Effects of Mutations of the αHis45 Residue of *Vibrio harveyi* Luciferase on the Yield and Reactivity of the Flavin Peroxide Intermediate†

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ABSTRACT: This work was undertaken to investigate the functional consequences of mutations of the essential αHis45 residue of *Vibrio harveyi* luciferase, especially with respect to the yield and reactivity of the flavin 4a-hydroperoxide intermediate II. A total of 14 luciferase variants, each with a different single-residue replacement for the αHis45, were examined. These variants showed changes, mostly slight, in their light decay rates of the nonturnover luminescence reaction and in their K_m values for decanal and reduced riboflavin 5′-phosphate ($FMNH_2$). All αHis45 mutants, however, showed markedly reduced bioluminescence activities, the magnitude of the reduction ranging from about 300-fold to 6 orders of magnitude. Remarkably, a good correlation was obtained for the wild-type luciferase, 12 αHis45-mutated luciferases, and six additional variants with mutations of other α-subunit histidine residues between the degrees of luminescence activity reduction and the dark decay rates of intermediate II. Such a correlation further indicates that the activation of the O−O bond fission is an important function of the flavin 4a-hydroperoxide intermediate II. Both αH45G and αH45W were found to bind near-stoichiometric amounts of $FMNH_2$. Moreover, each variant catalyzed the oxidation of bound $FMNH_2$ by two mechanisms, with a minor pathway leading to the formation of a luminescence-active intermediate II and a major dark pathway not involving any detectable flavin 4a-hydroperoxide species. This latter pathway mimics that in the normal catalysis by flavooxidases, and its elicitation in luciferase was demonstrated for the first time by single-residue mutations.

Bacterial luciferase is a flavin-dependent monooxygenase (hydroxylase) catalyzing the oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and a long-chain aliphatic aldehyde by molecular oxygen producing FMN, fatty acid, water, and visible luminescence ($\lambda_{max} \sim 490$ nm) with a quantum yield of 0.1−0.2. The $\alpha\beta$ luciferase heterodimer has a single $FMNH_2$ substrate site (*1*, *2*), an aldehyde substrate site, and a separate aldehyde inhibitor site (*3*). Individual subunits are catalytically active, but their specific activities are 4−5 orders of magnitude lower than that of the dimeric native luciferase (*4*, *5*). The crystal structure of the *Vibrio harveyi* luciferase apoprotein has been determined (*6*). However, the exact locations of the flavin and aldehyde sites have not yet been established. Several "essential" residues, mostly on the α-subunit, have been identified by chemical modification and mutational studies (see the Discussion in ref *7*, and references therein). Structural alterations of these residues result in a marked reduction of the extent of substrate binding, intermediate stability, or luminescence activity. However, the specific functional roles of these essential residues remain poorly understood.

Four oxygenated flavin species, namely, 4a-hydroperoxyFMNH (intermediate II) (*8*), 4a-peroxyhemiacetalFMNH (intermediate III) (*9*), 4a-hydroxyFMNH radical cation (intermediate $IV^{+\bullet}$) (*10*), and 4a-hydroxyFMNH (intermediate IV) (*11*, *12*), have been shown or proposed to be key intermediates in the luciferase-catalyzed luminescent oxidation of N1-deprotonated reduced FMN (Scheme 1). Many other flavo-monooxygenases have also been shown to involve 4a-hydroperoxyFMNH and 4a-hydroxyFMNH intermediates in their catalytic pathways. However, luciferase is unique in generating the 4a-hydoxyFMNH intermediate in an excited state for luminescence. Among these four oxygenated flavin intermediates of luciferase, 4a-hydroperoxyFMNH intermediate II is the best characterized. We have previously shown that mutations of either the αHis44 or the αHis45 residue of the *V. harveyi* luciferase lead to a reduction in activity of 4−6 orders of magnitude (*13*). Recently, we have demonstrated by the approach of chemical rescue that αHis44 functions as a catalytic base in the luciferase reaction (*7*). Specifically, this catalytic base participates in the reaction at a step after the formation of intermediate II. Following our long-term interest in the structure−function relationship of luciferase, this work was

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[1] Abbreviations: $FMNH_2$, reduced riboflavin 5′-phosphate; q, quantum.

