Probing the Functionalities of αGlu328 and αAla74 of *Vibrio harveyi* Luciferase by Site-Directed Mutagenesis and Chemical Rescue†

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ABSTRACT: This work aimed at identifying essential residues on the α subunit of *Vibrio harveyi* luciferase and elucidating their functional roles. Four conserved α-subunit residues at the proposed luciferase active site were initially mutated to Ala. Screening of the in vivo bioluminescence of cells expressing these mutated luciferases allowed the work to focus on αGlu328 for additional mutations to Phe, Leu, Gln, His, and Asp. *V. harveyi* luciferase is known to contain, at the same proposed active site, an unusual *cis*-peptide linkage between αAla74 and αAla75. To explore the structure−function relationship, luciferase variants αA74F and αA74G were constructed. The six αGlu328-mutated and the two αAla74-mutated luciferase variants were purified and characterized with respect to V_{max}, Michaelis constants, light and dark decays, quantum yield, and, for αE328F and αA74F, yield of the 4a-hydroperoxyFMN intermediate and the ability to oxidize aldehyde substrate. Results indicated that the structural integrities of both αGlu328 and αAla74 were essential to luciferase bioluminescence activity. Moreover, the essentiality of αGlu328 was linked to the acidic nature of its side chain. The low activity of αE328A was sensitive to chemical rescue by sodium acetate, an effect that was not reproduced by phosphate. The efficiency of activity rescue by acetate progressively increased at lower pH in the range from 6.0 to 8.0, supporting the interpretation of αGlu328 as a catalytic general acid. The rescuing effect of acetate was on a reaction step after the formation of the 4a-hydroperoxyFMN intermediate. The exact catalytic function of αGlu328 is unclear, but possibilities are discussed.

Bacterial luciferase catalyzes the oxidation of reduced flavin 5′-phosphate ($FMNH_2$)[1] and a long-chain aliphatic aldehyde by molecular oxygen to generate FMN, carboxylic acid, water, and bioluminescence. Key steps of the luciferase reaction include the oxidation of deprotonated $FMNH^-$ (intermediate I) to form the 4a-hydroperoxyFMN intermediate II, reaction of II with aldehyde to generate 4a-peroxyhemiacetalFMN intermediate III, the decay of III to form carboxylic acid and the excited 4a-hydroxyFMN emitter (intermediate IV*), the relaxation of IV* for bioluminescence, the return to ground-state IV, and the breakdown of IV to generate water and FMN (*1*, *2*). Among all known flavin-dependent monooxygenases (or hydroxylases), bacterial luciferase is unique in its light-emitting activity, slow turnover rate, and, hence, unusually stable intermediates (half-life in seconds or tens of seconds at 23 °C). Moreover, although the α-carbon main chains of the *Vibrio harveyi* luciferase α and *β* subunits are nearly superimposable in their three-dimensional structures (*3*, *4*), the heterodimeric luciferase has only a single active site (*5*, *6*), for which the atomic structure has not yet been determined. In comparison with other flavo-monooxygenases, bacterial luciferase provides special advantages as well as many intriguing challenges for structural and mechanistic studies.

Earlier hybridization studies using chemically (*7*) or mutationally (*8*) altered subunits indicate that the luciferase active site is primarily, or even completely, located in the α subunit. The general location of the *V. harveyi* luciferase active site in a cleft of the α subunit has been postulated (*3*, *4*), and this proposed site is consistent with a computational model of the flavin substrate site (*9*). Moreover, a growing body of site-directed mutagenesis studies has identified a number of essential residues in the postulated active site pocket, thereby providing a strong support to the original postulate. These essential residues on the α subunit include His45 for the formation of intermediate II (*10*, *11*), Arg107 (*3*, *12*) and Trp250 (*13*) for $FMNH_2$ and, in the latter case, aldehyde binding, Gly275 (*14*) for a critical flexibility of a unstructured loop as a possible active pocket gate (*15*), and Phe261 (*14*) for maintaining an essential hydrophobic active site environment. The identification of these essential residues was primarily based on the adverse effects of mutations on various aspects of luciferase activity rather than a molecular interpretation of their specific catalytic functions. In one case, a catalytic function as a general base has been established for the conserved αHis44 by chemical rescue studies (*16*). In general, much remains to be elucidated with respect to a detailed structural makeup of the luciferase active site and specific functionalities for active site essential residues.

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[1] Abbreviations: $FMNH_2$, reduced riboflavin 5′-phosphate; FMN, oxidized riboflavin 5′-phosphate; q, quantum; I_o, peak bioluminescence intensity (in q s^{-1}) of the luciferase-catalyzed single-turnover reaction; ϕ_o, overall quantum yield of the luciferase reaction; ϕ_{IV^*}, emission quantum yield of the excited luciferase emitter; k_L, rate constant of the exponential light decay of the single-turnover bioluminescence reaction; k_D, rate constant for the dark decay of intermediate II in the absence of aldehyde.

