Functional Consequences of Site-Directed Mutation of Conserved Histidyl Residues of the Bacterial Luciferase α Subunit†

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ABSTRACT: The available sequences for the different bacterial luciferases reveal five conserved histidyl residues at positions 44, 45, 82, 224, and 285 of the α subunit. Ten variants of *Vibrio harveyi* luciferase were obtained by selective site-directed mutations of these five histidines. The essentiality of αHis44 and αHis45 was indicated by 4–7 orders of magnitude of bioluminescence activity reductions resulting from the substitution of either histidine by alanine (αH44A or αH45A), aspartate (αH44D or αH45D), or lysine (αH45K). Moreover, αH44A and αH45A were distinct from the native luciferase in thermal stabilities. Mutations at the other three positions also resulted in activity reductions ranging from a fewfold to 3 orders of magnitude. Despite these widely different bioluminescence light outputs, mutated luciferases exhibited, in nonturnover in vitro assays, light emission decay rates mostly similar to that of the native luciferase using octanal, decanal, or dodecanal as a substrate. This is attributed to a similarity in the catalytic rate constants of the light-emitting pathway for the native and mutated luciferases, but various mutated luciferases suffer in different degrees from competing dark reaction(s). In accord with this interpretation, the bioluminescence activities of mutated luciferases showed a general parallel with the relative stabilities of their 4a-hydroperoxyflavin intermediate species. Furthermore, the drastically reduced bioluminescence activities for luciferases with the αHis44 or αHis45 substituted by aspartate, alanine, or lysine were accompanied by little or no activities for consuming the aldehyde substrate. However, on the basis of deuterium isotope effects of [1-^{2}H]decanal, a direct involvement of αHis44 or αHis45 in the abstraction of the aldehyde C1 hydrogen was not detected. The αH44A, αH44D, and αH44K variants showed surprisingly good yields (14–45%) of 4a-hydroperoxyflavin detectable by absorption spectroscopy. Moreover, the 4a-hydroperoxyflavin species of αH44A or αH44D exhibited distinct decay rates when determined by absorption changes and by decreases in bioluminescence capacity. These findings indicate that two forms of 4a-hydroperoxyflavin, with one active and the other essentially inactive in bioluminescence, can be formed by a single luciferase species.

Bacterial luciferase catalyzes a bioluminescent monooxygenation reaction utilizing reduced FMN ($FMNH_2$),[1] a long-chain aliphatic aldehyde, and oxygen as substrates. This enzyme is highly unusual in several respects among known flavin-dependent external monooxygenases (hydroxylases). Luciferase has a unique heterodimeric structure, designated $\alpha\beta$, with no detectable activity for either of the individual subunits. This enzyme requires $FMNH_2$ as a substrate which is provided in vivo through the activity of other enzymes. In comparison, other flavo-hydroxylases, with the sole exception of 2,5-diketocamphane monooxygenase (Taylor & Trudgill, 1984), require tightly bound FAD as a cofactor which is converted to the reduced form through their own catalysis using NAD(P)H as a co-substrate. Luciferase has an unusually slow turnover rate in association with several long-lived intermediates with half-lives in the order of seconds or longer at room temperature. Most striking, luciferase is unique among flavo-hydroxylases in its activity to catalyze a light-emitting reaction. Therefore, bacterial luciferase represents a particularly challenging system for structural and mechanistic studies.

Previous subunit hybridization studies (Meighen et al., 1971; Cline & Hastings, 1972) indicate that the luciferase α subunit (molecular weight ~41 000) participates directly in catalysis. While the β subunit (molecular weight ~37 000) may be involved in the binding of $FMNH_2$ (Meighen & Bartlet, 1980; Watanabe et al., 1982) and aldehyde (Tu & Henkin, 1983; Paquatte et al., 1988), the definitive function of this subunit remains poorly understood. *Vibrio harveyi* luciferase has seven cysteinyl residues on the α and six more on the β, and chemical modifications of a single α subunit cysteinyl residue result in marked luciferase inactivation (Nicoli et al., 1974). This "essential" cysteine at position 106 of the α-subunit polypeptide chain, however, is neither required for the binding of the $FMNH_2$ and aldehyde substrates (Paquatte & Tu, 1989) nor involved directly in chemical catalysis (Baldwin et al., 1989; Xi et al., 1990). It is proposed instead that this αCys106 has a critical role in maintaining luciferase in a functionally active conformation (Xi et al., 1990; Tu, 1991), since changing this cysteinyl residue to a bulkier function by chemical or mutational means greatly impedes the formation of or drastically destabilizes the 4a-hydroperoxyflavin intermediate essential to the bioluminescence activity (Paquatte & Tu, 1989; Xi et al., 1990; Tu, 1991).

There are a total of 11 histidyl residues on the α and 12 histidyl residues on the β. Inactivation of the *V. harveyi* luciferase has been demonstrated to result from chemical modifications of a single "essential" histidyl residue (pK_a 6.8) on the α subunit (Cousineau & Meighen, 1976). This histidyl residue can be partially protected against chemical modification by the binding of aldehyde or the 4a-hydroperoxyflavin to luciferase (Cousineau & Meighen, 1976). However, neither the location in the sequence of the α subunit nor any specific function has been identified for this "essential" histidyl residue. Inactivation of *V. harveyi* luciferase also results from modi-

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[1] Abbreviation: $FMNH_2$, reduced flavin mononucleotide.

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fications of an unspecified primary amino group on either the α or the β subunit (Welches & Baldwin, 1981).

