapidly than the wild type intermediate to yield FMN and H_2O_2.

An overview of our current understanding of the kinetic mechanism of the bacterial luciferase reaction is presented here. The rate constants developed in these studies are internally consistent; using these rate constants, one may analyze the validity of various assumptions that underlie methods commonly used by workers in the field.

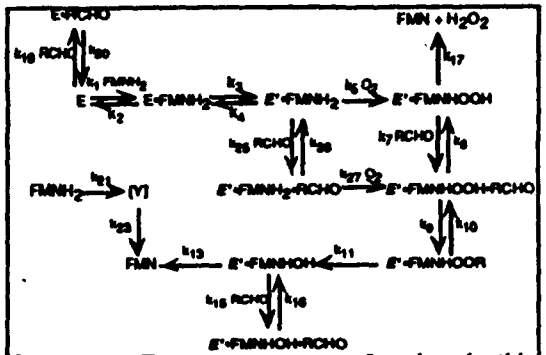
MATERIALS AND METHODS

Enzyme was purified from *E. coli* carrying the *luxAB* genes as described (6). Formation and decay of various intermediates in the reaction were monitored spectrophotometrically using a Hi-Tech Ltd. model SF-51 stopped flow instrument. Formation of the peroxyflavin intermediate was monitored at 380 nm, which is isosbestic with FMN. Formation of FMN was monitored at 445 nm. Light emission was monitored directly from the observation cell of the stopped flow device. Oxygen concentration was measured using an Orion pH meter equipped with an oxygen electrode. Unless otherwise stated, enzyme concentration was 75 μM, $FMNH_2$ concentration was 15 μM, and oxygen concentration was 120 μM. All measurements were made in 50 mM bis-tris HCl, pH 7.0 at 25±0.2°. All data were transferred to a

Silicon Graphics workstation and analyzed using modified forms of the KINSIM and FITSIM programs (4).

RESULTS AND DISCUSSION

The kinetic mechanism that we have determined for the bacterial luciferase-catalyzed bioluminescence reaction is depicted in Scheme I (4). The free enzyme can bind either $FMNH_2$ or the aldehyde. The E•$FMNH_2$ complex undergoes an



Scheme I. The rate constants referred to in this scheme are presented in Table 1.

Table I: Rate Constants for the Kinetic Model of Scheme I

	Wild Type	αC106V		Wild Type	αC106V
k_1	1.7×10^7 $M^{-1}s^{-1}$	1.0×10^7 $M^{-1}s^{-1}$	k_{15}	3000 s^{-1}	
k_2	1200 s^{-1}	1400 s^{-1}	k_{16}	0.06 s^{-1}	
k_3	200 s^{-1}	120 s^{-1}	k_{17}	0.10 s^{-1}	15 s^{-1}
k_4	14 s^{-1}	11 s^{-1}	k_{19}	9.1×10^5 $M^{-1}s^{-1}$	1.0×10^6 $M^{-1}s^{-1}$
k_5	2.4×10^6 $M^{-1}s^{-1}$	2.7×10^6 $M^{-1}s^{-1}$	k_{20}	5.8 s^{-1}	6.6 s^{-1}
k_7	1.9×10^7 $M^{-1}s^{-1}$	1.2×10^7 $M^{-1}s^{-1}$	k_{21}	4.7 s^{-1}	
k_8	120 s^{-1}	2200 s^{-1}	k_{23}	11.5 s^{-1}	
k_9	1.6 s^{-1}	13 s^{-1}	k_{25}	1.2×10^6 $M^{-1}s^{-1}$	
k_{10}	1.2 s^{-1}	1.4 s^{-1}	k_{26}	37 s^{-1}	
k_{11}	1.1 s^{-1}	0.02 s^{-1}	k_{27}	5.1×10^4 $M^{-1}s^{-1}$	
k_{13}	0.60 s^{-1}				

[a]pH 7.0, 25 °C. Note that k_{21} and k_{23} (4.7 and 11.5 s^{-1}, respectively) were determined by a fit of the time courses for the reaction of $FMNH_2$ with O_2 monitored at 380 and 445 nm to a sequential mechanism (Eq. 5; ref. 1).

isomerization step prior to reaction with O_2 to yield the 4a-peroxydihydroflavin intermediate (1) (*Initial* spectrum in Figure 1). In the absence of aldehyde, this intermediate decays in a first order "dark" reaction to yield FMN (*Final* spectrum in Figure 1) and H_2O_2 (3). These steps were elucidated in two sets of experiments. First, mixtures of enzyme and $FMNH_2$ in one syringe were mixed with various concentrations of O_2 (dissolved in buffer) in the other syringe and the rate of formation of the peroxyflavin monitored. The data were fit to a single exponential; the first order rate constant was directly proportional to the concentration of O_2, allowing determination of the

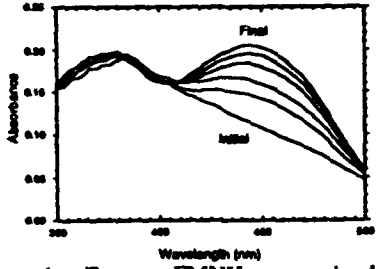


Figure 1. Enzyme•$FMNH_2$ was mixed with air-equilibrated buffer and absorbance spectra scanned. The first spectrum was scanned 7 ms after mixing. Subsequent spectra were scanned at 2.8 s intervals for a period of 25.8 s. Not all spectra are shown.

second order rate constant, k_5, of 2.4×10^6 $M^{-1}s^{-1}$. On the other hand, when enzyme and oxygen were mixed with $FMNH_2$, the rate of formation of the peroxyflavin reached a limiting rate at high [O_2] that was much less than k_5, indicating the existence of a slower isomerization step preceding reaction with O_2. By fitting these data to the steps shown in Scheme I, the rate constants k_3 and k_4 for the isomerization of the E•$FMNH_2$ complex were determined. In the absence of aldehyde, the peroxyflavin intermediate decayed to yield FMN and H_2O_2. Formation of FMN, monitored at 445 nm, occurred in a first order process with a rate constant of 0.1 s^{-1}.

The rate constants for the processes associated with aldehyde binding and generation of the light emitting species were estimated by monitoring the time-dependent onset and decay of light emission. These rate constants (k_7 through k_{11} in Scheme I) were determined by fitting of the data obtained when E'•$FMNH_2$ was mixed with O_2 and varying concentrations of decanal. We assume two steps leading to the emitter, the first as an equilibrium binding of aldehyde and the second involving reaction of the aldehyde with the peroxyflavin to yield the tetrahedral intermediate. Breakdown of the tetrahedral intermediate yields the 4a-hydroxyflavin, which we assume emits light on a nanosecond or faster timescale. The various bioluminescence time courses could be well fit with rate constants for k_7 through k_{11} of 1.9×10^7 $M^{-1}s^{-1}$, 120 s^{-1}, 1.6 s^{-1}, 1.15 s^{-1}, and 1.11 s^{-1}, respectively. Finally, we observed that the release and dehydration of the flavin hydroxide to yield FMN was dependent on the concentration of aldehyde, suggesting that aldehyde could bind to the enzyme•hydroxyflavin complex and prevent the dehydration step. The aldehyde dependence of the rate of formation of FMN required us to incorporate the binding and release steps k_{15} and k_{16}, respectively.

The free enzyme can also bind aldehyde, but the E•aldehyde complex appears to be unable to bind $FMNH_2$ (4). If enzyme is incubated with aldehyde prior to injection of $FMNH_2$, apparent inhibition results due to the competing reaction of free $FMNH_2$ with molecular oxygen (Fig. 2). When enzyme with O_2 and varying concentrations of aldehyde was mixed with $FMNH_2$ and the formation of the peroxyflavin monitored at 380 nm, the reaction was clearly biphasic. At low aldehyde concentrations, the time courses were the same as for reaction of aerobic enzyme with $FMNH_2$. In the presence of very high concentrations of aldehyde, the reaction appeared the same as for reaction of $FMNH_2$ with O_2 in the absence of enzyme. The same result was observed whether the reaction was monitored at 380 nm or at 445 nm. These observations demonstrate that the enzyme•aldehyde complex which forms at high aldehyde concentration cannot bind $FMNH_2$ and explain the mechanism of aldehyde substrate inhibition described earlier (9). Monitoring of the inhibition of the bioluminescence activity by high aldehyde allowed us to determine the rate constants for binding and release of aldehyde by free enzyme, k_{19} and k_{20}, to be 9.1×10^5 $M^{-1}s^{-1}$ and 5.8 s^{-1}, respectively.

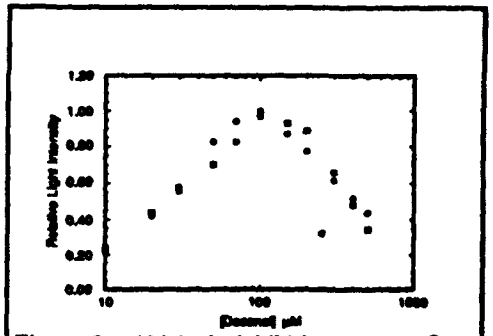


Figure 2. Aldehyde inhibition curve. Open squares represent experimental data. Reactions were initiated by injection of $FMNH_2$ into solutions of enzyme, aldehyde and O_2. The initial maximum light intensity is given in the figure. The closed symbols are calculated values based on the rate constants in Table I and the kinetic mechanism in Scheme I.

High aldehyde concentrations were found to decrease the rate of formation of the peroxyflavin intermediate as well, when the enzyme was incubated with decanal and

$FMNH_2$ prior to mixing with O_2, suggesting that aldehyde could bind to the E'•$FMNH_2$ complex and thereby alter the rate of reaction with O_2. When enzyme•$FMNH_2$ and varying concentrations of decanal were mixed with O_2, the reaction monitored at 380 nm was strongly biphasic. At low concentrations of aldehyde, the fast phase predominated, while at high aldehyde concentration, the slow phase predominated. Furthermore, when the O_2 concentration-dependence of the rate of reaction of O_2 with E•$FMNH_2$•aldehyde was investigated, the apparent second order rate constant determined was about 100-fold less than for the same reaction in the absence of aldehyde. These results demonstrated the existence of the ternary complex. To fit the data, it was necessary to include both the equilibrium binding of aldehyde to E'•$FMNH_2$ and the direct reaction of O_2 with the resulting ternary complex. These processes are indicated by k_{25}, k_{26} and k_{27} in Scheme I.

The commonly used method to determine the $FMNH_2$ binding affinity of bacterial luciferase is the "dithionite assay" described by Meighen and Hastings (10). In this method, a solution of enzyme and rMN is reduced and O_2 removed by addition of a small amount of dithionite. The bioluminescence reaction is initiated by injection of air equilibrated aldehyde. By varying the concentration of FMN (and therefore of $FMNH_2$) and determining the flavin concentration dependence of the initial maximum light intensity, a Lineweaver-Burk plot may be constructed and the K_m for flavin determined. It has been commonly assumed that for the bacterial luciferase reaction, the K_m so determined is in fact a K_d. In the dithionite assay, the enzyme and $FMNH_2$ equilibrate. When O_2 is injected, two reactions occur. The enzyme-bound flavin reacts with O_2 to form the peroxyflavin which goes on to yield light. Unbound flavin reacts with O_2 to yield FMN and H_2O_2, such that no turnover occurs. The initial maximum light intensity is then taken as diagnostic of the amount of E•$FMNH_2$ complex at the time of O_2 injection, and it is assumed that the rate of reaction of free flavin is sufficiently fast that no additional flavin binding can occur. However, the rate of reaction of O_2 with enzyme bound flavin is fast (k_5 in Table 1), and the rate of reaction of the free flavin with O_2 is comparatively slow (k_{21} and k_{23}). These results call into question the assumption that the K_m for this reaction is a true K_d. Based on the rate constants given in Table 1, the calculated K_d for $FMNH_2$ is 4.9 μM. By using Scheme I and the rate constants in Table 1, we have calculated bioluminescence profiles for flavin concentrations ranging from 0.1 μM to 10 μM. Plotting of the resulting data and fitting to the Michaelis-Menten equation yields a value of 3.44 μM for K_m. For most purposes, these numbers are in reasonable agreement. However, it should be stressed that the binding of $FMNH_2$ is a two step process, since the initial complex isomerizes (k_3 and k_4 in Scheme I and Table 1).

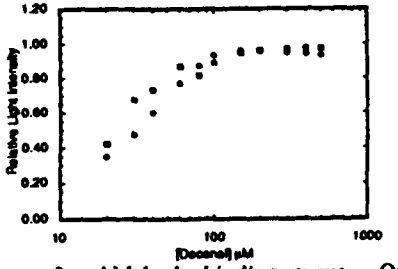


Figure 3. Aldehyde binding curve. Open squares represent experimental data from reaction of E•$FMNH_2$ with aerobic decanal in which the concentration of aldehyde was varied. In this assay format, no inhibition was apparent at high aldehyde concentrations. The filled circles represent calculated values based on Scheme I and the rate constants in Table 1.

The results of this analysis also demonstrate why the bioluminescence reaction performed by the dithionite method is not sensitive to aldehyde substrate inhibition, while the normal flavin injection assay is sensitive to aldehyde inhibition (compare Figs. 2 & 3). In the former assay format, the enzyme-flavin complex forms without exposure to aldehyde. The reaction with O_2 is fast, and at high flavin concentrations, there is effectively no free enzyme to bind to aldehyde. In the flavin injection assay, the enzyme first equilibrates with aldehyde prior to injection of $FMNH_2$. Any enzyme that is complexed with aldehyde at the time of $FMNH_2$ injection is effectively removed from the reaction, since it has to dissociate from the aldehyde before it can bind the flavin. The excess reduced flavin is at the same time being removed by reaction with O_2.

Finally, the results of our experiments demonstrate clearly that the reaction catalyzed by bacterial luciferase is complex. Light is one of the final products of the reaction, and it is a valuable experimental parameter with which to monitor the overall reaction. However, it is not possible to adequately investigate all aspects of this complex reaction by monitoring bioluminescence. It is essential to perform a thorough investigation of the kinetic mechanism prior to developing hypotheses regarding the effects of inhibitors, mutations, or other modifications of the enzyme or its substrates.

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FOLDING AND ASSEMBLY OF THE SUBUNITS OF BACTERIAL LUCIFERASE

M.M. Ziegler[2], A.C. Clark[2], J.F. Sinclair[2], A.F. Chaffotte[1,2,4], M.E. Goldberg[4] and T.O. Baldwin[1,2,3]

[1]*Center for Macromolecular Design and Departments of* [2]*Biochemistry & Biophysics and* [3]*Chemistry, Texas A&M University, College Station, TX 77843-2128, U.S.A.*
[4]*Unité de Biochimie Cellulaire, Institut Pasteur, 28 rue du Dr. Roux, Paris 75724 (CEDEX 15), France*

INTRODUCTION

Bacterial luciferase is a flavin monooxygenase, catalyzing the reaction

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + light.$$

The enzyme has a heterodimeric structure, with two nonidentical but homologous subunits, α and β (1,2). The subunits can be dissociated and resolved by ion exchange chromatography in 5 mol/l urea (3), yielding the unfolded chains (in 5 mol/l urea) which can be refolded together to yield the active heterodimer (4-6). The subunits obtained by chromatography in urea can be refolded separately, but the very low activity observed for the individual refolded subunits was attributed to incomplete resolution of the subunits (3). Recently, the individual subunits of *V. harveyi* luciferase have been expressed separately in *E. coli* (7) and purified under native conditions (8). With a sensitive photometer and no possibility of contamination with the other subunit (since the expression system included only one gene or the other), it was discovered that both of the individual subunits had low but authentic catalytic activity, about 0.0005% that of the heterodimeric enzyme (8,9). Except for the low specific activities, the catalytic parameters (substrate binding affinities, substrate inhibition by aldehyde, and kinetics of light emission in the standard single-turnover assay) of the separate α and β preparations are very similar to that of the heterodimer (8). The α and β subunits folded separately *in vivo* (7) or *in vitro* (5) fail to form the high specific activity heterodimer upon mixing either under non-denaturing conditions or after treatment of the recombinant subunits with 5 mol/l urea followed by dilution together out of the urea. However, treatment of the recombinant subunits with a stronger denaturant (5 mol/l guanidine HCl) followed by dialysis into 5 mol/l urea and then dilution out of the urea resulted in formation of the high specific activity αβ species (8). Thus the native recombinant α and/or β species is resistant to unfolding in 5 mol/l urea, but is susceptible to unfolding in 5 mol/l guanidine HCl. These results suggested that formation of the heterodimer *in vivo* must occur by interaction of transient species of α and/or β which differ in conformational stability from the stable forms of the subunit(s) which result from folding separately.

MATERIALS AND METHODS

Luciferase was expressed in *Escherichia coli*; the enzyme was purified and assayed and unfolding/refolding procedures were carried out at 18 °C in 50 mmol/l or 400 mmol/l phosphate (as specified), pH 7.0, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, as previously described (4-6).