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This work was initiated as a continuation of our effort to elucidate critical structural and mechanistic properties of luciferase. On the basis of the selection criteria of conservation in the polypeptide sequences of luciferases from several luminous bacterial strains and the presence in the proposed active site pocket, five residues on the α subunit were targeted for initial mutational studies. Such an approach enabled us to focus on the conserved αGlu328 for additional mutations. The major portion of this study involved characterizations of the properties of six αGlu328-mutated luciferase variants and chemical rescue of the activity of one such mutant by acetate. Results of these studies indicated a catalytic role for αGlu328 as a general acid.

V. harveyi luciferase also contains an unusual non-proline *cis*-peptide between αAla74 and αAla75 (*4*). A possible functional role of this *cis*-peptide has been suggested (*4*, *17*). A close proximity of αAla75 and the *cis*-amide linkage to FMN and the αCys106 residue present at the active site has been reported, but the mutation of αAla75 to glycine resulted in very little luciferase inactivation (*4*, *17*). In this work, we also constructed and characterized αA74G and αA74F mutants. Effects of these mutations on luciferase activity, yields of intermediates, kinetics, and quantum yield of the single-turnover bioluminescence reaction were analyzed. Results indeed indicated an essential role of αAla74 in luciferase reaction.

MATERIALS AND METHODS

Materials. Plasmid DNA was purified with Wizard SV Plus Miniprep kits from Promega. A Quickchange site-directed mutagenesis kit and competent cells of *Escherichia coli* JM101 were purchased from Stratagene. Oligonucleotide primers were made by MWG Biotech. FMN, decanal, and sodium hydrosulfite were all purchased from Sigma. DEAE-cellulose DE52 was from Whatman. Dithiothreitol was from Promega. DEAE-Sepharose resin was purchased from Pharmacia. All decanal solutions were prepared in absolute ethanol shortly before experiments. Unless stated otherwise, 50 mM phosphate (P_i) buffer, pH 7.0, with molar fractions of 0.39 sodium monobase and 0.61 potassium dibase in deionized water was used as the standard buffer.

Luciferase Mutants. *V. harveyi luxAB* genes encoding the luciferase $\alpha\beta$ heterodimer were harbored in a pUC19 vector and expressed in *E. coli* JM101. A site-directed mutagenesis kit from Stratagene was used to perform mutation in *luxA*. The codon AAC of αAsn5 was modified to GCC for alanine (αN5A); also, codons ACT and TAT of αThr9 and αTyr10, respectively, were changed to GCT to obtain αT9A and αY10A. The codon GCA encoding αAla74 was modified to TTT and GGA for phenylalanine (αA74F) and glycine (αA74G), respectively. The codon GAA of αGlu328 was replaced with GCA, TTC, CAA, CAT, GAT, and CTA for alanine (αE328A), phenylalanine (αE328F), glutamine (αE328Q), histidine (αE328H), aspartic acid (αE328D), and leucine (αE328L), respectively. All mutated genes were confirmed by sequencing by the Lone Star Lab. Plasmids harboring the genes for the wild-type luciferase or the mutated luciferase variants were each transformed into *E. coli* JM101 cells following the protocol from Stratagene.

Enzyme Purification and Luciferase Activity Assays. Transformed cells were grown at 37 °C for 16−20 h for the overexpression of wild-type or mutated luciferases following procedures reported previously (*10*). Cells were harvested at $OD_{600} \approx 4$, and the expressed luciferase was purified as previously described (*10*). In all cases, luciferase samples so obtained had ≥85% homogeneities based on patterns of sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The luciferase bioluminescence activity was measured at 23 °C, using a calibrated homemade luminometer, in 50 mM P_i buffer at, unless specified otherwise, pH 7.0 by the single-turnover assay with FMN reduced by dithionite or Cu(I) (*6*, *18*). Decanal was used as the standard cosubstrate. In such a single-turnover assay, the bioluminescence quickly reached a peak within 1−2 s and then decayed exponentially (rate constant designated k_L) for ≥2 orders of magnitude of light decrease. Luciferase activity (υ) was measured as the peak intensity (I_o in q s^{-1}). Michaelis constants for decanal (K_{mA}) and $FMNH_2$ (K_{mF}) and V_{max} were determined from double-reciprocal plots of results obtained from substrate titration experiments. The total quantum produced in a single-turnover reaction was determined by integrating the intensity over the entire time course of the bioluminescence reaction. The overall quantum yield (ϕ_o) is defined as the number of quantum produced per molecule of the limiting component used in the assay. When saturating substrates were used, luciferase was the limiting component in such a single-rurnover reaction.

Dark Decay of 4a-Hydroperoxy-FMN Intermediate II. The formation of intermediate II was initiated by mixing a slight excess amount of sodium hydrosulfite (dithionite) into 1 mL of 50 mM P_i buffer containing 50 μM FMN and luciferase. The initial reduction of FMN was indicated by the bleaching of the yellow color. The subsequent reappearance of yellow color after 3−5 s of mixing under air indicated the completion of the oxidation of the excess free $FMNH_2$. During this time, the luciferase-bound $FMNH_2$ was also converted to intermediate II. After different times of incubation at 23 °C, 100-μL aliquots were withdrawn for activity assays at 23 °C by mixing with a solution containing a saturating level of decanal. The dark decay rate of intermediate II (rate constant designated k_D) was determined by a semilogarithmic plot of observed I_o versus time of incubation of the intermediate II sample.

