J. Biochem. **142,** 539–552 (2007)
doi:10.1093/jb/mvm155

Luciferase from *Vibrio campbellii* is more thermostable and binds reduced FMN better than its homologues†

Chutintorn Suadee[1], Sarayut Nijvipakul[1], Jisnuson Svasti[1], Barrie Entsch[2,3], David P. Ballou[2] and Pimchai Chaiyen[1,*]

[1]Department of Biochemistry and Center for Excellence in Protein Structure & Function, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand; [2]Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, 48109-06060, USA; and [3]School of Biological, Biomedical and Molecular Sciences, University of New England, Armidale, NSW, 2351, Australia

Received June 8, 2007; accepted August 11, 2007; published online August 30, 2007

A new luciferase from *V. campbellii* (Lux_Vc) was cloned and expressed in *Escherichia coli* and purified to homogeneity. Although the amino acid sequences and the catalytic reactions of Lux_Vc are highly similar to those of the luciferase from *V. harveyi* (Lux_Vh), the two enzymes have different affinities toward reduced FMN ($FMNH^-$). The catalytic reactions of Lux_Vc and Lux Vh were monitored by stopped-flow absorbance and luminescence spectroscopy at 4°C and pH 8. The measured K_d at 4°C for the binding of $FMNH^-$ to Lux_Vc was 1.8 μM whereas to Lux_Vh, it was 11 μM. Another difference between the two enzymes is that Lux_Vc is more stable than Lux_Vh over a range of temperatures; Lux_Vc has $t_{1/2}$ of 1020 min while Lux_Vh has $t_{1/2}$ of 201 min at 37°C. The superior thermostability and tighter binding of $FMNH^-$ make Lux_Vc a more tractable luciferase than Lux_Vh for further structural and functional studies, as well as a more suitable enzyme for some applications. The kinetics results reported here reveal transient states in the reaction of luciferase that have not been documented before.

Key words: bioluminescence, flavin, luciferase, monooxygenase, *Vibrio campbellii*.

Abbreviations: C_1, reductase component of *p*-hydroxyphenylacetate hydroxylase from *Acinetobacter baumannii*; $FMNH^-$, reduced form of FMN; HPA, *p*-hydroxyphenylacetate; K_d, dissociation constant; k_{obs}, apparent rate constant; Lux, luciferase; Lux_Vc, luciferase from *V. campbellii*; Lux_Vh, luciferase from *V. harveyi*; Lux:FMN, complex of luciferase and oxidized FMN; Lux:$FMNH^-$, complex of luciferase and reduced FMN.

Bacterial bioluminescence is a phenomenon caused by luciferase (Lux), an enzyme catalyzing the reaction of O_2 with reduced FMN and a long-chain aliphatic aldehyde, and resulting in oxidized FMN, water, and a fatty acid, with concomitant emission of blue–green light (Fig. 1) (*1*, *2*). This reaction has been found mainly in the three genera, *Vibrio*, *Photobacterium*, and *Xenorhabdus*, and the enzymes from only five species have been isolated and studied: *Vibrio(Photobacterium) fischeri*, *Vibrio(Beneckea) harveyi*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, and *Photorhabdus (Xenorhabdus) luminescens* (*3–5*). The first four species are found in marine environments, while the last species is found in terrestrial habitats (*6–9*). All of these enzymes catalyze the same chemical reactions and are heterodimers composed of an α subunit with a molecular mass of 40–52 kDa and a β subunit with a molecular mass of 36–41 kDa (*1*, *6*, *10–16*). The amino acid sequences of all these enzymes are similar (50–60% identity). However, the kinetics of light decay among luciferases from different species varies, with the luciferase from *V. harveyi* (Lux_Vh) having the slowest light decay kinetics (~0.12 s^{-1} at 30°C). The rest have considerably faster rates of decay of ~1 s^{-1} at 30°C (*17–19*). Variation has also been found in the spectrum of light emitted from different bacterial strains. In *V. fischeri* strain Y-1, a yellow fluorescent protein (YFP) was reported as an accessory protein that formed a transient complex with Lux and shifted the emitted light to longer wavelengths (*17*, *20–22*). A similar accessory protein called lumazine was isolated from *P. phosphoreum* and *P. leiognathi*. In contrast to YFP, lumazine actually shifts the emitted light to shorter wavelengths (*23–25*).

Studies have shown that $FMNH^-$ is the first substrate binding to Lux, and then oxygen reacts rapidly with the bound $FMNH^-$ to form a C(4a)–peroxyflavin (C(4a)–$FMNHOO^-$) (*26–29*). In the absence of aldehyde substrate, this intermediate slowly eliminates H_2O_2 to yield FMN with no emission of visible light (*26–27*). When present, aldehyde binds and reacts with the bound C(4a)-$FMNHOO^-$ to form a flavin–C(4a)–peroxyhemiacetal adduct (C(4a)–FMNHOOR), which then decomposes to form the carboxylic acid product and an excited flavin intermediate that emits blue–green light (*30*, *31*). The species emitting light was shown to be an excited state C(4a)–hydroxyflavin (C(4a)–FMNHOH) (*30*, *32*), which, after emitting light, dehydrates to yield FMN. It should be noted that the fluorescence quantum yield of

†Nucleotide sequence data reported are available in the GenBank database under the accession number EF394780.

*To whom correspondence should be addressed. Tel: +662-201-5607, Fax: +662-354-7174, E-mail: scpcy@mahidol.ac.th

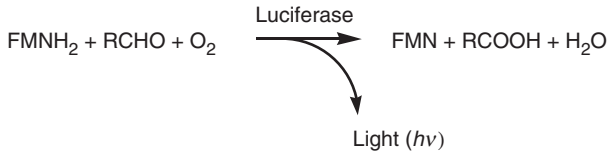

Fig. 1. **The reaction of bacterial luciferase.**

flavin analogues mimicking the luciferase emitter is very low (less than 10^{-5}) (*33*, *34*). It has been shown that specific properties of the luciferase active site are major factors in increasing the quantum yield of the flavin, because the fluorescence of 5-decyl-4a-hydroxy-4a,5-dihydroriboflavin-5′-phosphate is markedly increased when bound to the luciferase (*35*). Therefore, knowledge of the binding interactions between the flavin substrate and luciferase is crucial for understanding the molecular basis underlying the light emitting reaction.

The crystal structure of Lux_Vh at 1.5 Å resolution shows that each subunit folds into a single domain made up of an eight-stranded β/α TIM barrel motif (*36*). The structure obtained had no flavin bound due to its low affinity for the flavin substrate, and hence the precise location of the active site is not yet known (*36*, *37*). Based on molecular docking and site-directed mutagenesis studies, the active site is predicted to be at the C-terminal part of the TIM barrel of the α-subunit (*36–39*). Site-directed mutagenesis experiments have shown that changes of several residues in this area affect K_m values for $FMNH^-$ as well as the light yield of Lux_Vh (*18*, *26*, *40–49*).

Here, we report the identification and isolation of a bacterial luciferase that efficiently catalyzes the light-emitting reaction, binds more tightly to the $FMNH^-$ substrate, and is more thermostable than Lux_Vh. Therefore, the novel enzyme is a good candidate for obtaining crystals with coherent flavin binding. Details of the cloning, expression, and characterization of this luciferase from *Vibrio campbellii* (isolated from the southern seashore of Thailand) are described.

MATERIALS AND METHODS

Materials—All laboratory chemicals used were of analytical grades and mostly purchased from Sigma Chemical Co. Dodecyl aldehyde was from Aldrich and sodium dithionite was from Fluka. Sephadex G-200 and DEAE-Sepharose were from Pharmacia. Restriction endonucleases (*Nde*I, *BamH*I), DNA polymerase, and DNA modifying enzymes were purchased from New England Biolabs. Proofreading DNA polymerase (*Pfu*) and the T-A cloning vector (pGEM-T) were from Promega. The expression plasmid (pET-11a) was from Novagen, and DNA and plasmid preparation kits were obtained from Qiagen. FMN was prepared by conversion of FAD to FMN with snake venom from *Crotalus adamanteus* (*50*, *51*). This provided FMN that was highly pure. Commercial FMN is frequently only ~70% pure. In brief, FAD (2.5 mg/ml) and venom (50 μg/ml) in 20 mM potassium phosphate buffer, pH 7.0 were incubated overnight in the dark. The reaction mixture was loaded onto a C18 Sep-Pak® cartridge (Waters) pre-equilibrated with 20 mM potassium phosphate buffer pH 7.0 and the cartridge was then washed with 10 mM potassium phosphate buffer pH 7.0. FMN was eluted from the column with water and the solution was lyophilized. The FMN obtained was analyzed by thin-layer chromatography (Silica gel 60 F_{254} Merck) using *n*-butanol/glacial acetic acid/water (3.75: 4.75: 1.5 v/v). FMN, FAD, and riboflavin were used as standards.

The reductase component of *p*-hydroxyphenylacetate (HPA) hydroxylase (C_1) (*51*) was kindly provided by Dr. Jeerus Sucharitakul, Faculty of Dentistry, Chulalongkorn University, Thailand, and pAM1582UE9 used for preparation of p*lux_Vh* was a generous gift from Prof. Kazuki Terauchi, Division of Biological Science, Nagoya University, Japan and Prof. J. Woodland Hastings, Department of Molecular and Cellular Biology, Harvard University, USA.

Concentrations of the following compounds were determined using known extinction coefficients at pH 7.0: NADH, $\varepsilon_{340} = 6.22\,mM^{-1}\,cm^{-1}$; HPA, $\varepsilon_{277} = 1.55\,mM^{-1}\,cm^{-1}$ (*52*); FAD, $\varepsilon_{450} = 11.3\,mM^{-1}\,cm^{-1}$; FMN, $\varepsilon_{450} = 12.2\,mM^{-1}\,cm^{-1}$; C_1, $\varepsilon_{450} = 12.8\,mM^{-1}\,cm^{-1}$ (*51*).

Isolation and Identification of Light-emitting Organisms—Seawater obtained from the southern seashore of Thailand was inoculated into artificial seawater medium (ASW) (*19*) and then maintained at room temperature (28–30°C) for cultivation of luminous organisms. The luminous cultures were transferred several times by diluting and spreading onto agar plates of ASW. Luminescent colonies were selected and re-grown on ASW agar plates to obtain pure cultures. Each bacterial strain was classified for its species designation by comparing its 16S rDNA sequence against the known 16S rDNA genes in the database (*53*, *54*). Genomic DNA from each isolated luminous bacterial strain was extracted and purified using Qiagen DNA purification kits. Partial 16S rDNA was amplified from genomic DNA by PCR using a proofreading polymerase (*Pfu*) with the nucleotides 5′-AGAGTTTGATCCTGGCTCAG-3′ (sense strand: fD1) and 5′-ACGGTTACCTTGTTACGACTT-3′ (anti-sense strand: rP1) corresponding to positions 6-25 and 1492-1512 on the 16S rDNA of *V. vulnificus*, respectively. The resulting PCR product (1.4 kb) was then directly sequenced using primers fD1, rP1, and Int1 (5′-TACGGGAGGCAGCAGTGGGGAATA-3′ corresponding to the sequence 351-374 of the 16S rDNA of *V. harveyi*). A nucleotide similarity search was done using the BLAST program provided by GenBank.

