Functional Implications of the Unstructured Loop in the $(\beta/\alpha)_8$ Barrel Structure of the Bacterial Luciferase α Subunit†

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ABSTRACT: Bacterial luciferase catalyzes the conversion of $FMNH_2$, a long-chain aliphatic aldehyde, and molecular oxygen to FMN, the corresponding carboxylic acid, and H_2O with the emission of light. The light-emitting species is an enzyme-bound excited state flavin. The enzyme is a heterodimer ($\alpha\beta$) of homologous subunits each with an $(\beta/\alpha)_8$ barrel structure. A portion of the loop in the α subunit that connects β strand 7 to α helix 7 is disordered in the crystal structure. To test the hypothesis that this loop closes over the active site during catalysis and protects the active site from bulk solvent, a mutant was constructed in which the 29 residues that are disordered in the 2.4 Å crystal structure were deleted. Deletion of this loop results in a heterodimer with a subunit equilibrium dissociation constant of 1.32 ± 1.25 μM, whereas the wild-type heterodimer shows no measurable subunit dissociation. This mutant retains its ability to bind substrate flavin and aldehyde with wild-type affinity and can carry out the chemistry of the bioluminescence reaction with nearly wild-type efficiency. However, the bioluminescent quantum yield of the reaction is reduced nearly 2 orders of magnitude from that of the wild-type enzyme.

Bacterial luciferase is a heterodimer (*1*) of homologous subunits (*2*) that converts $FMNH_2$, a long-chain aliphatic aldehyde, and O_2 to FMN, the corresponding carboxylic acid, and water, with the emission of blue-green light (*3*). When the first crystal structure of the enzyme was solved in 1995, it was apparent that both subunits were $(\beta/\alpha)_8$ barrels and that a portion of the polypeptide that connects β strand 7 to α helix 7 on the α subunit was disordered (*4*). This portion of the polypeptide had been previously reported to be exquisitely protease-sensitive (*5*). Cleavage in this region by any of a number of proteases rapidly inactivates the enzyme (*6*, *7*). This loop is protected from proteolytic cleavage upon binding of $FMNH_2$ (*8*, *9*), suggesting that the loop is no longer mobile and accessible but rather becomes ordered and unavailable to proteases. This region of the polypeptide is the longest contiguous sequence of highly conserved residues in the enzyme and is also highly conserved among all of the known bacterial luciferases (*3*). Although the active site of luciferase has not been demonstrated structurally, many lines of evidence point to a single active site on the α subunit. Nearly all $(\beta/\alpha)_8$ enzymes have active sites at the C-terminal end of their β barrel domains (*10*), which in the luciferase α subunit lies just beneath the mobile loop.

The glycolytic enzyme triosephosphate isomerase is the archetypical $(\beta/\alpha)_8$ barrel. Studies of triosephosphate isomerase have shown an 11-residue loop that closes over the active site of the enzyme, protecting a highly reactive enediol intermediate that is produced in the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (*11*). Mutation of this loop can result in failure to protect the intermediate, with consequent elimination of the phosphate group from the enediol intermediate and production of methylglyoxal, a compound toxic to cells (*12*).

The light-emitting species in the luciferase reaction is the excited state of an enzyme-bound flavin (thought to be the 4a-hydroxyflavin) (*13*). The enzyme environment has been demonstrated to influence the color and bioluminescence quantum yield of the reaction (*14*). On the basis of the known role of the loop in triosephosphate isomerase, we hypothesized that the mobile loop in luciferase might be similarly required for a high chemical yield or quantum yield or both, i.e., that one or more reaction intermediates might be protected by a section of polypeptide that closes over the active site.

To test this hypothesis, we constructed a mutant in which residues 262−290 of the α subunit, the residues not observable in the 2.4 Å crystal structure (*4*), were deleted by excision of the corresponding codons from the recombinant *lux*A gene. This paper reports the characterization of the loop-deleted enzyme with respect to its ability to fold and assemble, as well as bind substrates and support the chemistry of the bioluminescence reaction.

EXPERIMENTAL PROCEDURES

Cloning of the $\alpha_{\Delta 262-290}\beta$ *Heterodimer.* Deletion of residues 262−290 inclusive from the α subunit was accomplished by PCR, resulting in a subunit that is only two residues longer than the β subunit. The heterodimeric luciferase expressed by this strain is designated $\alpha_{\Delta 262-290}\beta$. The PCR was performed using a Seamless Cloning kit (Stratagene, La Jolla, CA) on a Perkin-Elmer GeneAmp 2400. The plasmid pJHD500 (*15*) was amplified with the following primers: 5′-GGCTCTTCAGACACCAATCGCCGAATTG-3′ and 5′-CAACTCTTCTGTCGGCATTCACGTATGA GTCGTACCAATG-3′. The resulting PCR product was digested with Eam1104I (Stratagene, La Jolla, CA) and ligated. The ligation mix was transformed into the XL1 blue

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MRF′ cell line provided by Stratagene. Plasmids were isolated and screened by restriction analysis. Two potential mutants were selected and sequenced, both of which contained the desired sequence. The plasmid containing the loop deletion was named pαdel1, and the transformed cell line was designated JMS66.