Stopped-Flow Spectrophotometry. An Olis RSM 1000 stopped-flow spectrophotometer (On-line Instrument Systems) was used to monitor the luciferase-catalyzed oxidation of $FMNH_2$. FMN (50 μM) was prepared in 50 mM P_i buffer containing 10 mM EDTA and was made anaerobic by repeated evacuation and equilibration with nitrogen in an airtight flask. FMN was then reduced by long-wavelength UV and withdrawn into an airtight stopped-flow syringe. An air-saturated 50 mM P_i buffer containing 135 μM luciferase was prepared in a second syringe. The oxidation of $FMNH_2$ at 23 °C was initiated by mixing 120 μL of solution from each syringe. Absorption changes at 445 and 382 nm were monitored over time. For a direct comparison of the time course of ΔA_{382} with that of ΔA_{445}, data observed at each wavelength were normalized as $\Delta A/\Delta A_\infty$, where ΔA and ΔA_∞ are the absorbance increases from time zero to, respectively, a given time point after the mixing and the completion of the overall oxidation.

Aldehyde Consumption. A standard curve of the decanal concentration versus light intensity was first constructed as

described previously (*10*). A decanal consumption reaction was performed by injecting 1 mL of buffer containing 50 μM $FMNH_2$ [reduced by Cu(I)] into an equal volume of buffer containing 1 μM decanal and 20 μM of a desired luciferase species. Upon completion of the luminescence reaction, 20-μL aliquots of the sample were withdrawn, and each was used as the aldehyde source for the determination for unconsumed decanal. A control reaction was conducted similarly but with the Cu(I)-reduced $FMNH_2$ completely reoxidized before injection into the solution containing luciferase and decanal.

Chemical Rescue of α*E328A.* Sodium acetate solutions were prepared in 50 mM P_i buffer at pH between 6.0 and 8.0. For most experiments, a 1-mL buffer containing αE328A and acetate as a rescue reagent was incubated at 23 °C for 10 min. The bioluminescence reaction was initiated by the injection of 1 mL of buffer containing 0.01% decanal and 50 μM $FMNH_2$, reduced by Cu(I) (*18*). Experiments were also carried out to examine whether the recovery of αE328A activity was due to the rescuing effect of acetate on the formation of intermediate II or a step after the II formation. Intermediate II was first formed with αE328A and the dithionite-reduced $FMNH_2$ (*6*) under aerobic conditions in 50 mM P_i, pH 7.0, as described above. The preformed intermediate II was then mixed with an equal volume of the same P_i buffer containing saturating decanal and 0 or 400 mM sodium acetate for measurements of bioluminescence activity. Alternatively, the same level of sodium acetate was included in the initial solution containing luciferase and $FMNH_2$ for intermediate II formation. The bioluminescence activity of the preformed intermediate II was then determined by mixing with a buffer containing saturating decanal but no acetate. The final concentrations of αE328A in all samples were at 0.1 μM.

RESULTS

Site-Directed Mutagenesis. On the basis of conservation in the primary sequences of the α subunit of luciferases from *V. harveyi*, *Kryptophanaron alfredi*, *Vibrio fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, and *Xenohabdus luminescens* (*19*) and their presence near the proposed active site pocket, αAsn5, αThr9, αTyr10, and αGlu328 were targeted for mutational studies to investigate their possible functional roles. Each of these residues was initially mutated to an alanine. αAla74 for the *cis*-peptide bond formation was also mutated to phenylalanine to test the essentiality of this residue or the *cis*-peptide to luciferase activity. Screening of the in vivo luminescence intensities (initiated by the addition of decanal) indicated that cells expressing the luciferase variants αA74F and αE328A were extremely low in emission, whereas cells expressing the wild-type luciferase and all other luciferase variants were apparently similar in emission intensity. Hence, residues αGlu328 and αAla74 were chosen for additional mutations, with the former mutated to phenylalanine, leucine, histidine, glutamine, and aspartate and the latter to glycine.

General Kinetics Properties of α*Glu328 and* α*Ala74 Mutants*. All αGlu328- and αAla74-mutated luciferases were purified for characterization of their general kinetic properties (Table 1). When the wild-type anionic αGlu328 was mutated to a bulky hydrophobic phenylalanine, the V_{max} of αE328F was decreased by 5 orders of magnitude. Substantial but lesser degrees of inactivation (by 2−3 orders of magnitude of reductions of V_{max}) of luciferase were also observed when αGlu328 was mutated to a smaller hydrophobic residue leucine or alanine, or to glutamine to abolish the side-chain negative charge, or to a less acidic histidine. However, a remarkable recovery to 39% of the wild-type luciferase activity was obtained with αE328D when the carboxyethyl side chain of αGlu328 was replaced by the aspartate carboxymethyl side chain. Changes in K_{mA} or K_{mF} were observed for these αGlu328 mutants but none showed >6-fold changes from that of the native luciferase. Changes in K_{mA} were slightly more variable than those in K_{mF}. Therefore, mutational effects on V_{max}/K_{mA} were also analyzed and found to be similar to those on V_{max} (Table 1). In comparison with the wild-type luciferase, a decrease in V_{max}/K_{mA} by 5 orders of magnitude was found for αE328F, whereas decreases by 3−4 orders of magnitude were detected with αE328L, αE328A, αE328Q, and αE328H. Once again, a substantial 13% of V_{max}/K_{mA} was retained by αE328D. Similar analyses were carried out for αA74F and αA74G. Mutation of αAla74 to a bulky phenylalanine resulted in >10-fold increases in K_{mA} and K_{mF} and reductions by 2 and 3 orders of magnitude in, respectively, V_{max} and V_{max}/K_{mA}. αA74G was found to have a normal K_{mA} and a nearly normal K_{mF}. Both V_{max} and V_{max}/K_{mA} of αA74G were reduced to ∼40% of that of the wild-type luciferase.