Cloning of the Gene Encoding Luciferase from V. campbellii—The amino acid sequences of subunits of fatty acid reductase (LuxD and LuxE) from the *lux* operon of *Photobacterium phosphoreum* (NCMB844) (*55*), *Photobacterium leiognathi* (ATCC25521) (*13*), *Vibrio harveyi* (*15*, *56*), and *Vibrio fisheri* (MJ-1) (*11*) were used for multiple sequence alignment (ClustalW version v1.83, http:/www.ebi.ac.uk/clustalw/). Regions conserved among all species were used for designing degenerate primers. Because the genes encoding LuxD *(luxD)* and LuxE (*luxE)* flank the gene encoding both subunits of the luciferase (*luxAB*) at the 5′ and 3′ ends, the PCR product resulting from using these degenerate primers should

cover the entire *luxAB* gene. The sense strand primer corresponding to the region in *luxD* was designed to be 5′-(A/G)TIGTI(C/T)TI(C/A)GIAA(C/T)TT(C/T)TA(C/T)CA-3′ (fluxD), while the anti-sense strand corresponding to the region in *luxE* was designed to be 5′-A(A/G)I(G/C)(A/T)IATIAC(A/G)TA(C/T)TT(A/G)AACCA-3′ (rluxE). The PCR reaction using these primers and the genomic DNA of *V. campbellii* as a template resulted in a product of ~2.5 kb. The product was subcloned into a T-A cloning vector (pGEM-T), and analyzed for its sequence. The result indicated that this 2.5-kb PCR product contained the entire gene *luxAB*.

The plasmid for expression of Lux_Vc was constructed by amplifying the full-length gene of *luxAB* from *V. campbellii* genomic DNA using a proofreading polymerase (*Pfu*) and primers 5′-AGGAAATCATATGAAATTTGGAA-3′ (sense strand) and 5′-CCTTCAGGATCCGTTAAACGTTACG-3′ (anti-sense strand). The primers were designed to incorporate the recognition sites (underlined) of *Nde*I into the sense strand and *BamH*1 into the anti-sense strand. The resultant PCR product was then treated with *Nde*I and *BamH*1, purified by agarose gel electrophoresis, and ligated into the pET-11a expression vector that had been previously digested with *Nde*I and *BamH*1. The ligation product was designated as p*lux_Vc*, and was transformed into the expression host *E. coli* strain BL-21 (DE3). Colonies containing p*lux_Vc* were identified from LB/ampicillin agar plates, and p*lux_Vc* was verified to contain the correct gene *luxAB* from *V. campbellii* by sequencing analysis.

The plasmid for expression of luciferase from *V. harveyi* (Lux_Vh) was constructed by amplifying the *lux_Vh* gene from pAM1582UE9 using a proofreading polymerase and primers 5′-AAGGAAATCATATGAAATTTGG-3′ (sense strand with *Nde*I restriction site (underlined) and ATG for in-frame translation initiation) and 5′-TGAGGGGATCCCATGAAAATC-3′ (anti-sense strand with *BamH*1 restriction site (underlined)). The resulting PCR product was treated with *Nde*I and *BamH*1, and then purified by agarose gel electrophoresis. The purified DNA was ligated into pET-3a pre-digested with *Nde*I and *BamH*1 to generate the *plux_Vh* plasmid for protein expression. The plasmid was transformed into *E. coli* strain BL-21(DE3) and positive clones were selected by using LB/ampicillin agar. The sequence of the insert was analyzed to verify that p*lux_Vh* contained the same sequence as the gene of *lux_Vh* (accession code P07739 for LuxB (*12*) and P07740 for LuxA (*10*)).

Expression and Purification of Luciferase—E. coli cells harbouring p*lux_Vc* and p*lux_Vh* were cultured in sterile Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml). The culture was maintained at 37°C using an incubator shaking at 250 rpm until the OD_{600} reached ~1.2. Then, the temperature of the culture was changed to 25°C and IPTG was added to a final concentration of 1 mM. When the culture reached an OD_{600} ~4.0, cells were harvested by centrifuging at $5000 \times g$ at 4°C for 8 min and then stored frozen at −80°C until used. The expression under the conditions described above generated a high fraction of soluble cellular protein as Lux (about 50%) and typically yielded 3.5 g of cell paste per 1 L of culture.

Unless otherwise specified, all purification procedures were performed at 4°C. Frozen cell paste was thawed and suspended in 50 mM sodium phosphate at pH 7.0, containing 60 μM phenylmethanesulfonylfluoride, and 0.3 mM EDTA. Cells were disrupted by sonication (Sonic Vibra_cell™; Model VCX750) and then centrifuged at $100\,000 \times g$ for 60 min. Cell debris was discarded and the clear lysate was used for purification. This crude extract was loaded onto a DEAE-Sepharose column, previously equilibrated with 100 mM NaCl in 10 mM sodium phosphate buffer pH 6.2. The column was eluted with a 2 l linear gradient of 100–250 mM NaCl in 10 mM sodium phosphate buffer pH 6.2. Fractions containing luciferase were identified using a coupled assay with the flavin reductase (C_1), combined, and concentrated by Centriprep® YM–30 (30 kDa cut off). Absorption at 280 nm was used to estimate the amount of protein in each fraction. At this stage, the purity of Lux_Vc was judged to be greater than 98% by analysis with 12% SDS–PAGE, and the purified enzyme was kept in 30 mM MOPS buffer pH 7.0 at -80°C until used. For Lux_Vh, the partially purified enzyme from DEAE-Sepharose chromatography was applied onto a Sephadex G-200 gel filtration column pre-equilibrated with 100 mM sodium phosphate buffer pH 7.0 and the enzyme was eluted with the same buffer. From 12% SDS–PAGE analysis, the purity of Lux_Vh was estimated to be greater than 98%. The purified Lux_Vh was stored in 30 mM MOPS buffer pH 7.0 at −80°C until used.

Protein Properties—Molecular Mass of the Native Enzyme

The molecular weight (M_r) of the native enzyme was determined using a Pharmacia FPLC gel filtration chromatography apparatus equipped with a Superdex 200 HR 10/30 column (Pharmacia). Proteins were eluted with 150 mM NaCl in 50 mM sodium phosphate buffer, pH 7.0 with a flow rate of 0.5 ml/min. The Superdex column was calibrated with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The void volume (V_o) of the column was determined as the elution volume of Blue dextran (2000 kDa). The molecular weight of luciferase was determined from the ratio of V_e/V_o (V_e is the elution volume) and a calibration line that was constructed by plotting the logarithm of the known molecular masses of protein standards versus their respective V_e/V_o values.

Molecular Mass of the Enzyme Subunits

The subunit molecular weight was determined by both MALDI-TOF mass spectrometry and 12% SDS–PAGE. For SDS–PAGE analysis, the molecular weight markers used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The protein bands were developed by staining the gel with Coomassie brilliant blue. A calibration curve was generated by plotting the logarithm of molecular weight of the standards versus the relative mobility (R_f = distance migrated by protein/distance migrated by dye). The molecular weight of luciferase subunits was calculated by comparing the R_f

of each protein with those of molecular weight standards. Based on the amino acid sequences, the molecular masses of the α and β subunits were calculated using the ProtParam tool on the ExPaSy Proteomics server (*57*). With the same software, the extinction coefficients of both luciferases from *V. campbellii* and *V. harveyi* were calculated to be $\varepsilon_{280} = 79.6\,mM^{-1}\,cm^{-1}$.

Spectroscopic Studies—UV-visible absorbance spectra were recorded with a Hewlett Packard diode array spectrophotometer (HP 8453A), or a Shimadzu 2501PC double beam spectrophotometer. Light emission and fluorescence measurements were carried out with a Shimadzu RF5301PC spectrofluorometer. These instruments were equipped with thermostated cell compartments.

Enzyme assays—Light generated from the luciferase reaction was used to determine the enzyme activity, and the reductase (C_1) (*51*) was used to supply a constant amount of $FMNH^-$ in the assay reaction. The intensity of light emission was monitored at 490 nm by a spectrofluorometer with an emission slit width of 3 nm, using no excitation source. The coupled assay was typically performed at 25°C in 1.2 ml of 50 mM sodium phosphate buffer pH 7.0 containing 20 μM FMN, 100 μM HPA, 100 μM NADH, 40 μM dodecanal (freshly prepared), 0.5–1 μM luciferase, and excess C_1 (1, 3, and 5 μM). The reaction was initiated by injecting 0.6 ml of a solution containing NADH and dodecanal into 0.6 ml of a solution containing FMN, C_1, and dodecanal. The light intensity rapidly increased and then decayed within 3 min. Total photons from each assay were calculated by integrating the area beneath the luminescent trace and the value was converted into quanta units by correlating the quantum yield of the luminescent reaction to that of luminol (0.0124 photon/one molecule of luminol) using a protocol described previously (*58*, *59*).

Rapid Reaction Experiments—The kinetics of Lux_Vc and Lux_Vh reactions were investigated using stopped-flow techniques (Hi-Tech Scientific instruments, models SF-61, SF-61SX, or SF-61DX) at 4°C using both absorbance and luminescence. The optical path lengths of the observation cells were 1 cm. Xenon or tungsten lamps were used as the light sources for absorbance measurements, and the lamp was turned off during luminescence monitoring. The stopped-flow apparatus was made anaerobic by flushing the flow system with an oxygen-scrubbing solution consisting of 400 μM glucose, 1 mg/ml glucose oxidase (15.5 unit/ml), and 4.8 μg/ml catalase in 50 mM sodium phosphate pH 7.0. The oxygen-scrubbing solution was allowed to stand in the flow system overnight and was then thoroughly rinsed with an anaerobic solution of 50 mM sodium phosphate pH 8.0, at 4°C before starting experiments. Typically, an anaerobic solution containing luciferase in complex with reduced FMN ($FMNH^-$) was prepared in a tonometer that was connected to one syringe, and this was mixed with an oxygen-containing solution from the other syringe of the stopped-flow instrument. In one glass tonometer, a solution containing 36 μM FMN and 72 μM Lux_Vc (2-fold excess) or 180 μM Lux_Vh (5-fold excess) in 50 mM sodium phosphate pH 8.0 was made anaerobic. Oxygen from the tonometer was removed by alternatively evacuating and flushing with oxygen-free argon or nitrogen that had been passed through an oxygen removal column (Oxyclear) using a gas train. This procedure was repeated for 10–12 cycles. The absorption spectrum of FMN in the solution was recorded and the FMN was stoichiometrically reduced with aliquots from a sodium dithionite solution (~10 mM in 100 mM potassium phosphate pH 8.0) from a Hamilton titrating syringe attached to the tonometer. The absence of absorbance peaks around 380 and 450 nm indicated that the FMN was fully reduced. The enzyme was mixed with buffer (50 mM sodium phosphate pH 8.0) containing various concentrations of oxygen with or without the dodecanal substrate in the stopped-flow instrument. Solutions with various concentrations of oxygen were prepared by equilibrating buffer with air or with certified oxygen/nitrogen gas mixtures. Details of individual stopped-flow experiments are described in the figure legends and text. Kinetic data were analyzed using Program A (developed by Chun-Jen Chiu, Rong Chang, Joel Dinverno, and Dr David P. Ballou, University of Michigan). This software uses the Marquardt algorithm to abstract from kinetic traces apparent rate constants (k_{obs}) and amplitudes for parallel or sequential mechanisms, by obtaining the best fit to experimental data. Determination of the second-order rate constants for oxygen reacting with luciferase were carried out by fitting plots of k_{obs} versus concentrations of oxygen using a linear least squares algorithm that is included in the KaleidaGraph software (Synergy Software).