Protein Expression and Purification. $\alpha_{\Delta 262-290}\beta$ was overexpressed in JMS66 by the method described by Thomas and van Tilburg (*16*). Cells were pelleted by centrifugation at 5000 rpm for 30 min and resuspended in 1.25 mL of lysis buffer/g of cell pellet. Lysis buffer (pH 7.0) contained 50 mM phosphate, 1 mM EDTA, 1 mM DTT, and 100 mM NaCl. The bacterial cell suspension was lysed with a French press, and the crude lysate was centrifuged at 13000 rpm for 25 min. The supernatant was brought to 30% saturation with ammonium sulfate and centrifuged at 13000 rpm for 25 min. The resulting supernatant was brought to 75% saturation by further addition of ammonium sulfate. After centrifugation of the 75% ammonium sulfate preparation, the pellet was redissolved in 100 mM phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialyzed protein was applied to a 500 mL DEAE-Sephadex A-50 column and eluted with a 4 column volume linear gradient from 100 mM to 1 M phosphate buffer, pH 7.0 (1 mM EDTA, 1 mM DTT). Fractions were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and stained with Gelcode (Pierce Chemicals, Dallas, TX). Select fractions were pooled, concentrated, and dialyzed against 100 mM phosphate buffer. The dialyzed protein was applied to a Q-Sepharose column and eluted with a linear gradient from 100 to 900 mM phosphate buffer. Fractions were again analyzed on an SDS−PAGE gel stained with Gelcode, and selected fractions were pooled, concentrated, and dialyzed against 50 mM phosphate buffer, pH 7.0 (1 mM EDTA, 1 mM DTT). The purified luciferase was stored at approximately 15 mg/mL at −80 °C. Wild-type luciferase was purified as described previously (*17*, *18*).

Determination of the Extinction Coefficient of $\alpha_{\Delta 262-290}\beta$. The molar extinction coefficient at 280 nm was calculated to be 77500 M^{-1} cm^{-1} by the method of Pace et al. (*19*).

Bioluminescence Activity in Vitro. Bioluminescence activity was determined by the flavin injection method (*20*). The enzyme was incubated in buffer containing 1-decanal in a vial in a luminometer. The reaction is initiated by injection of 1 mL of $FMNH_2$ prepared by exposing 50 μM FMN with 2 mM EDTA to fluorescent light in 1 mL translucent syringes. Reduction was judged to be complete when the solution became colorless. Light production was measured with a Turner Designs (Mountain View, CA) model TD-20e luminometer, and peak luminescence was recorded.

Circular Dichroism Spectroscopy. CD spectra were acquired with an Aviv Instruments 202 SF spectropolarimeter (Aviv Instruments, Lakewood, NJ). Protein solutions were dialyzed against 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Protein concentrations were adjusted to 2.6 μM for the near-UV spectra and to 0.26 μM for the far-UV spectra by diluting with the dialysate. The near-UV CD data were obtained using a 1 cm path-length cuvette, and the far-UV data were obtained in a 0.2 cm cuvette. Five scans were collected and averaged, the buffer spectra were subtracted, and the data were converted to mean residue ellipticity by the equation:

$$\mathrm{MRE} = \frac{\Theta}{(10Cnl)}$$

where C is the molar concentration of protein, n is the number of residues, and l is the path length in centimeters.

Analytical Ultracentrifugation. Equilibrium ultracentrifugation data were obtained using a Beckman (Palo Alto, CA) XL-I analytical ultracentrifuge. The protein was first dialyzed exhaustively against 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, and 100 mM NaCl. Six sector cells were loaded with protein at initial loading concentrations ranging from 1.36 to 11 μM. Cells were centrifuged at speeds of 16000−22000 rpm at 18 °C until they reached equilibrium, approximately 16 h. The data were then fit to both a single sedimenting model and a monomer−dimer interacting model. The following model, which describes the behavior of a monomer−dimer interacting system, was the one that best fit the data.

$$A_r = A_0 \exp\{[M(1-\nu\rho)\omega^2/2RT][r^2 - r_0{}^2]\} + (A_0)^2 K_a \exp\{[M(1-\mathrm{v}\rho)\omega^2/2RT][r^2 - r_0{}^2]\} + E$$

where A_r = the absorbance at any radial position, r = the radial position, A_0 = the absorbance at the reference radial position, r_0 = the reference radial position, K_a = the association constant, ν = the partial specific volume of the sedimenting species, ρ = solvent density, ω = angular velocity, R = the ideal gas constant, and T = absolute temperature. The partial specific volume of $\alpha_{\Delta 262-290}\beta$ was calculated on the basis of the per residue values of Cohn and Edsall (*21*) and determined to be 0.731 mL/g. Buffer density was 1.00635 g/mL, measured at 18 °C using a Mettler Toledo DE50 density meter. Goodness of fit was judged by distribution of residuals. The K_d reported is the average of 12 data sets.

Bioluminescence Emission Spectra in Vivo. Bioluminescence emission spectra were obtained using an SLM 8000C spectrofluorometer. Strains of *Escherichia coli* transformed with a plasmid encoding the appropriate luciferase, either the wild-type (IGC1) or the $\alpha_{\Delta 262-290}\beta$ (JMS66), were grown in Terrific Broth to an OD of 4−5. Bioluminescence was initiated by adding 4 μL of decanal to 1 mL of liquid culture while stirring in a 1 × 1 cm quartz cuvette. The emission monochromator was used to scan the luminescent sample from 400 to 600 nm, without any excitation light. Spectra were all normalized to the same maximum.

Aldehyde Binding. Aldehyde binding was measured by quantitating the enzyme's ability to produce light at various concentrations of decanal as described by Holzman and Baldwin (*22*). Decanal stock solutions were made to 10 mM and 10 μM by adding decanal to water and sonicating in two 10 s pulses. Solutions were stored under N_2 and diluted into assay buffer immediately prior to use. Enzyme and aldehyde were incubated for 30 s prior to each assay. Assays were initiated by injection of 1 mL of photoreduced FMN (see activity assay section above for details). Each assay was repeated three times, and the average value was plotted. Data were fit to the following Michaelis−Menten equation that takes into account decanal inhibition of the enzyme at high concentration:

$$\nu = \frac{\nu_m}{1 + K_d/[S] + [S]/(\alpha K_d)}$$

where ν_m is the maximal rate that would be attained in the absence of substrate inhibition, K_d is the dissociation constant of the E(RCHO) complex, and α is the interaction constant indicating the effect the first bound substrate molecule has on the second (*22*).