Table 1: General Kinetic Properties of Wild-Type Luciferase and Variants

	K_{mA} (μM)	K_{mF} (μM)	relative V_{max}	relative V_{max}/K_{mA}
wild type	1.6	0.6	1	1
αE328F	4.5	3.9	6.6×10^{-5}	2.2×10^{-5}
αE328L	9.7	0.3	2.5×10^{-2}	4.1×10^{-3}
αE328A	3.3	0.3	8.8×10^{-3}	4.1×10^{-3}
αE328Q	2.3	0.2	6.9×10^{-3}	4.8×10^{-3}
αE328H	9.5	0.2	4.3×10^{-3}	7.2×10^{-4}
αE328D	5.0	0.4	3.9×10^{-1}	1.3×10^{-1}
αA74F	17.3	8.7	1.9×10^{-2}	1.0×10^{-3}
αA74G	1.6	1.5	3.7×10^{-1}	3.8×10^{-1}

Light and Dark Decays and Quantum Yield. The overall quantum yield (ϕ_o) of luciferase can be determined from the total quanta emitted in the entire time course of a single-turnover reaction. Qualitatively similar to the mutational effects on V_{max} and V_{max}/K_{mA}, the most severe reduction in ϕ_o was with αE328F; the smallest decrease was with αE328D, and in-between effects on ϕ_o were detected for all other αGlu328 mutants (Table 2). However, when compared quantitatively with effects on V_{max} or V_{max}/K_{mA}, less pronounced decreases in ϕ_o were observed for αE328D and, considerably more so, for all other αGlu328 mutants. Because the luminescence relaxation of the excited emitter IV* to ground state is very fast, the rate-limiting step in the luminescence reaction is the formation of IV*. At saturating aldehyde, k_L for the exponential light decay in a single-turnover reaction provides a measure of the rate constant for the formation of the IV* from intermediate III (*20*). In comparison with the wild-type luciferase, the k_L was reduced by only 30% for the homologous mutant αE328D, whereas all other αGlu328-mutated variants showed considerably lower k_L (Table 2). The dark decays of intermediate II formed with all αGlu328 mutants were also examined at 23 °C (Table 2). The least stable intermediate II was obtained from

Table 2: Comparison of Wild-Type with Mutant Luciferases with Respect to Light Decay, Quantum Yield, Dark Decay, and Yield of Intermediate II and Aldehyde Consumption at 23 °C

enzyme	relative ϕ_o	relative k_L	relative k_D	yield of II[a] (%)	decanal oxidation[b] (%)
wild type	1	1[c]	1[d]	92	84 ± 1
αE328F	3.0×10^{-3}	3.1×10^{-2}	3.00	63	20 ± 6
αE328L	4.2×10^{-1}	6.1×10^{-2}	1.51		
αE328A	2.5×10^{-1}	2.3×10^{-2}	0.89		
αE328Q	5.6×10^{-1}	1.5×10^{-2}	0.56		
αE328H	2.9×10^{-2}	6.8×10^{-2}	1.08		
αE328D	6.7×10^{-1}	7.0×10^{-1}	1.78		
αA74F	4.3×10^{-2}	4.4×10^{-1}	1.75	68	92 ± 1
αA74G	6.1×10^{-1}	6.1×10^{-1}	1.54		

[a] Based on stopped-flow data obtained at 23 °C as shown in Figure 1. The usual errors were ±2−3%. [b] Determined in triplets. [c] 13.1 min^{-1} for the wild-type enzyme. [d] 2.07 min^{-1} for the wild-type luciferase.

αE328F, whereas relatively low levels of stabilization or destabilization of intermediate II were detected for other αGlu328 mutants.

In comparison with wild-type luciferase, relatively low levels of changes were detected for αA74F and αA74G with respect to k_L and k_D of intermediate II (Table 2). Both αA74F and αA74G had markedly lower ϕ_o than the wild-type luciferase. Similar to mutational effects on V_{max} and V_{max}/K_{mA}, the replacement of αAla74 by Phe resulted in a more pronounced reduction of ϕ_o than that detected with αA74G (Table 2).