Determination of the Dissociation Constant (K_d) for Binding FMN to Luciferase—Various concentrations of Lux_Vc or Lux_Vh (40, 80, 150, and 200 μM) in 50 mM sodium phosphate pH 8.0 were mixed with 10 μM FMN. Under equilibrium condition, the bound and free FMN were separated using an ultrafiltration device (Centriprep® YM–30, Amicon). The centriprep was centrifuged at 200 × *g*, 4°C for 5 min to obtain a filtrate of ~800 μl. The filtrate and retentate were analyzed for the amount of free and bound FMN, respectively. A plot of the amount of enzyme-bound FMN against free enzyme concentration used represents the binding isotherm for luciferase binding to FMN.

RESULTS

Identification of Luminous Bacteria—The species of luminous organisms isolated were identified on the basis of their 16S rDNA sequences as described in Materials and Methods. Most of the identified organisms belonged to *Photobacterium* or *Vibrio* genera, and the one chosen for this study was identified as *Vibrio campbellii*. The strain is a coccobacillus Gram-negative bacterium with 16s rDNA sequence 100% identical to the sequence of *Vibrio campbellii* (ATCC 25920) (*3*, *53*, *54*, *60*, *61*). The *in vivo* luminescent spectrum of *V. campbellii* has its emission peak at 495 nm (Fig. 2). The *in vivo* luminescence characteristics of *V. campbellii* have never been reported, nor has the luciferase from this species been isolated (*53*, *60–62*).

Sequence Analysis of LuxAB from V. campbellii—A complete sequence of *lux_Vc* was deposited in the

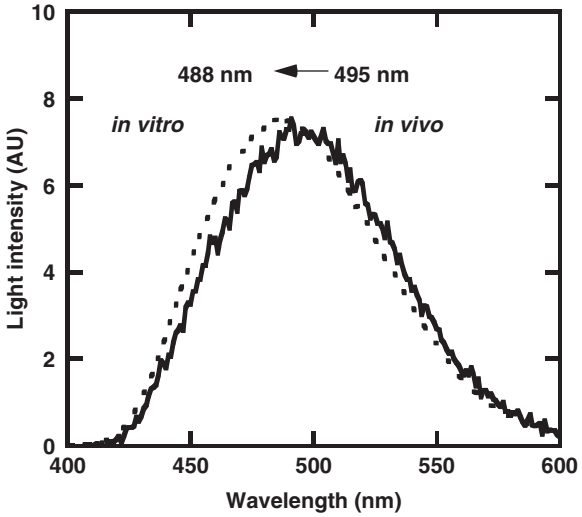

Fig. 2. **Luminescence of luciferase from *V. campbellii*.** The solid line represents an *in vivo* emission spectrum of *V. campbellii* obtained from recording light emitted from the bacterial culture. The *in vitro* spectrum (dotted line) was obtained by recording the steady-state light produced from the reaction of purified Lux_Vc, 20 μM FMN, 2 μM C_1, 100 μM HPA, 40 μM dodecanal, and 100 μM NADH in 1.2 ml of 50 mM sodium phosphate buffer, pH 7.0. The spectra were recorded at 25 ± 1°C using the spectrofluorometer without light input. The solid line was multiplied by an arbitrary factor to normalize the two spectra so that the emission shapes can be compared. The *in vitro* Lux_Vc light emission is blue-shifted ~ 7 nm compared to that of *in vivo*.

GenBank database (accession number EF394780). The gene had two open reading frames, with the first encompassing nucleotides 1 to 1068 that encoded the α subunit of luciferase (LuxA), and the second including nucleotides 1095 to 2069 that encoded the β subunit of luciferase (LuxB) (Fig. 3). The open reading frames of LuxA and LuxB are arranged in a head-to-tail direction and are separated by 26 non-coding nucleotides. The translation of the open reading frames revealed polypeptides of 355 amino acids for LuxA and 324 amino acids for LuxB with calculated molecular weights of 40.15 and 36.41 kDa, respectively. These molecular weights are consistent with the subunit molecular weights of Lux_Vc determined by SDS–PAGE (40 and 36 kDa for the α and β subunits) and mass spectrometry (40.02 and 36.27 kDa). The native molecular weight of Lux_Vc is 76.56 kDa from analyses of sequence and is consistent with the result from gel filtration chromatography (67.5 kDa). Thus, the native protein structure is an αβ heterodimer like Lux_Vh. Sequences of the two subunits of Lux_Vc share 30% identity. Comparison of Lux_Vc to Lux_Vh (*10*, *12*) shows that the enzymes share 98.8% sequence identity and are only different by 3 amino acids on the α subunit and 5 amino acids on the β subunit (Fig. 3). Lux_Vc shares 57% sequence identity with Lux from *V. fischeri* (*63*) and ~52% identity with Lux from *Photobacterium* species (*13*, *55*) (data not shown). Since the sequence of Lux_Vc is highly homologous to that of Lux_Vh, the overall three-dimensional structure of Lux_Vc must also be very similar to that of Lux_Vh. Therefore, the structure of Lux_Vh (PDB accession code 1LUC, (*36*)) was used to locate the probable sites of these 8 amino acid residues (Fig. 4). Each subunit of Lux_Vh folds into a single domain of a TIM barrel $(\beta/\alpha)_8$ motif where the C-terminal part of the α-subunit barrel was proposed to be the flavin binding site (indicated by the arrow in Fig. 4) (*38*, *39*, *48*). The three amino acids that are different on the α subunit are $68T_{Vh}/K_{Vc}$ in the loop prior to β3-strand, $238R_{Vh}/K_{Vc}$ in the α7a-helix, and $321D_{Vh}/N_{Vc}$ in the turn before β8-strand (Figs. 3 and 4). The 5 amino acids that are different on the β subunit are $20V_{Vh}/I_{Vc}$ in the α1-helix, $102A_{Vh}/V_{Vc}$ in the β4-strand, $193R_{Vh}/K_{Vc}$ in the loop between β6-strand and α6-helix, $256S_{Vh}/P_{Vc}$ in the loop prior to α7b-helix, and $265G_{Vh}/V_{Vc}$ in the α7b-helix (Figs. 3 and 4). The α-subunit residues, $68T_{Vh}/K_{Vc}$ and $321D_{Vh}/N_{Vc}$, are located at the N-terminal part of the barrel while 238 R_{Vh}/K_{Vc} is at the protein surface and the outer sphere of the proposed binding pocket (Fig. 4). The β-subunit residue $102A_{Vh}/V_{Vc}$ is at the N-terminal part of the β-subunit barrel, while $193R_{Vh}/K_{Vc}$ and $256S_{Vh}/P_{Vc}$ are at the C-terminal region of the barrel. Residue $265G_{Vh}/V_{Vc}$ is located on the protein surface while $20V_{Vh}/I_{Vc}$ of the α1-helix is partially buried inside the protein. Therefore, the 8 amino acids of Lux_Vc that are different from those of Lux_Vh do not reside within the area proposed to be critical for binding flavin to Lux_Vh.

Expression, Purification, and Characterization of Lux_Vc and Lux_Vh—The plasmids p*lux_Vc* and p*lux_Vh* were transformed into *E. coli* BL21 (DE3) to express Lux_Vc and Lux_Vh, respectively. It was found that expression of each of the genes was optimal when IPTG (1 mM) was added to the *E. coli* cultures at OD_{600} ~1.2 and the temperature was kept at 25°C during Lux production. In both cases, cells were harvested when their OD_{600} ~4.0, which typically yielded ~30 g of cell paste from 8.4 l of cell culture. Lux_Vc and Lux_Vh were purified according to the procedures described in Materials and Methods. Only one chromatographic step was required for Lux_Vc purification and 1.6 g of the purified protein with a specific activity of 2.85×10^{14} quanta/mg was obtained from 8.4 l cell culture. For Lux_Vh purification, a second step of chromatography was required to yield 0.96 g of purified enzyme from 8.4 l culture. This procedure differs from that described in (*58*) only in the DEAE-Sepharose chromatography, which was carried out at pH 6.2 instead of 7.0.

Recombinant Lux_Vc and Lux_Vh are capable of carrying out *in vitro* light-emitting reactions with spectra as shown in Fig. 2. A luminescent emission spectrum from the reaction of Lux_Vc coupled to the reaction of a flavin reductase (see Materials and Methods) shows a maximum intensity at 488 nm (dotted line in Fig. 2). When the same concentration of Lux_Vh was employed under the same conditions, a luminescent emission spectrum with shape and intensity similar to that of Lux_Vc was obtained (data not shown).

Reactions of Lux_Vc:FMNH⁻ and Lux_Vh:FMNH⁻ with Oxygen—A solution of $FMNH^-$ (18 μM) and Lux_Vc (36 μM) in 50 mM sodium phosphate, pH 8.0, was mixed in the stopped-flow apparatus at 4°C with 50 mM sodium phosphate buffers, pH 8.0, containing

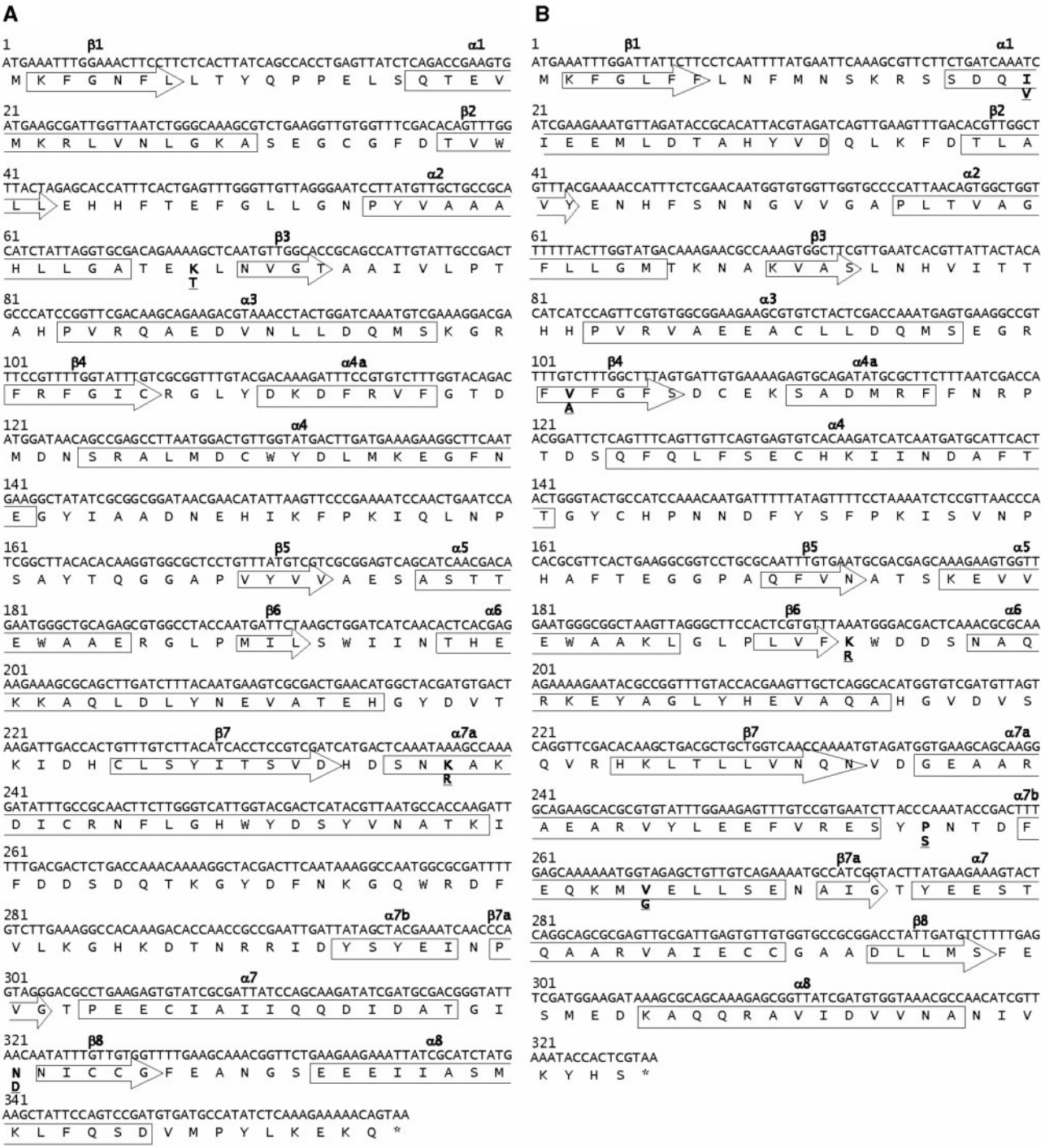

Fig. 3. **Complete nucleotide and amino acid sequences of Lux from *V. campbellii* and predicted secondary structures.** Panel A represents the sequence of the α subunit of Lux_Vc while panel B represents that of the β subunit. The upper line represents the nucleotide sequence while the translated amino acid residues with predicted secondary structures based on the structure of Lux_Vh (*36*) are represented in the lower line. Amino acid residues of Lux_Vh that are different from those of Lux_Vc are specified in the underlined and bold letters beneath the Lux_Vc sequence. Broad arrow-like forms display β strands and rectangular boxes indicate α-helical regions. Remaining residues are parts of random coils. Asterisks (*) indicate a stop codon.