RESULTS AND DISCUSSION

The far uv circular dichroism spectrum of the luciferase heterodimer suggests that the enzyme is fully unfolded in 5 mol/l urea (4). The kinetics of reappearance of luciferase activity upon dilution out of 5 mol/l urea (Fig. 1) illustrate several points. First, there is a concentration-independent lag of about 3 min (Fig. 1A), reflecting first-order folding steps of the individual subunits preceding the heterodimerization-competent species (shown as α_i and β_i in Scheme 1). If both subunits are permitted to refold separately

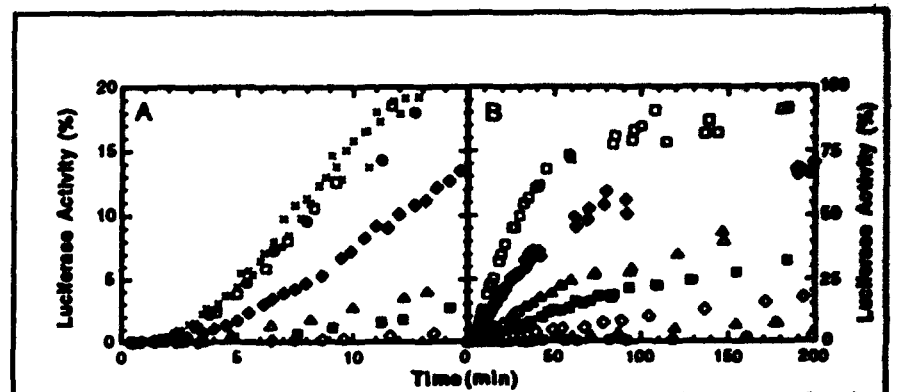
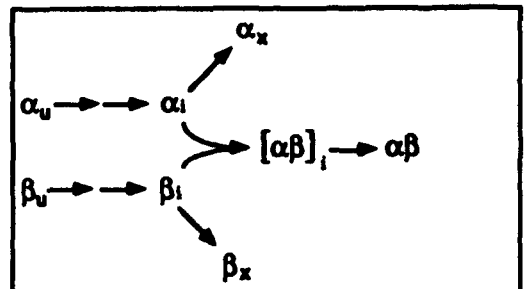


Figure 1. Effect of luciferase concentration on rate and extent of recovery of active enzyme. The enzyme was denatured in 5 mol/l urea and after initiation of refolding by rapid 50-fold dilution of the enzyme into renaturation buffer (50 mmol/l phosphate), the time course of formation of active luciferase was monitored by removal of aliquots for assay (4). The initial 15 min are expanded in Panel A, and a longer time course is shown in Panel B. Protein concentrations in the refolding mixtures were 0.2 μg/ml (○), 0.4 μg/ml (▲), 0.8 μg/ml (◇), 2.0 μg/ml (■), 4.0 μg/ml (△), 10 μg/ml (◆), 20 μg/ml (□), 50 μg/ml (×), 100 μg/ml (●). Percent recovery is expressed relative to the activity of a native control sample at each concentration diluted into the same renaturation buffer, 0.1 mol/l in urea, and incubated for the same period of time.

for several minutes prior to mixing, there is no lag in appearance of activity (5). Second, the rate of appearance of active enzyme following the lag is concentration-dependent up to about 20 μg/ml, presumably reflecting the second-order formation of the heterodimer. Finally, at higher concentrations the rate appears to become concentration-independent (Fig. 1B). This result, together with the results of secondary dilution experiments in which activity recovery continued at the higher rate for about 1.5-2 min following 10-fold dilution (4), led us to postulate a first-order (isomerization) step in the folding pathway following formation of the heterodimer, by which an inactive αβ species is converted to the active heterodimer (Scheme 1). Refolding of the individual subunits for several hours or longer prior to mixing resulted in markedly reduced yields of active heterodimer (5), consistent with the observation that subunits produced separately in *E. coli* (folded *in vivo*) had assumed heterodimerization-incompetent structures (7,8).



Scheme 1. Kinetic mechanism of folding and assembly of bacterial luciferase subunits (4,5).

The kinetics experiments (4,5) suggested a folding mechanism (Scheme 1), in which (first-order) folding steps for the individual subunits precede the (second-order) heterodimerization step, whose product $[\alpha\beta]_i$ is inactive and undergoes an isomerization to the active αβ species. The formation of the heterodimerization incompetent, 5M-urea resistant form of α and/or β (α_x and/or β_x) is a slower process observable in the absence of the other subunit.

The interpretation of the kinetics experiments was in good agreement with the 3-state model proposed based on equilibrium experiments (6), involving isomerization of the native heterodimer to a nonnative heterodimer $[\alpha\beta]_i$ and dissociation of the nonnative heterodimer to yield unfolded subunits (Fig. 2). Equilibrium data (circular dichroism signal at 222 nm, intrinsic fluorescence, and activity as a function of urea concentration) were fit to the three-state model using a Macintosh version of Nonlin (Robelko Software, Carbondale, IL) (6). The α and β subunits were assumed to be identical in this treatment. The fitting yielded highly consistent results, both between spectral methods and with different protein concentrations. Using average values from the experimental data for $\Delta G_1^{H_2O}$ [4.52 kcal/mol], $\Delta G_2^{H_2O}$ [19.7 kcal/mol], m_1 [2.38 kcal·mol^{-1}·(mol/l)$^{-1}$] and m_2 [3.99 kcal·mol^{-1}·(mol/l)$^{-1}$], it is possible to calculate the equilibrium distribution of the three species, native enzyme, intermediate and subunits, at each urea concentration. The results of these calculations are shown in Fig. 2.

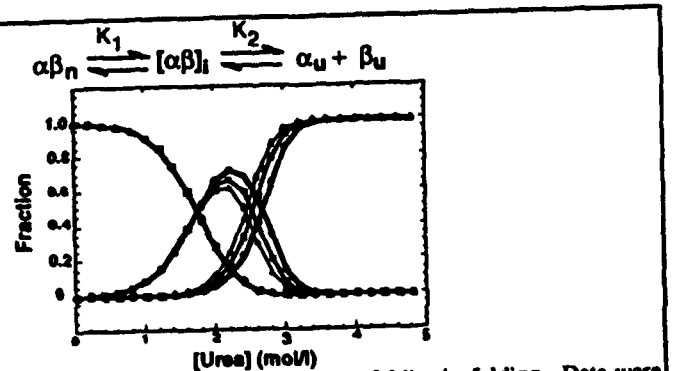


Figure 2. Three-state equilibrium in bacterial luciferase folding/unfolding. Data were calculated for three different protein concentrations, 25 mg/ml, 10 mg/ml, and 5 mg/ml. The fraction of protein in the intermediate form is highest for the highest protein concentration, about 65% at 2.3 mol/l urea and 25 mg/ml total protein. $\alpha\beta_n$, native heterodimer; $[\alpha\beta]_i$, inactive heterodimer; α_u and β_u, unfolded subunits.

Both experimental approaches yielded the same model for the folding-assembly reaction of the luciferase subunits. In the refolding reaction, the unfolded subunits undergo first order folding steps to yield heterodimerization-competent structures which assemble to form an inactive heterodimer, $[\alpha\beta]_i$. This inactive form then isomerizes to yield the active heterodimeric enzyme. Under nondenaturing conditions in 50 mmol/l phosphate, pH 7.0 and 18°, the equilibrium constant K_1 for the conversion of the native heterodimer to $[\alpha\beta]_i$ was 4.03×10^{-4}. This equilibrium is concentration independent; under these conditions, the ratio of active to inactive heterodimer is about 2500 to 1. For the dissociation of $[\alpha\beta]_i$ to the unfolded subunits, the equilibrium constant K_2 was 1.60×10^{-15} mol/l. The overall equilibrium dissociation constant for the conversion of the active heterodimer to free subunits should therefore be about 6.43×10^{-19} mol/l under these conditions.

When the individual subunits were allowed to refold to completion independently prior to mixing, the native heterodimeric structure did not form (5,7,8). Investigation of the stability properties of the subunits synthesized and folded individually in *E. coli* demonstrated that the β subunit assumes a structure that is insensitive to unfolding in 5 mol/l urea (Sinclair and Baldwin, unpublished). This unexpected observation demonstrates a hysteresis in the equilibrium unfolding of the β subunit: the αβ structure

is completely unfolded in 5 mol/l urea, and the β subunit stays unfolded under these conditions. However, the native free β subunit isolated from *E. coli* does not unfold in 5 mol/l urea.

The effects of ionic strength on the rates of protein folding have not been investigated in detail, while the effects of ionic strength on protein stability have been investigated for numerous proteins. It is well known that multivalent anions stabilize the bacterial luciferase structure (10). We have begun to investigate the effects of ionic strength and specific phosphate ion effects on the rate of refolding of the luciferase subunits. When luciferase was refolded at low concentration from urea in buffer at high phosphate concentration, the rate of activity recovery and the final yield of activity recovered were markedly enhanced; at 2 μg/ml, the recovery was 100%, and the rate was essentially the same as the rate with 50 μg/ml in low ionic strength buffer (Fig. 3). The ionic strength/phosphate effects appear to be primarily on the rate of folding of the individual subunit(s) prior to dimerization, as suggested by the decreased lag in the presence of high salt. Based on our model (Scheme 1), at low protein concentration, the rate of formation of active enzyme is limited by the rate of heterodimer formation, while at high protein concentration, the rate of formation of active enzyme is limited by the first order isomerization of $[\alpha\beta]_i$ to αβ. If the effect of high salt were to accelerate the rate of formation of the heterodimerization-competent subunit species α_i and β_i, then the concentrations of these species at early times would be higher, leading to a faster rate of formation of $[\alpha\beta]_i$. This hypothesis is consistent with the observed shorter lag phase in the presence of high salt (Fig. 3). Phosphate had a more marked effect than the same ionic strength of NaCl.

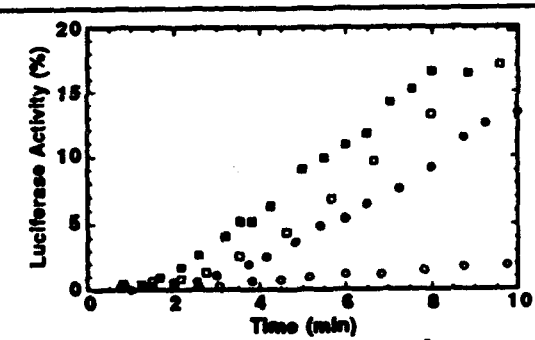


Figure 3. Effect of phosphate concentration on rate of recovery of active enzyme at low and high protein concentration. The enzyme was denatured in 5 mol/l urea and after initiation of refolding by rapid 50-fold dilution of the enzyme into renaturation buffer, the time course of formation of active luciferase was monitored by removal of aliquots for assay (4). Only the initial 10 min are shown. Protein concentrations in the refolding mixtures were 2.0 μg/ml (open symbols) and 50 μg/ml (closed symbols), in renaturation buffer at 50 mmol/l phosphate (circles) or 400 mmol/l phosphate (squares). Percent recovery is expressed relative to the activity of a native control sample at each concentration diluted into the same renaturation buffer, 0.1 mol/l in urea, and incubated for the same period of time.

The luciferase subunits are highly charged and anionic (1,2). In the early phases of protein refolding, condensation of the unfolded structure would be impeded by charge-charge repulsion. This presumed inhibitory effect would be partially overcome by Debye-Hückel screening of the charged groups on the protein, allowing for a faster condensation of the unfolded protein. The lag phase that is apparent in both Figs. 1 and 3 was protein concentration independent. The lag was shown to be due to first order folding steps involving both of the individual subunits (5). The fact that high ionic strength reduced the duration of the lag indicates that the effect of salt is primarily on

the first order folding steps of the individual subunits. At this time, it appears that the most likely explanation of this effect is charge shielding.

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L.J. Kricka, and P. Stanley
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TWO CYSTEINYL RESIDUES CONSERVED IN ALL INSECT LUCIFERASES ARE NOT ESSENTIAL FOR THE ACTIVITY OF *LUCIOLA MINGRELICA* FIREFLY LUCIFERASE

Galina D. Kuntzova[1] and Thomas O. Baldwin
Center for Macromolecular Design and Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843, USA

INTRODUCTION

At the present time amino acid sequences of four firefly luciferases: *Photinus pyralis* (1), *Luciola cruciata* (2), *L. lateralis* (3), *L. mingrelica* (4) and of the bioluminescent click beetle *Pyrophorus plagiophthalamus* (5) are known. The luciferases from the fireflies and the clickbeetles are clearly homologous (Fig.1). The *Luciola* luciferases are about 80% identical in amino acid sequence throughout, the strongest similarity being within the carboxyl terminal half of the molecule; alignment from residue 200 to the carboxyl terminus shows more than 90% identity. A comparison between the *L. mingrelica* and *P. pyralis* luciferases shows about 67% identity, while the *L. mingrelica* luciferase has only 43% identity with the clickbeetle (green) luciferase.

The regions of amino acid sequence that are the most stringently conserved in protein families are those associated with the active center and, in the case of multimeric proteins, the subunit interface. Comparisons of the encoded amino acid sequences (Fig.1) have revealed an apparent homology between all insect luciferases and several Mg^{2+}ATP, CoA-utilizing enzymes, which share a common catalytic reaction mechanism (6-8). Long-chain acyl-CoA synthetases and plant 4-coumarate:CoA ligases catalyze the formation of acyl-CoAs from ATP, CoA, and carboxylic acids. Acyl-AMPs are the intermediates formed in the first reaction. Peptide antibiotic synthetases, gramicidin S synthetase 1 and tyrocidine synthetase 1 also catalyze the formation of amino-acyl-AMP from ATP and phenylalanine in the first reaction, and transfer the aminoacyl moiety to an SH group on the enzymes (not to the SH group of CoA as in the other enzymes) to yield covalently bound thiolester-linked phenylalanine in the second reaction.

The amino acid compositions of beetle luciferases are very similar. The total numbers of charged residues are essentially the same for all insect luciferases. The major differences are in the numbers of tryptophanyl and cysteinyl residues. The luciferase from *L. mingrelica* and the luciferases from *L. cruciata* and *L. lateralis* have a single tryptophanyl residue, which is conserved in all beetle luciferases, while the others have 2-3 residues in different positions. The *P. pyralis* luciferase has 4 cysteinyl residues, the *Luciola* luciferases have 7-8 and the clickbeetle luciferases have 13 cysteinyl residues. Only two cysteinyl residues, in positions 82 and 393 (for *L. mingrelica*) are conserved in all beetle luciferases, and residue 393 is conserved in long-chain acyl-CoA synthetase and in plant 4-coumarate:CoA ligase.

Alter and DeLuca reported that the thiols of *P. pyralis* luciferase are not essential for the bioluminescence reaction (9). They showed at least two slow reacting thiols (reaction with the thiol-directed reagents N-ethylmaleimide and DTNB) which when modified resulted in red light emission and a decreased quantum yield, suggesting that these thiols may be located at or near the active site. Based on the assumption that the two cysteinyl residues conserved in all beetle luciferases and related enzymes might be those studied by DeLuca, we constructed two mutants of the *L. mingrelica* luciferase, Cys82-Ala and Cys393-Ala and investigated their properties in comparison with wild type enzyme.

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[1] *Present address: Promega Corporation, 2800 Woods Hollow Rd, Madison WI, 53711, USA*

MATERIALS AND METHODS

The cloning, sequencing, overexpression and purification of the luciferase from *L. mingrelica* have been described in our previous paper (4). One of the plasmid vectors described there, pGK2, a pBluescript plasmid carrying *L. mingrelica* luciferase cDNA with a modified 5'-nontranslated region for optimized expression, was used for mutagenesis and as a source of wild-type and mutant luciferases. Enzyme assays and purification were performed essentially as described previously (4); modifications are mentioned in the text. Wild-type and mutant luciferases used in these studies were about 90% pure.

Oligonucleotide-directed site-specific mutagenesis and sequence analysis The codons encoding Cys-82 and Cys-393 in the wild-type luciferase in pGK2 were modified to Ala codons. Twenty-one base oligonucleotides corresponding to the coding region of the luciferase cDNA were synthesized with the desired nucleotide mismatches (GC instead of TG) at the central position thus creating a new BalI site for the C82A mutant and eliminating a BglI site for the C393A mutant. Mutagenesis was performed as described by Kunkel et al. (10). All mutations were confirmed by DNA sequence analysis.