K_d for $FMNH_2$. K_d values for $FMNH_2$ with the wild-type and $\alpha_{\Delta 262-290}\beta$ luciferases were determined by the dithionite assay method (*23*). Dithionite solutions were prepared as described by Tu and Hastings (*24*). The enzymes were incubated with 1 mL of FMN in 50 mM phosphate buffer, pH 7.0 at 24 °C. The flavin was reduced by the addition of 10 μL of a 30 mg/mL dithionite solution. Bioluminescence activity was initiated by the injection of 1 mL of a 0.01% suspension of decanal in 50 mM phosphate, pH 7.0. At the higher flavin concentrations, the FMN was reduced photochemically in the presence of 2 mM EDTA, and bioluminescence was initiated by injection of the $FMNH_2$ into a mixture of enzyme, decanal, and oxygen. Each measurement was taken in triplicate. The final enzyme concentrations for these experiments were 400 nM for the wild type and 2.6 μM for $\alpha_{\Delta 262-290}\beta$.

$FMNH_2$ Binding by the Equilibrium Method. Reduced flavin binding was monitored by a decrease in intrinsic protein fluorescence as a function of increasing concentrations of $FMNH_2$. Fluorescence data were obtained using an SLM 8000C spectrofluorometer. The enzyme concentration was held constant at 6.5 μM (3.25 μM final concentration). One milliliter of a 6.5 μM solution of luciferase was placed in the sidearm of an anaerobic cuvette, and 1 mL of FMN was placed in the bottom of the anaerobic cuvette. Oxygen was removed from the cuvette by vacuum, and the cuvette was flushed with argon. The entire cuvette was then placed in a light box, and the flavin was allowed to photoreduce until the yellow color disappeared. The solutions were mixed by inverting the cuvette. Each sample was allowed to incubate for 30 s, after which time the intrinsic fluorescence emission was monitored from 300 to 450 nm with excitation at 280 nm. The spectral envelope was integrated, and values were normalized to protein in the absence of $FMNH_2$. Dissociation constants were determined by fitting data to a standard Langmuir binding isotherm.

Oxidized Flavin Binding. Fluorescence data were obtained using an SLM 8000C spectrofluorometer. The binding of oxidized FMN was monitored by quantitating the decrease in the FMN fluorescence as a function of increasing luciferase concentration. Samples were prepared to a final concentration of 10 μM FMN and various luciferase concentrations in pH 7.0 50 mM bis-tris [[bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane]. The fluorescent emission spectra of the samples were scanned from 470 to 620 nm, with excitation at 450 nm. Spectra were integrated, and the total area was used to calculate the dissociation constant from the equation (*26*):

$$\frac{F_{FMN}}{F_{FMN} - F_{obs}} = \left(\frac{f_{FMN}}{f_{FMN} - f_{E-FMN}}\right)\frac{K_d}{[E]} + \frac{f_{FMN}}{f_{FMN} - f_{E-FMN}}$$

where F_{FMN} is the fluorescence of 10 μM FMN, F_{obs} is the observed fluorescence of sample with luciferase, f_{FMN} is the intrinsic fluorescence of the free FMN, f_{E-FMN} is the intrinsic fluorescence of the enzyme−FMN complex, K_d is the dissociation constant for the FMN, and [E] is the total enzyme concentration.

Decanoic Acid Analysis. The carboxylic acid produced by enzymatic oxidation of decanal substrate was quantitated by gas chromatography. Standard flavin injection assays were performed in 10 mL volumes. Protein concentrations were held constant at 50 μM. The resulting assay mixture was immediately prepared for GC analysis by removing a 1 mL aliquot and adding 200 μL of 200 μg/mL undecanoic acid in methanol, an internal standard. The sample was then acidified by the addition of 50 μL of 4 M H_2SO_4. The acidified mixture was extracted by addition of 1.0 mL of CH_2Cl_2 with constant shaking for 3 min. The organic layer was transferred to another tube, and 0.4 mL of MSTFA was added. The mixture was warmed for 5 min, and an aliquot was injected into the GC. A Hewlett-Packard Model 5880 gas chromatograph equipped with an Alltech Associates Inc. EC-5 column and a flame ionization detector was used for the analysis. Helium at 12 psi column pressure was used as the carrier gas. It was found that the decanoic acid adduct had a retention time of 6.49 min. Quantitation of carboxylic acid was determined by comparison to the internal standard.

RESULTS

After purification of the $\alpha_{\Delta 262-290}\beta$ mutant luciferase, in vitro assays showed that the loop-deleted enzyme produced approximately 1% of the light of the wild-type enzyme/mg of dimer. The specific activity of the wild-type enzyme was determined to be 1.07×10^{14} quanta/(s·mg) in 50 mM phosphate buffer, pH 7.0, agreeing well with previous determinations (*3*). The specific activity of $\alpha_{\Delta 262-290}\beta$ was determined to be 1.08×10^{12} quanta/(s·mg) of dimer at a protein concentration of 13 μM, or approximately 10 times the K_d under the same solution conditions (see below). Activity was reported as activity per milligram of dimer because, unlike the wild-type enzyme, the $\alpha_{\Delta 262-290}\beta$ has a measurable subunit dissociation equilibrium (see below).

Under native conditions, CD spectroscopy showed that $\alpha_{\Delta 262-290}\beta$ was folded with wild-type-like secondary structure (Figure 1). There is an increase in mean residue ellipticity at 222 nm, suggesting that per residue $\alpha_{\Delta 262-290}\beta$ has more α-helical character than does wild-type luciferase, as might be expected from deletion of a disordered region comprising nearly 10% of the α subunit sequence. The near-UV CD data suggest that the aromatic side chains are in nearly the same environment as are the corresponding residues in the wild-type enzyme.

