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Identification and Characterization of a Catalytic Base in Bacterial Luciferase by Chemical Rescue of a Dark Mutant†

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ABSTRACT: Mutation of the His44 residue of the α subunit of *Vibrio harveyi* luciferase to an alanine was known to reduce the enzyme bioluminescence activity by five orders of magnitude [Xin, X., Xi, L., and Tu, S.-C. (1991) *Biochemistry 30*, 11255−11262]. We found that the residual activity of the αH44A luciferase was markedly enhanced by exogenously added imidazole and other simple amines. The peak luminescence intensity in nonturnover assays was linearly proportional to levels of αH44A and the rescue agent, indicating a lack of significant binding under our experimental conditions. The rescue effect of imidazole was pH dependent and quantitatively correlated well with the amount of imidazole base. The rescue efficiencies of imidazole and amines were found to be regulated by both their molecular volume and pK_a. A Brønsted analysis revealed a β value of 0.8 ± 0.1. The enhancement of αH44A activity by imidazole took place after the formation of the flavin 4a-hydroperoxide intermediate. The predominant form of the flavin 4a-hydroperoxide intermediate generated by αH44A was inactive in bioluminescence, but was reactive with the aldehyde substrate for bioluminescence in the presence of imidazole. These findings, taken together, provide evidence for assigning a role for the αHis44 imidazole as a catalytic base in the luciferase reaction. This study provides the first characterization of a catalytic residue for bacterial luciferase and the first demonstration of the rescue of a histidine-mutated enzyme by exogenous imidazole and amines.

Bacterial luciferase, a flavin-dependent monooxygenase catalyzing the oxidation of $FMNH_2$[1] and a long-chain aliphatic aldehyde with concomitant emission of light (quantum yield ≅ 0.1), has been the subject of extensive enzymological studies (*1*−*5*). Nevertheless, much remains to be elucidated with respect to luciferase reaction mechanism and active site structure. As shown in a minimal scheme (Scheme 1), subsequent to the binding of one $FMNH_2$ to the luciferase $\alpha\beta$ dimer (*6*, *7*), molecular oxygen reacts with the 4a-carbon site of the bound reduced flavin to form an activated hydroperoxyflavin species (*8*, *9*) designated intermediate **II**. The aldehyde substrate then reacts with intermediate **II** to form intermediate **III**, proposed to be an FMN 4a-peroxyhemiacetal (*10*), which decays in a still poorly understood process to emit light and yield the final products carboxylic acid, FMN, and water.

Luciferase is a heterodimer with molecular masses of approximately 40 and 35 kDa for the α and β subunits, respectively. Recently, the crystal structure of bacterial luciferase has been solved (*11*, *12*). However, the active

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

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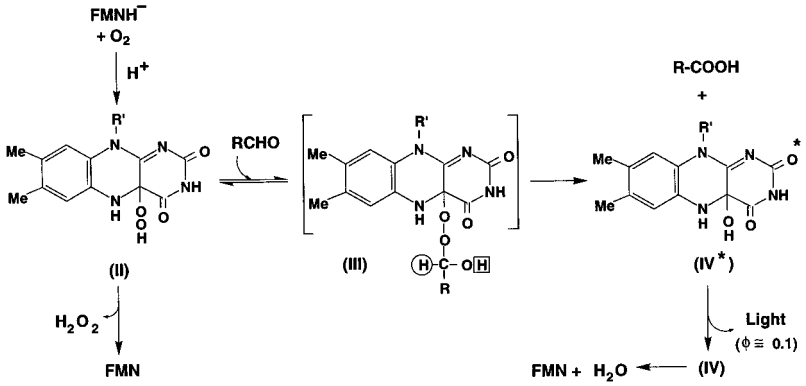
Scheme 1



site location has not been determined and is still the subject of speculation. Several "essential" amino acid residues of luciferase, mostly on the α subunit, have been identified through a series of chemical modification (*13*−*16*) and site-directed mutagenesis (*17*−*22*) studies. Modifications of these residues result in marked reductions in substrate binding affinity and/or luciferase bioluminescence activity. However, with respect to the chemical mechanism of luciferase, no luciferase residue has thus far been explicitly demonstrated to be essential to any specific catalytic function.