Purification of wild-type and mutant *L. mingrelica* luciferases from *E. coli* Purification of wild-type recombinant luciferase was performed from 9 liters *E. coli* culture carrying the recombinant plasmid pGK2 grown at 18°C until late log phase. Cells were harvested by centrifugation at 4°C and resuspended in the assay buffer (0.1 mol/l Tris-acetate, 10 mmol/l $MgSO_4$, 2 mmol/l EDTA, pH 7.8). After cell lysis the crude lysate was fractionated with ammonium sulfate and the fraction precipitating between 35%-75% saturation was collected and dialysed exhaustively against 0.05 mol/l Tris-acetate buffer (pH 7.8, 2 mmol/l EDTA, 5 mmol/l $MgSO_4$, 10% glycerol). The resulting sample was applied to a DEAE Sephadex A-50 column (5x20 cm; 300 ml bed volume in a 5x30 cm Bio-Rad Econo-Column) equilibrated with the same buffer, washed with 500 ml of starting buffer and then eluted with a linear gradient formed from 1 liter each of the starting buffer and the same buffer containing 80 mmol/l $MgSO_4$. The fractions with highest luciferase activity were collected, pooled, concentrated and applied to a column of Blue Sepharose (1.5x19cm) that had been equilibrated with 0.05 mol/l Tris-acetate buffer, pH 7.8, 2 mmol/l EDTA, 60 mmol/l $MgSO_4$ with 10% glycerol. Luciferase was eluted with 10 mmol/l ATP in the same loading buffer or the loading buffer but containing 20% ethylene glycol. SDS-polyacrylamide gel electrophoresis on a 10% gel showed 90% purity of the wild-type and mutant enzymes.

Bioluminescence emission spectra Emission spectra of luciferase-catalyzed bioluminescence were measured in the fluorometer (SLM Aminco) with the excitation lamp turned off. Emission was recorded from 520 to 620 nm after the emission intensity was determined to be constant.

Inactivation of luciferase with N-ethylmaleimide (NEM) The chemical modification of wild-type and mutant luciferases (0.1 mg/ml) with 0.1 mmol/l NEM was carried out in 0.1 mol/l phosphate buffer, pH 7.2 at 0°C. The concentration of NEM was determined by measuring the absorbance at 302 nm where ε_{302}=620 $M^{-1}cm^{-1}$. The reaction was stopped by addition of DTT to a final concentration of 1 mmol/l. Aliquots were taken during the course of the reaction and assayed for luciferase activity.

RESULTS AND DISCUSSION

Expression and purification of wild-type and mutant luciferases We used the same method to purify luciferase encoded by pGK2 that was developed by Devine et al. (4) for recombinant *L. mingrelica* luciferase encoded by pJGR. The latter plasmid encoded an enzyme with a 9 residue fusion of the amino terminus of the LuxC protein from *Vibrio fischeri*, while pGK2 encodes the native luciferase. Expression from pJGR was about 3-4 times higher than from pGK2. To ascertain if the higher expression might be due to the fusion with LuxC, we deleted the LuxC sequence from pJGR, creating a new plasmid, pGKR, which was otherwise identical to pJGR. The expression from pGKR was identical to pGK2, suggesting that the elevated expression from pJGR might be due to enhanced translational efficiency and/or greater stability of the fusion protein in *E. coli*. Wild-type luciferase from pGK2 bound more weakly to DEAE Sephadex than did the luciferase encoded by pJGR (4). Column chromatography of the wild-type enzyme resulted in two peaks of activity, one eluting essentially unretarded and the second eluting with a $MgSO_4$ gradient. Protein eluting in the second peak was pooled, concentrated and applied to a Blue Sepharose column in a buffer containing 10% glycerol. The wild-type protein was only slightly retarded by this column, eluting

after a peak of contaminating proteins. The C82A mutant behaved the same as wild-type luciferase; both eluted about 90% pure from the Blue Sepharose column. The C393A mutant luciferase chromatographed on the DEAE Sephadex the same as the wild-type and C82A mutant, but unlike wild-type and C82A luciferases the C393A enzyme bound tightly to the Blue-Sepharose column, requiring either 15 mmol/l ATP or 20% ethylene glycol for elution. These chromatographic properties suggest that the region of the enzyme that binds the Cibacron Blue dye may become more hydrophobic as a result of the C393A mutation.

Comparison of the activity and bioluminescence emission spectra of mutant and wild-type luciferases The specific activity of the mutant luciferases appeared to be very similar to the wild-type enzyme under the same conditions. Kinetic parameters of the mutant luciferases differed only slightly from the wild-type. The time course of light emission for the mutants was identical to that of the wild-type enzyme, with an initial peak of light followed by a slow decay. The bioluminescence emission spectra of the C82A and C393A mutants were identical to the wild-type spectrum, showing yellow-green emission with a maximum at 562 nm. The identity of kinetic properties of wild-type and mutant luciferases indicates that these two cysteinyl residues conserved in all insect luciferases are not essential for the catalytic activity of *L. mingrelica* firefly luciferase. These results do not rule out the possibility that one or both of these residues were the target of the modification experiments of Alter and DeLuca which yielded altered bioluminescence emission spectra (9). Modification of the reactive α106C residue of *V. harveyi* bacterial luciferase even with the small -SCH_3 group leads to complete inactivation of the enzyme (11), while mutation to Ala or Ser yields essentially wild-type activity (12).

Stability of wild type, C82A and C393A mutant luciferases. Firefly luciferases are rather labile enzymes *in vitro*. The luciferase isolated from *L. mingrelica* fireflies loses about 50% activity in 2-3 hours incubation at room temperature in the assay buffer (13). The stability of *L. mingrelica* luciferase depends on its concentration, and the differences may be attributed to differences in

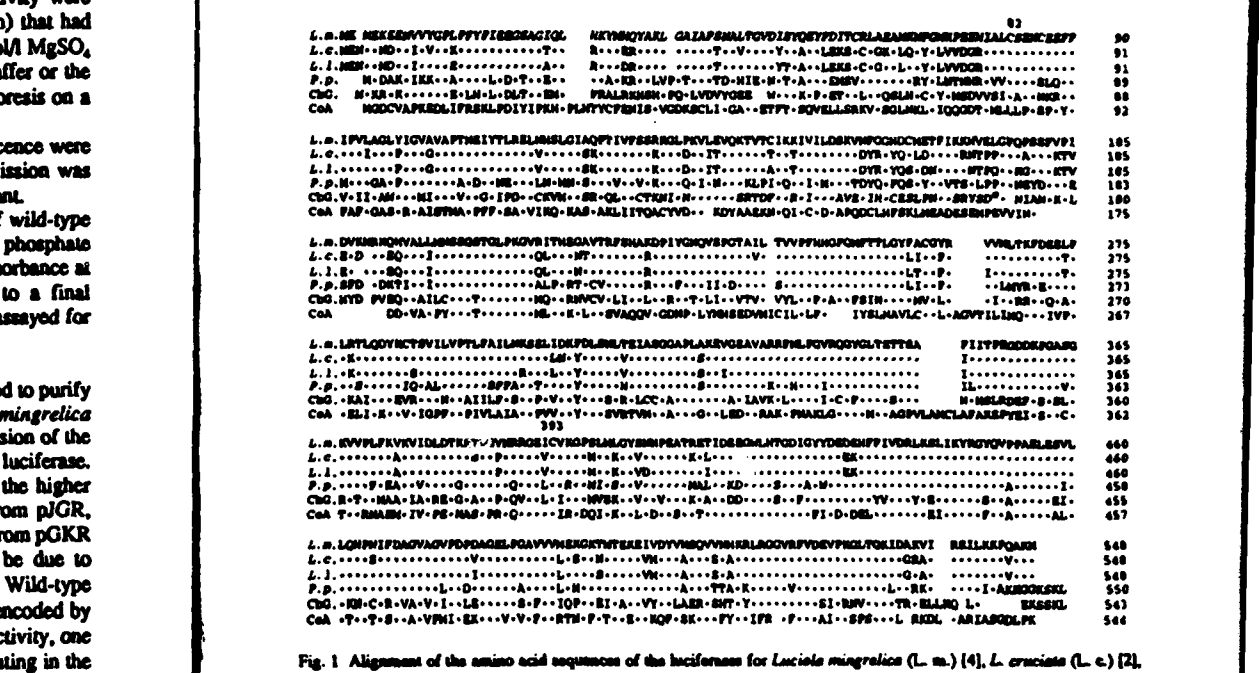

Fig. 1 Alignment of the amino acid sequences of the luciferases for *Luciola mingrelica* (L. m.) [4], *L. cruciata* (L. c.) [2], *L. lateralis* (L. l.) [3], *Photinus pyralis* (L. p.) [1] and the green-emitting strain of the clickbeetle (CbG) [5]. Also shown in this alignment is the 4-coumarate:CoA ligase (CoA) [7].

stability between the dimeric and monomeric forms of the enzyme. We investigated the stability of wild-type and mutant recombinant *L. mingrelica* luciferases under different conditions. The stability of the C393A mutant was similar to that of the wild-type enzyme, and like the wild-type, depended on the concentration of the enzyme (Fig.2). The stability of the enzyme was slightly concentration dependent in the range of 19 nmol/l - 1.4 μmol/l and increased significantly at higher concentration (17 μmol/l). The same dependence of stability on enzyme concentration was observed for the C82A mutant. Comparison of the stability of the wild-type and mutant luciferases at 22°C revealed that both mutants are slightly less stable than the wild-type enzyme under standard assay conditions with 10% glycerol (Fig.3). The stability of wild-type luciferase increased more than 10 fold if the buffer contained not only 10% glycerol but also 20% ethylene glycol (Fig.3). After 24 hours of incubation at room temperature the enzyme still retained 60% of its original activity. The stability of luciferase was also increased in 20% ethylene glycol without glycerol but not to the same extent. The most significant stabilization of wild-type luciferase was shown by bovine serum albumin (BSA). In assay buffer containing 1 mg/ml BSA, luciferase was incubated at 22°C for more than a month without any loss of activity (data not shown). In the buffer containing 10% glycerol and 20% ethylene glycol, the stability of the C393A mutant increased dramatically (100 times), making this mutant even more stable than wild-type enzyme under these conditions (Fig.3). Surprisingly the C393A and C82A mutant luciferases appeared to be somewhat more stable at 22°C than at 0°C, while the wild-type luciferase, like most proteins, is more stable at 0°C than at room temperature.

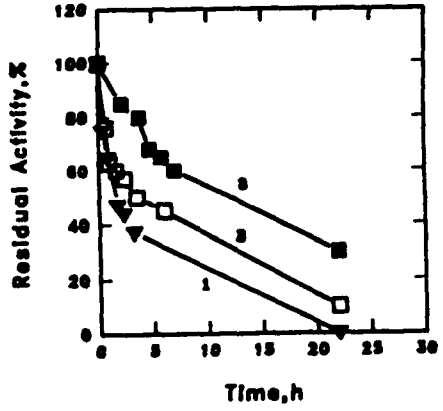

Fig. 2. The stability of the C393A mutant in 0.1 mol/l Tris acetate, pH 7.8, 2 mmol/l EDTA, 10 mmol/l $MgSO_4$, 22°C at different concentrations of enzyme: 19 nmol/l [1], 1.4 μmol/l [2] and 17 μmol/l [3]

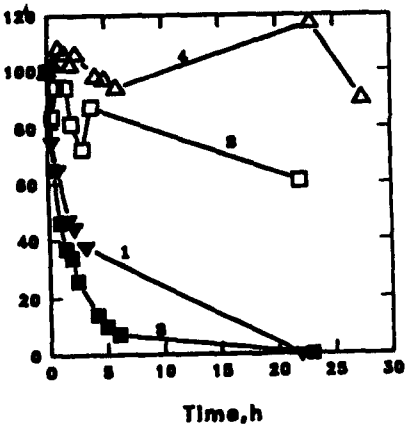

Fig.3. The stability of the wild-type [1,2] and C393A mutant [3,4] luciferases (9 nmol/l) at 22°C in 0.05 mol/l Tris-acetate, pH 7.8, 2 mmol/l EDTA, 60 mmol/l $MgSO_4$, 10% glycerol [1,3] or in the same buffer but with 20% ethylene glycol [2,4]

Inactivation of wild-type and mutant luciferases by N-ethylmaleimide (NEM). The C393A mutant luciferase appeared to be less sensitive to inactivation by NEM than the wild-type enzyme and C82A mutant (data not shown). Alter and DeLuca (9) found that *P. pyralis* luciferase with all SH-groups modified by methyl methanethiolsulfonate (S-methylluciferase) shows an anomalous time course of light emission. For the modified luciferase the initial peak of light was essentially lost and there was a gradual increase in light intensity for 10-15 min to a level that was constant for several minutes and then decayed (9). Furthermore, the modified enzyme emitted red light. None of these phenomena were observed for the Cys mutants reported here.

These results suggest that the cysteinyl residue at position 393 in wild-type luciferase resides in or near the active site of the luciferase. Neither C393 nor C82 is involved mechanistically in the bioluminescence reaction.

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KINETIC MECHANISM OF THE BACTERIAL LUCIFERASE REACTION

W. A. Francisco, H. M. Abu-Soud, A. C. Clark, F. M. Raushel and T. O. Baldwin

Center for Macromolecular Design and Departments of Chemistry and of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128

INTRODUCTION

We have undertaken a detailed, multidimensional investigation of the kinetic mechanism of the bacterial luciferase-catalyzed reaction (1-3). Luciferase is a heterodimeric enzyme with a single active center on the α subunit. While the individual subunits exhibit low but authentic bioluminescence activity (4, 5), the active form of the enzyme is the heterodimer. The β subunit is required for the high quantum yield reaction, but its precise function is unknown (6).

Light emission from the enzyme involves reaction of $FMNH_2$, an aliphatic aldehyde and O_2 on the surface of the enzyme to yield an excited state flavin and the carboxylic acid (6). One atom of the oxygen is found in the product carboxylate (7). It is assumed that the other atom from molecular oxygen is converted to water. FMN is the flavin product that is released following bioluminescence (8). It is known that the reaction proceeds through the intermediacy of the C4a-peroxydihydroflavin (9, 10) which can be distinguished from $FMNH_2$ by the characteristic absorbance at 380 nm (10). The formation of FMN can be monitored by absorbance at 445 nm. Bioluminescence resulting from formation of the excited flavin species can likewise be monitored. The lifetimes of singlet excited states are typically in the nanosecond range so that the intensity of light emission at any time is proportional to the rate of formation of the excited state. It has been proposed that the emitter in the bioluminescence reaction is the C4a-hydroxyflavin (11); the FMN product is produced by dehydration of the C4a-hydroxyflavin.

Several chemical mechanisms for the reaction of $FMNH_2$, O_2 and aldehyde have been proposed (6, 12). We favor a mechanism by which the proposed tetrahedral intermediate formed by reaction of the C4a-peroxyflavin with the aldehyde collapses to form the dioxirane and the C4a-hydroxyflavin (13; Fig. 1). The primary excited state suggested by this mechanism would be formed on the carboxylic acid product by collapse of the dioxirane. The C4a-hydroxyflavin would become excited by energy transfer from the primary excited state. In the presence of lumazine protein (14) or yellow fluorescence protein (15), the secondary emitter would likewise be excited by energy transfer.

The experiments reported here comprise a detailed investigation of the kinetic mechanism of the luciferase catalyzed reaction (1-3). All measurements were made under conditions of 25°, 50 mM Bis-Tris HCl, pH 7.0. The enzyme concentration was maintained at 75 μM for most

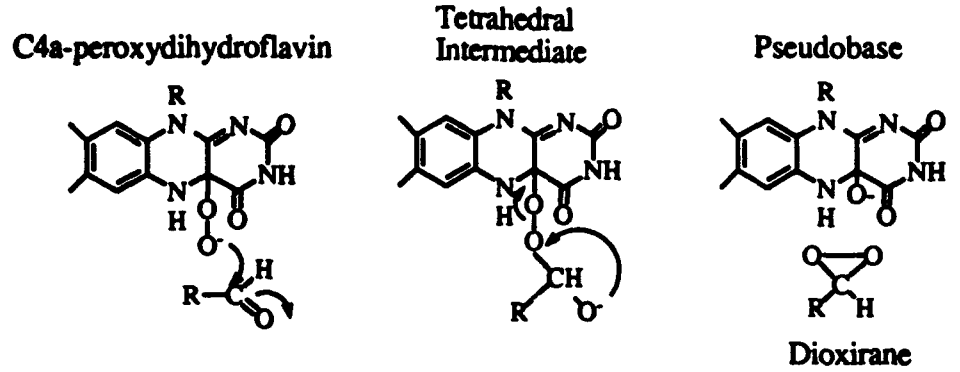
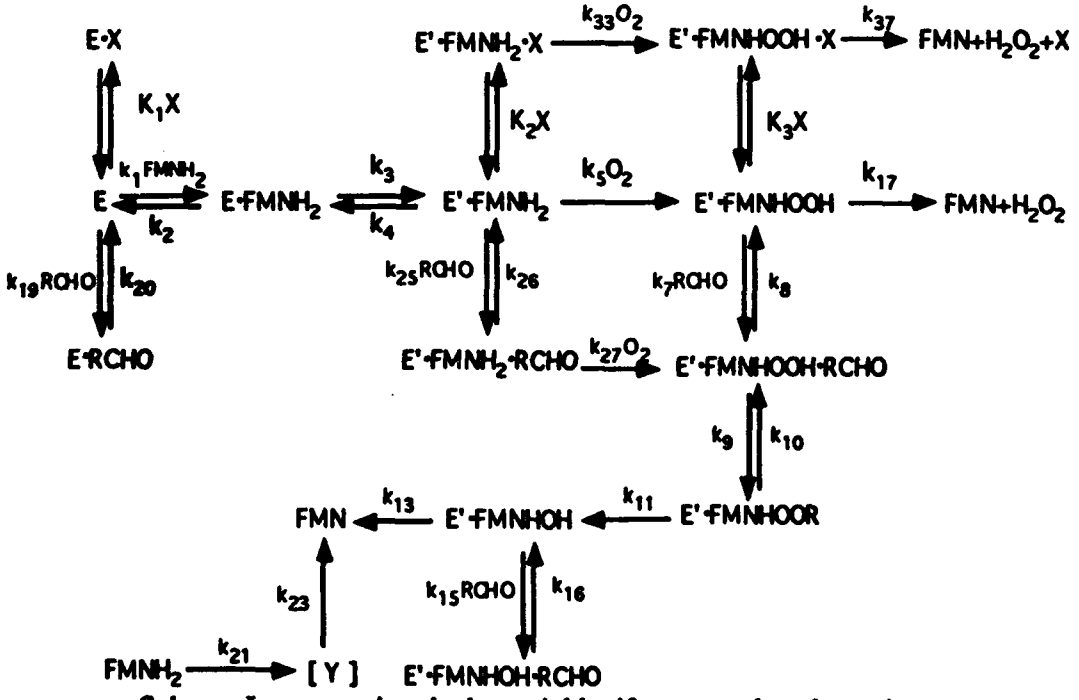


Figure 1. Schematic representation of the reaction of the flavin C4a peroxide with the aldehyde substrate to yield the proposed dioxirane intermediate and the C4a hydroxyflavin from a tetrahedral intermediate (E'-FMNHOOR in Scheme I) (13).

experiments, and the $FMNH_2$, aldehyde and O_2 concentrations were varied. The highest flavin concentration used was 15 μM. Experimental data were collected with a stopped flow spectrophotometer. Rate constants were determined either by fitting of the data to a specific equation or by simulation using KINSIM (16). The enzyme used in these experiments was purified from *Escherichia coli* carrying the *luxAB* genes from *Vibrio harveyi* on a pUC-derived plasmid. From this recombinant plasmid, we have been able to isolate about 1 gram of luciferase per liter of culture (17). The high level overproduction of luciferase was essential to the completion of this project, since the complete analysis required over 75 grams of enzyme. In some experiments, mutant forms of luciferase having mutations at position 106 of the α subunit were used. These mutant luciferases, αC106A, αC106S and αC106V, have been described previously (17-19).