a range of oxygen concentrations (Fig. 5). Kinetic traces at wavelengths between 310 and 550 nm were recorded for the first 40 s of the reaction. After 40 s, the reaction was monitored every 30 s for 150 min by recording spectra using the scanning mode of the stopped-flow instrument. Figure 5 shows that the reaction of the Lux:$FMNH^-$ complex with oxygen, as monitored at 390 nm, had 3 phases up to 10 s. The first phase (dead

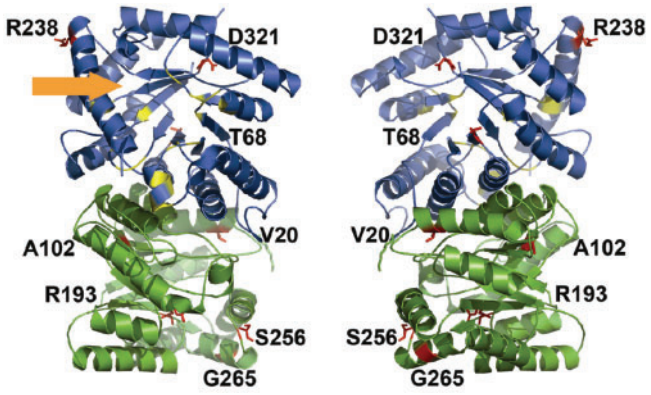

Fig. 4. **The structure of luciferase from *V. harveyi* (1LUC) (*36*) with locations of the amino acid residue differences in Lux_Vc.** The structure of Lux_Vh was generated with the program PyMOL based on the X-ray structure of Lux_Vh at 1.5 Å resolution deposited in the Brookhaven Protein Data Bank (PDB entry 1LUC) (*36*). The α subunit is shown in blue and the β subunit in green. The arrow indicates the area proposed to be the active site of the enzyme, the C-terminal part of the TIM barrel of the α subunit. The left picture shows the active site while the right picture was obtained from 180° anti-clockwise rotation of the left. The eight amino acid differences in Lux_Vc are represented in red. The three amino acid differences on the α subunit are $68T_{Vh}/K_{Vc}$, $238R_{Vh}/K_{Vc}$, and $321D_{Vh}/N_{Vc}$. The five amino acid differences on the β subunit are $20V_{Vh}/I_{Vc}$, $102A_{Vh}/V_{Vc}$, $193R_{Vh}/K_{Vc}$, $256S_{Vh}/P_{Vc}$, $265G_{Vh}/V_{Vc}$. Yellow colour indicates the positions of amino acids that have been identified from site-directed mutagenesis studies (*18*, *26*, *40–46*, *48–49*) and a model of the Lux_Vh-flavin complex (*39*) as important for luciferase reaction and $FMNH^-$ binding.

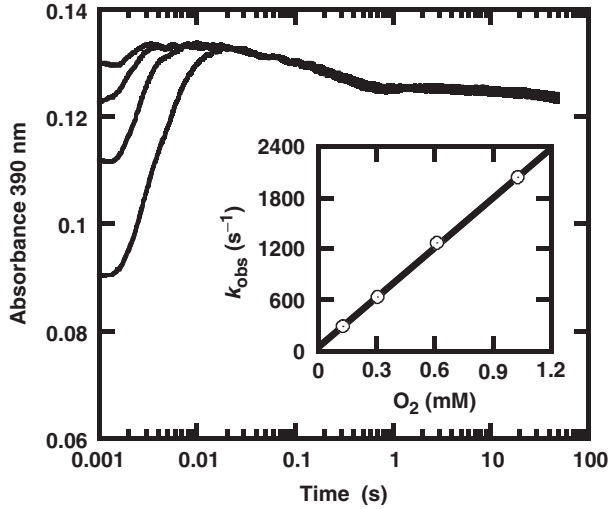

Fig. 5. **Absorbance traces of the reaction of Lux_Vc:$FMNH^-$ with oxygen.** An anaerobic solution of Lux_Vc (36 μM) and $FMNH^-$ (18 μM) in 50 mM sodium phosphate buffer, pH 8.0 was mixed with the same buffer containing oxygen at 0.13, 0.3, 0.61, or 1.03 mM (from lower to upper traces) at 4°C. Concentrations are as after mixing. Kinetic traces were monitored at 390 nm. The inset is a plot of the observed rate constants for the first phase versus oxygen concentration. The plot shows a linear relationship with the slope giving the second-order rate constant for reaction with oxygen of $1.95 \pm 0.1 \times 10^6\,M^{-1}s^{-1}$.

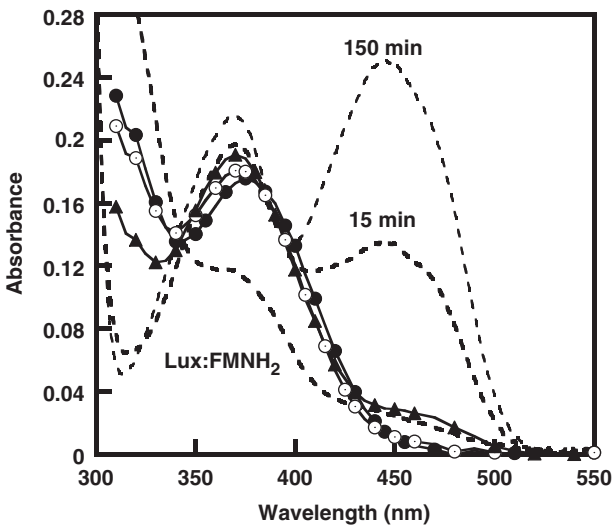

Fig. 6. **Spectra of intermediates formed during the reaction of Lux_Vc:$FMNH^-$ with oxygen.** Similar experiments to those in Fig. 5 were carried out and absorbance traces at various wavelengths between 310 and 550 nm were recorded (to 40 s). Spectra of the intermediates at different stages of the reaction were obtained from plotting the absorbance at 0.01 s (filled circles), 0.25 s (open circles), and 5 s (filled triangles). Dashed lines represent the reduced enzyme and spectra recorded at 15 and 150 min after initiation of the reaction.

time till 0.02 s) was very rapid and dependent on oxygen concentration. This phase was detected as an increase in absorbance in the range of 350–410 nm, and a slight decrease of absorbance at 450 nm. A plot of observed rate constants of this phase versus oxygen concentrations was linear and yielded a second-order rate constant of $1.95 \pm 0.1 \times 10^6\,M^{-1}s^{-1}$ (Inset of Fig. 5). When the absorbance at the end of this phase (reaction time of 0.01 s) was plotted for the range of 310–550 nm, a spectrum with an absorbance maximum at 375 nm (filled circle spectrum of Fig. 6) was obtained. Therefore, the first phase can be interpreted as formation of C(4a)-peroxy-FMN. The spectrum of this intermediate is similar to those of previously reported C(4a)-hydroperoxy flavins in the reaction of luciferase from *V. harveyi* (*27*, *29*, *32*, *64*), in chemical models for flavin-oxygen intermediates (*65*, *66*), in the class of single-component aromatic hydroxylases (*67–70*), as well as with cyclohexanone monooxygenase (*71*), and with two-component flavin-dependent hydroxylases such as *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* and *Pseudomonas putida* (*72*, *73*). The second phase of the reaction (0.02–0.7 s) was a decrease of absorbance at 390 nm with a rate constant of $13.6\,s^{-1}$ that was independent of oxygen concentration (Fig. 5). When the absorbance at the reaction time of 0.25 s was plotted, a spectrum with an absorbance maximum at 370 nm was obtained (Fig. 6, curve with open circles). This spectrum is very similar to the spectrum of the first intermediate, but is blue-shifted ~5 nm. The third phase (0.7–10 s) is detected as a small increase in absorbance in the range of 370–400 nm with an observed rate constant of $1.5\,s^{-1}$. At the end of this phase (5 s from the start of

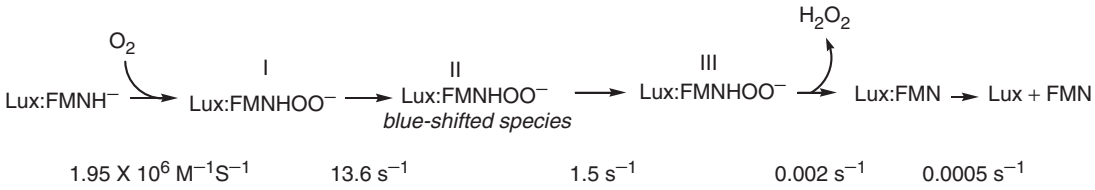

Fig. 7. **Kinetic mechanism of the reaction of Lux_Vc:FMNH$^-$ with oxygen.**

the reaction), the spectrum obtained by plotting the absorbance (Fig. 6, curve with filled triangles) is that of a third transient intermediate with an absorbance maximum at 370 nm (but a higher extinction than the second intermediate); it also has characteristics of a C(4a)-peroxy-FMN. The lower absorbance of the third intermediate in the range 310–350 nm is due to the concurrent reoxidation of dithionite. There is also a small increase in absorbance between 430 and 500 nm that can be explained by the reoxidation of a small fraction of reduced flavin not bound to the enzyme (consistent with the measured K_d for FMNH$^-$ shown below in Fig. 9). The reoxidation of flavin bound to the enzyme was slow, biphasic, and characterized by the appearance of an intense absorbance band with a maximum at 445 nm (Fig. 6). A plot of the time course of absorbance at 450 nm (from the spectra taken between 1 and 150 min) showed two phases of increase (data not shown). The initial phase corresponded to an observed rate constant of 0.002 s^{-1} and the final phase corresponded to a rate constant of 0.0005 s^{-1}. These slow phases are clearly involved with the loss of hydrogen peroxide from the C(4a)-peroxy-FMN to form FMN bound to the enzyme and the release of FMN from Lux. The scheme in Fig. 7 summarizes the kinetic data obtained in the reaction of oxygen with Lux_Vc:FMNH$^-$.