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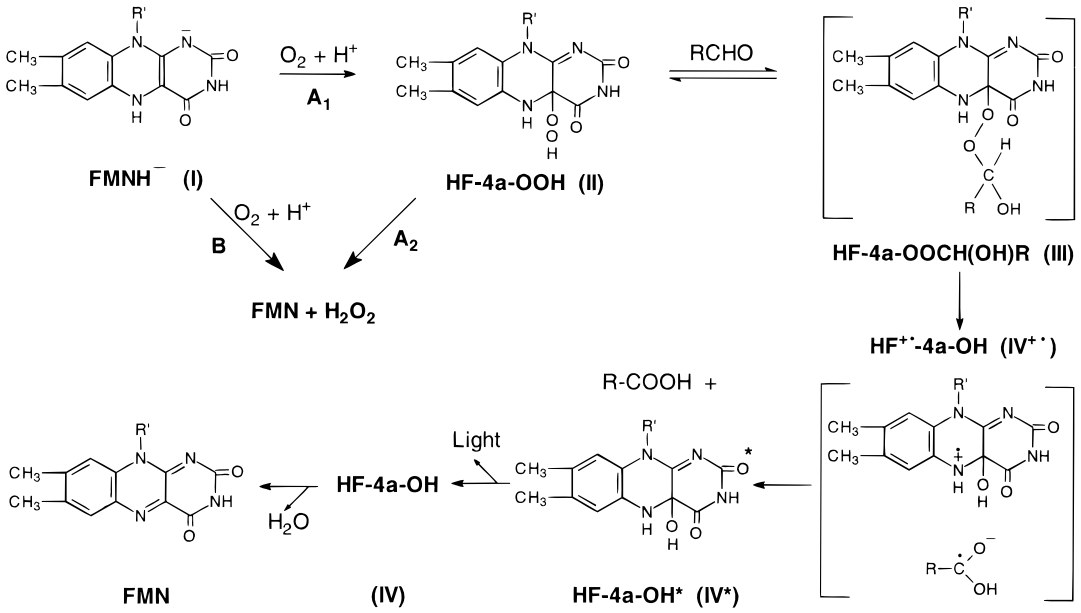
Scheme 1



carried out to investigate the functional consequences of mutations of the αHis45 residue of the *V. harveyi* luciferase, especially with respect to the effects on the yield and reactivity of intermediate II. Among other findings, two αHis45-mutated luciferase species were shown to each catalyze the oxidation of bound $FMNH_2$ by two pathways. A minor pathway (step A_1 of Scheme 1) generated the expected luminescence-active intermediate II, whereas a major and new pathway (step B of Scheme 1) was elicited that involved no detectable 4a-hydroperoxyFMNH intermediate. The latter pathway of reduced flavin oxidation mimics that in the normal catalytic reactions of flavooxidases. A good inverse correlation was also obtained between the luminescence intensity and the dark decay rate of intermediate II for the wild-type luciferase and variants mutated at the αHis45 or each of several other conserved αHis residues.

MATERIALS AND METHODS

Materials. Site-directed mutagenesis and DNA sequencing kits were purchased from United States Biochemical Corp. [α-^{35}S]dATP was a product of Du Pont. Wizard Plus Minipreps DNA purification systems, restriction endonucleases, and T_4 DNA ligase were purchased from Promega. X-Gal (isopropyl thiogalactoside) and dithiothreitol were from Fisher. DEAE-Cellulose DE-52 was a product of Whatman. FMN and decanal were from Sigma and Aldrich, respectively. [1-^{2}H]Decanal was obtained as described previously (*13*). Unless stated otherwise, 0.05 M phosphate (pH 7.0) was used as the standard buffer.

Luciferase Mutations. Single-stranded DNA from recombinant phage MTX1 (*14*) was used as a template to obtain variants of *V. harveyi* luciferase each bearing a single mutated amino acid residue at the αHis45 site. The wild-type codon CAC was mutated to CAG, TCC, GAC, AGA, CCT, ATG, TGT, TGG, GGG, AAA, TAC, CTC, GCC, and TTT for glutamine (αH45Q), serine (αH45S), aspartate (αH45D), arginine (αH45R), proline (αH45P), methionine (αH45M), cysteine (αH45C), tryptophan (αH45W), glycine (αH45G), lysine (αH45K), tyrosine (αH45Y), leucine (αH45L), alanine (αH45A), and phenylalanine (αH45F), respectively, with the corresponding luciferase variants specified in parentheses. A ~2.5 kb fragment containing mutated *luxAB* was obtained by *Hin*dIII−*Kpn*I digestion of the corresponding replicative form DNA of mutated MTX1. Each fragment was then subcloned into a *Hin*dIII−*Kpn*I-digested pUC19 vector to generate the desired recombinant plasmid. A recombinant plasmid containing the native *luxAB* was similarly constructed. All mutations were verified by dideoxy sequencing analysis of double-stranded DNA (*15*).

Luciferase Purification and Activity Assay. The expression of wild-type and mutated luciferases in *Escherichia coli* JM107 host cells harboring a desired recombinant plasmid was carried out as described previously (*13*). The expressed wild-type and mutated luciferases were purified (*16*) to purities of 60−93% on the basis of sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Bioluminescence activities were usually determined in the standard buffer at 23 °C by the dithionite assay (*16*) and/or the Cu(I) assay (*17*) with modification (*3*). Upon the initiation of these two nonturnover assays, the luminescence intensity rapidly rose to a peak within a few seconds. The light intensity subsequently decreased following a first-order process, with a rate constant referred to as k_{light} in min^{-1}, for about the first 2 orders of magnitude of light decay. Luciferase activity was determined as the initial peak luminescence intensity (I_o) with the observed arbitrary light unit converted to quanta per second (q s^{-1}) using a liquid light standard (*18*) for calibration. In addition, the total quantum output (Q in quanta) was also determined by integrating the area under the entire light decay curve. Values of maximal activity and K_m for a given substrate were determined with double-reciprocal plots of activity versus the concentration of the substrate in question using a saturating level of the other cosubstrate. In some cases, luciferase activities were determined using saturated [1-^{2}H]decanal or the control decanal. The deuterium isotope effects ($^D k$) were determined as the ratios of the light decay rate (k_{light}) obtained using the control decanal to that obtained using the deuterated decanal.