It has been known for some time that the wild-type luciferase has a very low subunit equilibrium dissociation constant. Since the association is so strong, it is difficult to measure the actual K_d but it is thought to be less than nanomolar (*27*; for a review see ref *3*). The strength of the subunit association in the $\alpha_{\Delta 262-290}\beta$ heterodimer was investigated using equilibrium analytical ultracentrifugation (Figure 2 and Table 1). The data demonstrated unambiguously that deletion of the mobile loop drastically affected the subunit equilibrium dissociation constant. Multiple samples, at different protein concentrations, were subjected

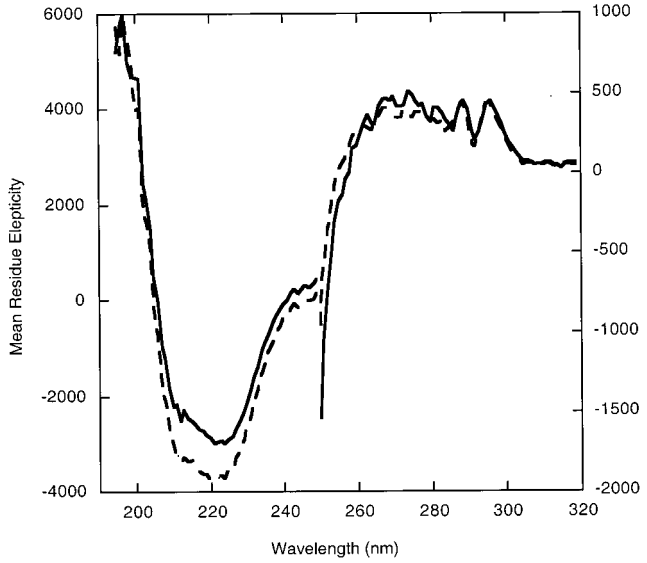

FIGURE 1: Near- and far-UV CD spectra of wild type and $\alpha_{\Delta 262-290}\beta$. CD scans of wild type (solid line) and $\alpha_{\Delta 262-290}\beta$ (dashed line) were obtained for both the near- and far-UV region. The concentration of protein for the far-UV spectrum (190−250 nm) was 0.26 μM. The concentration of protein used for the near-UV spectrum (250−330 nm) was 2.6 μM. Protein was equilibrated in 50 mM phosphate, pH 7.0 at 18 °C. Each spectrum represents the average of five scans, and ellipticity was converted to mean residue ellipticity.

to equilibrium ultracentrifugation at 18 °C. The data were fit using a monomer−dimer associating model where all terms were fixed, leaving only the equilibrium association constant to be mathematically determined. The average value of all data suggested an equilibrium dissociation constant of 1.32 ± 1.25 μM.

Using a fluorometer without excitation light, it was determined that both the wavelength of maximum emission intensity and the spectral envelope of the bioluminescence in vivo were unchanged by deletion of the disordered loop (Figure 3). When the data were normalized to the same maximum signal, the spectra of the mutant and wild-type cultures were superimposable with a maximum emission at 491 nm.

The low yield of light emission of the bioluminescence reaction catalyzed by the loop-deleted enzyme could be caused by a reduction in the ability of the enzyme to carry out the chemistry needed to populate the excited state, a reduction in efficiency of population of the excited state of that intermediate, and/or a reduction in quantum yield of the excited state itself. We thus undertook an investigation of the ability of the enzyme to carry out the reaction chemistry, including its ability to bind substrates $FMNH_2$ and aldehyde, and the efficiency with which it carried out the oxidation of the aldehyde to the carboxylic acid.

The ability of $\alpha_{\Delta 262-290}\beta$ to bind $FMNH_2$ was investigated by determining the ability of the enzyme to produce light as a function of $FMNH_2$ concentration (*23*) (Figure 4 and Table 2). The K_d values were determined to be 1.8 μM and 0.76 μM for $\alpha_{\Delta 262-290}\beta$ and the wild type, respectively, suggesting that the deletion caused no major change in binding affinity for $FMNH_2$ in the presence of the aldehyde substrate. These K_d values agree well with the values reported by Meighen and Hastings for the wild-type enzyme (*23*).

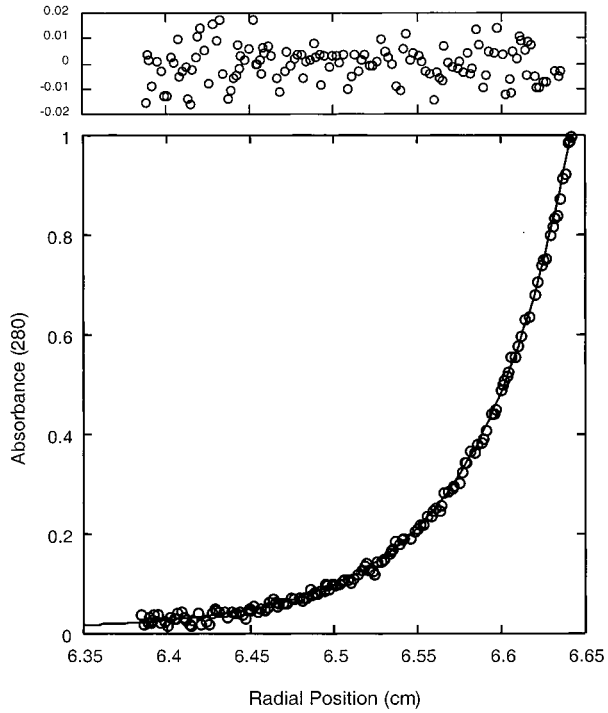

FIGURE 2: Representative example of equilibrium sedimentation ultracentrifugation of $\alpha_{\Delta 262-290}\beta$. This sample was centrifuged at 18000 rpm in 50 mM phosphate and 100 mM NaCl, pH 7.0 at 18 °C. After equilibrium was achieved (approximately 20 h), the absorbance at 280 nm was recorded as a function of radial position. The solid line is a best fit of a monomer−dimer interacting system to the data. The residuals (top panel) are the difference between the data and the curve fit. The random nature of the residuals indicates that the model is a good description of the data. The results of 12 other experiments are given in Table 1. The equilibrium constant, determined from the average of all measurements, for this association was 1.32 ± 1.25 μM.