Scheme I representing the bacterial luciferase-catalyzed reaction.

RESULTS AND DISCUSSION

Three spectroscopic signals were utilized to determine the kinetic mechanism of the bacterial luciferase reaction. Absorbance measurements at 380 nm allowed determination of the formation of E-FMNHOOH (10). Emission of visible light allowed measurement of processes occurring following addition of the aldehyde substrate, and absorbance measurements at 445 nm allowed detection of FMN formation from decay of E-FMNHOOH or from dehydration of the pseudobase, E-FMNHOH (8, 11). The time courses for the various transformations were determined as a function of the concentration of $FMNH_2$, O_2, aldehyde and enzyme. The minimal model that satisfies the complete data set is presented in Scheme I. The rate constants presented in Table I were progressively determined by fitting of the data to rate equations and by simulation of more complex reactions (1-3).

The reaction of $FMNH_2$ with O_2 to yield FMN and H_2O_2 in the absence of enzyme was monitored at 380 nm and at 445 nm. The data were fit to the sum of two consecutive first-order reactions (A→B→C) where the two rate constants are 4.7 s^{-1} and 11.5 s^{-1}; the order of the two rate constants, k_{21} and k_{23}, is arbitrary.

Table I: Rate Constants and Equilibrium Constants for the Model in Scheme I[a]

k_1	1.7×10^7 M^{-1} s^{-1}
k_2	1200 s^{-1}
k_3	200 s^{-1}
k_4	14 s^{-1}
k_5	2.4×10^6 M^{-1} s^{-1}
k_7[b]	1.9×10^7 M^{-1} s^{-1}
k_8[b]	120 s^{-1}
k_9[b]	1.6 s^{-1}
k_{10}[b]	1.2 s^{-1}
k_{11}[b]	1.1 s^{-1}
k_{13}	0.60 s^{-1}
k_{15}[b]	3.0×10^3 M^{-1} s^{-1}
k_{16}[b]	0.06 s^{-1}
k_{17}	0.10 s^{-1}
k_{19}[b]	9.1×10^5 M^{-1} s^{-1}
k_{20}[b]	5.8 s^{-1}
k_{21}	4.7 s^{-1}
k_{23}	11.5 s^{-1}
k_{25}[b]	1.2×10^6 M^{-1} s^{-1}
k_{26}[b]	37 s^{-1}
k_{27}[b]	5.1×10^4 M^{-1} s^{-1}
k_{33}[c]	7.7×10^4 M^{-1} s^{-1}
k_{37}[c]	0.004 s^{-1}
K_1[d]	3.9×10^3 M^{-1}
K_2[d]	6.1×10^3 M^{-1}
K_3[d]	3.5×10^4 M^{-1}

[a]Determined at pH 7.0, 25°C.
[b]Determined with *n*-decanal.
[c]Determined with *n*-decanol.
[d]Equilibrium constants determined with *n*-decanol.

Formation and Decay of the Peroxydihydroflavin Intermediate

The second-order rate constant (k_5) for the formation of E-FMNHOOH was determined by mixing E-$FMNH_2$ with varying concentrations of O_2. The change in absorbance at 380 nm could be fit to a single exponential. The resulting pseudo-first-order rate constants were linearly dependent on the O_2 concentration and the plot passed through the origin, indicating that the reaction is irreversible and that O_2 apparently does not bind to the enzyme prior to reaction. The slope of the linear plot gave the second-order rate constant of $2.4 \cdot 10^6$ $M^{-1}s^{-1}$.

The rate constants for formation of E-$FMNH_2$ were extracted by simulation from data obtained by mixing of either enzyme and $FMNH_2$ with O_2 or enzyme and O_2 with $FMNH_2$. The second order rate constant (k_5) for reaction of E-$FMNH_2$ with O_2 was known from the previous experiments, so it was not allowed to vary in the simulations. When increasing

concentrations of air-equilibrated enzyme were mixed with $FMNH_2$, the rate constant for formation of E-FMNHOOH reached a limiting value of about 85 s^{-1}, significantly below that observed when E-$FMNH_2$ was mixed directly with O_2. These results demonstrate that the initial complex of E-$FMNH_2$ does not react directly with O_2 until after a unimolecular reaction occurs yielding E'-$FMNH_2$. The rate constants k_1, k_2, k_3, and k_4 presented in Table I allowed the best simulation of the experimental data with the value of k_5 fixed at $2.4 \cdot 10^6$ $M^{-1}s^{-1}$.

The decay of E-FMNHOOH to yield FMN was monitored by absorbance at 445 nm. The time course following mixing of luciferase (75 μM) and $FMNH_2$ (15 μM) with O_2 (120 μM) fit a single exponential with a rate constant of 0.10 s^{-1} (k_{17} in Scheme I). The formation of E-FMNHOOH is complete within 10 ms under most experimental conditions. By comparison, the decay to yield FMN occurs on a time scale of many seconds.

Binding of Aldehyde to the Various Enzyme Species

In the presence of *n*-decanal, light emission is observed (Fig. 2). In the range up to about 500 μM *n*-decanal, increased aldehyde results in increased light emission when aldehyde and O_2 are mixed with E-$FMNH_2$. When the reagents are mixed, light emission rises rapidly to a peak and then decays exponentially over a period of several seconds. The decay rate is strongly dependent on the chainlength of the aldehyde and the source of the enzyme (6, 12).

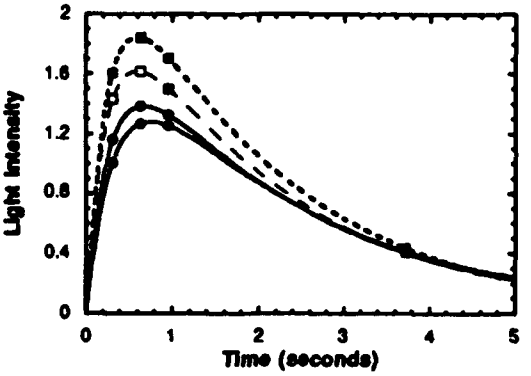


Figure 2. Effect of concentration of *n*-decanal on the peak light emission from reactions initiated by mixing E-$FMNH_2$ with aldehyde and O_2. The concentrations were 30 μM (●), 40 μM (○), 100 μM (□) and 500 μM (■).

When the reactions described above were carried out in the presence of *n*-decanal, numerous alterations were observed in the reaction time courses. When enzyme, $FMNH_2$ and aldehyde were mixed with O_2, the formation of E-FMNHOOH appeared biphasic at intermediate aldehyde concentrations, and could be fit to the sum of two exponentials; at very low concentrations of aldehyde, the time course approached that observed in the absence of aldehyde, while at very high aldehyde concentration, the rate of formation of E-FMNHOOH was again monophasic, but much slower (Fig. 3A). This observation suggested the existence of a ternary complex E'-$FMNH_2$-RCHO that reacts more slowly with O_2 than the binary complex E'-$FMNH_2$. The equilibrium constant for the formation of the ternary complex from E'-$FMNH_2$ and the associated rate constants were determined by analysis of the formation of the 380 nm chromophore as a function of *n*-decanal concentration. The same experiment done at constant enzyme (75 μM), $FMNH_2$ (15 μM) and *n*-decanal (500 μM) with O_2 varying from 120 μM to 600 μM allowed determination of the second-order rate constant for reaction of the ternary complex with O_2. This reaction (k_{27}) appeared to be about 100-fold slower than the reaction of O_2 with the binary complex.

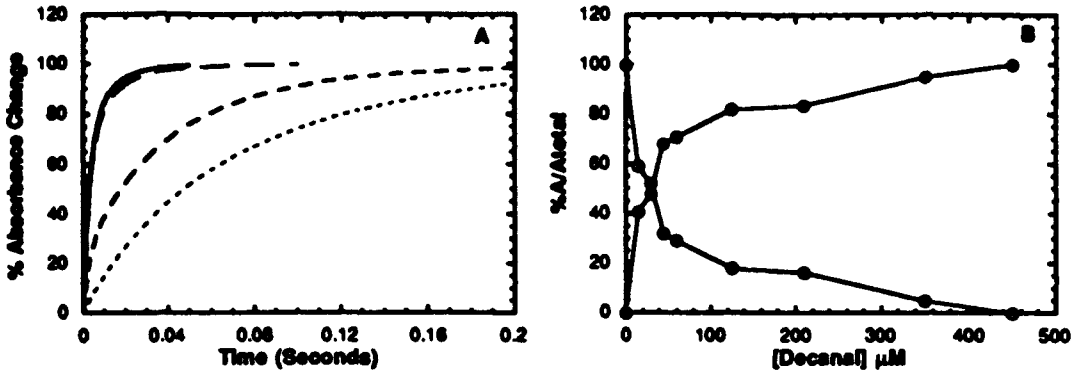


Figure 3. Effect of concentration of *n*-decanal on the time course of formation of the 380 nm chromophore. Panel A shows the % absorbance change versus time for reactions in which enzyme, $FMNH_2$ and various concentrations of aldehyde were mixed with O_2-containing buffer. The solid line represent the reaction with no aldehyde, the long dashed line is for 10 µM *n*-decanal, the intermediate dashed line is for 100 µM *n*-decanal, and the short dashed line is for 400 µM *n*-decanal. Panel B shows the effect of *n*-decanal concentration on the relative amplitudes of the fast phase (open symbols) and the slow phase (filled symbols) of the reactions depicted in Panel B.

Measurement of bioluminescence following mixing of enzyme, $FMNH_2$, O_2 and aldehyde allowed investigation of the processes from aldehyde binding through the formation of E-FMNHOH (k_7 through k_{11}). In addition, these measurements demonstrated the binding of aldehyde to the free enzyme (k_{19} and k_{20}) and confirmed the binding of aldehyde to E'-$FMNH_2$ to form the ternary complex E'-$FMNH_2$-RCHO. When E'-$FMNH_2$ was mixed with air-equilibrated aldehyde, light emission increased to a maximum about 1 s after mixing and decayed exponentially over the next 10 s. The peak intensity increased as the aldehyde concentration was increased up to about 100 µM, remaining constant thereafter. However, when enzyme, aldehyde and O_2 were mixed with $FMNH_2$, the peak light intensity decreased at aldehyde concentrations above about 100 µM. This phenomenon has been described as aldehyde inhibition (20), and is strongly dependent upon the chainlength of the aldehyde. Inhibition is virtually absent with *n*-heptanal and becomes progressively more pronounced as the aldehyde chainlength is increased. This behavior appears to be due to binding of aldehyde to the enzyme to form a binary E-RCHO complex that does not bind $FMNH_2$ (Fig. 4). The inhibition reflects the reaction of $FMNH_2$ with O_2 in solution, a competing process that consumes $FMNH_2$ that would otherwise react on the surface of the enzyme. The order of addition is therefore crucial to the process of inhibition. If E'-$FMNH_2$ is mixed with air equilibrated aldehyde, aldehyde inhibition is not observed, since O_2 reacts quickly with the flavin on the surface of the enzyme.

The formation of the product FMN following dehydration of the pseudobase, E'-FMNHOH, was detected by measurement of absorbance at 445 nm. Fixed concentrations of enzyme (75 µM) and $FMNH_2$ (15 µM) were mixed with various concentrations of air equilibrated *n*-decanal. At low concentrations of aldehyde, the formation of FMN was essentially complete after about 15 s, while in the presence of 500 µM *n*-decanal, the reaction became distinctly biphasic, with a fast phase with the same rate as that observed in low aldehyde concentrations, and a slow phase that continued to change after 50 s. These obser-

vations suggest that aldehyde binds to E'-FMNHOH and prevents dehydration of the pseudobase (k_{15} and k_{16}).

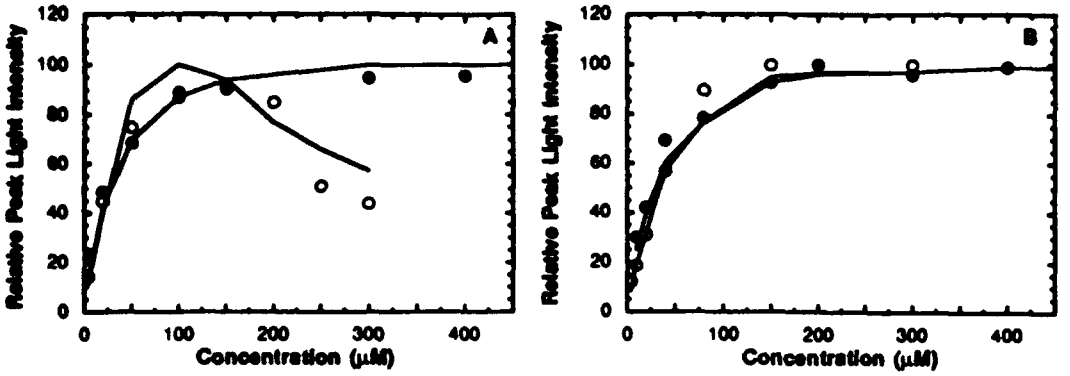


Figure 4. Effect of order of addition and aldehyde chainlength on aldehyde substrate inhibition. In Panel A, reactions were initiated by mixing enzyme, aldehyde and O_2 with $FMNH_2$. In Panel B, reactions were initiated by mixing enzyme and $FMNH_2$ with aldehyde and O_2. Filled circles represent the relative peak light emission with *n*-heptanal as substrate and open circles represent relative peak light intensity with *n*-undecanal as substrate. The symbols represent the experimental data and the lines were calculated based on the rate constants given in Table I.

Mode of Binding of Aliphatic Inhibitors

Luciferase is known to be inhibited by a variety of aliphatic compounds (21-23), including *n*-alkyl alcohols, carboxylic acids, amines and trifluoromethylketones. We have determined the effects of these compounds on the rate of formation of E-FMNHOOH (absorbance at 380 nm following mixing of E-$FMNH_2$ ± inhibitor with O_2) and on the rate of formation of FMN from E-FMNHOOH (absorbance at 445 nm following mixing of E-$FMNH_2$ ± inhibitor with O_2 ± inhibitor). The results of these experiments (3) demonstrate that the aliphatic inhibitors decrease the rate of reaction of O_2 with enzyme-bound $FMNH_2$ in the same manner as the aldehyde substrates, suggesting that the mode of inhibition by these compounds is similar to aldehyde substrate inhibition. Furthermore, these compounds decrease the rate with which the E-FMNHOOH intermediate decays to FMN and H_2O_2, demonstrating the existence of a ternary complex of E-FMNHOOH-inhibitor. Tu has demonstrated that *n*-decyl alcohol has a strong stabilizing influence on E-FMNHOOH and has used *n*-decyl alcohol as a buffer additive for isolation of E-FMNHOOH by column chromatography (22). Aldehyde binding to E-FMNHOH appears to stabilize the product complex and prevent or slow the dehydration reaction (k_{13}, k_{15} and k_{16}); inhibitor binding to E-FMNHOOH appears to exert a similar influence.