While useful information is revealed by these structural studies, clearly much remains to be explored concerning the structure–function relationship of bacterial luciferase. At present, advancement in this area is impeded by the lack of detailed structural information on luciferase from either X-ray crystallographic or high-resolution NMR studies. In order to better our understanding of luciferase structure and mechanism, we chose to examine the functional consequences of site-directed mutations of conserved histidyl residues on the α subunit of *V. harveyi* luciferase. The rationale for this approach is based on several lines of consideration. First, amino acid residues crucial to maintaining the critical conformation, substrate (cofactor) binding, or chemical catalysis are often highly conserved for a given type of enzyme from various sources (Bowie et al., 1990). Primary sequences have so far been reported for the α subunit of luciferase from *V. harveyi* (Cohn et al., 1985), *Vibrio fischeri* (Baldwin et al., 1987; Foran & Brown, 1988), *Photobacterium leiognathi* (Illarionov et al., 1988; Johnston et al., 1990), *Xenorhabdus luminescens* strains Hm (Johnston et al., 1990), Hb (Szittner & Meighen, 1990), and HW (Xi et al., 1991), and a symbiont of the flashlight fish *Kryptophanaron alfredi* (Haygood, 1990), thus providing a reasonable data base for sequence comparison. The choice of the α subunit for mutation is based on the prior knowledge of this subunit being directly involved in luciferase catalysis. The reported but yet uncharacterized identity and essentiality of a single histidyl residue on the α subunit make this type of residue an interesting target for probing further the luciferase structure–function relationship. Furthermore, an analysis of all the available α-subunit sequences reveals that only five histidyl residues are conserved (namely at positions 44, 45, 82, 224, and 285), thus markedly simplifying the scope of the intended comparative site-directed mutagenesis study.

In this work, we generated single mutations at positions 224 and 285 and multiple mutations at positions 44, 45, and 82 for the *V. harveyi* luciferase α subunit. These mutated luciferases were isolated and characterized. Two histidyl residues were identified as particularly critical to luciferase activity. Certain mutations at either of these two positions resulted in remarkable 5–7 order of magnitude reductions in activity, primarily due to enhanced efficiencies of competing dark pathways. Moreover, the ability of a single luciferase species to form two types of 4a-hydroperoxyflavin intermediates, with one active and the other inactive in bioluminescence, was demonstrated for the first time.

Experimental Procedures

Materials. Peptone and yeast extract were purchased from GIBCO. Ultrapure dNTP and cloning vectors M13mp19 and pBR322 were obtained from Pharmacia LKB Biotechnology Inc. Restriction endonuclease and T4 DNA ligase were products of New England Biolabs. T4 polynucleotide kinase and T4 DNA polymerase were from Pharmacia. X-Gal and IPTG were purchased from Fisher. [α-^{35}S]dATPαS (37 TBq/mmol) was obtained from Amersham. The Sequenase kit from United States Biochemical Corp. was used for both single- and double-strand DNA sequences. DEAE-cellulose DE-52 was purchased from Whatman. Ethoxyformic anhydride, decanal, octanal, dodecanal, tetradecanal, and copper(I) bromide were all from Aldrich. Dodecanol was obtained from MC&B Manufacturing Chemists. Sodium hydrosulfite, FMN, dithiothreitol, and ampicillin were from Sigma. [1-^{2}H]Decanal was obtained first through reduction of ethyl caprate by lithium aluminum deuteride (from Dtohler Isotope Chemicals) to form deuterated decanol and then by oxidation of the alcohol to aldehyde using pyridinium chlorochromate (Corey & Suggs, 1975).

Site-Directed Mutagenesis. A recombinant M13mp19 phage (designated MTX1) containing the *lux*A and *lux*B genes encoding the wild-type *V. harveyi* luciferase α and β subunits, respectively, (Xi et al., 1990) was used for site-directed mutagenesis. Five conserved histidyl residues on the α subunit were each mutated. In each case, a 21-base synthetic oligonucleotide, obtained by using a Biosearch 6800 automated DNA synthesizer, containing a necessary new codon for the desired mutation was used as a primer. A total of 10 mutated luciferases were constructed which are identified, in the expression of (mutant designation)/(original codon → mutant codon), as (αH44D)/(CAC → GAC), (αH44A)/(CAC → GCC), (αH44K)/(CAC → AAA), (α-45D)/(CAC → GAC), (αH45A)/(CAC → GCC), (αH45K)/(CAC → AAA), (αH82D)/(CAC → GAT), (αH82K)/(CAT → AAA), (αH224A)/(CAC → GCC), and (αH285A)/(CAC → GCC). The single-letter abbreviations used for amino acid residues are H for histidine, D for aspartate, A for alanine, and K for lysine. Procedures for mutating a single codon (Kunkel, 1985) and screening were essentially the same as described previously (Xi et al., 1990). All mutated phages were confirmed by dideoxy sequencing analysis (Sanger et al., 1977). Overexpression plasmids, designated as the pXH series, with each containing a desired *lux*A gene, were constructed as follows. First, *Hin*dIII–*Sma*I fragments from MTX1 containing the wild-type *lux*A and *lux*B genes were subcloned into the *Hin*dIII and blunted *Eco*RI sites of pBR322 to produce pXH0. Subsequently, DNA fragments containing the mutated *lux*A gene but not the native *lux*B gene were obtained by digesting the mutated MTX1 with *Hin*dIII and *Eco*RI. These fragments were subcloned into pXH0, pretreated with *Hin*dIII and *Eco*RI to remove the native *lux*A gene, to generate the pXH series of recombinant plasmids. The screening and overexpression of the desired clones in *Escherichia coli* HB101 were essentially the same as previously described (Xi et al., 1990). The nature of mutagenesis for each pXH was further confirmed by double-strand DNA sequencing (Haltiner et al., 1985).