Table 1: Sedimentation Equilibrium Ultracentrifugation of $\alpha\Delta_{262-290}\beta$

rotor speed (rpm)	loading concn (μM)	K_d (μM)	rotor speed (rpm)	loading concn (μM)	K_d (μM)
20000	6.38	0.28	22000	6.38	1.80
20000	5.80	2.00	22000	5.80	1.37
20000	5.15	0.98	22000	5.15	0.70
20000	3.86	1.37	22000	3.86	1.39
20000	2.60	2.52	22000	2.60	1.35
20000	1.29	1.00	22000	1.29	0.64

The $\beta\alpha 7$ loop of the α subunit of luciferase has been shown, by protection from proteolysis, to become ordered upon substrate binding and is thought to cover the active site of luciferase (*8*, *9*). Since it is known that the conformational flexibility of the loop is decreased by the binding of $FMNH_2$, we investigated the effect of deletion of the loop on the affinity of the enzyme for $FMNH_2$. By monitoring the decrease in the intrinsic fluorescence of the protein as a function of increasing $FMNH_2$, the K_d for $FMNH_2$ was determined to be 5.3 μM for wild type and 8.2 μM for $\alpha_{\Delta 262-290}\beta$ (Figure 5 and Table 2), again demonstrating no major effect of the deletion on affinity of the enzyme for reduced flavin.

Bacterial luciferase is known to bind FMN on the order of 100 times more weakly than the substrate $FMNH_2$ (*26*). The ability of $\alpha_{\Delta 262-290}\beta$ to bind FMN was determined by monitoring the intrinsic fluorescence of the FMN (Figure 6

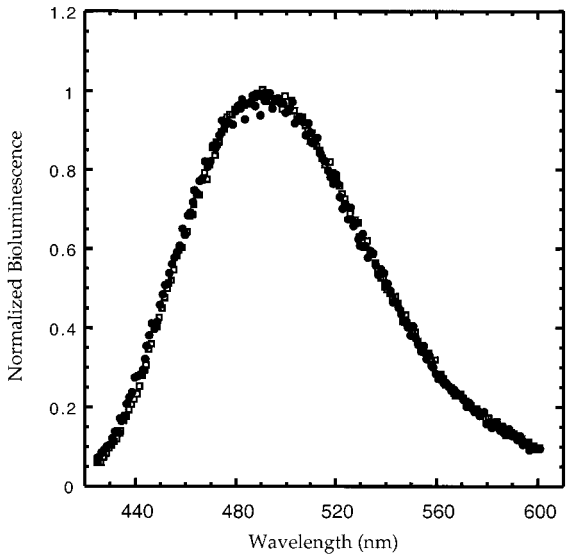

FIGURE 3: Bioluminescence emission spectra in vivo of wild type and $\alpha\Delta_{262-290}\beta$. Cultures of IGC1 (wild type) and JMS66 ($\alpha\Delta_{262-290}\beta$) were grown overnight. Cultures were diluted to an $OD_{600} = \sim 4$ and allowed to grow at 20 °C. Aliquots were removed and placed in a fluorometer with no excitation light, and the emission spectra of wild type (closed symbols) and $\alpha\Delta_{262-290}\beta$ (open symbols) were collected. Spectra were normalized to a maximum signal of 1. The maximum emission wavelength for both enzymes was determined to be 491 nm.

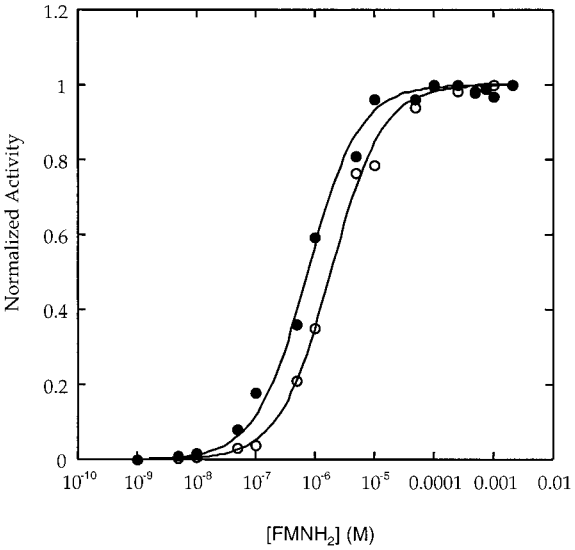

FIGURE 4: Initial velocity plots of wild type and $\alpha_{\Delta 262-290}\beta$ as a function of [$FMNH_2$]. Wild type (solid symbols) and $\alpha_{\Delta 262-290}\beta$ (open symbols) were incubated with increasing concentrations of $FMNH_2$. The FMN was reduced by dithionite (*18*) or by photoreduction (*3*). The initial maximum intensity of light production was monitored. Light production was monitored in a luminometer upon injection of a suspension of aldehyde. Each measurement was taken in triplicate, and the average data were plotted. Solid lines are best fits to the Michaelis−Menten equation. K_d values are reported in Table 2.

and Table 2). The $\alpha_{\Delta 262-290}\beta$ heterodimer was found to bind FMN with a K_d of 400 μM, or approximately four times that of the wild-type enzyme.

Aldehyde is both a substrate and an inhibitor of bacterial luciferase (*22*, *28*, *29*). A form of the Michaelis−Menten equation that accounts for substrate inhibition (*22*) was fit to the data. The K_m for decanal was found to be 2.77 μM for the wild type and 1.47 μM for $\alpha_{\Delta 262-290}\beta$. The K_i for

Table 2: Flavin and Aldehyde Binding Constants[a]

enzyme	K_{dFMNH_2}[b] (μM)	K_{dFMNH_2}[c] (μM)	K_{dFMN}[d] (μM)	K_M[e] (μM)	K_i[e] (μM)
wild type	0.76	5.3	120	1.46	86.2
$\alpha_{\Delta 262-290}\beta$	1.87	8.4	400	2.77	116

[a] The error in the numbers presented is less than ±10% of the value. [b] K_d values were obtained from data in Figure 4. [c] K_d values were obtained from data in Figure 5. [d] K_d values for the FMN product were obtained from data shown in Figure 6. [e] Values for K_m and K_i were determined by fitting a form of the Michaelis−Menten equation that accounts for substrate inhibition to the data shown in Figure 7.