Aldehyde Consumption Assay. A decanal standard curve was constructed as described previously (*13*). One milliliter of the standard buffer containing 50 μM $FMNH_2$, reduced by the Cu(I) method, was injected into the same volume of standard buffer containing a limiting level of decanal (1 μM) and an excess amount of a desired luciferase species (14−30 μM). After the bioluminescence reaction reached completion, 20 μL aliquots of the sample were used for the determination of the amounts of remaining aldehyde as described previously (*13*). Control samples were similarly prepared and analyzed for all luciferases except that the Cu(I)-reduced $FMNH_2$ was first oxidized by O_2 before injection. Each sample was tested in triplicate.

Stability of Flavin 4a-Hydroperoxide Intermediates II. Intermediate II was first formed by reacting a desired luciferase species with 75 μM $FMNH_2$ (reduced by dithionite) under aerobic conditions. Aliquots (50 μL) were withdrawn after different incubation times at 23 °C, and each was injected into 1 mL of the standard buffer containing a saturating level of decanal to initiate the bioluminescence reaction. The decay rate of flavin 4a-hydroperoxide intermediate II (k_{II}) was determined by plotting $\log(I/I_o)$ versus time in which I_o and I are the emission peak intensities obtained upon mixing decanal with the intermediate II sample at time zero and a given time point, respectively, after its formation.

Binding of $FMNH_2$ by Luciferases. The extent of $FMNH_2$ binding by luciferase was determined by gel filtration (*19*). A 0.5 mL sample containing 10 mM phosphate (pH 7.0), a desired luciferase at 40 μM, and 40 μM $FMNH_2$ (reduced by dithionite) was applied to a 1 cm × 25 cm Sephadex G-25 column pre-equilibrated with 10 mM phosphate (pH 7.0) containing 40 μM $FMNH_2$ (obtained by photochemical reduction), 10 mM glucose, 10 mM EDTA, and 3 μg/mL glucose oxidase and catalase. Enzyme samples were eluted by the same buffer used for column equilibration. The total amount of $FMNH_2$ in each 1 mL fraction was determined by A_{445} after oxidation.

Stopped-Flow Spectrophotometry. The kinetics of the luciferase-catalyzed oxidation of $FMNH_2$ were determined in 0.2 M phosphate buffer (pH 7.0) at 23 °C using a Dionex D-110 stopped-flow spectrophotometer. Solutions containing 30 μM FMN, 10 mM EDTA, and a desired luciferase species at 250 μM were made anaerobic by 10 cycles of evacuation and equilibration with nitrogen gas. After transfer to a stopped-flow syringe, the flavin in the sample was reduced by irradiation using a long-wavelength UV lamp. Upon the solution being mixed with an equal volume of the same buffer containing 0.25 mM O_2 from the second syringe, changes in A_{450} and A_{382} were recorded as a function of time. Each experiment was repeated three to five times, and the results were averaged for data analysis. The kinetics of autoxidation of $FMNH_2$ were determined following the same procedures except that luciferase was excluded from the flavin solution.

RESULTS

General Catalytic Properties of αHis45 Variants. The αHis45 residue, previously mutated to alanine, aspartate, and lysine (*13*), was mutated to 11 additional residues. In comparison with the wild-type luciferase, all the αHis45 mutants exhibited only minor changes in their light decay rates. Consequently, their relative activities in terms of the peak luminescence intensity (I_o) were quite similar to those based on the total quantum output (Q). All αHis45-mutated luciferases showed markedly reduced bioluminescence activities, confirming the essentiality of this residue, which was reported previously (*13*). The exact nature of the mutation greatly affected the activity, ranging from an activity reduction of about 300-fold for αH45Q to a reduction that was close to 7 orders of magnitude for αH45F. With regard to the K_m for decanal, mutations of the αHis45 resulted in a 7-fold decrease for αH45C, insignificant or relatively small changes for most mutants, and about 4−6-fold increases for αH45M, αH45Q, and αH45L. Most of the αHis45 mutants also showed no change or only little change in their K_m for $FMNH_2$. However, the K_m for $FMNH_2$ was increased by about 90-, 200-, and 300-fold for αH45C, αH45Q, and αH45S, respectively. Interestingly, among all mutated luciferases, αH45Q and αH45S had the highest K_m for $FMNH_2$, but they were also among the most active ones in terms of luminescence activity.

