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

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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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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

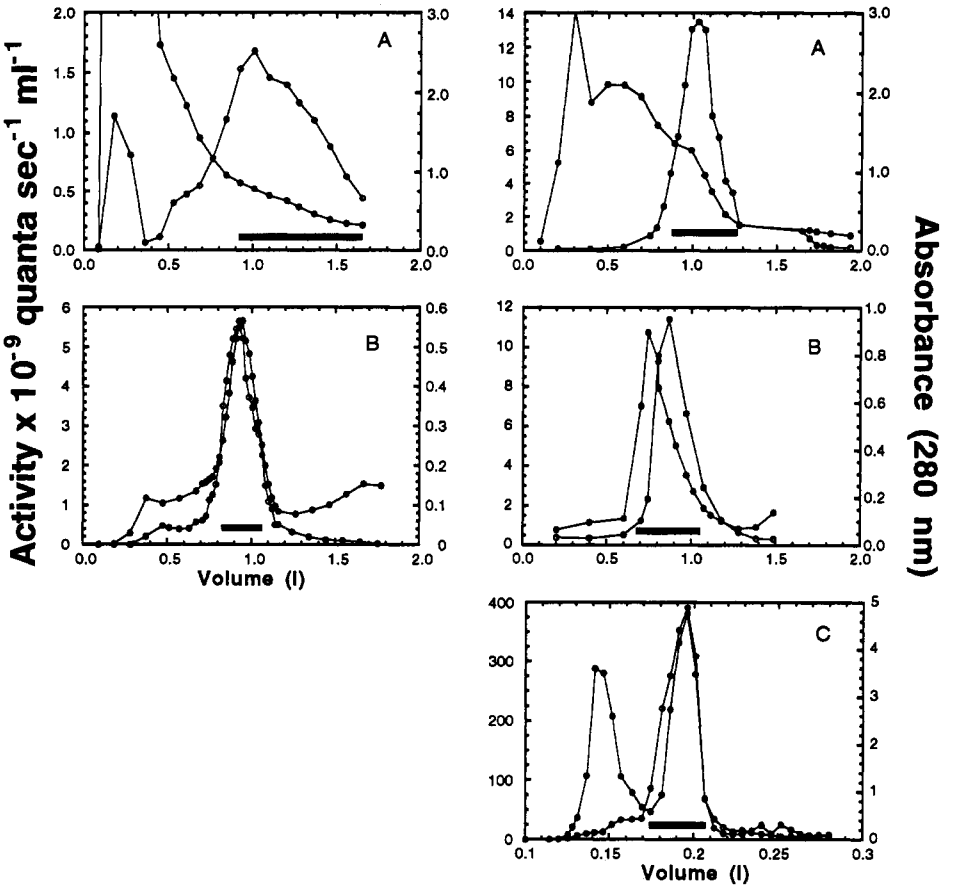

FIGURE 1: Column elution profiles for the α (left panel) and β (right panel) subunits. Bioluminescence activity (open symbols) and absorbance at 280 nm (closed symbols) are plotted against the elution volume for each column. (Left panel A) First DEAE-A50 column; (left panel B) second DEAE A-50 column; (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	5.8	45.4	1.6	75	81
second A-50	108	262	190.0	6.9	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

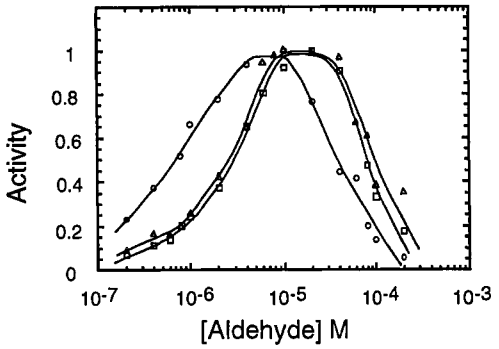

FIGURE 2: Effect of *n*-decanal concentration on bioluminescence activity. Peak light intensity was determined by the flavin injection assay in the presence of the indicated concentration of *n*-decanal. These values were then normalized to the same scale and plotted. The relative activities are shown 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.

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.

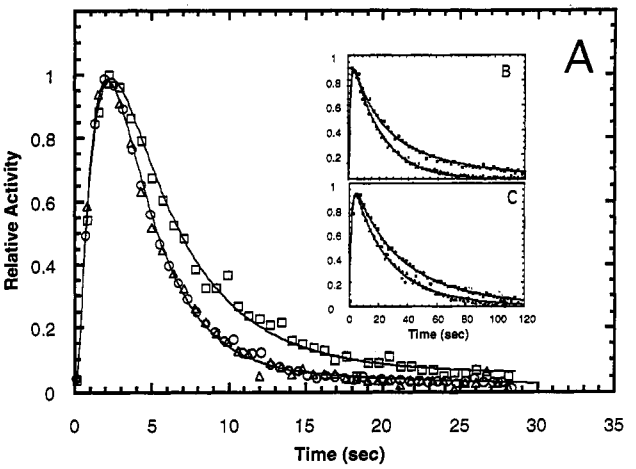

FIGURE 3: Time course of the bioluminescence reaction catalyzed by the heterodimer (circles), α subunit (triangles), and β subunit (squares). (Panel A) Light production using *n*-decanal as substrate. (Panel B) Light production using *n*-octanal as substrate. (Panel C) Light production using *n*-dodecanal 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.