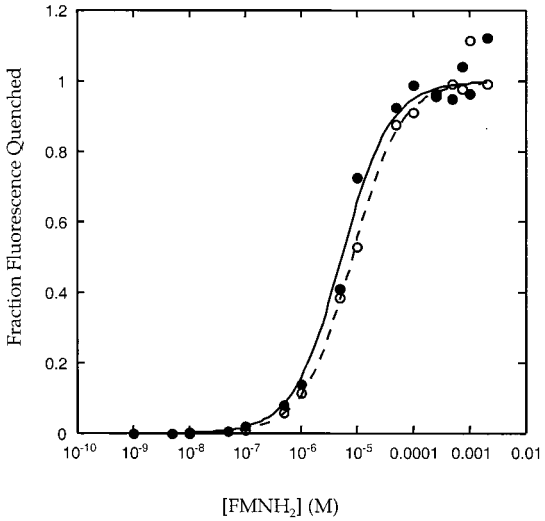

FIGURE 5: $FMNH_2$ binding by $\alpha\Delta_{262-290}\beta$. Binding of $FMNH_2$ was measured by monitoring the decrease in intrinsic tryptophan fluorescence of the enzyme. Samples were placed in an anaerobic cuvette, and the flavin was photoreduced. The spectral emission envelope was integrated from 300 to 450 nm; excitation was at 280 nm. Wild-type luciferase is represented by the solid circles and $\alpha\Delta_{262-290}\beta$ by the open symbols. The K_d values determined are reported in Table 2.

decanal was determined to be 86.2 μM for the wild type and 116 μM for $\alpha_{\Delta 262-290}\beta$ (Figure 7 and Table 2).

To determine whether $\alpha_{\Delta 262-290}\beta$ is capable of catalyzing the chemistry of the bioluminescence reaction with the same efficiency as the wild-type luciferase, the decanoic acid oxidation product of the aldehyde substrate was measured. A method was developed by which the amount of decanoic acid produced could be determined by extracting the acid, separating it by gas chromatography, and determining the amount using a flame ionization detector. The background level of decanoic acid was 0.80 μg/mL. The wild-type enzyme produced 1.04 μg/mL of decanoic acid, while $\alpha_{\Delta 262-290}\beta$ produced 0.67 μg/mL under the conditions assayed after subtracting the control of 0.80 μg/mL. This represents a decrease of ~30% in the amount of acid produced by $\alpha_{\Delta 262-290}\beta$ per milligram of dimer as compared to the wild-type enzyme.

DISCUSSION

It is known that $(\beta/\alpha)_8$ barrels are very stable structures and that mutation of the loops connecting the β strands to the α helices generally does not have any large effect on the overall structure of the protein. However, it was necessary to confirm that deletion of the disordered part of the $\beta\alpha 7$ loop in $\alpha_{\Delta 262-290}\beta$ does not perturb the tertiary structure. Far-

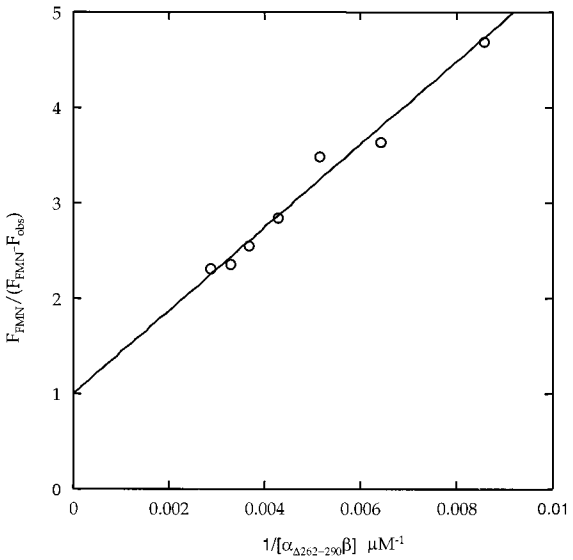

FIGURE 6: Binding of oxidized flavin by $\alpha_{\Delta 262-290}\beta$. The affinity of $\alpha_{\Delta 262-290}\beta$ luciferase for FMN was determined by quenching of the flavin fluorescence by increasing concentrations of the enzyme in 50 mM phosphate buffer, pH 7.0 at 18 °C. The data were plotted as a function of the molar concentration of the enzyme as described in Results, and the line is a best fit to the equation in Experimental Procedures. The ordinate being equal to one indicates that the luciferase−FMN complex is nonfluorescent. The K_d determined was 400 μM, which is slightly more than the published value for the wild-type enzyme.

UV circular dichroism showed that, on a per residue basis, the $\alpha_{\Delta 262-290}\beta$ heterodimer has more α-helical structure than does the wild type, as would be expected following deletion of a disordered region comprising approximately 10% of the protein. The near-UV circular dichroism spectrum is characteristic of the environment of the chromophores in the protein. Comparison of the near- and far-UV circular dichroism spectra of the wild-type and $\alpha_{\Delta 262-290}\beta$ enzymes showed that there was no significant change in the overall structure or in the environment of the aromatic side chains, suggesting that the $\alpha_{\Delta 262-290}\beta$ heterodimer is folded and has similar architecture to that of the wild-type enzyme.

Despite the fact that each subunit alone will catalyze the bioluminescence reaction at levels several orders of magnitude below the activity of the heterodimer (*30*), the heterodimer is necessary for the high quantum yield reaction (*3*). In the late 1980s each subunit was produced alone in recombinant *E. coli* (*31*). Once purification schemes for each subunit were developed, their ability to produce light was determined. It was found that each subunit alone was able to produce low levels of light. The specific bioluminescence activity of the α subunit is 3×10^9 quanta/(s·mg), 5 orders of magnitude below the light yield of the heterodimeric enzyme, and that of the β subunit is 8×10^9 quanta/(s·mg), again far below the activity of the $\alpha\beta$ heterodimer (*30*).