Effect of Mutations at α106 on the Enzyme-Catalyzed Reaction

Luciferase is known to possess an "essential" thiol (24) that resides at position α106 (25). Modification of this residue with even the very small nonpolar -SCH_3 group renders the enzyme inactive (26). By site-directed mutagenesis, we demonstrated that this thiol is not

essential for activity (18). The αC106S, αC106A and αC106V variants were created and the enzymes analyzed and shown to be active in the bioluminescence reaction (17); the αC106S variant had essentially wild-type activity and appeared to be less sensitive to aldehyde substrate inhibition than the wild-type enzyme, implying that the mode of inhibition might be through formation of a thiohemiacetal (18), an hypothesis that we have since discounted (17). The same mutant luciferases have been studied in the laboratory of Tu (19) confirming the conclusion that the α106 cysteinyl residue is not essential for bioluminescence activity. Xi et al. (19) studied the reaction of the valine mutant with $FMNH_2$ and O_2 and concluded that the mutation converted luciferase from a flavin monooxygenase to a flavin oxidase. We have demonstrated that with the valine mutant, the E-FMNHOOH intermediate forms at essentially the same rate as for the wild-type (2), disproving the hypothesis of a mechanistic switch. The αC106V enzyme, however, exhibits a reduced bioluminescence quantum yield due to a greatly increased (>100 fold) rate of decay of the E-FMNHOOH intermediate to yield FMN and H_2O_2 (k_{17}) (2). The instability of the C4a-hydroperoxyflavin intermediate for the valine mutant (2) probably accounts for the results of Xi et al. (19).

CONCLUSIONS

The results of these studies (1-3) comprise a set of rate constants defining the primary reactions catalyzed by the bacterial luciferase from *Vibrio harveyi*. These rate constants were determined under a single set of well-defined experimental conditions. It is clear from the complexity of the reaction that few valid conclusions can be drawn about the effects of inhibitors, mutations, buffer conditions, etc., on the reaction without performing a detailed kinetic analysis. The discovery of an isomerization of the E-$FMNH_2$ complex to yield the O_2-reactive E'-$FMNH_2$ was unexpected, but is consistent with reports of a two step mechanism for binding of $FMNH_2$ to the enzyme of *Photobacterium phosphoreum* (27) and a conformational change that occurs in the *Vibrio harveyi* enzyme during the catalytic cycle (28).

The mechanism of aldehyde substrate inhibition appears to reside simply in the ordered binding of substrates (1-3). If enzyme and aldehyde are mixed prior to addition of $FMNH_2$, $FMNH_2$ binding cannot occur until after aldehyde release. The inhibition is due to loss of the free $FMNH_2$ to reaction with O_2 prior to binding to the enzyme. Formation of the ternary complex E-$FMNH_2$-RCHO reduces the rate of formation of E-FMNHOOH, but does not greatly reduce the bioluminescence quantum yield. Oxygen can react directly with the complex, albeit at a reduced rate, and if the aldehyde temporarily dissociates, O_2 can react with the E'-$FMNH_2$ very rapidly (1).

The rate constants shown in Table I allow simulation with high precision of the various reaction time courses that occur on the *V. harveyi* enzyme. These results should serve as a foundation for investigations into the details of the chemical mechanism of bacterial luciferase.

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KINETIC CONTROL OF FOLDING AND ASSEMBLY OF HETERODIMERIC BACTERIAL LUCIFERASE

T. O. Baldwin, M. M. Ziegler, J. F. Sinclair, A. C. Clark and A.-F. Chaffotte

Center for Macromolecular Design and Departments of Chemistry and of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128

Introduction

Bacterial luciferase is a heterodimeric enzyme ($\alpha\beta$) with a single active center that resides primarily if not exclusively on the α subunit (1, 2). The role of the β subunit is not known, but it is required for the high quantum yield of the bioluminescence reaction. The individual α and β subunits exhibit an authentic bioluminescence activity with a very low quantum efficiency (3, 4). Over 20 years ago, Hastings and colleagues demonstrated that the α and β subunits could be resolved by anion exchange chromatography in urea-containing buffers (5, 6). Mixing of the subunits and dilution of the urea resulted in renaturation of the enzyme. The low level of bioluminescence activity observed upon refolding of the individual subunits was attributed to incomplete chromatographic resolution of the subunits (5). More recently, we expressed the two subunits independently from recombinant plasmids in cultures of *Escherichia coli* (7). We were surprised to find that (a) the individual subunits do exhibit low but authentic bioluminescence activity (3, 4), and that (b) mixing of the α and β subunits produced in separate cultures of *E. coli* did not lead to formation of the biologically active $\alpha\beta$ heterodimer, even after prolonged incubation, indicating that proper assembly of of $\alpha\beta$ requires folding in the same cell (4, 7). Following unfolding of the α and β subunits with urea or guanidine HCl, the subunits recombined upon dilution of the denaturant, demonstrating the covalent integrity of the recombinant subunits. These observations suggested that the formation of the $\alpha\beta$ heterodimer *in vivo* might constitute a kinetic trap (8); under conditions of folding that preclude heterodimerization, an alternative structure(s) appears to form that is not in equilibrium with the conformations of the subunits that interact to form the heterodimer. To test this hypothesis, we have undertaken an analysis of the folding and assembly of the luciferase enzyme by both equilibrium and kinetic techniques (4, 9-11). We have used both the wild-type luciferase and mutants at position βD313 that exhibit a strong kinetic defect in the refolding reaction. We conclude that our hypothesis was correct: the β subunit, when allowed to fold independently of α, forms a hyperstable β_2 structure that does not unfold in 5 M urea. The structure of the carboxyl terminal region of the β subunit appears to play a critical role in the process of both heterodimerization and homodimerization, but it has little or no effect on the structure, stability or activity of the heterodimer once it is formed. These observations suggest that the native form of a protein need not be at a global

energy minimum. Rather, it would appear that the native structure of a protein must be kinetically accessible and possess sufficient conformational stability to exist on a biological time scale.

Results and Discussion

When bacterial luciferase from *Vibrio harveyi* is placed in 5 M urea, 50 mM phosphate, pH 7.0 and 18°, denaturation is complete within a few seconds. The unfolded protein has no detectable bioluminescence activity and a far UV circular dichroism spectrum indicative of a random structure (9). Rapid 50-fold dilution of the unfolded protein into buffer without urea with a final protein concentration of 5-25 μg/ml leads to refolding of active enzyme with a high yield (9; Fig. 1). At concentrations above 50 μg/ml, the yields are reduced due to aggregation, while at low concentrations, the yield is compromised due to apparent competing folding reactions of the individual subunits (9, 10). The latter observation is consistent with the inability of individual subunits produced from recombinant plasmids to associate to form the active heterodimer (4, 7). One would expect each individual subunit to fold into a heterodimerization-incompetent form similar to that formed upon folding *in vivo*. Under dilute refolding conditions, the first order processes involving the individual subunits would become apparent, whereas at higher concentrations, the second order heterodimerization process would predominate.

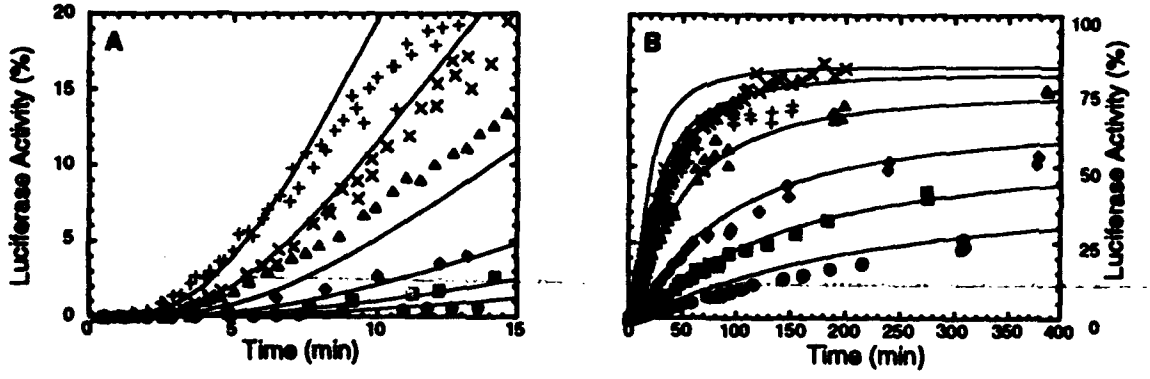

Figure 1. Time courses for the recovery of luciferase activity following 50-fold dilution of protein from 5 M urea into 50 mM phosphate, pH 7.0, 18°. Both panels depict the results of the same experiments, but with different time axes. Each data point represents a single enzyme activity determination in which an aliquot of the refolding reaction mix was diluted into assay buffer and the luciferase activity determined by rapid injection of $FMNH_2$. Refolding reactions typically were monitored for a period of 20-24 hours. The solid lines are calculated time courses based on the kinetic model and rate constants given in Figure 2. Each curve was calculated with the same set of rate constants but different protein concentrations. The final protein concentrations were 1.0 μg/ml (●), 2.0 μg/ml (■), 4.0 μg/ml (◆), 10 μg/ml (▲), 25 μg/ml (X), and 50 μg/ml (+).

When the unfolded protein was diluted into buffer and luciferase activity followed as a function of time, a lag phase of about 3-4 min was observed that did not change significantly with protein concentration (9). Following the lag, the enzyme activity increased at a rate that was strongly dependent on the protein concentration up to 10-20 μg/ml (9). At lower

concentrations, the rate of recovery of enzyme activity appeared to be limited by the second order rate of association of the α and β subunits. However, at higher concentrations, the rate became independent of protein concentration, suggesting that a first order step following heterodimerization was rate limiting. To test this possibility, we initiated a refolding reaction at 50 μg/ml, and after 6 min, diluted the refolding protein 10 fold to a final concentration of 5 μg/ml (9). At the higher concentration, the rate of recovery was concentration-independent, while at 5 μg/ml, the rate was strongly concentration dependent. When the refolding protein was diluted to the lower concentration, the refolding reaction continued at the same (fast) rate for a period of about 1 min before slowing to the rate expected for protein refolding at 5 μg/ml. This observation demonstrated the existence of an inactive heterodimeric species on the folding pathway. The inactive heterodimer formed quickly at the high protein concentration and slowly isomerized to the active conformation. Upon dilution, this process continued until the concentration of the intermediate decreased to a level at which the overall rate became limited by the rate with which the α and β subunits interacted to form the intermediate.

When the α and β subunits were separated by anion exchange chromatography in urea-containing buffers and allowed to refold separately (10), several interesting features emerged. First, if the subunits were allowed to refold for 4 min or more prior to mixing, no lag was observed, indicating that the lag is due to first order folding steps involving the individual subunits. Second, if the subunits were allowed to refold overnight prior to mixing, essentially no active luciferase was formed. This observation was consistent with the observed decrease in yield at low refolding concentrations, discussed above, and the reported inability of the subunits from recombinant *E. coli* to assemble (4, 7).

To better understand the cause for the failure of the subunits produced independently in *E. coli* to assemble, we have purified and studied subunits from that source (4). The separate subunits have circular dichroism spectra in the near ultraviolet which are indicative of packing of the aromatic residues, and far ultraviolet spectra indicative of well-ordered secondary structure. Comparison of the sum of the spectra of the two subunits with that of the heterodimer (4) indicates that the secondary and tertiary structures of the separate subunits are similar to the structures in the heterodimer. Comparison of the intrinsic fluorescence of the individual subunits with that of the heterodimer indicates some alteration in the environment of several tryptophanyl residues (4). The α subunit has 6 tryptophanyl residues (12) and the β subunit has 2 (13). The intrinsic fluorescence of the β subunit is extremely low, indicating a strong quenching of the fluorescence in the folded protein, while the intrinsic fluorescence of the α subunit is about twice that of the αβ enzyme, demonstrating that the final packing of residues within the heterodimer results in significant quenching of the fluorescence of tryptophanyl residues within the α subunit (4).

Subunits from luciferase, separated by column chromatography in urea and refolded independently by dilution from the urea, had circular dichroism spectra identical to those of the recombinant subunits. However, when the recombinant β subunit was placed in 5 M urea, it did not unfold, as shown by the fact that the CD spectrum did not change. The α subunit, however, unfolded rapidly in 5 M urea. Unfolding of the β subunit did occur in 6 M guanidine HCl (4); when the β subunit unfolded in 6 M guanidine HCl was dialyzed into 5 M urea, it remained unfolded. Dilution into buffer resulted in refolding into a structure that was stable in 5 M urea.

The strong hysteresis in the unfolding and refolding of the β subunit is clearly indicated in the rate constants for the process. When unfolded β subunit is diluted into buffer containing α subunit, it will fold with α to form active enzyme (10). If α subunit is added to the refolding β at various times, the amount of available β subunit decreases dramatically over a period of hours. The rate of loss of heterodimerization-competent β subunit is second order, suggesting the possibility of a homodimerization process. Likewise, the formation of the urea-stable form of the β subunit, monitored by circular dichroism in 5 M urea, following dilution from urea is second order, with an apparent second-order rate constant very similar to that for loss of heterodimerization competence. Recombinant β subunit was analyzed by analytical ultracentrifugation and found to be a dimer, as suggested by the kinetics of formation of the heterodimerization incompetent species. The α subunit, however, appears to be monomeric.

These observations offer an explanation for the inability of the recombinant subunits to assemble into the heterodimeric structure. The β subunit forms a stable homodimer that is not available for interaction with the α subunit. The formation of the active αβ structure appears to be kinetically preferred, but in the absence of α, β will self-associate in a very slow reaction to form a homodimer that is stable indefinitely in 5 M urea.

Under equilibrium conditions, we have shown that the luciferase unfolds by a three state process (11). When luciferase is introduced into urea-containing buffers (0-6 M) and allowed to incubate at 18° for 24 hours, the protein at high concentrations of urea appears to be completely unfolded as determined by fluorescence and circular dichroism. At intermediate urea concentrations (ca. 2.5 M) an intermediate structure is formed in high yield that is heterodimeric but inactive, has a reduced negative CD signal at 222 nm, and has an increased intrinsic fluorescence (11). The conversion from the native protein into this intermediate is independent of the protein concentration, but the conversion from this intermediate to the unfolded state is concentration dependent (11). We have therefore proposed a three-step unfolding mechanism; the equilibrium constant at 18°, 50 mM phosphate, pH 7.0 for the $\alpha\beta \rightarrow \alpha\beta_i$ interconversion was determined to be 4×10^{-4}, and for the $\alpha\beta_i \rightarrow \alpha + \beta$ equilibrium, 1.6×10^{-15} M. The overall equilibrium constant for the unfolding reaction under these conditions was 6.4×10^{-19} M.

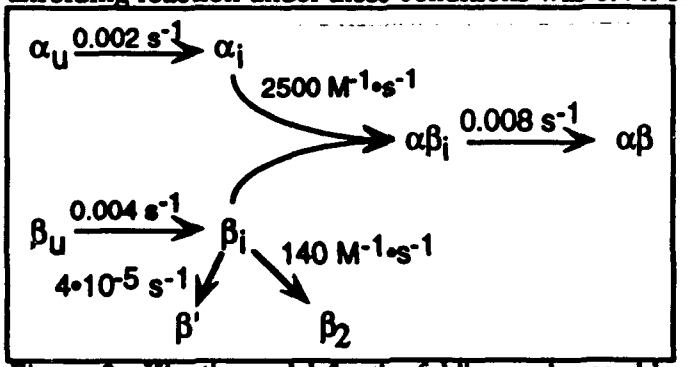

Figure 2. Kinetic model for the folding and assembly of the subunits of bacterial luciferase. The rate constants were determined by a combination of direct experimental measurements and simulation of the refolding data presented in Figure 1 (9).