Table 1: Bioluminescence Activities and Kinetic Properties of Wild-Type and αHis45-Mutated Luciferases[a]

luciferase	I_o/I_{oWT}	Q/Q_{WT}	k_{light} (min^{-1})	K_m (μM)		k_{II}[b] (min^{-1})
				$FMNH_2$	decanal	
wild-type	1.0	1.0	23.6	0.5	2.8	3.0
αH45Q	3.3×10^{-3}	2.6×10^{-3}	18.4	101.2	13.1	5.8
αH45S	3.3×10^{-4}	2.4×10^{-4}	25.9	156.1	6.8	8.5
αH45D	2.5×10^{-4}	2.7×10^{-4}	20.9	2.4	2.9	7.3
αH45R	1.6×10^{-4}	1.3×10^{-4}	24.1	0.9	3.6	7.6
αH45P	1.4×10^{-4}	1.0×10^{-4}	23.9	0.5	4.8	8.2
αH45M	1.1×10^{-4}	6.6×10^{-5}	27.6	0.5	10.6	8.1
αH45C	5.7×10^{-5}	4.2×10^{-5}	22.7	43.1	0.4	10.8
αH45W	4.7×10^{-5}	2.6×10^{-5}	19.5	0.7	4.1	10.1
αH45G	3.9×10^{-5}	2.3×10^{-5}	25.1	0.4	2.0	10.5
αH45K	1.8×10^{-5}	1.7×10^{-5}	21.6	3.6	2.4	11.7
αH45Y	6.6×10^{-6}	6.3×10^{-6}	24.4	0.6	1.8	14.4
αH45L	3.7×10^{-6}	2.6×10^{-6}	24.1	0.5	16.5	13.2
αH45A	6.6×10^{-7}	7.1×10^{-7}	20.1	1.8	8.4	
αH45F	1.5×10^{-7}	1.5×10^{-7}	23.2	1.3	5.0	

[a] Determined at 23 °C and pH 7.0 using decanal as the aldehyde substrate. I_o/I_{oWT} and Q/Q_{WT} were normalized to compensate for differences in the purity of luciferase samples. [b] Determined by following the decreases in the luminescence activity.

Stability of 4a-HydroperoxyFMNH Intermediates. In addition to reacting with aldehyde in the light-emitting monooxygenation pathway, intermediate II also undergoes a dark decay to generate FMN and H_2O_2. The decay rates of intermediate II (k_{II}) were determined at 23 °C for the wild-type luciferase and 12 of the αHis45 variants (Table 1) but not for αH45F or αH45A, whose extremely low bioluminescence activities posed technical difficulties for the intended measurement. Intermediate II of the wild-type luciferase was more stable than those derived from the luciferase variants. Using data from Table 1 for the wild-type luciferase and 12 luciferase variants, a close inverse correlation was obtained between the relative luminescence activity and the intermediate II dark decay rate constant k_{II} (Figure 1). Moreover, such a correlation is not limited to mutations of the αHis45 residue. When the published data of I_o/I_{oWT} and k_{II} for αH44A, αH44D, αH44K, αH82D, αH82K, αH224A, and αH285A (*13*) were included in such

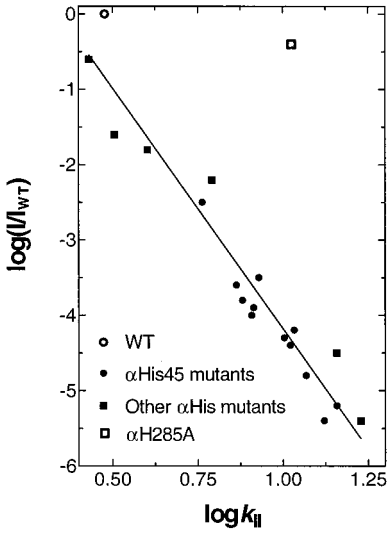


FIGURE 1: Correlation of the relative luminescence activities with the dark decay rate constants of the flavin 4a-hydroperoxide intermediates formed by the wild-type *V. harveyi* luciferase and variants with single-residue mutations of various conserved histidines. Data for I_o/I_{oWT} and k_{II} for the wild-type luciferase (○) and all the αHis45-mutated luciferases (●) are from Table 1, and those for αH285A (□) and αH44A, αH44D, αH44K, αH82D, αH82K, and αH224A (■) are taken from the report by Xin et al. (*13*).