Luciferase Purification and Activity Assay. Wild type luciferase was purified from *V. harveyi* cells, and mutant luciferases were each isolated from *E. coli* HB101 cells harboring the desired pXH recombinant plasmid following literature procedures (Hastings et al., 1978). On the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the wild-type luciferase was about 95% pure and the purities of all mutated luciferases were in the range of 85–95%. Bioluminescence activities were determined at 23 °C by a modified dithionite assay (Tu & Hastings, 1975) and/or copper(I) assay (Lei & Becvar, 1991). Unless stated otherwise, the standard buffer of 0.1 M potassium phosphate (KP_i), pH 7.0, was used for all reactions involving luciferase. Activities were measured at various concentrations of $FMNH_2$ or aldehyde at a saturating level of the cosubstrate. Values of the maximal activity and K_m were determined by double-reciprocal plots. The former values are expressed either as total light output (Q) in quanta (q) or as the peak light intensity (I_0) in $q \cdot s^{-1} \cdot A_{280}^{-1} \cdot mL^{-1}$ of the enzyme sample.

Inactivations of Luciferases by Ethoxyformic Anhydride and Heat. Wild-type and mutant luciferases (3–8 μM) were each mixed with 6×10^{-4} M ethoxyformic anhydride in 0.1 M KP_i, pH 6.0, at 0 °C. Aliquots (10 μL) were withdrawn at different times for the measurement of the remaining ac-

Table I: Comparison of Bioluminescence Activities,[a] Kinetic Properties,[a] and Stabilities for Wild-Type and Mutated Luciferases

luciferase	$I_0/I_{0,WT}$[b]	Q/Q_{WT}[b]	light decay rate (min^{-1})			K_m (μM)			k_{inact} (min^{-1}) by ethoxyformic anhydride[d]	E_a of thermal inactivation (kcal·mol^{-1})[f]
			octanal	decanal	dodecanal	$FMNH_2$[c]	decanal	tetradecanal		
wild type	1.0	1.0	3.0	19.3	3.1	0.7	11.1	0.1	0.18	68
αH44D	3.1×10^{-5}	3.2×10^{-5}	2.4	18.5	2.5	1.4	51.8	1.7		
αH44A	3.7×10^{-6}	6.6×10^{-6}	1.7	10.8	34.0	9.2	25.0			59
αH44K	1.8×10^{-2}	1.9×10^{-2}	5.4	18.3	5.6	41.0	38.2	1.7	0.11	
αH45D	1.4×10^{-4}	1.6×10^{-4}	3.3	16.1	2.5	4.2	40.3	1.2	0.22	
αH45A	3.9×10^{-7}	3.8×10^{-7}	4.9	19.8	0.8	2.2	83.5			78
αH45K	2.6×10^{-5}	2.8×10^{-5}	4.6	17.5	4.8	2.4	53.0	0.4		
αH82D	2.8×10^{-1}	3.1×10^{-1}	3.3	16.9	2.7	2.4	39.1		0.21[e]	
αH82K	7.1×10^{-3}	7.8×10^{-3}	2.8	16.1	2.4	20.0	25.0			
αH224A	2.8×10^{-2}	3.0×10^{-2}	2.5	17.5	2.4	0.7	28.8		0.21	
αH285A	3.7×10^{-1}	5.4×10^{-1}	4.1	13.1	5.5	2.9	69.9		0.30	

[a] Determined at 23 °C, pH 7.0. [b] Using decanal as a substrate. I_0 and Q are the peak emission intensity (in q·s^{-1}·A_{280}^{-1}·mL^{-1}) and the total quantum output, respectively. The subscript WT refers to the wild-type luciferase. [c] Using decanal as a co-substrate. [d] Determined at 0 °C in 0.1 M P_i, pH 6.0, using 0.6 mM ethoxyformic anhydride. [e] Corrected for $k = 0.12$ min^{-1} for spontaneous inactivation. [f] Determined in 0.35 M P_i, pH 7.0. The apparent k_{inact} at 55 °C were 0.72, 0.62, and 0.89 min^{-1} for the wild-type, αH44A, and αH45A luciferases, respectively.

tivity by the Cu(I) assay. In a separate experiment, selected luciferase samples in 0.35 M KP_i, pH 7.0, were each incubated at several different temperatures in the range of 47.5–55 °C, and the decrease in enzyme activity was determined as a function of time as described above. Energies of activation (E_a) for thermal inactivation were determined by plots of the logarithm of the apparent first-order rate constants of inactivation so determined against the reciprocal of the corresponding absolute temperatures according to the Arrhenius equation.

[1-^{2}H]Aldehyde Deuterium Isotopic Effect. Activities of wild-type and mutated luciferases were determined by the dithionite assay at 23 °C in the standard buffer containing 50 μM $FMNH_2$ and saturated [1-^{2}H]decanal or the control decanal (at about 0.2 mM). The deuterium isotope effects (^Dk) were determined as the ratios of the light decay rate for the control decanal over that for the deuterated decanal.

Aldehyde Consumption Assay. A standard curve was first constructed by correlating the bioluminescence activities of the wild-type *V. harveyi* luciferase (1.2 μM) with the amounts of tetradecanal (ranging from 0 to 25 nM) used in the Cu(I) assay with 50 μM $FMNH_2$ as a co-substrate. To test the ability of mutated luciferases to consume aldehyde as a substrate, a limiting level of tetradecanal (1 μM) was mixed with an excess amount of a desired luciferase (8–27 μM) in 1 mL of the standard buffer. The bioluminescence reaction was initiated at 23 °C by the injection of 1 mL of buffer containing 50 μM $FMNH_2$, reduced by the Cu(I) method, and was allowed to reach completion. Control samples were similarly prepared for all luciferases tested except that the Cu(I)-reduced $FMNH_2$ was first reoxidized by O_2 before the injection. Aliquots (20 μL) of the samples and the controls were withdrawn, each added as the aldehyde source to 1 mL of buffer containing 1.2 μM wild-type luciferase, and incubated for about 10 min to allow aldehyde reequilibrium, and the bioluminescence activity was initiated and measured under conditions identical to that for the construction of the tetradecanal standard curve. The amount of remaining tetradecanal in each sample and control was then determined by comparing the observed bioluminescence activity against the aldehyde standard curve.