In physical organic chemical studies, a powerful way to probe the transition-state structure in general acid/base-catalyzed reactions is the construction of a Br*ϕ*nsted plot in which the log *k* of the reaction is determined under conditions in which the electronic property of the catalyst is systematically varied. However, a major barrier for applying this approach to enzymatic reactions is that the catalytic moieties are integral parts of the enzyme structure and therefore cannot be easily varied in a systematic way. An elegant work by Toney and Kirsch (*23*) demonstrated that inactivation of aspartate aminotransferase due to the mutation of the catalytic Lys258 residue to an alanine can be overcome by exogenously added small molecule amines. This enabled them to systematically vary the amines to establish the first true Br*ϕ*nsted analysis of an enzymatic reaction. Following this pioneering work, a number of reports have further demonstrated the feasibility of chemical rescue as a general approach to identify catalytic residues and to establish structure−activity relationships for enzymatic reactions. Additional accounts of chemical rescue studies are included in the Discussion. In this report, we describe the application of chemical rescue in a study of bacterial luciferase catalysis. Evidence is shown to indicate a role for the His44 residue of the α subunit as a catalytic base. This provides the first identification of a catalytic residue for bacterial luciferase and the first demonstration for the restoration of activity to a histidine-mutated enzyme by exogenously added imidazole and amines. Moreover, to our knowledge, this work is also the first chemical rescue study in the families of luciferases and flavoenzymes and the second example for oxygenases.

EXPERIMENTAL PROCEDURES

Materials. Imidazole, 1-methylimidazole, 2-methylimidazole, decanal, and copper(I) bromide were all from Aldrich. Sodium hydrosulfite (dithionite), FMN, dithiothreitol, ampicillin, and all small molecule amines were obtained from Sigma. Amines were purchased as the hydrochloride salts, except for ammonia, obtained as ammonium phosphate in dibasic form. Peptone and yeast extract were purchased from GIBCO. DEAE−cellulose and DEAE−Sephadex were products from Pharmacia Biotech.

Luciferase and Assays. *Vibrio harveyi* luciferase and the αH44A mutant were generated and overexpressed in *Escherichia coli* and purified following the methods described previously (*20*). Stocks for both enzyme samples were kept in 0.3 M sodium−potassium phosphate, pH 7.0. On the basis of sodium dodecyl sulfate−polyacrylamide gel electrophoresis, both native luciferase and αH44A were about 95% pure. Bioluminescence activities were determined at 23 °C under nonturnover conditions by the copper(I) assay (*24*) or a modified dithionite assay (*25*), all using decanal as the aldehyde substrate. A liquid light standard (*26*) was used to convert observed emission intensity to unit of quantum per second (q s^{-1}). For both nonturnover assays mentioned above, luciferase activities were based on the initial peak of emission intensity (I_o) expressed in q s^{-1}, equivalent to the observed maximal initial rate of the reaction. Unless stated otherwise, the standard buffer of 0.1 M potassium phosphate (KP_i), pH 7.0, was used for all luciferase reactions.

Chemical Rescue of αH44A. Imidazole or amine was freshly prepared in 0.1 M KP_i at a desired pH and concentration. Chemical rescue experiments were carried out using two methods. In one case, a 1-mL buffer solution containing enzyme and a rescue reagent was incubated at 23 °C for 1 min and the luciferase activity was determined by the injection of 1 mL of buffer containing 50 μM $FMNH_2$ (reduced by the copper(I) assay method) and 0.8 mM decanal. Alternatively, enzyme was first mixed with 50 μM $FMNH_2$ (reduced by a slight excess dithionite) in 0.1 mL of buffer. Immediately thereafter, a 1-mL standard buffer containing a desired concentration of imidazole (or other amine) and 0.8 mM decanal was injected to initiate the bioluminescence. Unless stated otherwise, final concentrations of rescue agent are cited throughout the text.