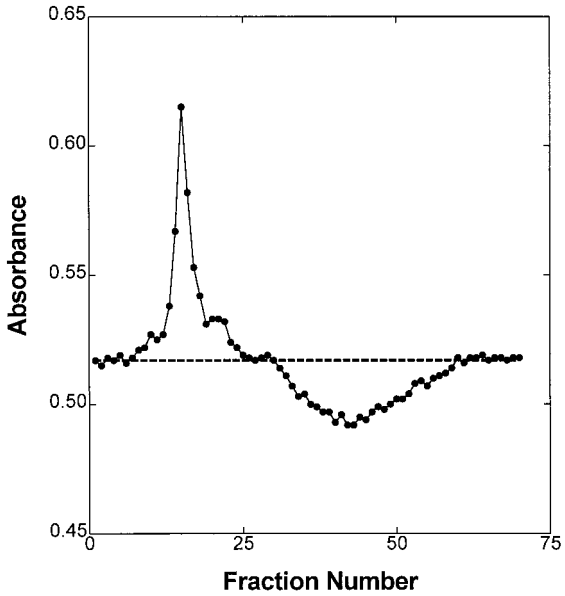


FIGURE 2: Binding of $FMNH_2$ by αH45W luciferase as shown by gel filtration. Experimental conditions are described in Materials and Methods. The total amount of $FMNH_2$ in each 1 mL fraction was determined by A_{445} after oxidation. The dashed line indicates the A_{445} level of the equilibration solution.

an analysis, the same correlation remained applicable for all mutants except αH285A (Figure 1).

[1-^{2}H]Aldehyde Deuterium Isotope Effects. The bioluminescence of wild-type luciferase is coupled with the oxidation of aldehyde to the corresponding carboxylic acid. Using decanal deuterated at the C1 position as a substrate, the bioluminescence reaction catalyzed by the wild-type luciferase shows a kinetic isotopic effect of 1.4−1.7 on the basis of either the peak intensity or the light decay rate (*9*, *20*−*22*). Previously, Xin et al. (*13*) showed that the ^Dk values of αH45D, αH45K, and αH45A for [1-^{2}H]decanal were similar to that of the wild-type luciferase. The deuterium kinetic isotope effects of [1-^{2}H]decanal on the other 11 αHis45 mutants in Table 1 were also tested, and were all found to have ^Dk values of 1.4−1.7.

Aldehyde Consumption. αH45D, αH45K, and αH45A were previously found to lack any significant aldehyde consumption ability, while almost 100% of the aldehyde can be consumed by the wild-type luciferase under the same conditions (*13*). αH45R, αH45W, αH45G, αH45Y, and αH45F were tested in this study for their ability to oxidize the aldehyde substrate. The control sample for the nonenzymatic oxidation of $FMNH_2$ exhibited a background of $4 \pm 1\%$ aldehyde disappearance. In comparison, the αH45R, αH45W, αH45Y, and αH45F showed no significant additional consumption of aldehyde (ranging from 4 ± 2 to $6 \pm 4\%$ without background subtraction), whereas αH45G was associated with, at best, a marginally detectable level of aldehyde consumption ($9 \pm 4\%$ without background subtraction).

Binding of $FMNH_2$ by αH45W and αH45G. Using 40 μM αH45W and $FMNH_2$ in the starting sample for molecular sieve chromatography at 23 °C, the molar ratio of bound $FMNH_2$ to total αH45W was quantified to be 0.83 on the basis of the areas of the leading peak and the trailing trough in Figure 2. When the experiment was repeated with αH45G, a molar ratio of 0.76 was found for bound $FMNH_2$ to total αH45G.

Stopped-Flow Measurements of $FMNH_2$ Oxidation. On the basis of low but detectable bioluminescence activities, mutants tested in this work were all active in generating essential 4a-hydroperoxyFMNH intermediate II. Their dark decay rates could be determined by the delayed aldehyde addition assay (Table 1). Similar to the wild-type luciferase, the intermediate II species formed by these mutants were markedly stabilized by low temperatures and/or complex formation with dodecanol (data not shown). Using a rapid molecular sieve method at 4 °C in the presence of a saturating dodecanol level, wild-type intermediate II can be isolated in near-stoichiometric amounts (*23*). However, no detectable II was obtained with the same method when αH45A, αH45D, or αH45K were used (*13*). In this study, the time courses of $FMNH_2$ oxidation catalyzed by αH45W and αH45G were investigated by stopped-flow spectroscopy and compared with those of autoxidation and catalytic oxidation by wild-type luciferase. αH45W and αH45G were chosen for such an investigation because their K_m values for $FMNH_2$ were similar to that of the wild-type luciferase (Table 1) and their near-stoichiometric binding of $FMNH_2$ (Figure 2). The kinetics of autoxidation (*24*) and luciferase-catalyzed oxidation (*25*) of $FMNH_2$ are complex. The stopped-flow experiments in this study were aimed at determining the intermediacy of 4a-hydroperoxyFMNH in the reactions catalyzed by αH45W and αH45G. Detailed analyses of reaction steps and rate constants were not intended.