Stopped-Flow Measurement of $FMNH_2$ Oxidation Catalyzed by Luciferase. Luciferase intermediate III has been previously detected by kinetic and absorption spectroscopic measurements (*21*). The ground-state transient intermediate IV has also been characterized by absorption and fluorescence (*22*, *23*). The best-characterized intermediate is II, formed from bound $FMNH^-$ and oxygen. II was first isolated at −20 °C in 50% ethylene glycol−phosphate buffer (*24*) and characterized along with its decay product by absorption (*24*, 25) and fluorescence (*26*) spectroscopy. Intermediate II was also isolated in aqueous media at 0−4 °C, with absorption and fluorescence properties essentially identical to those determined at −20 °C (*27*, *28*). In this work, we focused on the stopped-flow determination of yields of intermediate II by luciferase mutants following the analysis established previously in our studies on other luciferase variants (*11*, *14*). FMN has substantially higher absorption than $FMNH_2$ in the range of 360−500 nm. When no intermediate II is involved in the oxidation of $FMNH_2$ to FMN, signals of ΔA_{382} and ΔA_{445} would follow the same time course and reach the maximal level at the same time. In contrast, the luciferase-catalyzed reaction involves first a rapid formation of intermediate II from $FMNH_2$ and O_2 and then a slow decay of II to FMN. The formation of II is characterized by a large increase in A_{382} and a very small increase in A_{445}. Because II and FMN share an isosbestic point at 382 nm, the decay of II is associated with a marked increase of A_{445} but no further changes in A_{382}. In the case of 100% yield of intermediate II, the total increase in A_{445} consists of 9% ΔA_{445} from the rapid $FMNH_2 \rightarrow$ II and 91% ΔA_{445} associated with the slow II → FMN. In the oxidation of $FMNH_2$ to FMN involving a partial yield of intermediate II, the formation of II is completed when the plateau of the maximal ΔA_{382} is first reached. However, due to the simultaneous direct oxidation of $FMNH_2$ to FMN, the ΔA_{445} detected at this time would be >9% of the total increase of A_{445} obtainable upon the complete conversion to FMN. A comparison of ΔA_{445} at the completion of the rapid formation of II and that at the end of the slow decay to FMN would then provide a means for the determination of the yield of II.

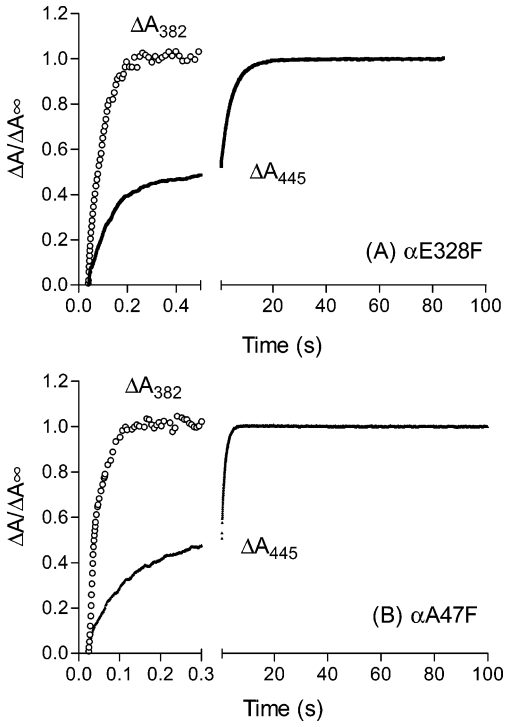


FIGURE 1: Time courses of $FMNH_2$ oxidation catalyzed by αE328F and αA74F. An anaerobic solution of 50 mM phosphate buffer, pH 7.0, containing 50 μM $FMNH_2$ was mixed with an equal volume of air-saturated buffer containing 135 μM αE328F (A) or αA74F (B) at 23 °C in an Olis stopped-flow spectrophotometer. The changes in ΔA_{382} (○) and ΔA_{445} (—) were monitored over time. For direct graphic comparisons, the absorbance changes were displayed as $\Delta A/\Delta A_\infty$, where ΔA and ΔA_∞ are the increases in absorbance from time zero to, respectively, a given time point after the mixing and upon the completion of the reaction.

The times courses of ΔA_{382} and ΔA_{445} for the oxidation of $FMNH_2$ catalyzed by αE328F and αA74F are shown in Figure 1. In both cases, the formation of II was indicated by the fast rise in ΔA_{382} to a maximum accompanied by a partial increase in ΔA_{445}. This was followed by a much slower phase during which ΔA_{445}, but not ΔA_{382}, continued to increase until the completion of the FMN formation. These data enabled us to calculate the yields of intermediate II in the αE328F- and αA74F-catalyzed reaction to be, respectively, 63 and 68% (Table 2). Using the wild-type luciferase as a positive control (stopped-flow data not shown), the yield of II was determined to be 92% (Table 2), similar to previously reported values (*11*, *14*).

Aldehyde Consumption. The formation of the light-emitting intermediate IV* is coupled to the oxidation of aldehyde to carboxylic acid in the bioluminescence reaction (*29*). Using a limiting amount of decanal and excess amounts of luciferase and $FMNH_2$, αA74F was able to consume 92 ± 1% of decanal, similar to the 84 ± 1% consumption by the wild-type luciferase, but αE328F consumed only 20 ± 6% of the decanal substrate (Table 2).