Preparation and Stability of the Flavin Hydroperoxide Intermediate II. For stability measurements at 23 °C, the desired II was first formed by reacting a wild-type or mutated luciferase with 50 μM $FMNH_2$ (reduced by dithionite) and 0 or 0.1 mM dodecanol as a stabilizing agent (Tu, 1979) under aerobic conditions. Aliquots (20–50 μL) were withdrawn after different times of standing at 23 °C, and each was injected into 1 mL of buffer containing a saturating level of decanal to initiate the bioluminescence. The decay rate of II (k_{II}) was then determined by a semilogarithmic plot of I/I_0 versus time in which I_0 and I are the bioluminescence activities at time zero and any given time point during the incubation, respectively. The intermediate II samples so obtained may contain some luciferase-bound flavin neutral semiquinone (Kurfürst et al., 1982). But this latter species is inactive in bioluminescence upon addition of aldehyde (Kurfürst et al., 1982) and, hence, did not interfere with our determination of II decay. For some experiments, the desired II samples for selected luciferase species (at 2.5 mg/mL) were formed and isolated at 4 °C by the Sephadex G-25 column/centrifugation method as described previously (Tu, 1986) and were found to be free from flavin semiquinone contaminations. Each isolated intermediate II sample was immediately transferred to an optical cuvette, precooled to and kept at 4 °C, and the absorption spectral changes were measured as a function of time until reaching completion. The decay rate of II was determined from a semilogarithmic plot of $\Delta A/\Delta A_0$ versus time in which $\Delta A = A_\infty - A$ and $\Delta A_0 = A_\infty - A_0$. A_0, A, and A_∞ are defined as the absorbance at 460 nm of the sample immediately after isolation, at any given time point during the decay, and at the completion of the decay, respectively. From time to time during the absorption spectroscopic measurement at 4 °C, 20-μL aliquots were withdrawn from the very same intermediate sample with a precooled Hamilton syringe and each was injected into 1 mL of buffer containing saturated tetradecanal to initiate the bioluminescence reaction at 23 °C. The stability of the bioluminescence-active II was determined by the semilogarithmic plot of I/I_0 versus time as described above.

Results

The five conservative histidyl residues on the α subunit of all bacterial luciferase species with known sequences correspond to positions 44, 45, 82, 224, and 285 of the *V. harveyi* luciferase α subunit. We have carried out single mutations of αHis224 and αHis285 and multiple mutations of each of αHis44, αHis45, and αHis82 for the *V. harveyi* luciferase. Bioluminescence activities and general kinetic properties of 10 such mutated luciferases were determined and compared with those of the wild-type luciferase (Table I). Mutations of these five conservative histidyl residues all resulted in re-

Table II: Decay Rates of 4a-Hydroperoxyflavin Intermediate II, at 23 °C, Formed by the Wild-Type and Mutated Luciferases in the Absence or Presence of Dodecanol as a Stabilizing Agent

	k_{II}[a] (min^{-1})									
stabilizing agent	wild type	αH44D	αH44A	αH44K	αH45D	αH45K	αH82D	αH82K	αH224A	αH285A
−dodecanol	3.3	14.4	16.9	4.0	7.1	13.4	2.7	6.2	3.2	10.6
+dodecanol	0.08	0.08	0.89	0.64	0.53	0.05	0.05	0.07	0.05	0.61

[a] Determined by following the time-dependent decrease, at 23 °C, in the remaining 4a-hydroperoxyflavin intermediate II active in bioluminescence upon reacting with decanal.

duced bioluminescence activities, but the degrees of inactivation were highly sensitive to the position of the histidyl residue that was mutated and the nature of the amino acid substituent. Among the five conserved histidyl residues, αHis44 and αHis45 appeared to be most crucial to the expression of luciferase bioluminescence activity. On the basis of either the initial peak intensities (I_0) or the total light output (Q) using decanal as a substrate, the wild-type luciferase bioluminescence activity was reduced from only a factor of 2–3 for αH82D and αH285A to a remarkable 6 orders of magnitude or more for αH44A and αH45A. Values of the K_m of the 10 mutated luciferases for $FMNH_2$, decanal, and tetradecanal were generally larger than those of the wild-type enzyme by a factor of (with the expections of the 30- and 60-fold larger K_m of αH82K and αH44K, respectively, for $FMNH_2$) 17 or less. The apparent first-order decay of light emission and the peak intensity observed in the in vitro assays for the wild-type luciferase are both known to be sensitive to the chain length of the aldehyde. In comparison with the wild-type luciferase, a similar pattern of aldehyde chain-length dependence and relatively small changes in the rates of light decay were observed for all mutated luciferases using octanal, decanal, and dodecanal as substrates (Table I). αH44A and αH45A are two notable exceptions. The former showed an 11-fold faster and the latter exhibited a 4-fold slower light decay rate than that of the native luciferase with dodecanal as a substrate. Peak intensities observed for the mutated luciferases using different aldehydes also followed the same pattern as that for the wild-type luciferase (Figure 1). In general, higher values of I_0 were detected with decanal and tetradecanal than with octanal and dodecanal. It was also noted that the peak intensities observed for αH285A were somewhat less sensitive to aldehyde variation, and αH82K exhibited an unusually high peak intensity with octanal.

At 0 °C in 0.1 M P_i, pH 6, the αH82D luciferase was found to be labile, exhibiting a half-life of 6 min corresponding to an apparent first-order rate constant of 0.12 min^{-1} for the spontaneous inactivation. Neither the wild-type luciferase nor any other mutated luciferases showed any decrease in activity under identical conditions for a period of at least 30 min. The αH82D luciferase, however, had a markedly improved stability at pH 7.0; no significant inactivation was observed for over 1 day at 0 °C and neutral pH in 0.05 M P_i.