*Decay Rate of Intermediate **II***. αH44A was first reacted with 50 μM $FMNH_2$ (reduced by a slight excess dithionite) in the standard buffer precooled on ice. The solution was immediately passed through a small Sephadex G-25 column pre-equilibrated and eluted with the ice-cooled standard buffer. The luciferase 4a-hydroperoxy-FMN intermediate **II** was separated from free flavin, dithionite, and its oxidized products and was obtained in the void volume. The decay rate of the isolated intermediate **II** was determined at 6 °C by measuring the time course of the increase of A_{450} associated with the formation of the FMN decay product. In addition, the time courses of the decay of the biolumines-

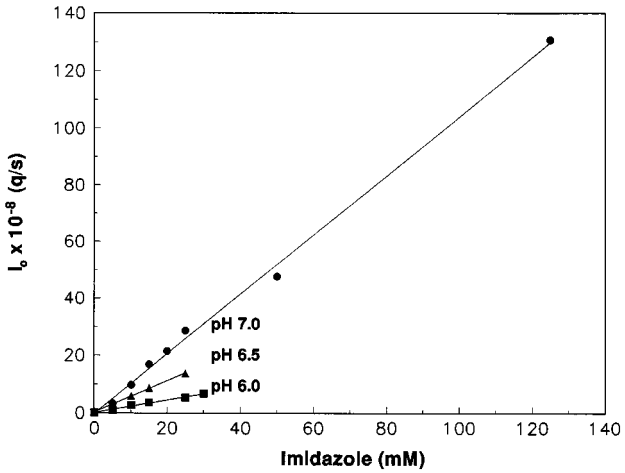


FIGURE 1: Dependence of the imidazole-assisted bioluminescence intensity on the amount of imidazole. Using 0.13 mg of αH44A per assay, the peak luminescence intensity was determined at pH 6.0, 6.5, and 7.0 at varying concentrations of imidazole as indicated.

cence-active intermediate **II** were determined by taking 0.1-mL aliquots of the isolated intermediate **II** sample after different times and injecting into 1 mL of standard buffer (kept at 23 °C) containing saturating decanal and 0 or 90 mM imidazole.

RESULTS

Chemical Rescue of αH44A by Imidazole. Replacement of αHis44 of the *V. harveyi* luciferase by alanine was previously shown to decrease the bioluminescence activity to $<10^{-5}$ of that for the wild type luciferase (*20*). In this investigation, we found that the bioluminescence activity of the αH44A dark mutant was markedly enhanced by the exogenously added imidazole. Using 0.18 mg of αH44A luciferase, the peak light intensity (I_o) of 2×10^7 q s^{-1} was enhanced about 200- and 540-fold by the addition of 50 and 125 mM imidazole, respectively. In comparison, the αH44A activity was not significantly enhanced by histidine and was only elevated 6- and 8-fold by 1-methylimidazole and 2-methylimidazole, respectively, at a final concentration of 50 mM of these rescue agents. Under the nonturnover assay conditions employed, the light decay for the αH44A enzyme followed an apparent first-order time course in the absence ($k_{dec} = 0.35$ min^{-1}) or presence of 50 mM ($k_{dec} = 0.86$ min^{-1}) or 125 mM ($k_{dec} = 1.1$ min^{-1}) imidazole. Taking both I_o and the light decay rate into consideration, the total quantum output was enhanced about 80- and 170-fold by 50 and 125 mM imidazole, respectively. At 50 mM imidazole, the peak luminescence intensities observed were linearly proportional to the amount of αH44A up to the highest level of 0.18 mg per assay. Enzyme-free control samples showed no significant luminescence by reacting $FMNH_2$ and decanal with O_2 in the absence or presence of imidazole. As another control, imidazole at 50 mM was found ineffective in stimulating the wild-type luciferase activity.