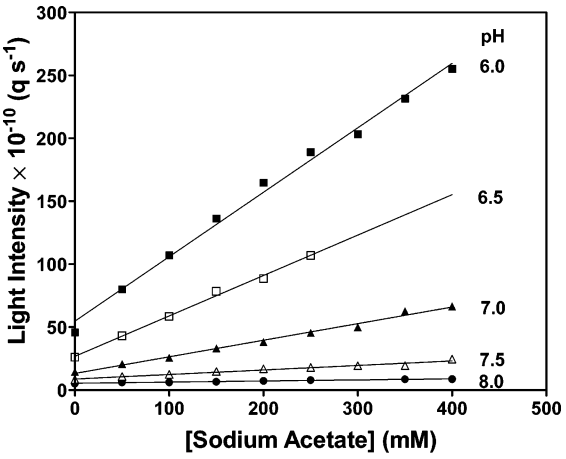


FIGURE 2: Enhancement of αE328A bioluminescence activity by sodium acetate and effects of pH. The peak luminescence intensities were determined for αE328A at 23 °C in 50 mM phosphate (at pH 8.0, 7.5, 7.0, 6.5, and 6.0) containing different final concentrations of acetate as indicated.

Table 3: Effect of Sodium Acetate in Relation to Reaction Steps

treatment[a]	$I_o \times 10^{-10}$ (q s^{-1})
1. (E + $FMNH_2$ + O_2 → II) → (decanal)	4.9 ± 0.4
2. (E + $FMNH_2$ + O_2 → II) → (sodium acetate + decanal)	10.6 ± 1.6
3. (E + sodium acetate + O_2 + $FMNH_2$ → II) → (decanal)	11.5 ± 2.1

[a] Reactions were carried out in 50 mM P_i, pH 7.0, at 23 °C using a modified dithionite assay. Each reaction solution contained 0.1 μM of the αE328A enzyme and, in treatments 2 and 3, 200 mM of acetate (all final concentrations).

Chemical Rescue of αE328A by Sodium Acetate. The finding that αE328D retained 39% of the V_{max}, whereas all other αGlu328 mutants had activities 2−5 orders of magnitude lower than that of the wild-type enzyme, suggests a plausible functional role of the acidic side chain of αGlu328. As an independent test, chemical rescue of the αE328A activity by sodium acetate was investigated. First, the activity of αE328A in 50 mM phosphate at pH 7.0 was found to increase linearly at higher concentrations of acetate tested up to 400 mM (Figure 2). At the same pH 7.0, increases from 50 mM to 1 M of the phosphate buffer containing no acetate did not result in any enhancement of αE328A activity. Effects of acetate on αE328A activity were also examined at four other pH values between 6.0 and 8.0, and results are included in Figure 2. Higher efficiencies of activity rescue (as indicated by steeper slopes of the plots in Figure 2) were obtained at lower pH values. Using the V_{max} of the wild-type luciferase at pH 6.0 as a reference, the activity of αE328A was rescued to 10% V_{max} by 400 mM acetate at pH 6.0.

The activity-rescuing effect of acetate with respect to catalytic steps was investigated at pH 7.0 (Table 3). As a control, intermediate II was first formed by reacting αE328A with dithionite-reduced $FMNH_2$ under aerobic conditions. The preformed intermediate sample was injected into an equal volume of solution containing saturating decanal to initiate the bioluminescence (treatment 1, Table 3). A second sample was treated identically except that the preformed intermediate II was mixed with an equal volume of decanal solution containing 400 mM acetate. The observed emission intensity was doubled in this case (treatment 2, Table 3). For a third sample, 400 mM acetate was included in the enzyme solution for the formation of intermediate II. Upon subsequent mixing with a buffer containing saturating decanal but no acetate, the same 2-fold bioluminescence activity enhancement was observed (treatment 3, Table 3). If the activity-rescuing effect of acetate was on a step leading to the formation of intermediate II, activity enhancement should be observed with only treatment 3 but not treatment 2, a prediction contradicted by our findings. Therefore, our results support the conclusion that acetate enhanced the bioluminescence activity of αE328A at a catalytic step after the formation of intermediate II.