FMN shares an absorption isosbestic point at 382 nm with luciferase intermediate II (*13*). At this wavelength, the level of absorption by FMN and intermediate II is about twice that by $FMNH_2$. At 445 nm, both $FMNH_2$ and intermediate II have very weak absorption levels whereas FMN absorbs strongly. Hence, the initial formation of intermediate II in the oxidation of $FMNH_2$ should be accompanied by a significant rise in A_{382} with little increase in A_{445}. The

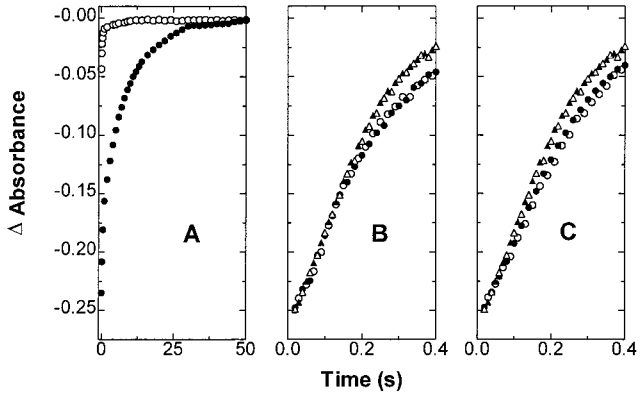


FIGURE 3: Time courses of $FMNH_2$ autoxidation and the oxidation catalyzed by wild-type luciferase, αH45W, and αH45G. For panel A, an anaerobic 0.2 M phosphate buffer (pH 7.0) containing 30 μM $FMNH_2$, 10 mM EDTA, and 0.25 mM wild-type luciferase was mixed in a stopped-flow spectrophotometer at 23 °C with an equal volume of the same buffer equilibrated with air. The oxidation of $FMNH_2$ was followed by monitoring changes in ΔA_{382} (○) and ΔA_{445} (●) using the completely oxidized final solution as the reference. For panels B and C, the time courses of ΔA_{382} (○) and ΔA_{445} (●) are shown for experiments using αH45W (B) or αH45G (C) in place of the wild-type luciferase under otherwise identical conditions. The autoxidation of $FMNH_2$ was also assessed by mixing the same reduced flavin solution in the absence of luciferase with air-equilibrated buffer, and the time courses of ΔA_{382} (△) and ΔA_{445} (▲) are shown in both panels B and C. For a direct graphic comparison, ΔA signals in panel B were multiplied by 1.85 (△), 1.21 (●), or 1.64 (○), and signals in panel C were normalized similarly.

subsequent decay of intermediate II to FMN should be associated with a sizable increase in A_{445} with no significant change in A_{382}. These characteristics were indeed observed in the wild-type luciferase-catalyzed oxidation of $FMNH_2$ (Figure 3A). The A_{382} showed a fast rise and remained at a plateau, whereas most of the rise in A_{445} occurred more slowly after the A_{382} plateau was reached. On the other hand, if flavin 4a-hydroperoxide is not involved or accumulated to any significant extent in the $FMNH_2$ oxidation, the time course for the increase in A_{445} should parallel that of A_{382}. Such characteristics were observed in the autoxidation of $FMNH_2$ (Figure 3B,C). For αH45W (Figure 3B) and αH45G (Figure 3C), the kinetics for the signal changes in A_{445} and A_{382} were essentially the same and were both slightly slower than those of the autoxidation. Therefore, the major pathway for the αH45W- and αH45G-catalyzed oxidation of $FMNH_2$ apparently did not involve any detectable flavin 4a-hydroperoxide intermediate. The oxidation of $FMNH_2$ to the FMN final product in the wild-type luciferase-catalyzed reaction was markedly slower than those by autoxidation or by the αH45W- or αH45G-catalyzed reaction.

DISCUSSION

The αHis45 mutants examined in this study exhibited drastically reduced luminescence activities but only slightly different light decay rates. Since these variants were distinct from the wild-type enzyme with respect to at least one of the three parameters of k_{II} and the K_m for $FMNH_2$ and decanal (Table 1), their low levels of luminescence activity and the wild-type-like light decay rates were not due to contamination by trace amounts of the native luciferase.

The luminescence activities of these variants were highly dependent on the nature of the mutation but showed no obvious correlation with the size or charge of the residue replacing the native αHis45. However, for the wild-type luciferase and 19 mutants, a good inverse correlation was obtained between the luminescence intensity and the intermediate II dark decay rate for all except the αH285A mutant (Figure 1). The generation of the excited 4a-hydroxyFMNH emitter (IV*) originates from the fission of the O−O bond of III. Intermediate II also undergoes a competing dark decay via the scission of the C4a−O bond (step A_2 of Scheme 1). While the O−O bond strength of intermediate III has not been directly determined, earlier chemical studies show that the O−O bond of the flavin 4a-hydroperoxide is the most activated among a number of peroxides (*26*). Moreover, the free energy of the O−O bond fission of flavin 4a-hydroperoxide is ∼15 kcal/mol in comparison with ∼21 kcal/mol for the less efficient C4a−O bond homolysis (*26*, *27*). Therefore, both earlier chemical studies and our enzymatic investigation (Figure 1) indicate that the activation of the O−O bond in II and, by inference, III for fission over the scission of the C4a−O bond is an important function of these flavin intermediates in the luciferase reaction.