Because we found that the $\alpha_{\Delta 262-290}\beta$ loop-deleted enzyme had a measurable subunit dissociation, activity assays of the mutant were done at concentrations that significantly populated the dimer. The specific activity of the $\alpha_{\Delta 262-290}\beta$ heterodimer was about 1% that of the wild-type heterodimer (discussed above). From the equilibrium subunit dissociation constant, determined here by analytical ultracentrifugation to be about 1.32 μM, it was calculated that at the protein concentration used for these assays ~70% was dimeric.

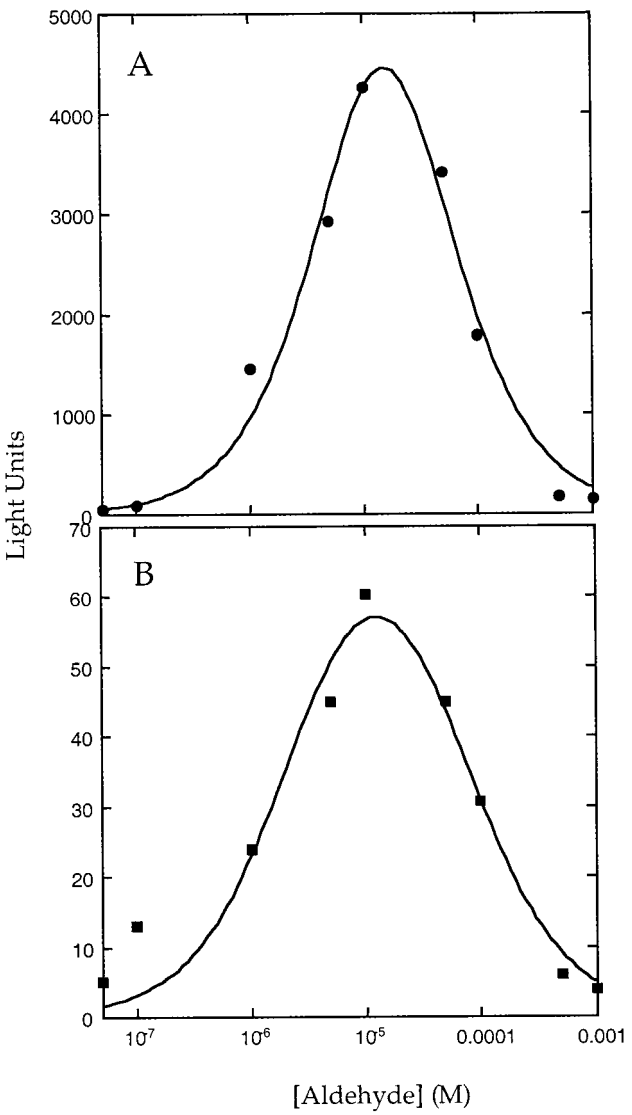

FIGURE 7: Aldehyde binding by wild-type luciferase and $\alpha\Delta_{262-290}\beta$ luciferase. K_m and K_I for wild-type enzyme (A) and $\alpha\Delta_{262-290}\beta$ (B) were determined by the effect of aldehyde concentration on bioluminescence activity. Assay mixtures contained 2.6 mM enzyme and 50 mM photochemically reduced $FMNH_2$. A form of the Michaelis−Menten equation that accounts for aldehyde inhibition at high substrate concentrations (*22*) was fit to the data. The data fitting provided the values found in Table 2.

Ideally, the protein concentration would be such that the entire population is dimeric, but at concentrations necessary to achieve a >90% dimeric population, $\alpha_{\Delta 262-290}\beta$ formed an aggregate and precipitated. The specific activity of the $\alpha_{\Delta 262-290}\beta$ heterodimer was considerably higher than that of the individual wild-type subunits but significantly lower than that of the wild-type enzyme.

The emission spectra of the *E. coli* producing wild-type enzyme and cells producing $\alpha_{\Delta 262-290}\beta$ were compared. Although the emission intensity from the $\alpha_{\Delta 262-290}\beta$-containing cells was low, the emission envelope could be measured and was found to be identical to that of the wild-type luciferase. Despite the absence of the mobile loop, the mutant enzyme emits weak luminescence of the same wavelength as the wild-type enzyme.

Bacterial luciferase binds $FMNH_2$ to form a 1:1 complex (*23*, *32*). Several methods have been used to monitor $FMNH_2$ binding. Meighen and Hastings determined the binding

affinity of the enzyme for $FMNH_2$ by a bioluminescence-based method (*23*) and reported a dissociation constant of 0.8 μM for wild-type luciferase and $FMNH_2$. These authors showed that there is only one binding site for $FMNH_2$ on the heterodimeric enzyme, as did Becvar and Hastings (*32*). The former authors employed a kinetic method to measure K_d while the latter employed an equilibrium method. Although the $\alpha_{\Delta 262-290}\beta$ loop-deleted enzyme had a decreased ability to produce light, the light produced was sufficient to monitor $FMNH_2$ binding. Assays were done at a concentration of protein such that the dimer was maximally populated without promoting aggregation. Light production was measured using a standard luminometer after luminescence was initiated by injection of flavin. The values for K_d determined from these experiments were 0.76 μM for the wild type and 1.87 μM for $\alpha_{\Delta 262-290}\beta$ (Table 2), suggesting no major effect of the loop deletion on $FMNH_2$ binding. In the present study $FMNH_2$ binding was also monitored both by bioluminescence activity and by a biophysical approach in which quenching of the protein's intrinsic fluorescence was monitored as a function of increasing $FMNH_2$ concentration. The value of the K_d for the wild-type enzyme is slightly higher than that previously reported, which may be due to use of a different method for monitoring binding. However, the $FMNH_2$ binding of the loop-deleted enzyme, monitored by the same method, agreed well with that of the wild type.