Imidazole as a Catalytic Base. For a fixed amount of the αH44A enzyme at 0.13 mg per assay, the bioluminescence peak intensity was linearly dependent on the concentration of the imidazole catalyst up to the highest level of 125 mM tested at pH 7.0 (Figure 1). Moreover, the efficiency of imidazole in enhancing the activity of the αH44A enzyme was pH dependent, with higher efficiencies at higher pH's within the range of 6.0−7.0 (Figure 1). Quantitatively, the effect of pH on the extent of activity rescue by imidazole can be correlated to the amount of the base form of imidazole following the relationship (*23*)

$$k_o = \frac{k_b[\text{imidazole}]_{total}}{1 + [H^+]K_a} + k_e \qquad (1)$$

in which K_a is the association constant for the imidazole acid−base equilibrium, k_o is the observed rate constant (in s^{-1}) for the luciferase light-emitting reaction, k_e is the k_o in the absence of imidazole, and k_b is a rate constant (in M^{-1} s^{-1}) for the emission catalyzed by the imidazole base. As defined above, k_o, k_e, and k_b are all based on rates of quantum emission rather than rates of other product formation by taking the quantum yield of the luciferase reaction into consideration. When normalized to the same amount of luciferase per reaction, eq 1 can be transformed to eq 2

$$I_o = \frac{k'_b[\text{imidazole}]_{total}}{1 + [H^+]K_a} + I_e \qquad (2)$$

where I_o is the observed peak light intensity and I_e is the I_o in the absence of imidazole. Moreover, I_o, I_e, and k'_b are all related to the corresponding k_o, k_e, and k_b by the same conversion factor. Therefore, k'_b (hence k_b) can be conveniently determined from plots of I_o against total imidazole concentration according to eq 2. Using data shown in Figure 1 and $pK_a = 6.95$ for imidazole (*27*), the k_b for imidazole base catalysis was determined to be $(2.0 \pm 0.1) \times 10^{-4}$ M^{-1} s^{-1} (Table 1). Moreover, theoretical ratios of the slope at one pH over that for another pH were calculated (on the basis of eq 1 or 2 and $pK_a = 6.95$) to be 2.0, 2.6, and 5.2 for $S_{7.0}/S_{6.5}$, $S_{6.5}/S_{6.0}$, and $S_{7.0}/S_{6.0}$, respectively, where S refers to the slope and the subscript refers to the pH. These theoretical ratios correlated well with the experimentally determined values of 1.9, 2.6, and 4.8 for $S_{7.0}/S_{6.5}$, $S_{6.5}/S_{6.0}$, and $S_{7.0}/S_{6.0}$, respectively (Figure 1).

Table 1: Rate Constants[a] (k_b) for Imidazole- and Amine-Assisted Bioluminescence by αH44A and Properties[b] of the Catalytic Agents

catalytic agent	pK_a	molecular volume (Å^3)	k_b (M^{-1} s^{-1})
imidazole	7.0	64.9	2.0×10^{-4}
methylamine	10.6	43.6	2.5×10^{-2}
ethylamine	10.6	61.7	8.1×10^{-3}
propylamine	10.5	80.7	3.7×10^{-3}
ethanolamine	9.5	69.3	2.4×10^{-4}
ammonia	9.2	25.4	3.5×10^{-3}
2-fluoroethylamine	9.0	65.9	2.5×10^{-4}
cyanomethylamine	5.3	53.5	2.5×10^{-7}

[a] Luciferase activities were measured by the Cu(I) assay at pH 7.0, 23 °C. Values of k_b were determined as described in the text. [b] The pK_a values for imidazole (*27*) and amines (*23*) are those reported previously. The molecular volume for imidazole was calculated using the method of Toney and Kirsch (*23*). Molecular volumes for amines were also calculated the same way and are essentially the same as reported previously (*23*).