Five selected luciferase variants, each corresponding to the mutation of a different position of the five conserved histidyl residues, were treated with 0.6 mM ethoxyformic anhydride at 0 °C and pH 6, and their apparent first-order constants of inactivation (k_{inact}) are shown in Table I. In comparison with the wild-type luciferase, similar or slightly faster rates of inactivation were observed for αH45D, αH82D (after correction for the autoinactivation), αH224A, and αH285A. In contrast, αH44K showed a rate of inactivation slower than that of the wild-type luciferase. The apparent first-order rate constants of inactivation by thermal denaturation were also determined for the wild-type enzyme and the two least active luciferase variants in 0.35 M P_i, pH 7.0, at several temperatures over the range of 47.5–55 °C. While similar values of k_{inact} were found at 50 °C for the wild-type enzyme (0.15 min^{-1}), αH44A (0.16 min^{-1}), and αH45A (0.14 min^{-1}), significantly different rates of inactivation were detected at 55 °C in the order of αH45A (0.89 min^{-1}) > wild type (0.72 min^{-1}) > αH44A (0.62 min^{-1}). Hence, the corresponding values of energy of activation (E_a) for thermal inactivation (Table I) were also in the order of αH45A > wild type > αH44A.

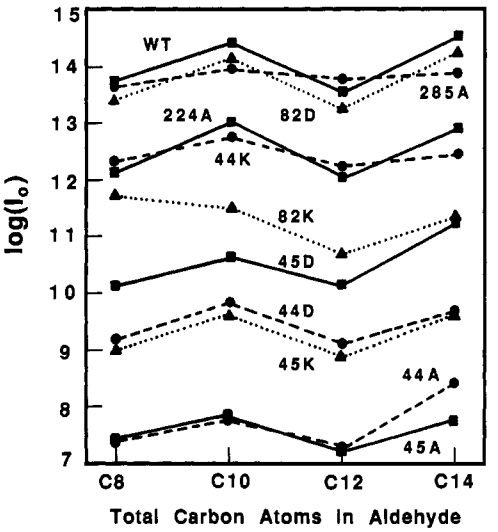


FIGURE 1: Bioluminescence peak intensities of wild-type and mutated luciferases using different aldehyde substrates. The peak intensities (in $q \cdot s^{-1} \cdot A_{280}^{-1} \cdot mL^{-1}$) were measured at 23 °C by the dithionite assays as described under Experimental Procedures for the wild-type (WT) and mutated luciferases. The designations for luciferase variants are the same as that shown in Table I, except that the first two letters are omitted for simplicity.

Decanal, dodecanal, and tetradecanal deuterated at the C1 position are known to exhibit deuterium kinetic isotope effects of 1.4–1.7 on the basis of either the peak light intensity or the rate of light decay of the *V. harveyi* luciferase-catalyzed reaction (Shannon et al., 1978; Tu et al., 1987). Using the dithionite assay to avoid aldehyde inhibition, deuterium isotope effects of [1-^{2}H]decanal on the light decay rates were observed to be in the range of 1.3–1.7 for all six of the αHis44- or αHis45-mutated luciferases, quite similar to an isotope effect of 1.6 for the wild-type enzyme.

The 4a-hydroperoxyflavin intermediate II of the wild-type *V. harveyi* luciferase is unusually stable (Hastings & Gibson, 1963; Hastings et al., 1973; Tu, 1979) and can be markedly stabilized further by complexing with non-aldehyde aliphatic compounds such as long-chain alcohols (Tu, 1979, 1982). The decay rates of the intermediate II species, which were active in bioluminescence upon reacting with decanal, were determined for all luciferase variants except αH45A (Table II). The exceedingly low bioluminescence activity of αH45A posed technical difficulties for an accurate determination of II stability by activity assays. While the stabilities of II for αH44K, αH82D, and αH224A were comparable to that of the wild-

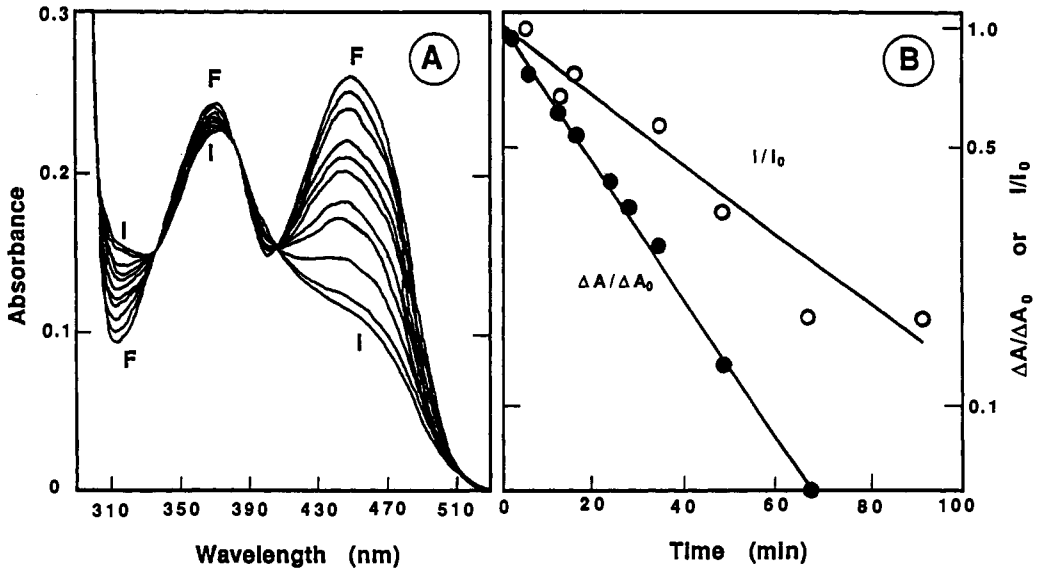


FIGURE 2: Decays of αH44A intermediate II at 4 °C as detected by absorption spectroscopy and bioluminescence capacity. Panel A: Absorption spectra of the αH44A intermediate II sample immediately after isolation (I) and the final FMN product (F) are shown along with transient spectra detected during the decay at 0.2, 5.7, 11.6, 15.8, 23.2, 28.0, 34.4, 49.0, and 66.3 min after the isolation of II. Panel B: The decay of intermediate II was determined by following the absorbance changes at 460 nm (●) and the decreases in capacity to emit bioluminescence upon reacting with decanal added secondarily (○). The ordinate of panel B is in a logarithmic scale.

type enzyme, the decay rates of II for the rest of luciferase variants were 2–5-fold faster than that of the native luciferase (Table II). For the native and mutated luciferases, complexation with dodecanol resulted in significant but various degrees of stabilization of II (Table II).