When the same reaction above was carried out with Lux_Vh, similar absorbance changes were obtained (data not shown), but only when 90 μM of Lux_Vh was used with 18 μM FMNH$^-$ (Fig. 8). When using a ratio of Lux_Vh:FMNH$^-$ of 2:1, nearly 25% of FMNH$^-$ oxidized as free flavin as shown by the increase of absorbance at 450 nm within the first 10 s after mixing for the Lux_Vh reaction; when the same concentration of Lux_Vc was used, only about 7% of FMNH$^-$ reacted as free flavin (Fig. 8). This indicates that the dissociation constant for binding of FMNH$^-$ to Lux_Vh is greater than that of Lux_Vc. Our finding here is also consistent with previous investigations of Lux_Vh at 25°C in (*74*) where 75 μM of Lux_Vh was required to bind 15 μM FMNH$^-$. Thus, a significant difference between Lux_Vc and Lux_Vh is their affinities for the FMNH$^-$ substrate.

Binding of FMNH$^-$ to Lux_Vc and Lux_Vh—Although binding of Lux_Vc to FMNH$^-$ caused some perturbations in the FMNH$^-$ spectrum (data not shown), this change was too small to be used for accurate determination of the K_d for binding with FMNH$^-$. We therefore used the formation of the C(4a)-peroxy-FMN intermediate as described in the previous section to estimate the K_d values for the Lux_Vc and Lux_Vh complexes. A solution of FMNH$^-$ was mixed in the stopped-flow

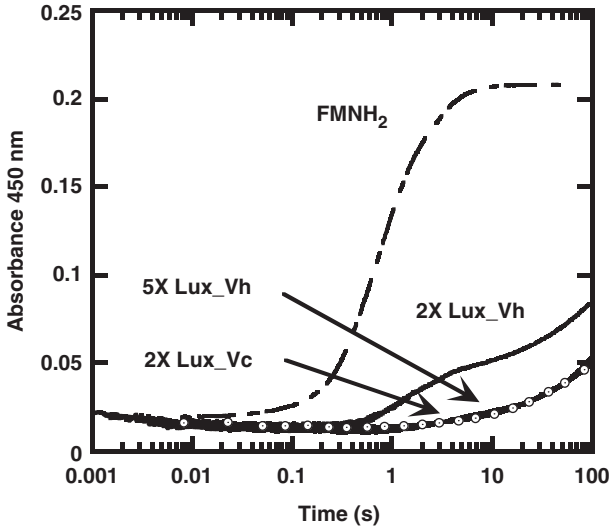

Fig. 8. **Reaction traces showing differences in binding affinity of Lux_Vc and Lux_Vh with FMNH$^-$.** Experiments were performed by mixing an anaerobic solution of Lux_Vh (36 and 90 μM) or Lux_Vc (36 μM) and FMNH$^-$ (18 μM) in 50 mM sodium phosphate buffer, pH 8.0 containing 0.13 mM oxygen in the stopped-flow spectrophotometer at 4°C. All concentrations are reported as after mixing. The control reaction without any enzyme present (18 μM FMNH$^-$) is also shown. In the reaction with Lux_Vh:FMNH$^-$ in a ratio of 2:1 (solid line), ~25% of FMN oxidized without binding to Lux_Vh. Almost no free FMN was observed in the reaction with Lux_Vh:FMNH$^-$ in a ratio of 5:1 (lowest solid line), and with Lux_Vc:FMNH$^-$ in a ratio of 2:1 (open circles).

spectrophotometer at 4°C with buffer solutions containing oxygen and various concentration of Lux_Vc or Lux_Vh (Fig. 9). In this experiment, the C(4a)-FMN-peroxide intermediate was completely formed at reaction times ranging from 0.2 to 0.8 s (high to low enzyme concentration); these times are much longer than those for intermediate formation in Fig. 5, implying that the rate of binding of FMNH$^-$ to Lux must limit the reaction with oxygen. The oxygenated intermediate formed, as measured by the absorbance at 380 nm, is directly correlated with the amount of Lux productively bound to FMNH$^-$ (Equation a). A K_d value for the binding of Lux to FMNH$^-$ can be calculated according to Equation b. The amplitude changes of the first phase (ΔA380) versus the total concentration of FMNH$^-$ were plotted to extrapolate the maximum change at 380 nm ($\Delta A380_{max}$), which represents the absorbance change if all of the

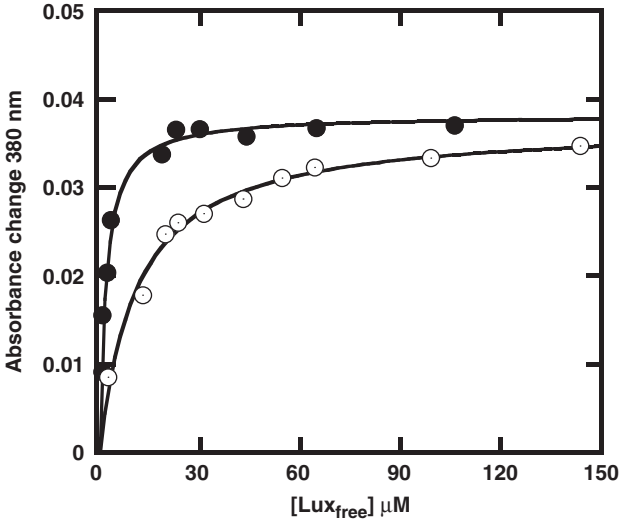

Fig. 9. **Thermodynamics of complex formation between luciferase and FMNH$^-$.** A solution of FMNH$^-$ (7 μM) in 50 mM sodium phosphate buffer, pH 8.0 was mixed with the same buffer containing 0.13 mM oxygen and various concentrations of Lux_Vc or Lux_Vh (4, 8, 16, 24, 28, 36, 48, 60, 70, 105, and 150 μM) in the stopped-flow spectrophotometer at 4°C. All concentrations are given as after mixing. The amount of C(4a)-FMN-peroxide formed, as noted by the absorbance at 380 nm, was obtained from the amplitude changes of the first phase in the reaction. This value directly correlates with the amount of Lux_Vc or Lux_Vh binding to FMNH$^-$ and was used for plotting against the concentration of free enzyme. The plot represents the binding isotherm of the Lux:FMNH$^-$ complex and yielded the K_d value of 1.8 ± 0.5 μM for Lux_Vc:FMNH$^-$ (filled circles) and 11 ± 1 μM for Lux:Vh:FMNH$^-$ (open circles).

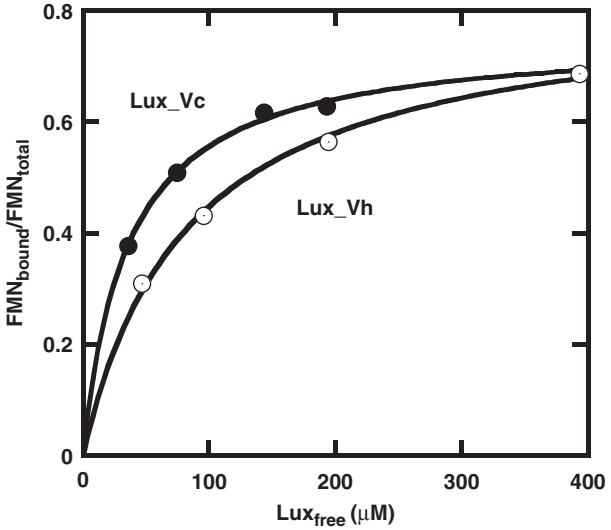

Fig. 10. **Thermodynamics of complex formation between luciferase and FMN.** The experiments are described in Materials and Methods. Direct plots of the bound FMN versus free Lux_Vc (filled circles) or Lux_Vh (open circles) in this figure represent the binding isotherm. The K_d values calculated from each plot are 36 ± 3 μM for Lux_Vc and 83 ± 8 μM for Lux_Vh.

FMNH$^-$ were bound to Lux (data not shown). At each Lux concentration used, the ratio of $\Delta A380/\Delta A380_{max}$ represents a fraction of saturation for FMNH$^-$ binding to Lux. This ratio can be used for calculating the amount of bound and free Lux according to Equation c. A plot of the amplitude changes of the first phase versus the concentration of free Lux was plotted (Fig. 9) and this represents the binding isotherm of Lux and FMNH$^-$. This plot yields K_d values for the complexes of Lux_Vc:FMNH$^-$ and Lux_Vh:FMNH$^-$ of 1.8 ± 0.5 μM and 11 ± 1 μM, respectively.

$$[\text{Lux}]_{free} + [\text{FMNH}^-]_{free} \rightleftharpoons [\text{Lux} : \text{FMNH}^-] + O_2 \longrightarrow [\text{Lux} : \text{FMNHOO}^-] \quad \text{(a)}$$

$$K_d = \frac{[\text{Lux}]_{free}[\text{FMNH}^-]_{free}}{[\text{Lux} : \text{FMNHOO}^-]} \quad \text{(b)}$$

$$[\text{Lux}]_{free} = [\text{Lux}]_{total} - \frac{dA380}{dA380_{max}}[\text{FMNH}^-]_0 \quad \text{(c)}$$

Binding of FMN to Lux_Vc and Lux_Vh—To obtain K_d values of the two luciferases for FMN, solutions of FMN were mixed with various concentrations of each of the two enzymes in 50 mM sodium phosphate, pH 8.0 and 4°C. The free FMN was separated using an ultrafiltration device as described in Materials and Methods. Plots of bound FMN versus free Lux (Fig. 10) were used to calculate the K_d values for Lux_Vc:FMN (36 ± 3 μM) and Lux_Vh:FMN (84 ± 8 μM). Thus, Lux_Vc binds more tightly than Lux_Vh to FMN as well as to FMNH$^-$.

Luminescence Kinetics—An anaerobic solution of Lux_Vc and FMNH$^-$ in 50 mM sodium phosphate buffer pH 8.0 was mixed in a stopped-flow apparatus at 4°C with the same buffer containing 0.13 mM oxygen and dodecanal. Light that was generated during the reaction was detected with a photomultiplier tube attached to the stopped-flow apparatus. Light emission occurred in two distinct phases (Fig. 11, panel A). An initial burst that accounts for only 1–2% of total light emission occurred between 0.1 and 3 s (note the logarithmic scale of the *X*-axis), while most of the light was emitted much more slowly and reached maximum intensity at ~80 s after mixing (Fig. 11, panel A). Decay of the light emission continued until ~2000 s and is consistent with the rate constant of 0.0025 s^{-1}, which is due to the decay of Lux_Vc:FMNHOH to form Lux_Vc:FMN as found in the stopped-flow absorbance traces. When the reaction was repeated at 25°C (Fig. 12A), the initial burst of light was present, but not as obvious. We postulate that the spike of light may be due to a small population of Lux (perhaps Lux in a different multimeric state) that catalyzes a faster reaction than the bulk of enzyme. The results showed that the light emission of both species of luciferase was faster with increasing temperature (Fig. 12A); however, the increase for the major population may be more sensitive to temperature than that of the minor species, causing both emission envelopes to