A kinetic model depicting the folding and assembly of the *V. harveyi* luciferase subunits is presented in Fig. 2. The species α_u and β_u indicate the unfolded subunits which refold through first order processes to form structures, α_i and β_i, that can associate to form the inactive heterodimer $\alpha\beta_i$. The inactive heterodimer isomerizes to form the active heterodimer αβ. The subunit species α_i appears to be the form of α that is isolated directly from lysates of *E. coli* that carry the *luxA* gene (4). The rate constant for the first order process $\alpha_u \rightarrow \alpha_i$, which undoubtedly involves numerous steps, was estimated as the rate constant for the slowest step in the formation of the

native circular dichroism signal at 222 nm (Chaffotte, Ziegler and Baldwin, unpublished). Likewise, the rate constant for the $\beta_u \rightarrow \beta_i$ process was estimated from stopped-flow CD measurements. The estimates of these rate constants were then varied from the measured values to allow an optimal fit to the lag observed in the experimental data for recovery of activity following dilution from urea (Fig. 1) (9). The final values giving the best fits to the experimental data were very close to the values determined from the CD kinetic data. The first order rate constant for the $\alpha\beta_i \rightarrow \alpha\beta$ isomerization was initially estimated from the kinetics of the shift that occurred in the rate of formation of $\alpha\beta$ upon dilution from 50 μg/ml to 5 μg/ml (9). The rate constant was then varied to obtain the optimum fit to the experimental data, including the secondary dilution experiments. The second order rate constant for the homodimerization of the β subunit was measured using two approaches. First, the rate with which refolding β subunit became heterodimerization-incompetent was determined over a range of concentrations and the data fit to a second order mechanism. Second, the rate with which refolding β subunit formed the 5 M urea-insensitive structure was determined over a range of concentrations and the data fit to a second order mechanism. Both approaches gave similar values for the second order rate constant. The heterodimerization rate constant was determined by simulation. When we attempted to fit the data presented in Figure 1 using these 5 rate constants, we were able to simulate the early and intermediate portions of the curves quite satisfactorily, but at later times, the simulations invariably continued to give a slow increase in activity that was not demonstrated by the data. To account for the flattening of the time courses of activity recovery at later times of refolding, we have introduced a first order conversion of $\beta_i \rightarrow \beta'$, a monomeric form of β subunit that is incompetent to heterodimerize. With the addition of this step, we have been able to fit the experimental data quite well (Fig. 1). It is this proposed first-order step that results in reduced yield of active enzyme at lower protein concentrations. The variance of the 50 μg/ml data from the simulation is due, we believe, to aggregation of folding intermediates that occurs at the higher protein concentrations, which has not been incorporated into Fig. 2.

Sugihara and Baldwin (8) have described β subunit termination mutants that appear to fold and assemble correctly at lower temperatures into proteins that have normal activity and stability, but at higher temperatures fail to assemble into the heterodimer. Based on the properties of these mutants, it was proposed that the carboxyl-terminal region of the β subunit must play a critical role in the folding and assembly reaction, but have little or no effect on the activity or stability of the successfully folded product (8). We have designed a series of mutants at position β313 based on the original termination mutants. The mutants, βD313A, βD313N, βD313G, and βD313P, all exhibit kinetic defects in the refolding reaction. However, they display the same conformational stability as the wild-type protein; in fact, the asparaginyl and alanyl mutants are slightly more stable than the wild-type protein. The prolyl mutant has the strongest kinetic defect of the four mutant enzymes. The lag phase in recovery of activity is the same as for the wild type, indicating that the process $\beta_u \rightarrow \beta_i$ is the same. It appears that the heterodimerization rate constant is much lower for the mutant than for the wild-type protein; the time courses of activity recovery for the βD313P mutant can be satisfactorily fit to the model in Fig. 2 by changing only the heterodimerization rate constant. Likewise, the homodimerization rate constant of the prolyl mutant appears to be extremely low or non-existent. Examination of the prolyl mutant β subunit by analytical ultracentrifugation showed it to be monomeric. The βD313P mutant β subunit does not fold into a 5 M urea hyperstable structure, but rather folds into a structure without significant near-

ultraviolet circular dichroism, suggestive of a molten globule-like structure. It appears that the proposed $\beta_I \rightarrow \beta'$ reaction for the wild-type protein also occurs for the βD313P mutant β subunit, whereas the homodimerization reaction does not occur.

Conclusions

It appears that the folding of luciferase subunits into the biologically active αβ structure is a kinetically-determined process. The slow formation of ββ leads to a hyperstable structure that does not catalyze the high quantum yield reaction. The observation of mutant proteins exhibiting kinetic defects in the folding reaction is entirely consistent with this hypothesis. It thus appears that the native structure of a protein must (a) be kinetically accessible and (b) have sufficient conformational stability to exist on a biological time scale. Alterations in the amino acid sequence may alter the kinetic pathway such that alternative structures become kinetically accessible. Clearly, in a folded protein there is substantial conformational flexibility, but it is unlikely that all conformations are in equilibrium under native conditions.

Acknowledgements

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corresponding author: Baldwin
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Nature Structural Biology (in press, May 1994 issue)

Kinetic partitioning during protein folding yields multiple native states

James F. Sinclair, Miriam M. Ziegler and Thomas O. Baldwin

The prevailing view in the field of protein folding holds that the native state is the most stable structure possible. A corollary of this thermodynamic hypothesis is that the native state is in equilibrium with all other conformations of the protein. We have found an example of a protein that may exist in two different states, both of which may be regarded as 'native', but which cannot equilibrate on a timescale that is biologically meaningful. We propose that the active conformation of this protein is at only one of several possible energy minima, and that during the process of refolding *in vitro* — and we assume folding *in vivo* — the choice of which state the polypeptide finally attains is determined by kinetic partitioning between folding pathways.

Center for Macromolecular Design and the Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128, USA

Correspondence should be addressed to T.O.B.

Protein folding is generally thought to be under thermodynamic control, implying that the stable, compact, 'native' structure of a polypeptide comprises an ensemble of conformations at a global energy minimum[1,2]. In the process of conversion from one state to another, the pathway that will predominate will be the one with the lowest activation energy, which will have the highest rate. For a reaction under thermodynamic control which yields two or more products, the ratios of the products will be determined by their relative free energies. For a reaction under kinetic control, the ratio of the final concentrations of the various products will be determined by the ratio of the rate constants for the reactions by which these products are formed. *In principle*, the products of a kinetically determined reaction will in time equilibrate, but if the activation barriers are high enough to cause kinetic partitioning, the time required to achieve equilibrium may be too long to be of biological significance.

The role of kinetic considerations in the determination of the folding pathway and/or the finally folded state has been discussed[3], but the examples so far studied are generally dismissed as curiosities; the possibility that kinetic control could play an important role in protein folding is not widely accepted. Our previous studies on the folding and assembly of bacterial luciferase *in vivo*[4] and *in vitro*[5-8] led us to propose that kinetic control plays a crucial role in determining the final conformation of this enzyme. We regard subunit assembly to be an integral part of protein folding. Many proteins are composed of multiple folding domains; interactions between such domains differ from interactions between subunits of a multimeric protein primarily in the covalent continuity

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Luciferase structure and assembly

Bacterial luciferase is a heterodimeric ($\alpha\beta$) flavin monooxygenase that catalyzes a light-emitting reaction in certain bacteria, found primarily in marine environments. Previous studies have defined both the kinetics of formation of active $\alpha\beta$ luciferase from urea-unfolded subunits[3,4] and the equilibrium unfolding of the enzyme[5]. We have shown that the formation of active $\alpha\beta$ structure *in vivo* requires that the two subunits fold within the same cell at the same time[3]. Furthermore, the individual subunits allowed to fold independently *in vitro* assume conformations that appear unable, upon mixing, to assemble to form the biologically active heterodimer[4,5]. Both subunits, folded independently either *in vivo* at 25 °C or *in vitro* at 18[illegible]°C, were shown by near UV circular dichroism (CD) spectroscopy to form stable structures with well-defined tertiary packing of aromatic side chains[6].

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Here we demonstrate that the inability of the separately folded subunits to assemble into the heterodimer is the consequence of folding of the β subunit into a heterodimerization-incompetent form which has a homodimeric (β_2) structure. The luciferase β subunit thus has at least two options as it folds within the cell (Fig. 1). It can heterodimerize with the α subunit, if α is available, to form active $\alpha\beta$ enzyme. Alternatively, it can homodimerize to form a β_2 structure which has only marginal bioluminescence activity ($< 10^{-5}$ that of the heterodimer)[8,10]. Under all conditions tested, including different ionic strength, pH and temperature, the $\alpha\beta$ and β_2 species do not achieve equilibrium, but appear to be separated by a large activation barrier. We propose that the biologically active conformation of the luciferase β subunit (the conformation it assumes in the $\alpha\beta$ heterodimer) is at one of several possible energy minima, and that during the process of refolding *in vitro* (and we assume folding *in vivo*) the choice of which conformation the polypeptide finally attains is determined by kinetic partitioning between folding pathways.

The heterodimerization-incompetent form of the β subunit

Previous studies established that

[redacted] either the α or the β subunit (or both) has been permitted to fold separately *in vivo*[3] or *in vitro*[4] is impaired in its ability to assemble into the heterodimer. In order to quantitate heterodimerization-incompetence and to establish which subunit was responsible for the phenomenon, the native and unfolded α and β subunits were permitted to refold in all four possible combinations (Table 1). The individual luciferase α and β subunits were produced in soluble form using an overexpression system in recombinant *Escherichia coli* grown at 22°C and purified as previously described[6]. The results of the refolding and assembly experiment show that the separately folded β subunit cannot assemble into active heterodimer with either the native α subunit or the refolding α subunit, whereas the refolding β subunit mixed with either native α or refolding α assembled into the active $\alpha\beta$ form with good yield (Table 1).

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To determine the characteristics of the folded β subunit that prevent heterodimerization, we analyzed the

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protein by analytical ultracentrifugation and by unfolding and refolding in buffer solutions containing urea or guanidinium chloride. The results of equilibrium ultracentrifugation (Fig. 2) demonstrate that the β subunit produced and folded in *E. coli* without the α subunit assumes a dimeric structure. Nonlinear least squares fits of the data to a single exponential equation indicate that β_2 is a single sedimenting species with a molecular weight of ~72,000 M_r. The molecular weight of the β subunit, determined from the sum of the molecular weights of the residues in the encoded amino acid sequence, is 36,349 (ref. 11). It thus appears that the β subunit, allowed to fold without α, can assume a homodimeric structure which cannot associate with α to form the active heterodimer.

Prolonged incubation of β_2 with α subunit results in no detectable (active) αβ dimer, suggesting that on the time scale of the experiments (weeks), the β_2 species does not dissociate, and thus cannot come to equilibrium with αβ. We therefore investigated the unfolding and refolding of the β_2 species in buffers containing urea (Fig. 3). The near UV CD spectrum of folded β is the same in 5 M urea as it is in buffer (Fig. 3*a*), even after prolonged (> 48 h) incubation of the folded protein in urea. Once the protein is unfolded by treatment with 9 M urea, it remains unfolded when the urea concentration is decreased to 5 M, demonstrating that the folded and unfolded conformations can coexist in 5 M urea without any detectable interconversion between the two forms. The β subunit in the αβ dimer is rapidly (< 30 sec) and fully unfolded when placed in 5 M urea[5]. To further evaluate this apparent hysteresis, the CD of the protein at 222 nm — monitoring primarily secondary structure — has been examined (Fig. 3*b*). The heterodimer is able to establish equilibrium between the native, intermediate, and denatured conformations whether starting from the native or denatured state[7], and appears to be fully unfolded in 5 M urea[5,7]. The β_2 homodimer shows a strong hysteresis between unfolding protein and refolding protein. There is no decrease in signal for β at concentrations of urea in which the αβ enzyme is largely unfolded[7], suggesting that the rate of unfolding of β_2 is very slow under the conditions employed, so that equilibrium is not achieved. Incubation of the protein for 18 h at 18°C in buffers containing ≥ 6 M urea did result in apparent unfolding, but dilution of the unfolded protein into buffers at a lower urea concentration did not result in refolding. This apparent hysteresis (Fig. 3*b*) in the unfolding and refolding processes of β_2 is in marked contrast to the reversible equilibrium unfolding and refolding of the αβ luciferase[7]. Since the failure of β and β_2 to interconvert on an experimentally achievable timescale prevented direct measurement of the equilibrium constant and thus the conformational stability of β_2, we obtained an estimate of the equilibrium constant by measuring the rate constants of formation and dissociation of β_2 (k_2 and k_{-2} in Fig. 1).

Folding and unfolding of β_2

We have used two approaches to investigate the rate of formation of the β_2 homodimer. First, we observe the

rate at which unfolded β becomes heterodimerization-incompetent during the process of refolding. Unfolded β subunit in 5 M urea is diluted from the urea and allowed to refold as described[6]. At various times, aliquots are removed and mixed with excess native α subunit and allowed to continue refolding overnight. The apparent rate of loss of heterodimerization competence shows a clear dependence on the concentration of the β subunit in the initial refolding mixture; the data in Fig. 4*a*, fit to a second order function, yields a value for k_1 (Fig. 1) of 167 ± 30 M^{-1} sec^{-1}. The second method is based on the observation (discussed above) that the β_2 structure is stable in 5 M urea. In a refolding experiment similar to that presented in Fig. 4*a*, we withdrew aliquots of the refolding protein at various times and increased the urea concentration to 5 M, conditions under which any β_2 that had formed should remain folded. We determined the rate of formation of β_2, the urea-stable species, by measuring the CD signal at 222 nm in 5 M urea of samples withdrawn at various times. Indeed, the data (Fig. 4*b*) fit well to a second order process with a rate constant (k_2, Fig. 1) of 200 ± 30 M^{-1} sec^{-1}, in good agreement with the experimental results presented in Fig. 4*a*. Since the difference between these two numbers is less than the error in the individual determinations, we conclude that loss of heterodimerization competence and formation of the urea-stable β_2 species occur with the same rate constant.

The native β_2, which has two tryptophanyl residues per polypeptide chain[11], has an intrinsic fluorescence emission spectrum blue-shifted relative to that of most folded proteins, with a maximum at 320 nm (Fig. 5, inset)[8]. The intensity of the fluorescence is quite low compared with that expected for a protein with two tryptophanyl residues[12] suggesting that the tryptophanyl residues of the β_2 species reside in a hydrophobic environment and that the fluorescence of these residues is quenched by interactions with other amino acid residues. The near UV CD spectrum of β_2 (Fig. 3*a*) demonstrates a well-organized packing of aromatic side chains, consistent with the intrinsic fluorescence emission spectrum[8]. Upon unfolding in 6 M guanidinium chloride (Fig. 5, inset) or in 9 M urea (data not shown), there is a red shift of the fluorescence to about 350 nm. This change in fluorescence occurred over the same urea concentration range as the change in CD signal shown in Fig. 3*b* (data not shown).

The unfolding of β_2, monitored by either fluorescence or CD, at 24 h following mixing with denaturant, occurs between 2–3 M guanidinium chloride. The rate of unfolding of the β_2 structure was determined by monitoring the increase in fluorescence following mixing of native protein with a series of guanidinium chloride concentrations (Fig. 5) using a stopped-flow mixing device attached to the fluorometer. Each reaction was fit to a single exponential to determine the rate constant for unfolding at each concentration of guanidinium chloride. Between 3–4 M guanidinium chloride, the unfolding reactions proceeded with readily measurable rates. The observed rate constants determined are the microscopic rate constants of unfolding, since there is no contribution from the refolding reaction under the condi-

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tions of the measurements. The data have been extrapolated to native conditions (no denaturant) to obtain an estimate of the first order rate constant of dissociation of β_2 (ref. 1) of 1.6×10^{-16} sec^{-1}. This rate is so slow that β_2, once formed, does not dissociate on a biological timescale under non-denaturing conditions.

The ratio of the rate constants (k_{-2}/k_2) yields an estimate of the dissociation equilibrium constant for the $\beta_2 \rightleftharpoons 2\beta$ process of about 10^{-16} M at 18 °C. This equilibrium constant corresponds to a free energy difference of about –21 kcal/mol^{-1}, close to the value of –24 kcal/mol^{-1} for the overall process $\alpha\beta \rightleftharpoons \alpha + \beta$, also determined at 18 °C (ref. 7). Considering the error involved in the extrapolation to zero denaturant to determine the rate of dissociation of the β_2 homodimer, as well as the error intrinsic to estimation of the equilibrium constant from the rate constants, we conclude that the conformational stabilities of the heterodimer and homodimer are not significantly different.

Based on measurements of the rates of refolding of luciferase ($\alpha\beta$) from unfolded subunits[5,6], we have estimated the second order rate constant for heterodimer assembly ($\alpha + \beta \rightarrow \alpha\beta$), k_1 in Fig. 1, to be about 2600 M^{-1} sec^{-1} (A.C. Clark, E.F. Waddill, A.-F. Chaffotte and T.O.B., manuscript in preparation). The second order rate constant k_2 for the competing homodimerization reaction ($\beta + \beta \rightarrow \beta_2$) reported here is about 180 M^{-1} sec^{-1}, over 10 times less than the rate constant for heterodimerization.

A large activation barrier

The β subunit refolding in the presence of α has at least two available folding pathways, the kinetically preferred heterodimerization pathway leading to the active αβ enzyme, and the slower homodimerization pathway leading to the β_2 species. Based on earlier investigations of the folding and assembly of the enzyme *in vivo*, we proposed that the assembly of the enzyme was under kinetic control[5,6]. We now understand the physical basis for the kinetically controlled folding reaction.

The experiments reported here demonstrate that the inability of the folded α and β subunits to assemble into active luciferase is due to the formation of a β_2 homodimer which is prevented from dissociation under non-denaturing conditions by a large activation barrier. The β_2 homodimer is not unfolded in 5 M urea, even after prolonged incubation, whereas treatment of the luciferase heterodimer with 5 M urea results in rapid unfolding of both subunits[5]. Both structures are stable under non-denaturing conditions on a biological timescale, so both conformations can be described as 'native'.