The mobile loop in the α subunit is thought to lie over the active site and has been shown to become ordered upon flavin binding (*8*, *9*). Despite the fact that this loop is associated with flavin binding, both methods of monitoring $FMNH_2$ binding suggested that the binding of $FMNH_2$ was not greatly affected by deletion of the loop.

The disordered loop also undergoes a change in conformation upon the binding of phosphate (*8*). It has been demonstrated that reduced riboflavin as a substrate supports only a very low quantum yield bioluminescence reaction, but the addition of phosphate greatly increases the quantum yield of the reaction with reduced riboflavin (*33*). These two observations suggest that the conformational change of the loop in response to phosphate binding might alter the active site environment to allow a high quantum yield reaction. The inferred inability of the loop to undergo a conformational change in the absence of phosphate would explain the low bioluminescence yield of the reaction with riboflavin, the same phenotype observed in the absence of the loop ($\alpha_{\Delta 262-290}\beta$). The decreased quantum yield for the wild-type enzyme with reduced riboflavin and for $\alpha_{\Delta 262-290}\beta$ may also be due to a requirement for the loop to protect the reaction intermediates.

Luciferase binds oxidized flavin approximately 100-fold more weakly than the reduced flavin (*26*). Investigation of the FMN binding properties of $\alpha_{\Delta 262-290}\beta$ showed a slightly lower affinity for FMN than that reported by Baldwin et al. (*26*) for the wild-type enzyme. The K_d for FMN determined for the wild-type enzyme at 18 °C is 120 μM (*26*). Using the same method as Baldwin et al. (*26*), we have determined the K_d for $\alpha_{\Delta 262-290}\beta$ to be 400 μM. Like the wild-type enzyme−FMN complex, the $\alpha_{\Delta 262-290}\beta$−FMN complex was nonfluorescent and the effect of the loop deletion on the affinity for FMN was minor.

The natural substrate for bacterial luciferase is myristic aldehyde, a 14-carbon aldehyde (*34*). However, in most laboratory experiments 1-decanal is used as the substrate because the 10-carbon aldehyde is more practical to use due to its lower melting point and greater solubility. The ability of $\alpha_{\Delta 262-290}\beta$ to bind decanal was compared to the wild type by assaying the enzymes in the presence of increasing amounts of decanal. Abu-Soud et al. (*25*) and Francisco et al. (*35*) proposed a mechanism that explains the earlier observations that high concentrations of aldehyde inhibit the enzyme's ability to undergo catalysis (*22*, *28*). The detailed kinetic analysis (*35*) fit a mechanism in which binding of aldehyde to the enzyme results in a complex in which the aldehyde must dissociate before the enzyme can proceed through productive catalysis. The detailed kinetic mechanism suggests that although the substrates of luciferase can bind in any order, the catalytically significant binding events must be ordered. The enzyme must first bind $FMNH_2$, then molecular oxygen, and finally the aldehyde.

The method of Holzman and Baldwin (*22*) was used to determine both the K_M and K_i for decanal for both the wild-type enzyme and $\alpha_{\Delta 262-290}\beta$ (Table 2). The K_M determined for the wild-type enzyme agreed well with the value of 1.1 μM from Holzman and Baldwin (*22*). The constants determined for the $\alpha_{\Delta 262-290}\beta$ enzyme were in good agreement with the wild-type values, showing that removal of the $\beta\alpha 7$ loop had no significant effect on the binding of 1-decanal.

The loop-deleted enzyme was found to bind both aldehyde and $FMNH_2$ with essentially the same affinity as does the wild-type enzyme. The cause of the decrease in bioluminescence specific activity could be an impaired ability of the enzyme to accomplish the oxidative chemistry of the reaction, an impaired efficiency of the population of the excited state of the flavin intermediate, or the quenching of emission of the excited state intermediate.

To investigate the chemical efficiency of the reaction, we measured the amount of carboxylic acid produced by the enzymes. A gas chromatography based method was developed by which the acid was extracted from the assay mixture, derivatized, separated by gas chromatography, and quantitated using a flame ionization detector. High concentrations of enzyme were used since the aldehyde spontaneously oxidized to carboxylic acid, providing a high background acid production in these experiments. The $\alpha_{\Delta 262-290}\beta$ loop-deleted enzyme produced about 70% the amount of carboxylic acid that the wild-type enzyme produced. It must be noted that, at the enzyme concentrations used, the mutant enzyme was about 75% dimeric. The requirement of the heterodimeric structure for high quantum yield bioluminescence (*3*) could explain most of the decrease in the amount of acid produced by the mutant. It appeared that the dimeric form of the mutant enzyme was able to produce the acid product with nearly the same efficiency as the wild-type enzyme. These observations suggest that the product of limited proteolysis of luciferase, apparently inactive in the bioluminescence reaction, should yield the carboxylic acid product of the flavin monooxygenase reaction as did the enzyme lacking the mobile loop. To our knowledge, the protease-inactivated enzyme has not been analyzed for acid production. An interesting difference between the enzyme lacking the disordered loop and the protease-inactivated enzyme is the fact that the subunits of the latter form do not appear to dissociate in the analytical ultracentrifuge at low concentration (*6*).

Because the enzyme lacking the mobile loop bound all substrates nearly as well as the wild-type enzyme and catalyzed the monooxygenase reaction with only slightly less efficiency than the wild-type enzyme, we concluded that the decreased bioluminescence quantum yield of the mutant enzyme was not the result of a decrease in chemical yield in the reaction. Rather, the decrease in bioluminescence yield in the absence of the α subunit mobile loop region must be due either to failure of the enzyme to efficiently populate the excited state of the flavin emitter in the reaction or to quenching of that excited state, possibly by increased solvent exposure in the absence of protection by the loop or both.

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