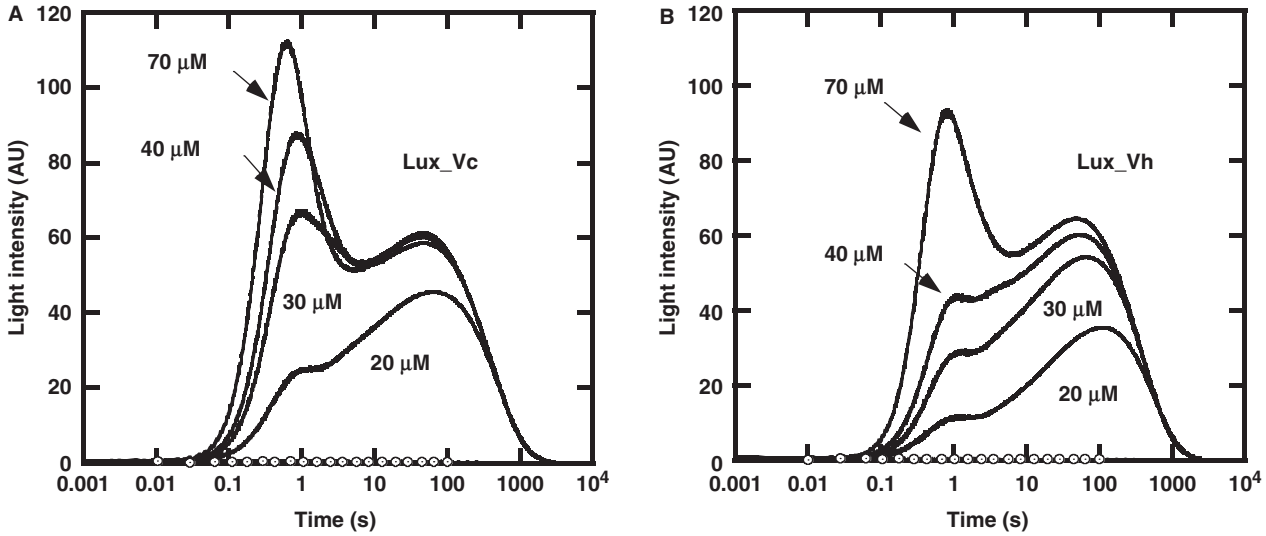

Fig. 11. **Luminescence kinetics of reactions of Lux_Vc:FMNH$^-$ (A) and Lux_Vh:FMNH$^-$ (B) with oxygen in the presence of dodecanal.** A solution of Lux_Vc (36 μM) or Lux Vh (90 μM) and FMNH$^-$ (18 μM) in 50 mM sodium phosphate buffer, pH 8.0 was mixed with the same buffer containing 0.13 mM oxygen and various dodecanal concentrations (20, 30, 40, 70 μM; lower to upper traces) in the stopped-flow spectrophotometer at 4°C. Concentrations are given as after mixing. Traces with open circles on both panels are controls without dodecanal. Light intensity was monitored by a photomultiplier tube attached to the stopped-flow machine.

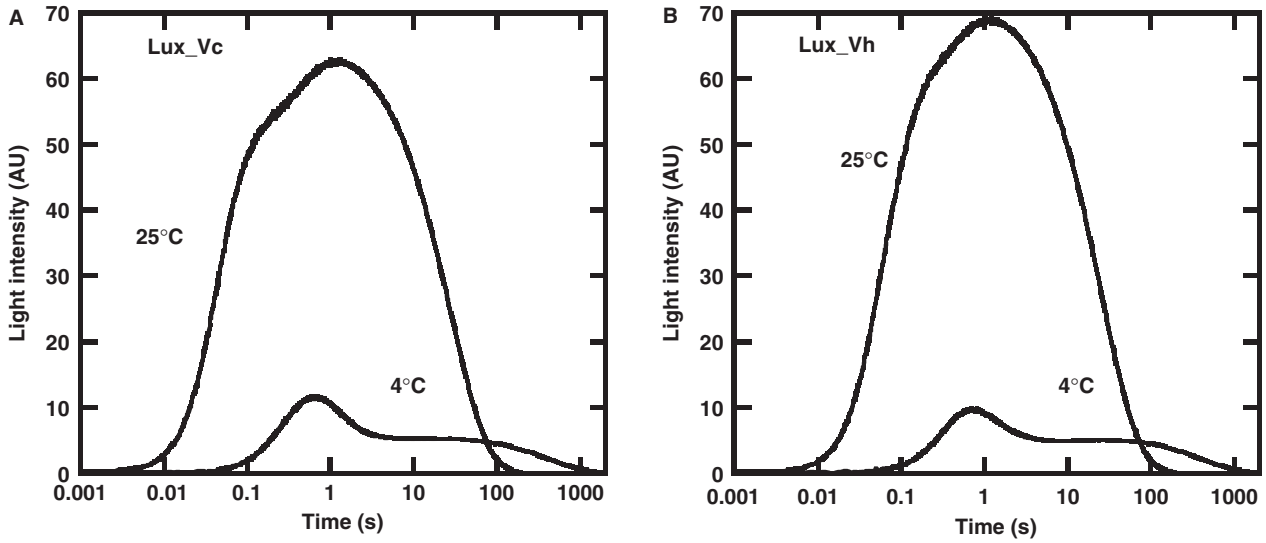

Fig. 12. **Luminescence kinetics of Lux_Vc and Lux_Vh at 4 and 25°C.** Experiments were performed by mixing a solution of Lux_Vc (16 μM) (A) or Lux_Vh (40 μM) (B) and FMNH$^-$ (8 μM) in 50 mM sodium phosphate buffer pH 8.0 with the same buffer containing 0.13 mM O_2 with 70 μM dodecanal. The concentrations given are after mixing. Light intensity was monitored as in the legend to Fig. 11.

blend, and partially mask the kinetics of the minor population. When the same types of reactions with Lux_Vh in a ratio of Lux_Vh:FMNH$^-$ of 5:1 were carried out at both 4 and 25°C, similar results were observed (Figs. 11B and 12B), including the initial spike of emission. At 25°C, formation and decay of the major luminescent species tended to blend with the initial spike like that with Lux_Vc. Therefore, it appears that this initial spike is a common characteristic of Lux_Vc and Lux_Vh.

Thermostability of Lux_Vc and Lux_Vh—Although the structures of the these enzymes must be very similar, we considered the notion that the difference in affinities for flavin for the two enzymes may be due to some structural stability differences that might be reflected in their thermal properties. Lux_Vc and Lux_Vh were incubated at various temperatures in 50 mM sodium phosphate buffer, pH 7.0. Aliquots were removed at time intervals and measured for light-emitting activity as described in Materials and Methods. Figure 13 shows that Lux_Vc is

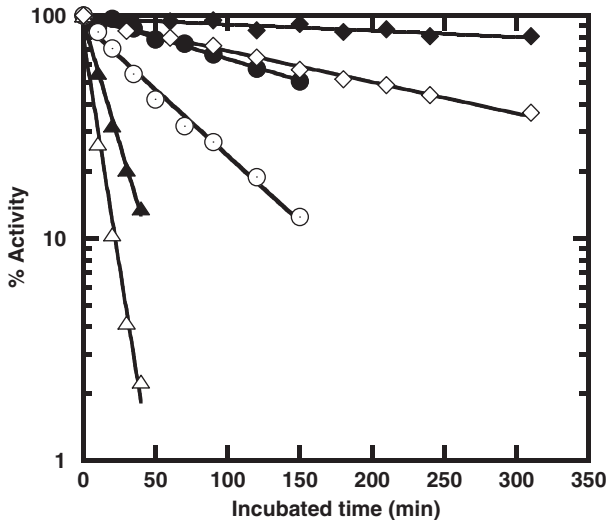

Fig. 13. **Thermal stability of Lux_Vc and Lux_Vh.** Lux_Vc or Lux_Vh (90 μM) in 50 mM sodium phosphate buffer, pH 7.0 was incubated at 37°C (diamonds), 40°C (circles), and 45°C (triangles). Activities of the enzymes at the designated time point were assayed as described in Materials and Methods. The activities of Lux_Vc are presented in filled symbols and of Lux_Vh are presented in open symbols.

more stable than Lux_Vh at 37, 40, and 45°C. The same pattern was also observed at other temperatures tested. Thus, the small differences in protein sequences do have interesting differential effects on the properties of the enzyme.

DISCUSSION

In this report, we describe the identification, cloning, expression in *E. coli*, ligand binding, kinetics of reactions with oxygen, and thermal stability properties of bacterial luciferase from *Vibrio campbellii* (Lux_Vc). The luminescence characteristics of *V. campbellii* (Fig. 2) have not been previously documented (*3*, *60*). In the genus *Vibrio*, *in vivo* luminescence has been reported for *V. harveyi*, *V. fischeri*, *V. logei*, *V. orientalis*, *V. splendidus*, and *V. cholerae* (*5*, *53*), but only the enzymes of *V. harveyi* (Lux_Vh) and *V. fischeri* have been isolated and studied. We have found that Lux_Vc is similar to Lux_Vh, but with at least two important differences. Lux_Vc has a higher affinity for $FMNH^-$ and is more thermostable than Lux_Vh. These differences suggest that Lux_Vc will be useful for future mechanistic studies and future applications. An example of the use of Lux_Vc in mechanistic studies is presented in this paper.

Sequence analysis of chromosomal DNA from *V. campbellii* showed that the *lux* operon is arranged in the sequential pattern *luxD, luxA, luxB*, and *LuxE*, just like the arrangement found in other *lux* operons (*5*, *75*). Compared to Lux_Vh, which is the most studied enzyme among bacterial luciferases, the only differences in sequence from Lux_Vc are in 8 amino acid residues (Figs. 3 and 4) that are located outside the proposed active site for Lux_Vh (*36*, *39*). The oxygen reactions of Lux_Vc in the absence and presence of aldehyde are similar to those of Lux_Vh (see Figs. 5, 11, and 12); thus, Lux_Vc can be classified as a slow light-decay type luciferase (*19*).

Our investigations have shown that Lux_Vc and Lux_Vh differ in their interactions with the flavin substrate. Lux_Vc binds to both oxidized and reduced FMN more tightly than does Lux_Vh (1.8 μM versus 11 μM for the binding of $FMNH^-$ and 36 μM versus 84 μM for the binding of FMN). The smaller K_d for Lux_Vc:$FMNH^-$ enables Lux_Vc to be studied in single turnover experiments with much less enzyme than with Lux_Vh (see Fig. 8). Although the interactions between the isoalloxazine ring and residues at the active site of the two enzymes are likely to be nearly identical based on the proposed structural interactions (*36*, *38*, *39*), the experimental differences suggest that the full extent of interactions with the flavin are still unknown. The smaller K_d for Lux_Vc:$FMNH^-$ also suggests that Lux_Vc is a superior enzyme to try in crystallization experiments to obtain information about flavin binding.

The results in Fig. 13 show that Lux_Vc and Lux_Vh are also different in their thermal stability *in vitro*, with Lux_Vc significantly more stable than Lux_Vh at all temperatures tested. Thus, the protein structural dynamics must be subtly different (*76*). We suggest that natural selection has resulted in the 8 amino acid residue differences in Lux_Vc compared to Lux_Vh in order to maintain the function of luciferase in the warmer temperatures of the tropical habitat from which *V. campbellii* was isolated. In other luminescent bacteria, optimum temperature for growth in each strain seems to be correlated with the thermal stability of the enzyme. *V. harveyi* can be grown between 10 and 37°C (*19*) and it has been shown that Lux_Vh is more thermostable and suitable for luciferase applications than the enzymes from *V. fischeri or Photobacterium sp.* (*5*). *Vibrio fischeri* can be grown between 4 and 25°C, *P. phosphoreum* between 0 and 25°C, and *P. leiognathi* between 4 and 35°C (*19*). It is also notable that the enzyme from *Photorhabdus luminescens*, a bacterium found in human wounds is more thermostable than Lux_Vh (*16*).