If the β subunit is unfolded by treatment of β_2 with 9 M urea, or isolated in the unfolded state by ion exchange chromatography of luciferase subunits unfolded in 5 M urea, it remains unfolded in the presence of urea under conditions that do not unfold β_2. At urea concentrations less than 2 M, the unfolded β subunit regains native structure. We have shown that the hysteresis is due to the extremely slow rate of homodimer dissociation, implying that a substantial energetic input is required to interconvert β subunit between the folded and unfolded conformations. A consequence of this large energy barrier is that the rates of folding and unfolding are very slow. The rate constant for the unfolding

reaction, estimated by extrapolation to 0 M guanidinium chloride, is 1.6×10^{-14} sec^{-1}, so slow that the half time for this reaction would be over one million years. The formation of the β_2 homodimer is also very slow, with a rate constant of about 180 M^{-1} sec^{-1}. This rate constant is much less than would be expected from diffusional limits, suggesting that there may be significant conformational rearrangements in the β subunit prior to or during the formation of specific contacts at the dimer interface. The simplest explanation for the observation of the same second order rate constant for loss of heterodimerization competent β subunit and appearance of urea-stable β_2 is that both heterodimerization and homodimerization involve the same β subunit intermediate. If the two second-order processes involve different forms of the β subunit, such forms must be in rapid equilibrium through some common intermediate.

By comparison of the near and far UV CD spectra of the αβ heterodimer with the sum of the spectra of the individual subunits purified from recombinant *E. coli*, we have concluded that the structures of the subunits folded independently are not grossly different from the structures of the subunits in the heterodimer[7]. High resolution structural information for the luciferase or its subunits is not currently available, so it is not possible to comment about structural differences between αβ and β_2. It is possible that the kinetic stabilization of the β subunit in the β_2 structure relative to the β subunit in the αβ structure could be due solely to differences in the subunit interface. However, the experiments reported here were developed to investigate the kinetic trap that occurs during the refolding reaction, not to place a structural interpretation on the kinetics and thermodynamics of the refolding reaction.

spectra

Kinetic partitioning

Protein folding occurs through a series of intermediate conformations, most of which have only a transient existence. It should be apparent that any pathway involving intermediates is subject to kinetic partitioning[15]. Amino acid substitutions in proteins may alter the folding pathway[4], such that the processes of mutation and natural selection work not only to define a stable folded structure, but also to define the pathway by which that structure is achieved. A corollary is that mutations in the coding sequence for the protein potentially can alter the folding pathway and cause the protein to fold into an incorrect structure. This proposal should be contrasted with the generally applied interpretation that mutations that decrease the yield of correctly folded protein do so by altering the free energy of the native structure, the unfolded polypeptide, or both. However, it is also possible for mutations to alter the folding pathway, allowing alternative structures to form, without altering the stability of the correctly folded structure. The product partition ratio could be altered by changes in the magnitude of the activation energy barriers between intermediates on (or off) the folding pathway.

(period)

implicit in this proposal

Kinetic partitioning has been shown to occur between the pathway leading to formation of the phage P22 tailspike trimer and off-pathway aggregation[16]. Numer-

n (lc)

aggregation[14]; numer-

ous mutants that can alter the apparent partition ratio have been isolated[15]. deletions and modifications of the carboxyl-terminal region of the luciferase β subunit can cause a temperature-sensitive folding phenotype[4]. It appears that these mutations lead to a partitioning of the folding β subunit into (at least) two conformational states, one that is capable of interacting with the α subunit to form the active αβ heterodimer, and the other that is heterodimerization-incompetent. Unlike the wild-type β subunit described here, the heterodimerization-incompetent form of these mutants is monomeric[4]. The αβ dimer formed from these altered β subunit constructs has normal activity and stability, leading to the conclusion that the carboxyl-terminal region of the β subunit plays a crucial role during the folding reaction, perhaps by stabilizing some intermediate, but has little or no effect on the activity or stability of the finally folded product[4] (A.C. Clark, E.F. Waddill, A.-F. Chaffotte and T.O.B., manuscript in preparation). These mutants show clearly that changes in the amino acid sequence of a polypeptide can alter the folding pathway. We propose that mutations of this type are probably fairly common, but have not been studied in any detail as they result in failure of the mutant protein to fold with high efficiency into the active conformation.

isolated[15] ⊙ Such mutants have also been reported for luciferase: deletions .....

Alternative pathways

In the case of the luciferase β subunit, the non-biological structure is observable and of interest because of the fact that it is known that the 'incorrectly folded' structure does not result from a mutation, and that the alternative folding pathway is the kinetic consequence of failure of the two polypeptides, α and β, to interact during folding[3,4,8]. Other proteins for which kinetic factors appear to determine the pathway or the final structure or both in folding include the α-lytic protease[16], the serpins[17], influenza hemagglutinin[18] and subtilisin BPN'[19]. For these polypeptides, the kinetic intermediates appear to be 'on pathway' intermediates and to have a crucial biological function. In the case of luciferase, however, the formation of β_2 is neither an 'on-pathway' process nor is it an 'off-pathway' aggregation. The observations presented here support the concept that a folding polypeptide chain can access one or more conformations which represent energy minima out of which the protein is unable to escape under physiological conditions. The biologically active conformation may be at the global free energy minimum, or it may be trapped in a local free energy minimum by a high activation energy barrier. There may be other pathways which lead to products of stability equal to or greater than that of the 'native' state. In the case of the folding and assembly of the subunits of luciferase, it would appear that the kinetically preferred pathway with the wild-type protein leads to the biologically active heterodimer.

We have demonstrated that by preventing the kinetically preferred reaction, other folding pathways can be explored by the polypeptide. It is clear that the assembly reaction is under kinetic control; the αβ and β_2 structures are not in equilibrium. The partition ratio is determined by their relative rates of formation, not by their relative stabilities.

corresponding author: Baldwin
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Methods

Preparation of luciferase and α and β subunits. Luciferase was purified from recombinant *E. coli* cells containing a plasmid carrying the *luxAB* genes from *Vibrio harveyi*, grown at 25 °C, as previously described[20,21]. Native α and β subunits were purified under native conditions[9] directly from *E. coli* cells containing plasmids carrying either the *luxA* or *luxB* gene from *V. harveyi*[9]. Growth was at 22 °C, at which temperature the subunits were overexpressed in soluble form[9]. The native subunits were never subjected to any urea treatment, and prior to the experiment each had never been exposed to the other subunit. Unfolded α and β subunits were obtained by denaturing luciferase (αβ) in 5 M urea and separating the unfolded subunits by ion exchange chromatography in 5 M urea[22]. Folded (native) subunits were in 50 mM phosphate buffer, 1 mM EDTA, 0.5mM DTT, pH 7.0. Unfolded subunits were in the same buffer 5 M in urea prior to initiation of refolding/assembly.

Subunit refolding/assembly. All refolding and assembly experiments *in vitro* were done at 18 °C, consistent with previous detailed kinetic and equilibrium studies of refolding[5,6]. The temperature used in these experiments is consistent with the marine habitat of the luminous bacterium *Vibrio harveyi*, from which the *luxA* and *luxB* genes were derived. Refolding of the subunits was initiated by 50-fold dilution from 5 M urea (or non-urea-containing buffer in the case of the native subunits) into 50 mM phosphate buffer, 1 mM EDTA, 0.5 mM DTT, 0.2% bovine serum albumin (BSA), pH 7.0, at 18 °C, with a residual urea concentration of 0.1 M (ref. 5).

Luciferase activity assays. Activity of the luciferase heterodimer was measured by a flavin injection assay in which the substrate $FMNH_2$ is injected into a solution of enzyme and the other two substrates, O_2 and aldehyde[20,23]. The subsequent light emission was measured with a Turner Designs TD20e luminometer.

Analytical ultracentrifugation. Samples of the β subunit used for analytical ultracentrifugation were dialyzed exhaustively against 200 mM phosphate, pH 7.0, containing 0.5 mM DTT. Analytical ultracentrifugation of native β subunit (0.1 mg ml^{-1}) was performed at 16,000 rpm and 23 °C, using a Beckman Optima XLA instrument. Equilibrium was established after 24 h, and at this time, the absorbance at 280 nm was determined as a function of radial position. The density of the buffer was determined to be 1.025 g ml^{-1} at 23 °C by the mechanical oscillator technique[24], and the partial specific volume of the protein was calculated to be 0.7234 ml g^{-1} from the amino acid composition[11,25]. The data were fit to the single exponential equation $A = A_0 \exp\{MW^2[(1-\bar{v}\rho)\omega^2/2RT](r_0^2-r^2)\}$ where A = absorbance at radial position r, A_0 = absorbance at the meniscus, r_0, MW = molecular weight, ω = angular velocity, $\bar{v}$ = partial specific volume, ρ = density, R = the ideal gas constant, and T = absolute temperature.

Spectroscopic methods. Bovine serum albumin was omitted from the refolding buffer for experiments involving spectroscopic methods. CD spectra were obtained with a Jasco J600A spectropolarimeter; samples were maintained at 18 °C. Folded β subunit was in 50 mM phosphate or in the same buffer with 5 M urea. The latter sample was incubated in 5 M urea for more than 48 h prior to recording the spectrum.

To obtain urea denaturation curves of β subunit, the initial samples were either native (in buffer without urea) or unfolded (in buffer with 9 M urea); these solutions were diluted into different concentrations of urea in 50 mM phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0, at 18 °C, final protein concentrations being 12 μg ml^{-1}. After approximately 24 h, CD at 222 nm was measured for each sample.

Determination of the second-order rate constant for homodimerization of β. Two methods were employed to determine the rate of formation of β_2. First, the rate of loss of

A_0 (subscript 0)

heterodimerization competence was determined at a variety of β subunit concentrations. Unfolded β subunit was diluted 50-fold into refolding buffer (above) plus 0.2% BSA. At various times, aliquots were withdrawn and mixed with excess native α subunit (75 μg ml^{-1}, or 1.87 μM final concentration in the same buffer). The bioluminescence activity was measured after 12 h to determine the final activity recovered, the maximum yield being limited by the concentration of heterodimerization-competent β subunit.

The second method relied upon the stability of β_2 in 5 M urea. At regular intervals following dilution of β subunit into refolding buffer without BSA, aliquots were removed and adjusted to 5 M urea, conditions under which β_2 is stable, but the β monomer unfolds[9]. Following equilibration at 18 °C for 24 h, the CD at 222 nm was recorded. From the signal for each sample and the signal of the native protein (homodimer) in 5 M urea, the fraction of urea-stable β subunit in each aliquot was determined.

Data from both experiments were fit to the second order rate equation $kt = 1/[\beta] - 1/[\beta]_0$ where k = second order rate constant, t = time in sec, $[\beta]$ = concentration of monomer, and $[\beta]_0$ = initial concentration of β subunit. A nonlinear least squares fit to the concentration of monomer was performed, and the fraction of each species was computed by dividing by the initial protein concentration. For the formation of the urea-stable state the fit was to the quantity $[\beta]_0 - [\beta]$.

Determination of the first order rate constant for β_2 dissociation into monomers. Fluorescence emission spectra of folded β in buffer (50 mM phosphate,1 mM EDTA, 0.5 mM DTT, pH 7.0) and unfolded β in 5 M urea under the same conditions were obtained with an SLM 8000 fluorometer at a protein concentration of 1 μM, with excitation at 280 nm. The first order rate constant for β_2 dissociation was determined at a number of different guanidinium chloride concentrations by stopped-flow fluorescence, using a rapid mixing device attached to the SLM fluorometer. Guanidinium chloride was used instead of urea because sufficiently high concentrations of the latter to do the unfolding experiment could not be achieved. The β_2 homodimer in buffer was injected against guanidinium chloride in a mixing ratio of 1 : 2.5 for a final protein concentration of 1 μM in each denaturant concentration (50 mM phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0, 18 °C). The change in fluorescence between the native and denatured state was followed at 365 nm using an excitation wavelength of 280nm. The change in signal could be fit to the equation $Y = Y_A[A] + Y_B([A]_0 - [A])$ with Y = fluorescence signal, Y_A = signal of dimer, Y_B = signal of product, $[A]_0$ = initial dimer concentration, and [A] = the concentration of dimer determined by the first order rate equation $[A] = [A]_0 e^{-kt}$, k = first order rate constant, and t = time in sec to obtain an observed first order rate constant for dissociation at each guanidinium chloride concentration.

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Table 1 Activity recovered after refolding of folded or unfolded β subunit mixed with folded or unfolded α subunit

Starting conditions[a]	Percent recovery[b]
Unfolded β + Unfolded α	100
Unfolded β + Folded α	60
Folded β + Unfolded α	<0.005
Folded β + Folded α	<0.005

[a] Unfolded and folded subunits were prepared as described in Methods.
[b] 100 percent is based on enzyme activity recovered 24 h after mixing unfolded α and unfolded β. Refolding/assembly was carried out at 18 °C for 24 h as described in Methods, with a final protein concentration of 50 μg ml⁻¹ (equimolar α and β). The appearance of luciferase heterodimer was followed by measuring bioluminescence activity as a function of time.

Fig. 1 Kinetic partitioning of luciferase β subunit during folding. β_u, unfolded β subunit; ⊕, active heterodimeric luciferase; ⊖, homodimer.

Fig. 2 Analytical ultracentrifugation of native ⊖ subunit (see Methods). The excellent fit of the data to the equation given in Methods indicates that ⊖ sediments as a single species with a molecular weight of 71,889, approximately the molecular weight of a β_2 homodimer. A sample was also analyzed with an initial loading concentration of 0.3 mg ml⁻¹ β subunit, with similar results (single sedimenting species of molecular weight 66,697).

Fig. 3 *a*, Near UV CD spectra of folded and unfolded β subunit. Folded β in 50 mM phosphate buffer (1) and in 5 M urea (2) have essentially the same spectrum. Unfolded β subunit in 5 M urea (3) showed little near UV CD, and remained unfolded under these conditions over long periods of time. *b*, Urea denaturation curves of β subunit (circles) and αβ luciferase (squares). Protein samples in buffer were mixed with urea at the indicated concentrations (open symbols) or were diluted from 9M urea to the indicated concentrations (filled symbols) (see Methods). Following incubation at 18 °C for ~24 h, the CD signal at 222 nm was recorded. Data for the equilibrium unfolding of luciferase (αβ at 25 μg ml⁻¹) are from ref. 7.

Fig. 4 Determination of the second order rate constant for β_2 formation at 18 °C. The concentrations of β subunit during refolding following dilution from 5 M urea were 0.13 μM (△), 0.26 μM (●), 0.52 μM (○), or 0.78 μM (■). *a*, loss of heterodimerization competence of refolding β subunit. *b*, appearance of 5 M urea-stable form of the β subunit (see Methods). The solid lines in panels ⊕ and ⊖ are fits to the second order rate equation $kt = 1/[\beta] - 1/[\beta]_0$ as described in Methods.

Fig. 5 Determination of the first order rate constant for β_2 dissociation into monomers. The first order rate constant for β_2 dissociation was determined at a number of different guanidinium chloride concentrations using stopped-flow fluorescence (see Methods). The observed first order rate constants determined by fitting the data (increase in fluorescence signal at 365 nm) to a single exponential equation are plotted as a function of guanidinium chloride concentration; extrapolation to 0 M guanidinium chloride yielded a value of 1.6×10^{-14} sec^{-1} for the rate constant for unfolding of β_2 homodimer under native conditions. Inset, Fluorescence emission spectra of folded and unfolded β subunit. Native β in buffer (- - -) has an emission maximum at about 320 nm. Unfolded β in 6 M guanidinium chloride (—) has a red-shifted emission maximum at about 350 nm. The vertical line shows the maximal difference between the folded and unfolded spectra at 365 nm, the wavelength at which the change in fluorescence during unfolding was monitored.