DISCUSSION

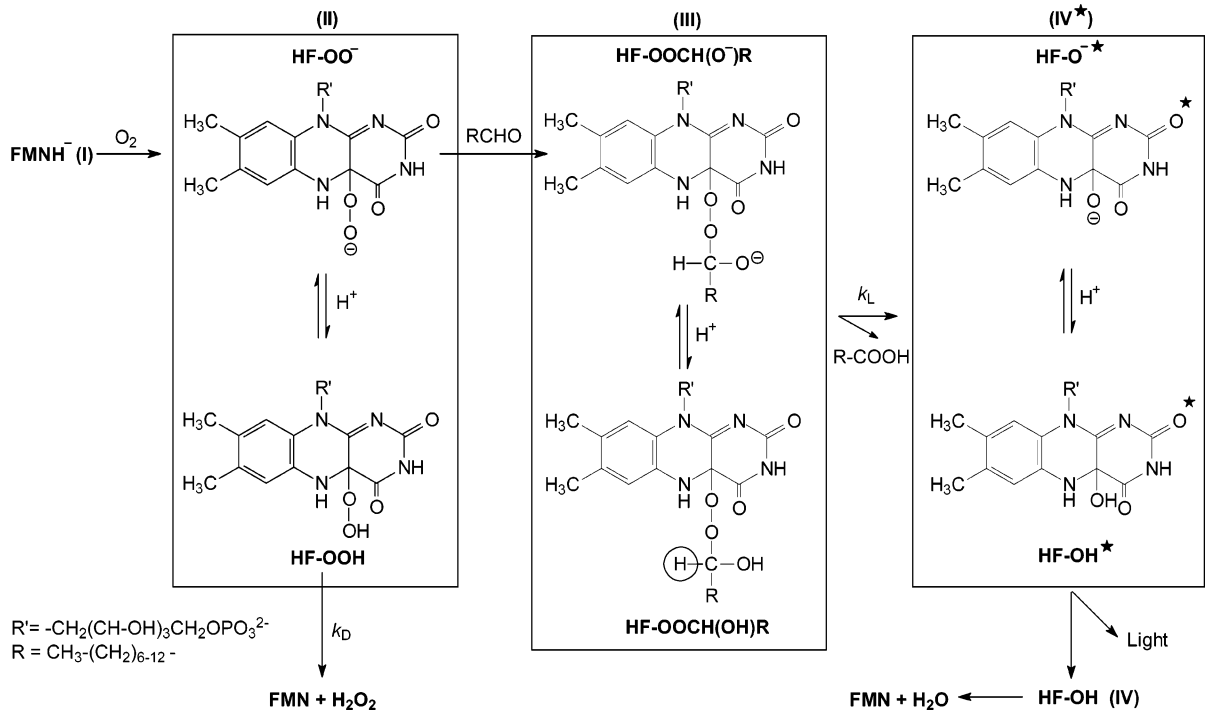
The major portion of this work focused on the identification of the conserved αGlu328 as an essential residue for *V. harveyi* luciferase and the characterization of its functionality. The essentiality of αGlu328 was clearly indicated by the findings that replacement of the acidic carboxyethyl side chain by the homologous carboxymethyl side chain of aspartate allowed the retention of 39% V_{max} and 13% V_{max}/K_{mA}, but mutations of αGlu328 to other nonhomologous residues shown in Table 1 resulted in reductions in V_{max} and V_{max}/K_{mA} by 3−4 orders of magnitude in most cases and by 5 orders of magnitude for the Glu → Phe mutation. In comparison with the wild-type luciferase, the k_L of the homologous mutant αE328D was only 30% lower, whereas all other αGlu mutants had their k_L reduced by 2 orders of magnitude, indicating that the acidic side chain of αGlu328 was important in regulating the rate-limiting step of the luciferase bioluminescence reaction. Due to the slower light decays of all of the αGlu328 mutants, the degrees of reductions in their ϕ_o values were less than those in V_{max} and V_{max}/K_{mA}. Qualitatively, the pattern of mutational effects on ϕ_o was similar to that on V_{max} and V_{max}/K_{mA}, with the most severe adverse effect observed with αE328F, the least reduction with αE328D, and in-between effects for the other nonhomologous mutants. For all parameters mentioned above, the charge rather than the size of the αGlu328 residue was the primary determinant of its functionality as evident from the finding that properties of αE328Q were similar to those of αE328A rather than the native luciferase or αE328D (Tables 1 and 2). All of the findings stated above, taken together, not only indicated an essential role of αGlu328 in luciferase activity but also suggested that the essentiality of αGlu328 is linked to the acidic nature of its side chain.

The specific functional roles of αGlu328 were examined by additional analyses. The overall quantum yield ϕ_o of the luciferase in vitro reaction can be described by the relationship

$$\phi_o = Y_{II}Y_{III}Y_{IV^*}\phi_{IV^*} \quad (1)$$

where each Y is the yield of an intermediate (defined by the subscript) and ϕ_{IV^*} is the emission quantum yield of the excited emitter IV*. The least active αE328F was examined on the basis of eq 1. Despite a much lower ϕ_o, αE328F was able to generate intermediate II at 63% yield. The yield of intermediate III is dictated by the partition between the reaction of II with aldehyde to form intermediate III in competition with the dark decay of II. Although αE328F

Scheme 1



had the least stable intermediate II among all αGlu328 mutants (Table 2), its dark decay rate under conditions of our test was only 3 times that for the wild-type luciferase. It is unlikely that αE328F would have a drastically reduced yield of III. The formation of IV* is coupled to the conversion of aldehyde to carboxylic acid (*29*). Hence, Y_{IV*} of αE328F can be estimated to be 20% on the basis of its ability to consume aldehyde as a substrate. Because the combined effect on Y_{II} and Y_{IV*} can account for only 1 of the 3 orders of magnitude decrease in ϕ_o for αE328F, mutation of Glu → Phe should lead to compromised ϕ_{IV*} and possibly also Y_{III}.