Our investigations into the transient state kinetics of the luciferase reaction at 4°C and pH 8 highlight some mechanistic features of both Lux_Vc and Lux_Vh that have not been documented previously. The luminescence kinetics at 4°C (Figs. 11 and 12) show an initial burst of light occurring between 0.1 and 3 s that accounts for only 1–2% of total light emission. We attribute this spike to a small population of luciferase having faster kinetics than the majority of the population. At 25°C, the kinetics of the main population of enzyme molecules may be accelerated more by temperature than that of the minor species, resulting in a kinetic profile that almost masks the kinetics of the minor population (Fig. 12). Transient kinetics studies on Lux_Vh (*30–32*, *77–78*) have been carried out at 4°C or lower, but the reactions were carried out after the C(4a)-peroxy-FMN intermediate had already been formed and isolated by gel filtration (*30*). Therefore, these studies did not document the kinetic changes we observe in the spectrum of the C(4a)-peroxy-FMN (Figs. 6 and 7) and the light-decay of the

minor population. Some transient state kinetic studies using absorbance (*74*, *79*, *80*) were carried out at 25°C and pH 7. It was found that C(4a)-peroxy-FMN formed with a second-order rate constant of $2.4 \times 10^6\,M^{-1}\,s^{-1}$ and decayed to eliminate H_2O_2 with a rate constant of $0.1\,s^{-1}$ (*74*). The rate of the latter reaction is much faster than that of Lux_Vc at 4°C, pH 8 reported here (Fig. 7). The extra phases in the reaction we reported were also not documented in the previous work. We suggest that the three consecutive transient intermediates with characteristics of C(4a)-peroxy-FMN we detected in the absence of aldehyde substrate (Fig. 6) are due to conformational rearrangements in the enzyme that cause subtle changes to the environment of the flavin. It will be fascinating to study the potential role of these changes in the complete chemistry of the light reactions. It is clear that Lux_Vc has great potential for more mechanistic studies.

The kinetic and thermodynamic constants for Lux_Vh in this report are different from some of the values reported in previous studies. These discrepancies can be attributed to differences in conditions or in methods employed. For example, in earlier studies, the affinity of $FMNH^-$ for Lux_Vh was often assumed to be equivalent to the K_m for $FMNH^-$, obtained from light-assay reactions in which the concentrations of $FMNH^-$ were varied and mixed with luciferase, aldehyde, and oxygen, and the emitted light was measured (*18*, *28*, *42*). The K_m for $FMNH^-$ in the wild-type Lux_Vh reaction was reported to be 0.6–0.7 μM at 25°C (*18*, *42*). We directly measured the affinity between $FMNH^-$ and Lux_Vh by monitoring the formation of C(4a)-peroxy-FMN, the compound resulting from the rapid-reaction of oxygen and the luciferase:$FMNH^-$ complex (Fig. 9). A similar method has previously been used to measure the K_d for binding of $FMNH^-$ to the oxygenase component of *p*-hydroxyphenylacetate hydroxylase (*73*). With this method, the binding of $FMNH^-$ to the enzyme can be measured without reference to the other substrate, aldehyde. In this report, the K_d value for Lux_Vh:$FMNH^-$ was 11 μM at 4°C. Thus, as is often the case, K_m and K_d values are clearly not at all equivalent.

In conclusion, our studies of bacterial luciferase from *V. campbellii* show that it clearly binds to reduced FMN more tightly and are more thermostable than the enzyme from *V. harveyi*. Lux_Vc opens exciting possibilities for further structural, functional, and kinetic studies of luciferase and may be of improved utility in technological uses of luciferase.

Funding was provided by The National Institutes of Health (GM64711 to D.P.B.); The Thailand Research Fund (RMU4880028 to P.C., RTA4780006 to J.S., the Royal Golden Jubilee Ph.D. program (PHD/0032/2545) to C.S. and (PHD/0211/2546) to S.N.); Faculty of Science, Mahidol University (to P.C.); Medical Scholar Program, Mahidol University (to S.N.). We thank Prof. Sandro Ghisla for valuable suggestions and Mr Janewit Wongratana for experimental assistance. We are grateful to Prof. J. Woodland Hastings and Prof. Kazuki Terauchi for their generous gift of the gene for luciferase from *V. harveyi*.

REFERENCES

1. Gunsalus-Miguel, A., Meighen, E.A., Nicoli, M.Z., Nealson, K.H., and Hastings, J.W. (1972) Purification and properties of bacterial luciferases. *J. Biol. Chem.* **247**, 398–404
2. Hastings, J.W. (1996) Chemistries and colors of bioluminescent reactions: a review. *Gene* **173**, 5–11
3. Baumann, P., Baumann, L., Bang, S.S., and Woolkalis, M.J. (1980) Reevaluation of the taxonomy of Vibrio, Beneckea, and Photobacterium: abolition of the genus Beneckea. *Curr. Microbiol.* **4**, 127–132
4. Hastings, J.W. and Nealson, K.H. (1977) Bacterial bioluminescence. *Ann. Rev. Microbiol.* **31**, 549–595
5. Meighen, E.A. (1991) Molecular biology of bacterial bioluminescence. *Microbiol. Rev.* **55**, 123–142
6. Colepicolo, P., Cho, K.W., Poinar, G.O., and Hastings, J.W. (1989) Growth and luminescence of the bacterium Xenorhabdus luminescens from a human wound. *Appl. Environ. Microbiol.* **55**, 2601–2606
7. Farmer, J.J., III, Jorgensen, J.H., Grimont, P.A., Akhurst, R.J., Poinar, G.O., Jr, Ageron, E., Pierce, G.V., Smith, J.A., Carter, G.P., Wilson, K.L., and Hickman-Brenner, F.W. (1989) *Xenorhabdus luminescens* (DNA hybridization group 5) from human clinical specimens. *J. Clin. Microbiol.* **27**, 1594–1600
8. Meighen, E.A. (1994) Genetics of bacterial bioluminescence. *Annu. Rev. Genet.* **28**, 117–139
9. Wilson, T. and Hastings, J.W. (1998) Bioluminescence. *Annu. Rev. Cell Dev. Biol.* **14**, 197–230
10. Cohn, D.H., Mileham, A.J., Simon, M.I., Nealson, K.H., Rausch, S.K., Bonam, D., and Baldwin, T.O. (1985) Nucleotide sequence of the *lux*A gene of *Vibrio harveyi* and the complete amino acid sequence of the alpha subunit of bacterial luciferase. *J. Biol. Chem.* **260**, 6139–6146
11. Engebrecht, J. and Silverman, M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**, 4154–4158
12. Johnston, T.C., Thompson, R.B., and Baldwin, T.O. (1986) Nucleotide sequence of the *lux*B gene of *Vibrio harveyi* and the complete amino acid sequence of the β subunit of bacterial luciferase. *J. Biol. Chem.* **261**, 4805–4811
13. Lee, C.Y., Szittner, R.B., and Meighen, E.A. (1991) The lux genes of the luminous bacterial symbiont, *Photobacterium leiognathi*, of the ponyfish. Nucleotide sequence, difference in gene organization, and high expression in mutant *Escherichia coli*. *Eur. J. Biochem.* **201**, 161–167
14. Mancini, J.A., Boylan, M., Soly, R.R., Graham, A.F., and Meighen, E.A. (1988) Cloning and expression of the *Photobacterium phosphoreum* luminescence system demonstrates a unique lux gene organization. *J. Biol. Chem.* **263**, 14308–14314
15. Miyamoto, C.M., Boylan, M., Graham, A.F., and Meighen, E.A. (1988) Organization of the lux structural genes of *Vibrio harveyi*. Expression under the T7 bacteriophage promoter, mRNA analysis, and nucleotide sequence of the luxD gene. *J. Biol. Chem.* **263**, 13393–13399
16. Szittner, R. and Meighen, E. (1990) Nucleotide sequence, expression, and properties of luciferase coded by lux genes from a terrestrial bacterium. *J. Biol. Chem.* **265**, 16581–16587
17. Eckstein, J.W., Cho, K.W., Colepicolo, P., Ghisla, S., Hastings, J.W., and Wilson, T. (1990) A time-dependent bacterial bioluminescence emission spectrum in an in vitro single turnover system: energy transfer alone cannot account for the yellow emission of *Vibrio fischeri* Y-1. *Proc. Natl. Acad. Sci. USA* **87**, 1466–1470
18. Lin, L.Y., Sulea, T., Szittner, R., Kor, C., Purisima, E.O., and Meighen, E.A. (2002) Implications of the reactive thiol and the proximal non-proline *cis*-peptide bond in the

