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 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 85-85%, 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
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 FMNH₂, O₂, 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.

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 FMNH₂ into a vial containing enzyme, n-decyl aldehyde and O₂ 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 following the native organism, V. harveyi. Enzyme concentrations were determined by absorbance at 280 nm, using an extinction coefficient of 0.94 (mg/ml)⁻¹ cm⁻¹ (Gunsalus-Miguel et al., 1972). The enzyme was assayed (22 °C) using a photomultiplier-photometer to detect the light emitted, with n-decyl aldehyde as the substrate, upon rapid injection of FMNH₂, photoreduced in a solution containing 2 mM EDTA (Hastings et al., 1978).

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 X the enzyme concentration desired for the refolding experiment. Refolding was initiated ("time 0") by a 1:50 dilution of the enzyme.

The abbreviations used are: FMNH₂, reduced flavin mononucleotide; DTT, dithiothreitol; BSA, bovine serum albumin.
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 H2O; approximately 15 s elapsed between the recorded time (dilution into assay buffer) and the actual initiation of the assay by injection of FMNH2.

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, and/or β, (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%.

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 Zotina, 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. 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.
was complicated by the fact that at higher concentrations, the yield (Figs. 3 and 4) 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 was 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 αβ, 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.

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 (A), 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.

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 sep-
parate 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 kinetic ally
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 pro-
cesses 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 sub-
units, 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 (e.g.,
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 pre-
dicted 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 fold-
ing 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 heterodi-
meter. 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 con-
centration-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.

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 ap-
ppeared 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 heteromer-
dimer 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 pos-
tulate the intermediacy of an inactive heterodimeric species,
(αβ), which was converted to active enzyme by one or more
(first-order) isomerization steps. By switching from condi-
tions (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 hetero-
dimer (Fig. 5). We have incorporated both first-order folding
steps for the individual subunits and an inactive heterodimer-
ic 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 de-
scribed 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, in-
volving intermediates on the pathway of folding of the indi-
vidual subunits, is inactive; formation of the active structure
requires one or more subsequent isomerization steps.
3) At low protein concentrations, the yield of active hetero-
dimer is compromised by competing first-order folding pro-
cesses 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 for-
mation of the active heterodimeric luciferase is a kinetically
controlled process. Under conditions that limit the ability of
the intermediate αi and βi structures to associate, the individ-
ual subunits appear to assume thermodynamically stable
structures (αi and/or βi) 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 heterodi-
meric structure of native luciferase may reside at a local
energy minimum with a lifetime, determined by high activa-
tion energies of interconversion, that is meaningful on a
biological time scale, rather than at the global energy mini-
mum that would prevail on a geologic time scale.