Rescue Effects of Other Amines and Brønsted Plot. Seven simple amines were also tested and found to be active in enhancing the light activity of αH44A (Table 1). At pH 7.0 and for each of these seven amines (up to 75 mM for propylamine and 250 mM for all others), the I_o increased as a linear function of the amine concentration qualitatively similar to that shown in Figure 1 for imidazole. Values of

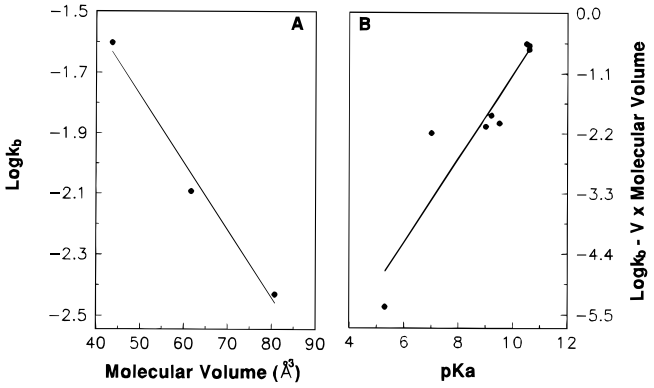


FIGURE 2: Effects of molecular volume and pK_a of imidazole and amines on the bioluminescence activity of αH44A. (panel A): On the basis of eq 3, the effect of amine molecular volume on log k_b was determined using data for methylamine, ethylamine, and propylamine from Table 1. (panel B): A Brϕnsted plot was also constructed as shown to evaluate the effect of pK_a. Data used for such a plot are from those in Table 1 and the term V was determined from the plot in panel A.

k_b for these amines are summarized in Table 1, along with values of their corresponding pK_a and molecular volume.

The efficacy of chemical rescue is sensitive to both molecular volume and pK_a of the rescue agent, and results shown in Table 1 were analyzed on the basis of eq 3 (*23*).

$$\log k_b = \beta(\mathrm{p}K_a) + V(\text{molecular volume}) + c \quad (3)$$

The dependence of the catalytic efficacy of the rescue agent on molecular volume can be shown by examining methylamine, ethylamine, and propylamine which have essentially the same pK_a but are quite different from one another in their molecular volumes. A plot of log k_b versus molecular volume for these three amines shows a linear relationship (Figure 2A), allowing the calculation of the steric term $V = -0.023$ in eq 3. On the basis of the data for all the rescue agents shown in Table 1 and taking the steric factor into consideration, a Brϕnsted plot of log $k_b - V$(molecular volume) as a function of pK_a was constructed (Figure 2B), giving a value of $\beta = 0.8 \pm 0.1$.

Catalytic Stage Affected by Imidazole. Several key intermediates in the luciferase reaction pathway are shown in Scheme 1. The question as to whether imidazole affects reaction steps prior to or after the formation of FMN 4a-hydroperoxide intermediate **II** was subjected to investigation. A modified dithionite assay method (*25*) was employed for the reduction of FMN. At 23 °C and using 0.17 mg of αH44A enzyme in a final solution of 2.5 mL of 0.1 M P_i, pH 7.0, a low level of 2.2×10^6 q s^{-1} was observed for the I_o without the addition of imidazole. The I_o was substantially and similarly enhanced when imidazole (final concentration 20 mM) was introduced either after (treatment 2) or before (treatment 3) the formation of intermediate **II** (Table 2). These results indicate that imidazole enhances the bioluminescence emission at reaction step(s) after the formation of the FMN 4a-hydroperoxide intermediate **II** rather than by increasing the yield of intermediate **II** under our experimental conditions.

*Identity of Reactive Intermediate **II** in the Rescued Bioluminescence.* Unlike the wild-type luciferase, the αH44A mutant has been shown to generate two forms of the intermediate **II** (*20*). The predominant form had a faster decay rate and was inactive in bioluminescence whereas a trace amount of a second form had a slower decay rate and was active in light emission. These earlier measurements were carried out using dodecanol as a stabilization agent for intermediate **II**. We have repeated these measurements at 6 °C in the absence of dodecanol under otherwise identical condition. Again, a faster decaying species ($k_{dec} = 0.45$ min^{-1}) was detected by the absorption changes at 450 nm, whereas a slower decaying form ($k_{dec} = 0.12$ min^{-1}) was detected by following the decreases in bioluminescence activity induced by secondary addition of aldehyde (Figure 3). The decay rate of the intermediate **II** species which was active in the imidazole-enhanced bioluminescence was also determined to be 0.46 min^{-1}, essentially identical to that for the faster decaying intermediate **II** species detectable by absorption signals.