Since αHis44 and αHis45 were exceedingly critical to the expression of the luciferase bioluminescence activity, we chose to examine further the properties of the six luciferase variants each with the αHis44 or αHis45 replaced by an aspartate, alanine, or lysine. The bioluminescence activity of the wild-type luciferase is known to be coupled with the monooxygenation of aldehyde to form the corresponding carboxylic acid. This is indicated by a proportionality between the quantum yield and the formation of carboxylic acid (Dunn et al., 1973), the markedly reduced yields of H_2O_2 from II in the presence of aldehyde (Hastings & Balny, 1975; Tu, 1982), and the 1.4–1.7 primary kinetic isotope effects of [1-^{2}H]-aliphatic aldehydes on the light emission intensity or decay rate (Shannon et al., 1978; Tu et al., 1987). Regarding the drastically reduced bioluminescence activities of the three αHis44-mutated and the three αHis45-mutated luciferases, the question as to whether any of these luciferase variants is capable of converting aldehyde to acid was examined. Using a limiting amount of tetradecanal (1 μM) and excess luciferase, almost 100% of the aldehyde was consumed by the wild-type luciferase in the in vitro bioluminescence reaction initiated with the Cu(I)-reduced $FMNH_2$. For the six luciferase variants mutated at αHis44 or αHis45, five of them did not show any significant aldehyde consumption in comparison with their respective controls in which the reduced flavin was first re-oxidized and then added to the sample solution containing enzyme and tetradecanal. Only the αH44D luciferase showed a marginal 12 ± 7% tetradecanal consumption. Therefore, these six αHis44- or αHis45-mutated luciferase variants retained little or no significant activities for the monooxygenation of aldehyde.

The αHis44- and αHis45-mutated luciferase variants were each reacted with $FMNH_2$ and O_2 and then subjected to molecular sieve column chromatography at 4 °C for the isolation of II as described under Experimental Procedures. As shown by absorption spectroscopy, no II was detectable for αH45D, αH45A, or αH45K whereas yields of 14, 45, and 20% were obtained for αH44D, αH44A, and αH44K, respectively (Table III). The yields of II of these three αHis44-mutated luciferases were only 2–6-fold lower than the 85–90% yields for the wild-type enzyme under similar conditions (Tu, 1979), but the bioluminescence quantum outputs for these mutated luciferases were several orders of magnitude lower than that of the wild-type luciferase (Table I). This prompted us to compare the decay rates of II by following absorption changes at 460 nm and by measuring the decreases in the capacity to emit bioluminescence upon reacting with decanal added secondarily. The absorption spectrum of the αH44A intermediate II sample immediately after isolation and the progressive spectral changes resulted from the decay to the final FMN product are shown in Figure 2A, exhibiting isosbestic points at 335, 383, and 405 nm. From such measurements, a decay rate constant of 0.04 min^{-1} was calculated (Figure 2B). However, a slower first-order decay rate constant of 0.02 min^{-1} was obtained as determined by decreases in the amount of II that was capable of emitting bioluminescence upon reacting with aldehyde added secondarily (Figure 2B; Table III). Similar measurements were also made for II obtained with αH44D, and the decay rate determined by the absorption

Table III: Yields and Stabilities of 4a-Hydroperoxyflavin Intermediate II Formed with Luciferase αHis44 Variants at 4 °C[a]

method of detection	yield of intermediate II[b] (%)			k_{II}[c] (min^{-1})	
	αH44D	αH44A	αH44K	αH44D	αH44A
bioluminescence				0.02	0.02
absorption	14	45	20	0.30	0.04

[a] 4a-Hydroperoxyflavin intermediate II samples were formed in 1 mL of buffer by reacting excess $FMNH_2$ with 33–50 nmol of luciferase in the presence of dodecanol as a stabilizing agent and were isolated by the Sephadex G-25 column/centrifugation method. [b] The yields of intermediate II were determined on the basis of absorbance changes at 460 nm between each initial isolate and the same sample upon the completion of decay, and each was calculated as the percent of the total molar amount of the starting luciferase. [c] Determined by following both the time-dependent decrease in the remaining intermediate II that was active in bioluminescence upon reacting with decanal and the changes in absorbance at 460 nm associated with the time-dependent decay of intermediate II to FMN.

changes (0.30 min^{-1}) was 15-fold higher than that detected by the decreases in bioluminescence capacity (Table III). These observations clearly indicate that certain luciferase species can form two different types of 4a-hydroperoxyflavin intermediate.