structure and function of *Vibrio harveyi* luciferase. *Biochemistry* **41**, 9938–9945
19. Nealson, K.H. (1978) Isolation, identification, and manipulation of luminous bacteria in *Methods in Enzymology* (Deluca, M.A, ed.) Vol. 57, pp. 153–166, Academic Press, New York
20. Daubner, S.C., Astorga, A.M., Leisman, G.B., and Baldwin, T.O. (1987) Yellow light emission of *Vibrio fischeri* strain Y-1: purification and characterization of the energy-accepting yellow fluorescent protein. *Proc. Natl Acad. Sci. USA* **84**, 8912–8916
21. Macheroux, P., Schmidt, K.U., Steinerstauch, P., Ghisla, S., Colepicolo, P., Buntic, R., and Hastings, J.W. (1987) Purification of the yellow fluorescent protein from *Vibrio fischeri* and identity of the flavin chromophore. *Biochem. Biophys. Res. Commun.* **146**, 101–106
22. Ruby, E.G. and Nealson, K.H. (1977) A luminous bacterium that emits yellow light. *Science* **196**, 432–434
23. Lee, J., Gibson, B.G., O'Kane, D.J., Kohnle, A., and Bacher, A. (1992) Fluorescence study of the ligand stereospecificity for binding to lumazine protein. *Eur. J. Biochem.* **210**, 711–719
24. Lee, J., O'Kane, D.J., and Visser, A.J. (1985) Spectral properties and function of two lumazine proteins from Photobacterium. *Biochemistry* **24**, 1476–1483
25. Lin, J.W., Chao, Y.F., and Weng, S.F. (1993) The lumazine protein-encoding gene in *Photobacterium leiognathi* is linked to the lux operon. *Gene* **126**, 153–154
26. Abu-Soud, H.M., Clark, A.C., Francisco, W.A., Baldwin, T.O., and Raushel, F.M. (1993) Kinetic destabilization of the hydroperoxy flavin intermediate by site-directed modification of the reactive thiol in bacterial luciferase. *J. Biol. Chem.* **268**, 7699–7706
27. Hastings, J.W. and Balny, C. (1975) The oxygenated bacterial luciferase-flavin intermediate. Reaction products via the light and dark pathways. *J. Biol. Chem.* **250**, 7288–7293
28. Meighen, E.A. and Hastings, J.W. (1971) Binding site determination from kinetic data. Reduced flavin mononucleotide binding to bacterial luciferase. *J. Biol. Chem.* **246**, 7666–7674
29. Tu, S.C. (1982) Isolation and properties of bacterial luciferase intermediates containing different oxygenated flavins. *J. Biol. Chem.* **257**, 3719–3725
30. Kurfuerst, M., Macheroux, P., Ghisla, S., and Hastings, J.W. (1987) Isolation and characterization of the transient, luciferase-bound flavin-4a-hydroxide in the bacterial luciferase reaction. *Biochim. Biophys. Acta* **924**, 104–110
31. Macheroux, P., Ghisla, S., and Hastings, J.W. (1993) Spectral detection of an intermediate preceding the excited state in the bacterial luciferase reaction. *Biochemistry* **32**, 14183–14186
32. Kurfurst, M., Ghisla, S., and Hastings, J.W. (1984) Characterization and postulated structure of the primary emitter in the bacterial luciferase reaction. *Proc. Natl. Acad. Sci. USA* **81**, 2990–2994
33. Ghisla, S., Massey, V., Lhoste, J.M., and Mayhew, S.G. (1974) Fluorescence and optical characteristics of reduced flavines and flavoproteins. *Biochemistry* **13**, 589–597
34. Kaaret, T.W. and Bruice, T.C. (1990) Electrochemical luminescence with N(5)-ethyl-4a-hydroxy-3-methyl-4a, 5-dihydrolumiflavin. The mechanism of bacterial luciferase. *Photochem. Photobiol.* **51**, 629–633
35. Lei, B., Ding, Q., and Tu, S.C. (2004) Identity of the emitter in the bacterial luciferase luminescence reaction: binding and fluorescence quantum yield studies of 5-decyl-4a-hydroxy-4a,5-dihydroriboflavin-5′-phosphate as a model. *Biochemistry* **43**, 15975–15982
36. Fisher, A.J., Thompson, T.B., Thoden, J.B., Baldwin, T.O., and Rayment, I. (1996) The 1.5-Å resolution crystal structure of bacterial luciferase in low salt conditions. *J. Biol. Chem.* **271**, 21956–21968
37. Baldwin, T.O., Christopher, J.A., Raushel, F.M., Sinclair, J.F., Ziegler, M.M., Fisher, A.J., and Rayment, I. (1995) Structure of bacterial luciferase. *Curr. Opin. Struct. Biol.* **5**, 798–809
38. Fisher, A.J., Raushel, F.M., Baldwin, T.O., and Rayment, I. (1995) Three-dimensional structure of bacterial luciferase from *Vibrio harveyi* at 2.4 Å resolution. *Biochemistry* **34**, 6581–6586
39. Lin, L.Y., Sulea, T., Szittner, R., Vassilyev, V., Purisima, E.O., and Meighen, E.A. (2001) Modeling of the bacterial luciferase-flavin mononucleotide complex combining flexible docking with structure-activity data. *Protein Sci.* **10**, 1563–1571
40. Hosseinkhani, S., Szittner, R., and Meighen, E.A. (2005) Random mutagenesis of bacterial luciferase: critical role of Glu175 in the control of luminescence decay. *Biochem. J.* **385**, 575–580
41. Huang, S. and Tu, S.C. (1997) Identification and characterization of a catalytic base in bacterial luciferase by chemical rescue of a dark mutant. *Biochemistry* **36**, 14609–14615
42. Li, C.H. and Tu, S.C. (2005) Active site hydrophobicity is critical to the bioluminescence activity of *Vibrio harveyi* luciferase. *Biochemistry* **44**, 12970–12977
43. Li, C.H. and Tu, S.C. (2005) Probing the functionalities of αGlu328 and αAla74 of *Vibrio harveyi* luciferase by site-directed mutagenesis and chemical rescue. *Biochemistry* **44**, 13866–13873
44. Li, H., Ortego, B.C., Maillard, K.I., Willson, R.C., and Tu, S.C. (1999) Effects of mutations of the αHis45 residue of *Vibrio harveyi* luciferase on the yield and reactivity of the flavin peroxide intermediate. *Biochemistry* **38**, 4409–4415
45. Li, Z. and Meighen, E.A. (1995) Tryptophan 250 on the α subunit plays an important role in flavin and aldehyde binding to bacterial luciferase. Effects of W→Y mutations on catalytic function. *Biochemistry* **34**, 15084–15090
46. Lin, L.Y., Szittner, R., Friedman, R., and Meighen, E.A. (2004) Changes in the kinetics and emission spectrum on mutation of the chromophore-binding platform in *Vibrio harveyi* luciferase. *Biochemistry* **43**, 3183–3194
47. Low, J.C. and Tu, S.C. (2002) Functional roles of conserved residues in the unstructured loop of *Vibrio harveyi* bacterial luciferase. *Biochemistry* **41**, 1724–1731
48. Moore, C., Lei, B., and Tu, S.C. (1999) Relationship between the conserved α subunit arginine 107 and effects of phosphate on the activity and stability of *Vibrio harveyi* luciferase. *Arch. Biochem. Biophys.* **370**, 45–50
49. Xin, X., Xi, L., and Tu, S.C. (1991) Functional consequences of site-directed mutation of conserved histidyl residues of the bacterial luciferase α subunit. *Biochemistry* **30**, 11255–11262
50. Duch, D.S. and Laskowski, M., Sr. (1971) A sensitive method for the determination of RNA in DNA and vice versa. *Anal. Biochem.* **44**, 42–48
51. Sucharitakul, J., Chaiyen, P., Entsch, B., and Ballou, D.P. (2005) The reductase of *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* requires *p*-hydroxyphenylacetate for effective catalysis. *Biochemistry* **44**, 10434–10442
52. Chaiyen, P., Suadee, C., and Wilairat, P. (2001) A novel two-protein component flavoprotein hydroxylase. *Eur. J. Biochem.* **268**, 5550–5561
53. Kita-Tsukamoto, K., Oyaizu, H., Nanba, K., and Simidu, U. (1993) Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **43**, 8–19

54. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697–703
55. Ferri, S.R., Soly, R.R., Szittner, R.B., and Meighen, E.A. (1991) Structure and properties of luciferase from *Photobacterium phosphoreum*. *Biochem. Biophys. Res. Commun.* **176**, 541–548
56. Johnston, T.C., Hruska, K.S., and Adams, L.F. (1989) The nucleotide sequence of the *lux*E gene of *Vibrio harveyi* and a comparison of the amino acid sequences of the acyl-protein synthetases from *V. harveyi* and *V. fischeri*. *Biochem. Biophys. Res. Commun.* **163**, 93–101
57. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. (2005) Protein identification and analysis tools on the ExPASy server in *The Proteomics Protocols Handbook* (Walker, J.M. ed.) pp. 571–607, Humana Press, New Jersey
58. Baldwin, T.O., Ziegler, M.M., Green, V.A., and Thomas, M.D. (2000) Overexpression of bacterial luciferase and purification from recombinant sources in *Methods in Enzymology* (Ziegler, M.M. and Baldwin, T.O., eds.) Vol. 305, pp. 135–152, Academic Press, New York
59. O'Kane, D.J., Ahmad, M., Matheson, I.B., and Lee, J. (1986) Purification of bacterial luciferase by high-performance liquid chromatography in *Methods in Enzymology* (Deluca, M.A. and McElroy, W.D., eds.) Vol. 133, pp. 109–128, Academic Press, New York
60. Baumann, P. and Baumann, L. (1977) Biology of the marine enterobacteria: genera Beneckea and Photobacterium. *Annu. Rev. Microbiol.* **31**, 39–61
61. Dorsch, M., Lane, D., and Stackebrandt, E. (1992) Towards a phylogeny of the genus Vibrio based on 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **42**, 58–63
62. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. (1994) *Bergey's Manual of Determinative Bacteriology*pp. 571–607 Lippincott Williams and Wilkins, Baltimore
63. Foran, D.R. and Brown, W.M. (1988) Nucleotide sequence of the LuxA and LuxB genes of the bioluminescent marine bacterium *Vibrio fischeri*. *Nucleic Acids Res.* **16**, 777.
64. Hastings, J.W., Balny, C., Peuch, C.L., and Douzou, P. (1973) Spectral properties of an oxygenated luciferase-flavin intermediate isolated by low-temperature chromatography. *Proc. Natl. Acad. Sci. USA* **70**, 3468–3472
65. Ghisla, S., Entsch, B., Massey, V., and Husein, M. (1977) On the structure of flavin-oxygen intermediates involved in enzymatic reactions. *Eur. J. Biochem.* **76**, 139–148
66. Kemal, C. and Bruice, T.C. (1976) Simple synthesis of a 4a-hydroperoxy adduct of a 1,5-dihydroflavine: preliminary studies of a model for bacterial luciferase. *Proc. Natl. Acad. Sci. USA* **73**, 995–999
67. Chaiyen, P., Brissette, P., Ballou, D.P., and Massey, V. (1997) Reaction of 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase with N-methyl-5-hydroxynicotinic acid: studies on the mode of binding, and protonation status of the substrate. *Biochemistry* **36**, 13856–13864
68. Chaiyen, P., Brissette, P., Ballou, D.P., and Massey, V. (1997) Unusual mechanism of oxygen atom transfer and product rearrangement in the catalytic reaction of 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase. *Biochemistry* **36**, 8060–8070
69. Entsch, B., Cole, L.J., and Ballou, D.P. (2005) Protein dynamics and electrostatics in the function of *p*-hydroxybenzoate hydroxylase. *Arch. Biochem. Biophys.* **433**, 297–311
70. Palfey, B.A., Ballou, D.P., and Massey, V. (1995) Oxygen activation by flavins and pterins in *Active Oxygen in Biochemistry* (Selver-stone Valentine, J., Foote, C.S., Greenberg, A., and Liebman, J.F., eds.) pp. 37–83, Chapman & Hall, London
71. Sheng, D., Ballou, D.P., and Massey, V. (2001) Mechanistic studies of cyclohexanone monooxygenase: chemical properties of intermediates involved in catalysis. *Biochemistry* **40**, 11156–11167
72. Chakraborty, S., Ortiz-Maldonado, M., Eschenburg, K., Entsch, B., and Ballou, D.P. (2005) *p*-Hydroxyphenylacetate-3-hydroxylase from *Pseudomonas aeruginosa* in *Flavins and Flavoproteins* (Nishino, T., Miura, R., Tanokura, M., and Fukui, K., eds.) pp. 161–166, ARchiTect Inc., Tokyo
73. Sucharitakul, J., Chaiyen, P., Entsch, B., and Ballou, D.P. (2006) Kinetic mechanisms of the oxygenase from a two-component enzyme, *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*. *J. Biol. Chem.* **281**, 17044–17053
74. Abu-Soud, H., Mullins, L.S., Baldwin, T.O., and Raushel, F.M. (1992) Stopped-flow kinetic analysis of the bacterial luciferase reaction. *Biochemistry* **31**, 3807–3813
75. Meighen, E.A. (1993) Bacterial bioluminescence: organization, regulation, and application of the *lux* genes. *FASEB J.* **7**, 1016–1022
76. Dill, K.A. (1990) Dominant forces in protein folding. *Biochemistry* **29**, 7133–7155
77. Becvar, J.E., Tu, S.C., and Hastings, J.W. (1978) Activity and stability of the luciferase-flavin intermediate. *Biochemistry* **17**, 1807–1812
78. Presswood, R. and Hastings, J.W. (1978) Oxygen dependent and independent steps in luciferase-$FMNH_2$ oxidation. *Biochem. Biophys. Res. Commun.* **82**, 990–996
79. Francisco, W.A., Abu-Soud, H.M., DelMonte, A.J., Singleton, D.A., Baldwin, T.O., and Raushel, F.M. (1998) Deuterium kinetic isotope effects and the mechanism of the bacterial luciferase reaction. *Biochemistry* **37**, 2596–2606
80. Francisco, W.A., Abu-Soud, H.M., Topgi, R., Baldwin, T.O., and Raushel, F.M. (1996) Interaction of bacterial luciferase with 8-substituted flavin mononucleotide derivatives. *J. Biol. Chem.* **271**, 104–110