Table 2: Effect of Imidazole in Relation to Reaction Steps

treatment[a]	I_o (q s^{-1})
1. (E + $FMNH_2$ + O_2) → **II** → + decanal	2.2×10^6
2. (E + $FMNH_2$ + O_2) → **II** → + imidazole + decanal	1.6×10^8
3. (E + imidazole + O_2) → + $FMNH_2$ → **II** → + decanal	1.1×10^8

[a] Reactions were carried out in 0.1 M P_i, pH 7.0 at 23 °C using a modified dithionite assay (*25*). Reactants included in the initial reaction solution were indicated by parentheses. Each reaction solution had a final volume of 2.5 mL containing 0.17 mg of the αH44A enzyme (E). The final concentration of imidazole was 20 mM for treatments 2 and 3.

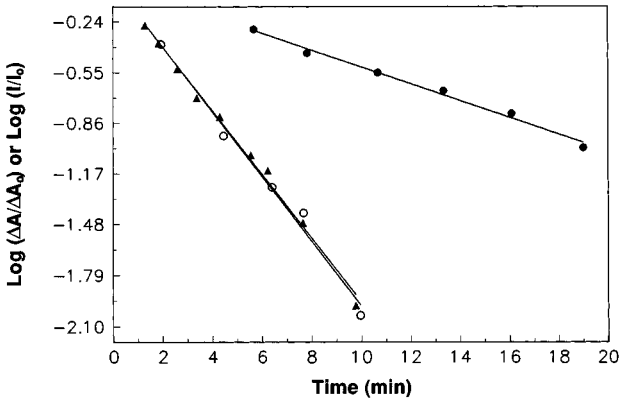
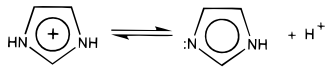


FIGURE 3: Decay kinetics of intermediate **II** species formed with αH44A. After isolation, the intermediate **II** was kept at 6 °C, and the decay kinetics were determined by following the changes in absorbance at 450 nm (▲) or bioluminescence peak intensity upon reacting with decanal at 23 °C in the absence (●) or presence (○) of 90 mM imidazole.

DISCUSSION

Mutation of the αHis44 residue of the *V. harveyi* luciferase to an alanine was previously shown to result in 5 orders of magnitude of activity reduction (*20*). In this study we found that the low activity of αH44A could be greatly enhanced by the addition of imidazole. Such an enhancement was dependent on the presence of the αH44A enzyme and linearly proportional to the αH44A concentration. In addition, the increases in light intensity at pH 6.0−7.0 were linearly proportional to the amount of imidazole up to the highest levels tested (Figure 1). Therefore, the enhancement of the light emission required the presence of both the αH44A luciferase and imidazole. However, on the basis of the linear dependence of emission enhancement on imidazole concentration, there was apparently no significant binding of imidazole by αH44A under our experimental conditions.