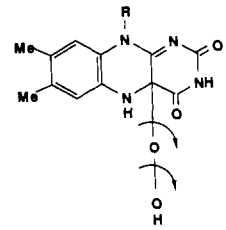
Discussion

Among the five conserved histidyl residues for the α subunit of bacterial luciferases, αHis44 and αHis45 are far more critical to the expression of the bioluminescence activity than the others. Substitution of alanine or aspartate for αHis44 or substitution of alanine, aspartate, or lysine for αHis45 resulted in reductions of bioluminescence activity by 4–7 orders of magnitude. However, the residual activities and kinetic patterns of all 10 mutated luciferases (Table I) do not show any clear correlations to the size or charge of the side chain of the amino acid substituents. On the other hand, the critical roles of αHis44 and αHis45 to luciferase activity can be rationalized on the basis of known sequences. There are a total of eight sequences reported thus far for luciferase α subunits, and an examination of these sequences reveals three highly conserved stretches corresponding to residues 36–60, 92–118, and 299–323. Among the five conserved histidines, only the αHis44 and αHis45 are within one of these three highly conserved sequence regions. Potentially important structural and/or catalytic functions for the residues 36–60 region in general and αHis44 and αHis45 in particular are thus indicated. In this regard, the thermal stabilities of αH44A and αH45A were somewhat different from the native luciferase (Table I), suggesting a conformational difference between each of the two luciferase mutants and the wild-type enzyme.

An attempt was made to determine the sequence location of the reported single "essential" histidine on the luciferase α subunit which could be preferentially modified by ethoxyformic anhydride resulting in luciferase inactivation (Cousineau & Meighen, 1976). If this still unidentified histidyl residue is among the five mutated in the present study, similar chemical modification of a luciferase variant containing an altered residue replacing this highly reactive histidine is expected to exhibit a slower rate of inactivation. Among the five luciferase variants with each corresponding to the mutation of a different conserved histidyl residue, only the αH44K showed a slower rate of inactivation by ethoxyformic anhydride than that of the wild-type enzyme. Such an observation suggests that the "essential" histidyl residue revealed by previous chemical modifications may be the αHis44.

The light decay rates of all mutated luciferases using octanal, decanal, or dodecanal (with the exceptions of αH44A and αH45A) as a substrate were quite similar to those of the native luciferase (Table I). Since a nonturnover assay was used, the observed light decay rate can be equated to the luciferase catalytic rate constant. It is striking that the total light outputs of the 10 luciferase mutants differed over a range of 6 orders of magnitude but their turnover rates for the bioluminescence reaction were different by about a factor of 10 or less. Since the 10 mutated luciferases were all distinct from the wild-type enzyme with respect to some of the parameters of characterization shown in Tables I and II, the apparent similarity in catalytic rate constants was not due to contaminations of trace amounts of the wild-type luciferase in the mutated enzyme samples. It has been previously demonstrated that the overall luciferase bioluminescence quantum yield is sensitive to multiple competing dark processes including nonproductive oxidation of bound reduced flavin that does not lead to the formation of active intermediate II, diminished production of the excited emitter due to either an enhanced

Scheme I



decay of intermediate II or hindered reactivity between intermediate II and aldehyde, and nonradiative relaxation(s) of the excited emitter (Tu, 1982). We propose that the mutated luciferases tested in this study suffer from various degrees of dark processes such as those identified above. Although their catalytic rate constants for the light-emitting pathway were similar to that of the native luciferase, their total light outputs could be little or drastically decreased depending on the efficiencies of the competing dark reactions.

The molecular basis for the widely different quantum yields of the 10 luciferase variants cannot be identified unambiguously at the present. However, our experimental findings indeed support the existence of enhanced efficiencies for some dark processes for mutated luciferases. The drastically reduced total light outputs for the six luciferase variants with mutations at αHis44 or αHis45 correlated with little or no detectable activities for aldehyde monooxygenation known to be coupled with the light-emitting pathway. For all flavin-dependent external monooxygenases (hydroxylases), the substrate hydroxylation activity (and hence the light-emitting activity for luciferase) is associated with the scission of the O–O bond of II whereas the H_2O_2-producing activity (i.e., the dark reaction for luciferase) is coupled with the scission of the C(4a)–O bond of II (Scheme I). The rate of luciferase II decay to form oxidized flavin and H_2O_2 is a measure of the fissility of the C(4a)–O bond. For the wild-type and mutated luciferases, reduced bioluminescence activities (Table I) are expected to result from a more efficient C(4a)–O bond scission over that of the O–O bond and are, with the exception of αH285A, indeed generally associated with faster rates of the II decay (Table II). Furthermore, as will be discussed below, the low-light-yield αH44A and αH44D apparently were able to form two types of II with the predominant one inactive in light emission.

Luciferase variants with mutations at αHis44 or αHis45 as described in this study showed little or no detectable activities for consuming aldehyde. The question as to whether αHis44 or αHis45 is directly involved in the oxidation of aldehyde was examined. With the wild-type luciferase, the observed primary deuterium kinetic isotope effects of 1.4–1.7 for 1-^{2}H-labeled aliphatic aldehydes on the light decay rate indicate that the abstraction of the C1 hydrogen of aldehyde contributes to the control of the bioluminescence reaction rate but there is at least one more slow step in the subsequent reaction pathway. If either αHis44 or αHis45 functions as a catalyzing base in the abstraction of the C1 hydrogen of aldehyde, the substitution of histidine by an amino acid residue which is a poor base is expected to make this hydrogen abstraction step much more rate-limiting in the overall bioluminescence reaction. In such a case, the deuterium isotope effect of 1-^{2}H-labeled aliphatic aldehydes should be significantly increased above the 1.4–1.7 level. Using luciferases with αHis44 or αHis45 mutated to alanine, aspartate, or lysine, the observed deuterium isotope effects for [1-^{2}H]decanal were all in the range of 1.3–1.7, with none of them being significantly larger than the isotope effect of 1.6 for the wild-type luciferase. On the basis of this ap-

proach, neither αHis44 nor αHis45 appeared to be crucial for the abstraction of the C1 hydrogen of aldehyde.