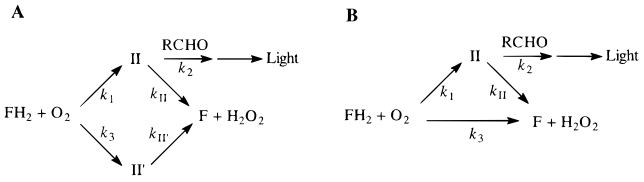
According to Scheme 1, a minimal relationship can be specified

$$\Phi_o = Y_{II}Y_{III}Y_{IV^+\bullet}Y_{IV^*}\Phi_{IV^*} \quad (1)$$

where Φ_o is the overall quantum yield of the luminescence reaction, each *Y* refers to the yield of a particular intermediate (indicated by the subscript) on the basis of its immediate precursor intermediate, and Φ_{IV^*} is the intrinsic emission quantum yield of excited intermediate IV*. For the mutants shown in Figure 1, increases in the value of k_{II} can reduce Y_{III}. However, the values of k_{II} vary by a factor of about 6, whereas the range of luminescence reduction covers about 5 orders of magnitude. Obviously, mutations have other functional consequences with respect to Y_{II}, $Y_{IV^+\bullet}$, Y_{IV^*}, and/or Φ_{IV^*}. As will be described in detail below, the yields of II for five αHis45 mutants were found to be quite low. In contrast, despite reductions in their luminescence activity of 2−6 orders of magnitude, αH44A, αH44D, and αH44K generated yields of II of at least 14−45% (*13*). αH45R, αH45W, αH45Y, αH45F, and αH45G were tested, and none was found to consume any significant amount of aldehyde in association with their oxidation of $FMNH_2$. Beyond this, this study does not allow a definitive quantification of Y_{III}, $Y_{IV^+\bullet}$, Y_{IV^*}, or Φ_{IV^*}. It is possible, however, for mutations to have significant and diverse effects on each of these terms. In this connection, the good inverse correlation between the luminescence activity and a single parameter k_{II} is remarkable in that k_{II} apparently serves as a universal (inverse) index of Φ_o for the wild-type luciferase and all but one of these αHis-mutated variants.

To account for the 5 order of magnitude variations in luminescence, the direct mutational effect on k_{II} (hence Y_{III}) must be amplified through effects on other Φ_o-regulating factors in eq 1. We hypothesize that mutations described herein could result in structural perturbation of the luciferase flavin site, making it less rigid and/or more solvent accessible. The C4a−O bond cleavage of flavin 4a-hydroperoxide models can be effectively retarded in hydrophobic media (*28*). The binding of a hydrophobic long-chain alcohol inhibitor (e.g., dodecanol) to the luciferase active site also

Scheme 2



markedly stabilizes intermediate II (*23*). Consequently, enhanced exposure of the flavin site by mutation could result in increased nonproductive C4a−O bond scission of II and, possibly, III. Moreover, the Φ_{IV^*} (0.16) of the wild-type luciferase emitter (*29*) is 4 orders of magnitude higher than the fluorescence quantum yield ($\leq 3 \times 10^{-5}$) of an N5-ethyl-4a-hydroxyflavin model (*30*), but the latter can be enhanced by freezing to an unspecified degree (*31*). Therefore, Φ_{IV^*} must be highly sensitive to the nature of IV* binding by luciferase. Mutations could result in critical perturbations of the microenvironment of the luciferase flavin site and, hence, markedly and diversely affect the value of Φ_{IV^*} potentially by up to 4 orders of magnitude.

The mechanism of $FMNH_2$ oxidation and the yield of II were examined for αH45G and αH45W. For the wild-type luciferase (Figure 3A), the formation of II was shown by the fast rise to an A_{382} plateau with little increase in A_{445} within the first second. The subsequent decay of II to FMN over the course of ∼50 s was evident from the slow and marked increase in A_{445} accompanied by only trivial changes in A_{382}. On the other hand, the nonenzymatic oxidation of $FMNH_2$ is autocatalytic (*24*), involving only a trace amount of the flavin 4a-hydroperoxide intermediate (*32*−*34*). Consequently, the reaction showed the same time courses for ΔA_{382} and ΔA_{445} (Figure 3B,C). Time courses for ΔA_{382} and ΔA_{445} were also essentially identical for the $FMNH_2$ oxidation in the presence of 125 μM αH45W (Figure 3B) or αH45G (Figure 3C). Using 40 μM αH45W or αH45G for molecular sieve chromatography, the binding of ∼0.8 mol of $FMNH_2$ per mole of luciferase was detected (Figure 2). Upon the solutions being mixed in the stopped-flow experiments, the samples contained 15 μM $FMNH_2$ and a concentration (125 μM) of αH45W or αH45G much higher than that in the molecular sieve experiments. Most of the $FMNH_2$ used in these stopped-flow experiments must be bound by αH45W or αH45G. Therefore, the major pathways for the αH45W- and αH45G-catalyzed oxidation of $FMNH_2$ did not involve any detectable flavin 4a-hydroperoxide species.