One important finding of this study is that mutations of the conserved αGlu328 affected the rate of quantum production (V_{max} or V_{max}/K_{mA}) by luciferase much more so than the overall quantum yield ϕ_o. This suggests that αGlu328 could have a true catalytic function in regulating the rate of the luciferase reaction. Therefore, the principle of chemical rescue (*30*) was employed to test the effect of sodium acetate on the activity of the dark mutant αE328A. Indeed, gradually increased bioluminescence activities were detected in the presence of increasing concentrations of acetate (Figure 2). Moreover, such an effect was sensitive to pH, with higher efficiencies of activity rescue at lower pH within pH 6.0−8.0. These results support the interpretation that αE328 functions as a catalytic general acid in the luciferase bioluminescence reaction. The response plots shown in Figure 2 are all linear up to 400 mM sodium acetate, indicating that no significant binding of acetate by the luciferase active site was detected under such conditions. The rescuing effect of acetate cannot be reproduced by phosphate up to 1 M at pH 7.0, hence ruling out increased ionic strength as the cause for activity enhancement. Interestingly, the intercepts on the ordinate show higher activities of αE328 at lower pH in the range from 8.0 to 6.0 in 50 mM P_i buffer without acetate. This pattern is distinct from the pH−activity profile of the wild-type luciferase, which shows an activity optimum at pH 6.7 with about 5 and 30% decreases at pH 7.0 and 6.0, respectively (*31*). Therefore, these findings further suggest that αE328 was also sensitive to the activity rescue by specific acid (H^+).

The acetate-rescuing effect was also shown to be on a reaction step after the formation of intermediate II (Table 3). Scheme 1 was formulated for the comparison of three possible points of involvement of αGlu328 as a catalytic acid as shown in the three boxes. The oxidation of luciferase-bound $FMNH^-$ generates intermediate II in the HF−OO^- state, which could undergo protonation to form HF−OOH (left box). We consider this unlikely to be the point for αGlu328 to act as a catalytic general acid for the reason that HF−OO^-, not HF−OOH, should be the active attacking species to react with aldehyde for the formation of the HF−OOCH(O^-)R intermediate III. The protonation states of intermediates III and IV* are not clear at the present, leaving open the possible participation of αGlu328 to facilitate the conversion of HF−OOCH(O^-)R to HF−OOCH(OH)R (middle box) and/or HF−O^-* to HF−OH* (right box). As discussed in a recent review (*2*), we presently favor a mechanism in which the removal of the circled proton in HF−OOCH(OH)R (shown in the middle box, Scheme 1) is coupled to the formation of HF−OH* as the excited emitter. Hence, HF−OOCH(O^-)R must first be protonated to HF−OOCH(OH)R, and this protonation could involve αGlu328 as a general acid catalyst. If our proposed generation of HF−OH* from HF−OOCH(OH)R (*2*) can be validated by future studies, then no general acid catalyst would be required after the formation of HF−OH* regardless of HF−OH* or HF−O^-* as the true emitter. However, if HF−O^-* is first formed from intermediate III and subsequently protonated to HF−OH* as the true emitter (right box), then the participation of a general acid catalyst, possibly αGlu328, at this point remains as a theoretical possibility that cannot be ruled out at the present.

V. harveyi luciferase contains an unusual αA74−αA75 *cis*-peptide bond (*4*). A computational model of the luciferase active site depicts this *cis*-amide linkage, the side chains of αAla75 and αCys106, and the FMN isoalloxazine ring as all closely packed (*9*). By analyses of luminescence activities and spectra of luciferase single and double mutants, evidence has been describe in support of a critical structural and, possibly, functional roles for the *cis*-peptide bond between αA74 and αA75 (*17*). Such a conclusion is supported by our characterization of the properties of αA74G and αA74F. Whereas αA75G retained 80−100% of the wild-type enzyme activity (*17*), the V_{max} and V_{max}/K_{mA} were reduced to 37−38% for αA74G and by 2 and 3 orders of magnitude, respectively, for αA74F (Table 1). Mutation of the αAla74 also resulted in slower rates for the rate-limiting step of IV* formation as indicated by 56 and 39% reductions in the light decay rates for αA74F and αA74G, respectively (Table 2). Moreover, 1 and 2 orders of magnitude decreases in ϕ_o were detected for αA74G and αA74F, respectively (Table 2). For αA74F, the yield of intermediate II was reduced by 32% but, once II was formed, the ability to react with decanal for IV* formation was fully retained. Therefore, the 2 orders of magnitude decrease in ϕ_o must be a consequence of not only a decreased Y_{II} but also reductions in Y_{III} and/or ϕ_{IV*}. It should be noted that mutations of αAla74 to glycine and phenylalanine do not necessarily abolish the ability of luciferase to form a *cis*-amide bond between the mutated residue and αAla75. In this connection, the occurrences of a Gly−Ala *cis*-peptide (*32*) and a Phe−Ala *cis*-peptide (*33*) in other enzymes have both been reported. Such a consideration notwithstanding, our characterizations of αA74F and αA74G clearly indicated that the structural integrity of the *cis*-peptide forming αAla74 is critical to luciferase bioluminescence with respect to peak emission intensity, rate-limiting step, and quantum yield.

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