In accord with the drastically reduced bioluminescence quantum yields, no II was isolatable for αH45D, αH45A, or αH45K as detected by absorption measurements. In contrast, 14–45% yields of intermediate II were detected by absorption spectroscopy using αH44D, αH44A, and αH44K under our experimental conditions (Table III), far higher than what were expected on the basis of bioluminescence measurements. Furthermore, the decay rates of II for αH44D and αH44A as determined by absorption changes were significantly faster than their corresponding decay rates deduced from decreases in the capacity to emit bioluminescence upon secondary additions of aldehyde (Figure 2; Table III). The simplest scheme to account for both the significant yield and the double decay rates of II formed with αH44D or αH44A is that each of these two luciferase variants can generate two forms of II which are not interconvertible and decay through independent pathways. The predominant species was essentially inactive in reacting with aldehyde for bioluminescence and underwent a faster decay as indicated by the absorption measurements. At the same time, a bioluminescence-active species was present in a trace amount with its decay detectable by the activity assay but not by the much less sensitive absorption spectroscopy. The exact difference between the two forms of intermediate II at the molecular level is not certain. At the present, we entertain the possibility that they may be stereoisomers with respect to the chiral 4a–C center.

This surprising finding that αH44A and αH44D can each generate two forms of II with different bioluminescence and decay properties is distinct from some earlier and recent reports. In sharp contrast to our observation, the II formed with the wild-type *V. harveyi* or *V. fischeri* luciferase shows identical decay kinetics when monitored similarly by absorption changes and decreases in bioluminescence capacity (Becvar et al., 1978). Our finding is also different from the earlier observation of a single II for the wild-type *V. harveyi* luciferase which can be photochemically transformed into a species having different fluorescence properties but essentially the same absorption spectrum and bioluminescence activity as those of the original II (Balny & Hastings, 1975; Tu, 1979). Recently, Ahrens et al. (1991) have observed that the decay of II of *V. harveyi* luciferase as determined by absorption spectroscopy is slower than the decay of a low-intensity endogenous light emission from the same sample. This endogenous light was attributed to the presence of a small amount of contaminating aldehyde and was observed, unlike our detection of bioluminescence capacities by secondary aldehyde additions at different times, directly from a II sample without any aldehyde addition. All flavo-hydroxylases, luciferase included, are capable of partitioning between a substrate-monooxygenation reaction and a nonproductive H_2O_2-formation pathway. The observation that αH44D and αH44A each apparently forms a bioluminescence-inactive II and a trace amount of bioluminescence-active II suggests that αHis44 is required for an efficient formation of the latter intermediate. Further investigations of αH44D and αH44A and the two apparently distinct II species may provide additional insights into the control of luciferase activity expression.

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Determination of Rates and Yields of Interchromophore (Folate → Flavin) Energy Transfer and Intermolecular (Flavin → DNA) Electron Transfer in *Escherichia coli* Photolyase by Time-Resolved Fluorescence and Absorption Spectroscopy†

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ABSTRACT: *Escherichia coli* DNA photolyase, which photorepairs cyclobutane pyrimidine dimers, contains two chromophore cofactors, 1,5-dihydroflavin adenine dinucleotide ($FADH_2$) and 5,10-methenyltetrahydrofolate (MTHF). Previous work has shown that MTHF is the primary photoreceptor which transfers energy to the $FADH_2$ cofactor; the $FADH_2$ singlet excited state then repairs the photodimer by electron transfer. In this study, we have determined the rate constants for these photophysical processes by time-resolved fluorescence and absorption spectroscopy. From time-resolved fluorescence, we find that energy transfer from MTHF to $FADH_2$ and FADH° occurs at rates of 4.6×10^9 and 3.0×10^{10} s^{-1}, respectively, and electron transfer from $FADH_2$ to a pyrimidine dimer occurs at a rate of 5.5×10^9 s^{-1}. Using Förster theory for long-range energy transfer and assuming $K^2 = {}^2/_3$, the interchromophore distances were estimated to be 22 Å in the case of the MTHF-$FADH_2$ pair and 21 Å for the MTHF-FADH° pair. Picosecond absorption spectroscopy identified an MTHF single state which decays to yield the first excited singlet state of $FADH_2$. The lifetimes of MTHF and $FADH_2$ singlets and the rates of interchromophore energy transfer, as well as the rate of electron transfer from $FADH_2$ to DNA measured by time-resolved fluorescence, were in excellent agreement with the values obtained by picosecond laser flash photolysis. Similarly, fluorescence or absorption lifetime studies of the folate-depleted enzyme with and without photodimer suggest that $FADH_2$, in its singlet excited state, transfers an electron to the dimer with 89% efficiency. The distance between $FADH_2$ and the photodimer was calculated to be ca. 14 Å.

The *cis-syn*-cyclobutadipyrimidine (pyrimidine dimer) is the major photoproduct produced in DNA by ultraviolet light (Wang, 1976). This form of UV damage is subject to repair by DNA photolyases, enzymes that bind to damaged DNA and split the cyclobutane ring of the dimer by utilizing the energy of near-UV or visible light. The enzyme isolated from *Escherichia coli* contains two chromophores, flavin adenine dinucleotide (Sanar & Sancar, 1984) and 5,10-methenyltetrahydrofolylpolyglutamate (MTHF;[1] Johnson et al., 1988). The flavin cofactor is apparently in the dihydro ($FADH_2$) form in vivo but becomes oxidized to the neutral radical form (FADH°) during purification (Jorns et al., 1984; Payne et al., 1987).

Previous work on the roles of two chromophores has shown that enzyme containing the flavin blue neutral radical is catalytically inert (Payne et al., 1987; Sancar et al., 1987).

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[1] Abbreviations: Pyr<>Pyr, T<>T, and U<>U, cyclobutadipyrimidines of the indicated compositions; *c-s*-T<>T, *cis-syn*-thymine dimer; MTHF, methenyltetrahydrofolate; E-$FADH_2$, E-$FADH_2$-MTHF, etc., photolyase containing the indicated chromophore(s); DTT, dithiothreitol; ϕ_F, ϕ_{eT}, ϕ_{ET}, and ϕ_{spl}, quantum yields for fluorescence, energy transfer, electron transfer, and splitting of cyclobutane ring, respectively.

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