It should be noted that a luminescence-active intermediate II can be formed by all the luciferase variants shown in Table 1. Since the major pathway of $FMNH_2$ oxidation by αH45G and αH45W would reach completion in about 1 s (Figure 3), it is apparent that the luminescence-active II derived from αH45G and αH45W (half-lives of ∼4 s, Table 1) must be formed by a different and minor pathway. Although the FMN formation by this major pathway was much faster than the corresponding dark decay of the luminescence-active II (Table 1), the former process was actually slightly slower than the autoxidation (Figure 3) and, hence, should not require any special new catalytic driving force to achieve such a rate of $FMNH_2$ oxidation.

Two schemes (Scheme 2A,B) can be proposed for the αH45W- and αH45G-catalyzed $FMNH_2$ oxidation. Common to both schemes is the fact that αH45G and αH45W can each catalyze a minor pathway leading to the formation of the luminescence-active II (at k_1) which either undergoes a dark decay (at k_{II}) or reacts with aldehyde (at k_2) to complete the light-emitting reaction. However, these two schemes differ in their major pathways of $FMNH_2$ oxidation. In Scheme 2A, flavin 4a-hydroperoxide is formed (at k_3) by both mutants as an obligatory intermediate but decays too fast for stopped-flow detection. The putative intermediate II (designated II′) in this major pathway must be distinct from the luminescence-active II in that the former lacks luminescence activity and must decay very fast (at $k_{II'}$) with a lifetime of no more than a few milliseconds. Possibly, the luminescence-active II and inactive II′ are stereoisomers at the chiral C4a site (*13*). The native luciferase active site allows only the attack of the flavin C4a site by oxygen from the correct direction in forming luminescence-active II, but conceivably, mutational perturbations of the active site could allow the formation of the active and inactive isomers with various yields. In Scheme 2B, the major pathway of FMN formation (at k_3) does not involve II and, as such, no longer follows the flavohydroxylase mechanism. Instead, it mimics the reduced flavin oxidation in normal catalysis by flavooxidases.

In chemical studies, the generation of flavin 4a-hydroperoxide (HF-4a-OO^- or HF-4a-OOH) from reduced flavin (FH^-) and O_2 involves an initial rate-limiting formation of a caged pair of neutral flavin semiquinone ($FH^\bullet$) and superoxide $O_2^{-\bullet}$ (*33*, *34*). The initial flavin semiquinone and superoxide pair are further suggested to be a paramagnetic complex which must undergo a poorly understood spin reversion to allow C4a−O covalent bond formation (*35*). Moreover, the lack of formation of flavin 4a-hydroperoxide by flavooxidases has been proposed to result from (a) the formation of anionic flavin semiquinone ($F^{-\bullet}$) which repels the $O_2^{-\bullet}$ or (b) the inability of flavooxidase to facilitate the spin inversion of the initial paramagnetic complex (*35*). These mechanistic possibilities could be applicable to the major pathway of reduced flavin oxidation by αH45G and αH45W as shown in Scheme 2B. However, the underlying principle which regulates the mechanism of reduced flavin oxidation still remains poorly defined for flavohydroxylases and flavooxidases in general and, in this case, for αH45G and αH45W.

Regardless of mechanistic details, αH45G and αH45W are the first examples of luciferase variants in which a major pathway of $FMNH_2$ oxidation involving no detectable flavin 4a-hydroperoxide intermediate was elicited. Several other luciferase variants also showed two pathways for $FMNH_2$ oxidation. Similar to αH45G and αH45W, these mutants generate small amounts of a luminescence-active II by a minor pathway. However, they differ from αH45G and αH45W in that a luminescence-inactive II can be formed in significant yields, allowing its isolation (*13*) and/or spectral characterization (*13*, *25*). This latter class of variants includes the subgroup of αH45A, αH45D, and αH45K (*13*) and, on the basis of distinct decay kinetics of two species of II (*14*, *36*), the subgroup of αC106A, αC106S, and αC106V (*25*).

The [1-^{2}H]decanal deuterium isotope effect of ^Dk (1.4−1.7) found for 11 αHis45 luciferase variants is quite similar to that reported for the wild-type luciferase (*9*, *20*−*22*) and αH44A, αH44D, and αH44K (*13*). If αHis45 functions in the abstraction of the C1−H of the aldehyde, its mutation to a functionally nonequivalent residue should significantly

reduce the rate of this isotope-sensitive step. Consequently, the expression of the deuterium isotope effect should be enhanced. However, our deuterium isotope study did not support such a function for αHis45.

Flavin 4a-hydroperoxide has never been identified as an intermediate in the normal catalytic pathway of any flavooxidase. However, glucose-bound neutral flavosemiquinone and superoxide generated by pulse radiolysis can react to form flavin 4a-hydroperoxide (*37*). While flavin 4a-hydroperoxide is a common intermediate for flavohydroxylases, it is apparently not formed in the oxidation of reduced *p*-hydroxybenzoate hydroxylase in the absence of the aromatic substrate (*37*). When these earlier findings are considered together with this study on αH45G and αH45W, it is clear that the intermediacy of flavin 4a-hydroperoxide is not an absolute criterion which distinguishes flavohydroxylases from flavooxidases.

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