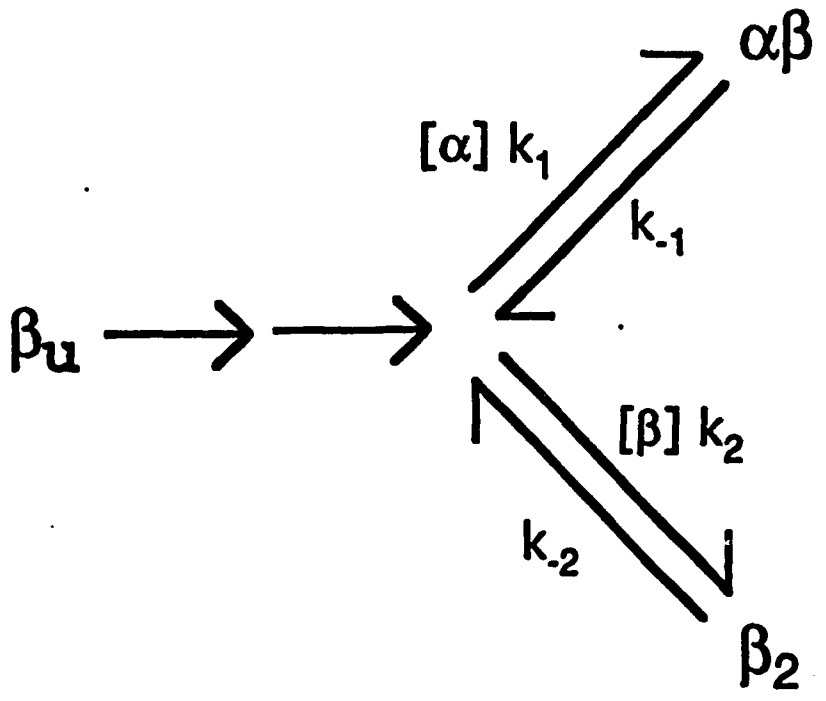
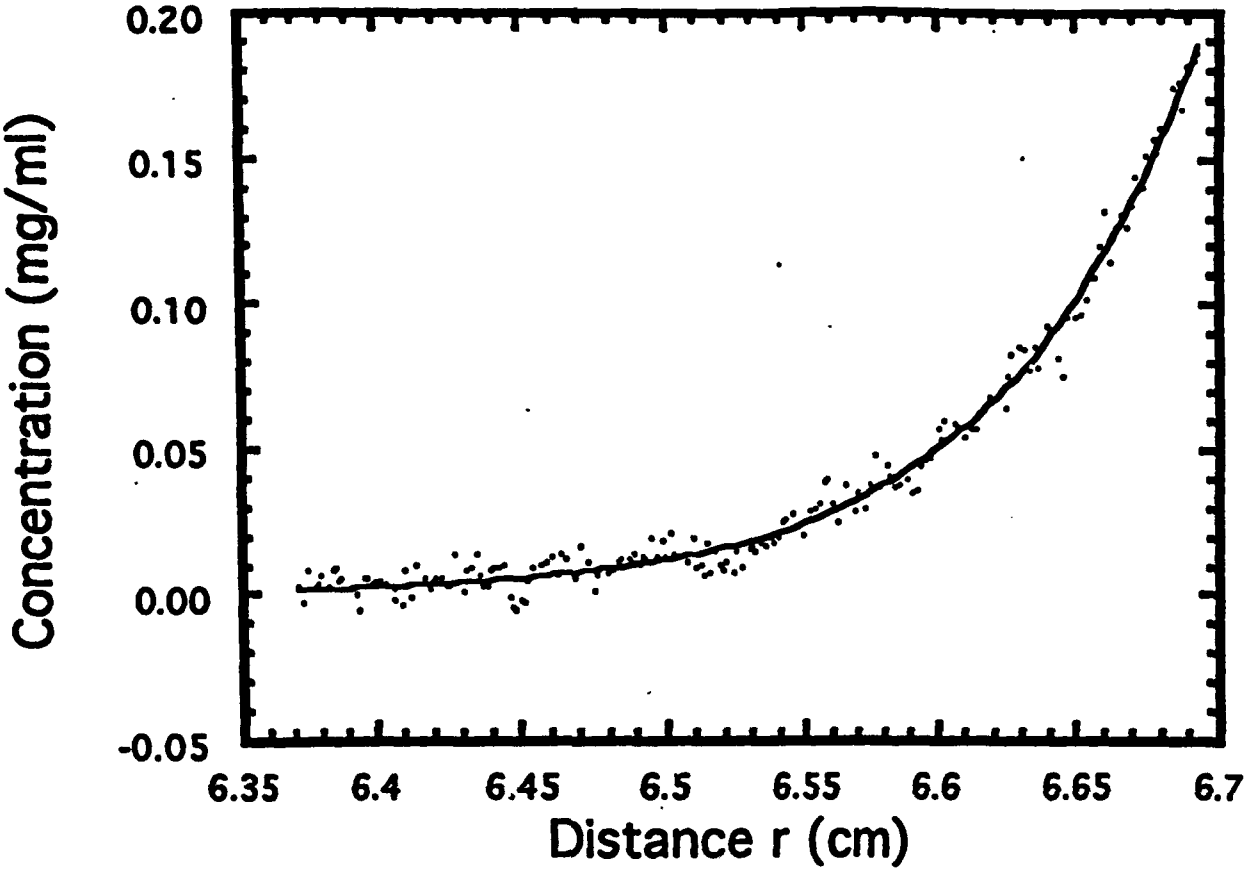
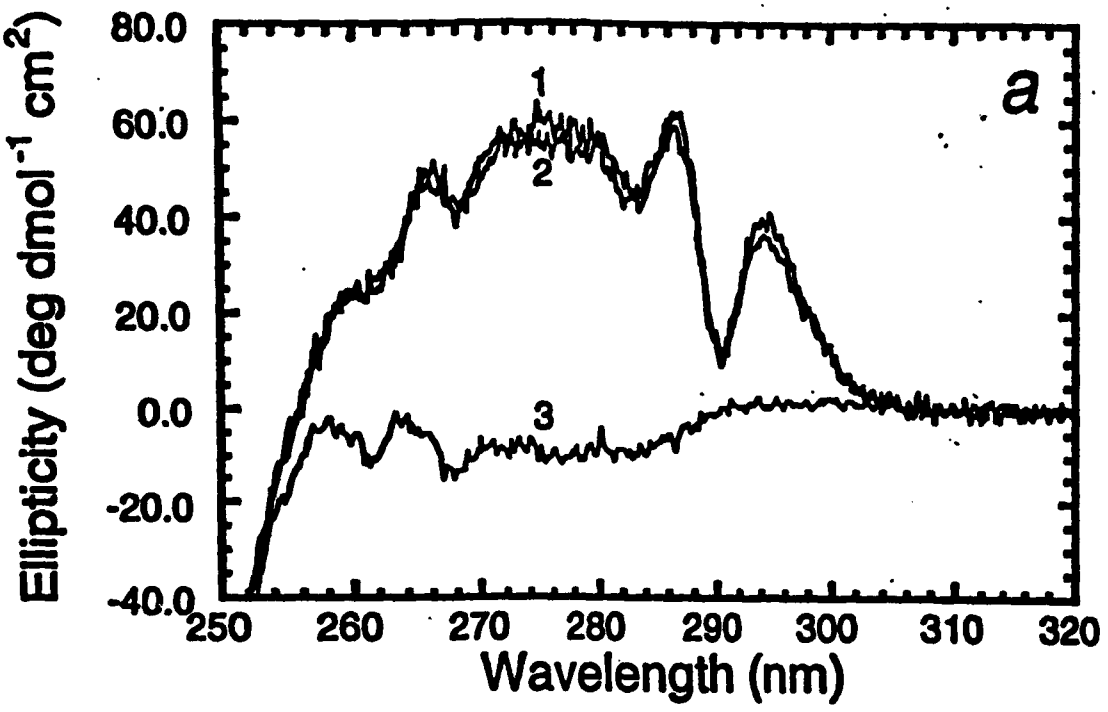
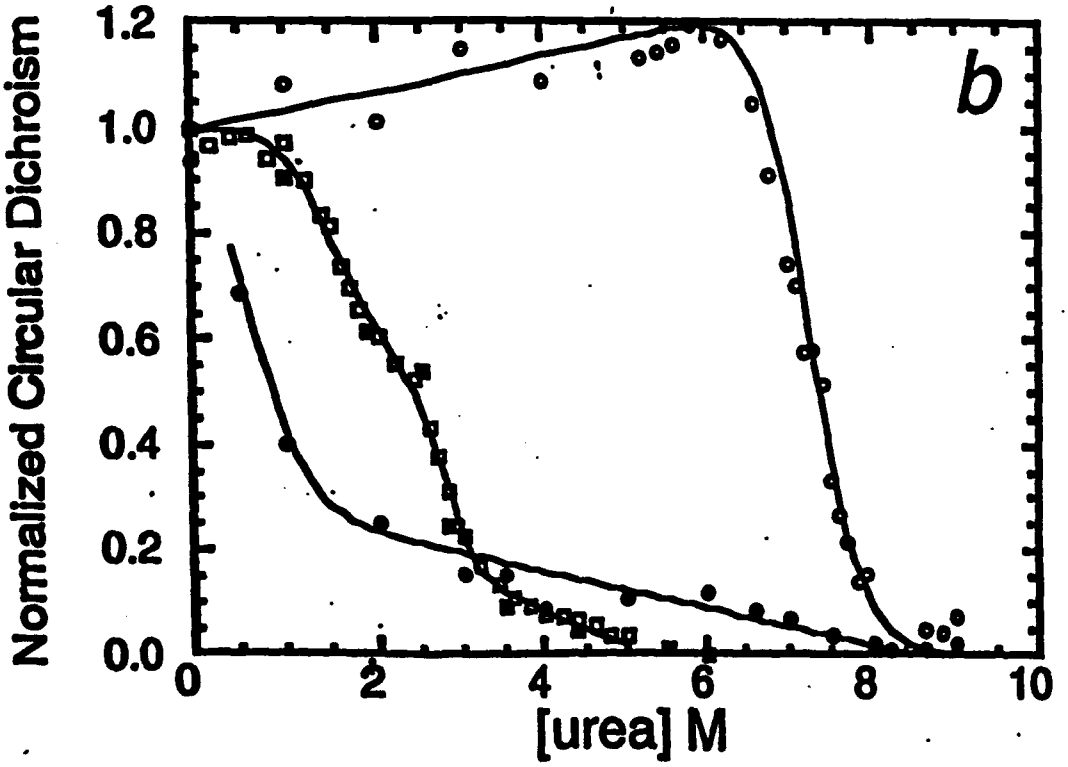


Fig.
~~Scheme~~ I. Kinetic partitioning of luciferase β subunit during folding. β_u, *unfolded* β *subunit*; αβ, active heterodimeric luciferase; β_2, homodimer.

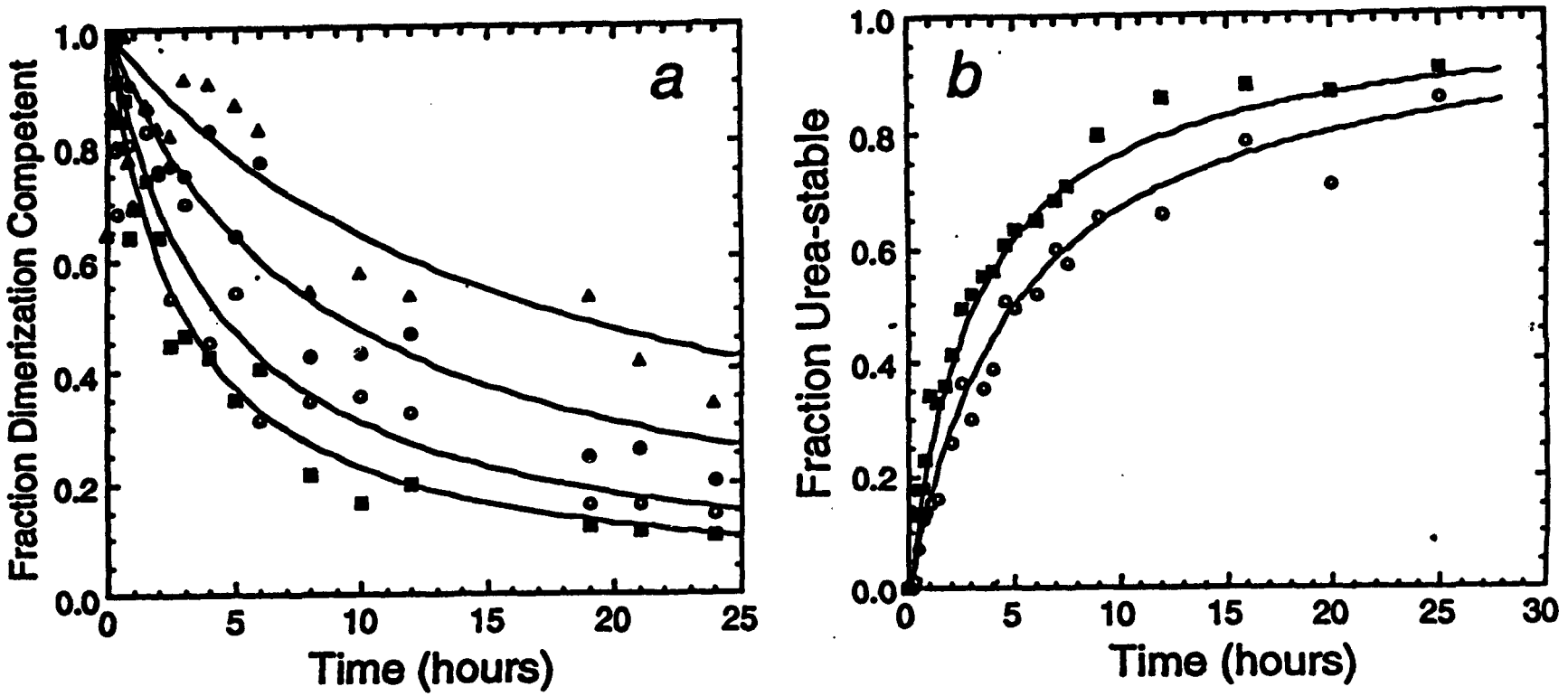


Sinclair, Ziegler, and Baldwin Fig. 12

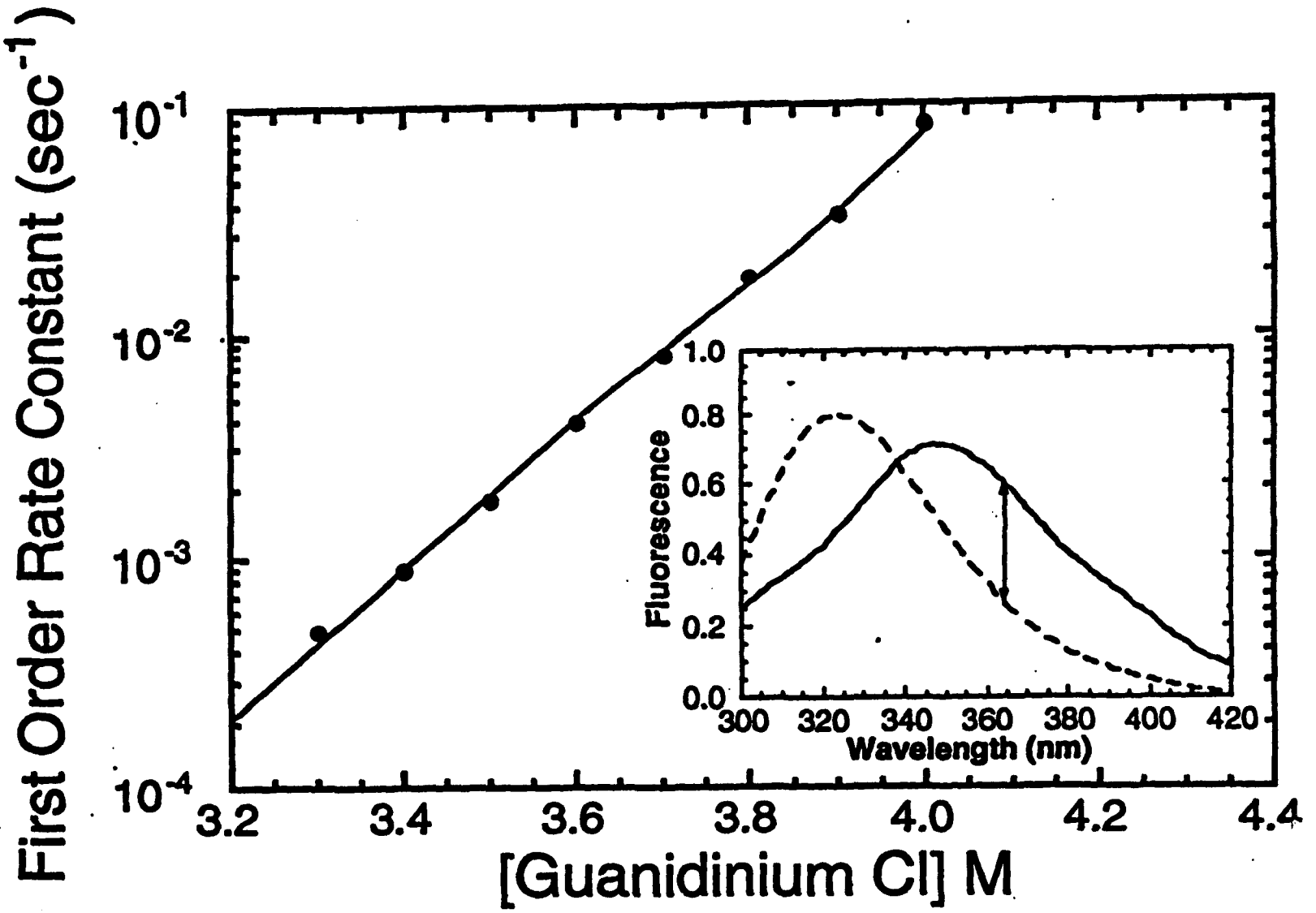




Sinclair, Ziegler, Baldwin Fig. 2 3



Sinclair, Ziegler, and Baldwin Fig. 3 4



Sinclair, Ziegler and Baldwin Fig. 4 S