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

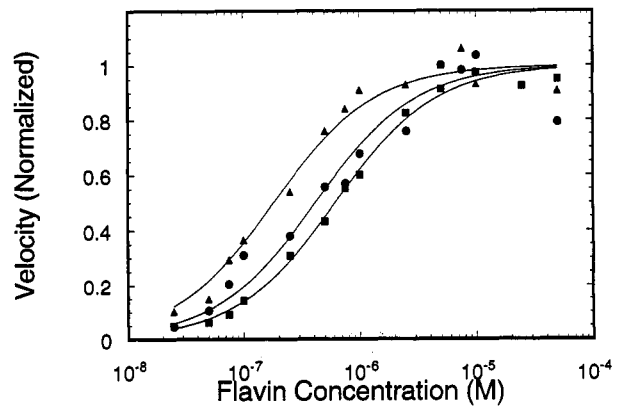

FIGURE 4: Interaction of luciferase and the α and β subunits with $FMNH_2$. Bioluminescence activity is plotted versus $FMNH_2$ concentration for the heterodimer (circles), the α subunit (triangles), and the β subunit (squares). The solid lines are the best fits of the data to the Michaelis–Menten equation. 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.

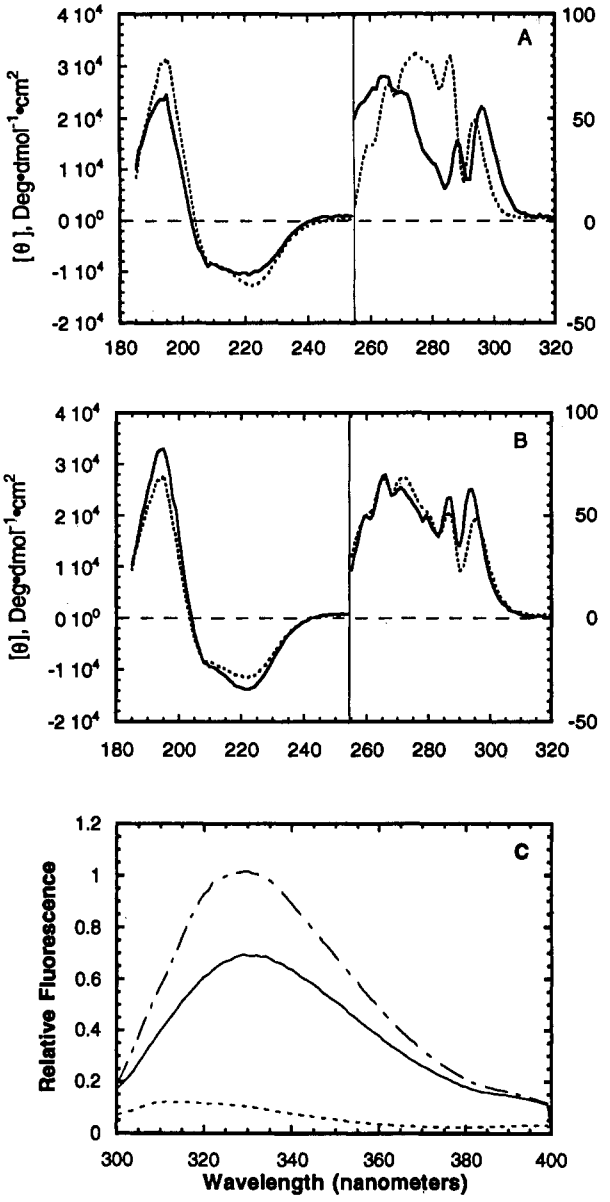

FIGURE 5: Circular dichroism and fluorescence emission spectra of luciferase, α subunit, and β subunit. (Panel A) Comparison of the circular dichroism spectra of equimolar concentrations of α subunit (—) and β subunit (- - -). (Panel B) Comparison of the sum of the spectra in panel A (- - -) to the circular 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).

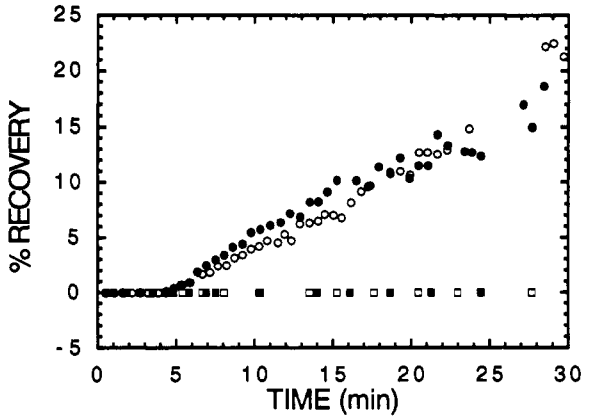

FIGURE 6: Time course of formation of the luciferase heterodimer following dilution of subunits from various solutions of denaturant at 18 °C. In the first experiment, equimolar amounts of α and β subunit were mixed in 5 M urea and diluted 50-fold to 0.1 M urea, and the activity was monitored as a function 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 ($\geq$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 $\geq$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.

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