Scheme 2



An important finding of this study is that the exogenous imidazole acted as a catalytic base in enhancing the αH44A activity. The effect of imidazole was greater at higher pH's within the range of pH 6.0−7.0. Moreover, the slopes of the plots at different pH's in Figure 1 could be interpreted according to eq 2, derived on the assumption that the imidazole base was the active species in enhancing bioluminescence. Using a pK_a of 6.95 for the acid−base equilibrium of imidazole (*27*) as shown in Scheme 2, the theoretical values of $S_{7.0}/S_{6.5}$, $S_{6.5}/S_{6.0}$, and $S_{7.0}/S_{6.0}$ all correlated well with the experimentally determined values (Figure 1). Therefore, the critical assumption that the imidazole base is the active species in enhancing the αH44A activity was validated. These findings allow us to infer that the imidazole group of the αHis44 residue in the wild-type *V. harveyi* luciferase functions similarly as a catalytic base in bioluminescence. In this connection, although the αHis45 residue is also highly critical to luciferase activity (*20*), imidazole did not have any detectable rescue effect on the activity of αH45A luciferase (Hui Li and Shiao-Chun Tu, unpublished results). The possibility that free imidazole could not diffuse into the cavity created by the αHis45 to alanine mutation cannot be eliminated. However, we favor the alternative interpretation that αHis45 is functionally distinct from αHis44. Although both residues are critical to bioluminescence, the FMN 4a-hydroperoxide intermediate **II** species could be isolated in substantial yields from luciferases with the αHis44 mutated to alanine, aspartate, or lysine but not from luciferases with the αHis45 residue similarly mutated (*20*). Moreover, the αH44K mutant was about 2% active as the wild-type luciferase and 4900-fold higher in activity than the αH44A enzyme (*20*). Probably the terminal amino group of the lysine side chain could partially replace the αHis44 imidazole as a catalytic base. In contrast, αH45K was 4 orders of magnitude less active than the native luciferase and only 70-fold more active than αH45A (*20*).

A series of amines were tested for their rescue effects on the αH44A activity. Such an endeavor was prompted by both our conclusion that αHis44 in the wild-type enzyme acted as a catalytic base and the finding that the αH44K mutant is markedly more active than αH44A (*20*). All seven amines tested showed significant effects in enhancing the αH44A activity (Table 1). Efficiencies of these amines and imidazole were sensitive to both their molecular volume and pK_a (Table 1, Figure 2A,B). On the basis of the eq 3, a Brϕnsted plot was constructed yielding a value of $\beta = 0.8 \pm 0.1$ (Figure 2B). This β value indicates that a transition state was formed containing close to a full positive charge at the protonation site. It also indicates that the luminescence reaction is highly sensitive to the basicity of the general base catalytic group.

At least three ground-state oxygenated flavin intermediates (namely **II**, **III**, and **IV** in Scheme 1) can be identified for the luciferase reaction. It is important to determine which reaction step(s) could be affected by the exogenous catalytic base. Using αH44A, the light activity was greatly and similarly enhanced when imidazole was added either after or before the formation of the FMN 4a-hydroperoxide intermediate **II** (Table 2). Therefore, the imidazole-assisted bioluminescence apparently resulted from reaction step(s) after the intermediate **II** formation rather than from increased yields of intermediate **II**.

The exact catalytic function of the added imidazole is still not fully understood. However, we entertain the following two hypotheses. The exogenous imidazole in the αH44A system and the αHis44 residue in the native luciferase system could first abstract a proton from the flavin peroxyhemiacetal intermediate **III** at the boxed position shown in Scheme 1 followed by a hydride transfer from the circled position according to a Baeyer−Villiger mechanism proposed some years ago (*10*) and resurrected recently (*28*). Alternatively, the catalytic base could be involved in facilitating the transfer of a proton from the circled position following an electron/charge transfer (a modified version of the chemically induced electron exchange) mechanism (*29*−*33*). In either case, this proposed catalytic role is consistent with the known deuterium isotope effect ($^{D}k = 1.4-1.7$) of C1-deuterated aldehydes on luciferase bioluminescence decay rate (*30*, *34*, *35*).

The αH44A and αH44D luciferases were each shown to generate two forms of intermediate **II** under conditions using dodecanol as a stabilization agent (*20*). In the present work, two forms of intermediate **II** were also detected in the absence of dodecanol (Figure 3). After the isolation of intermediate **II** formed with αH44A, the absorption changes accompanying the decay to FMN followed an apparent first-order process with $k_{dec} = 0.45$ min^{-1} at 6 °C. A bioluminescence-active intermediate **II** species, however, exhibited a first-order decay with $k_{dec} = 0.12$ min^{-1}. Since both processes were first-order in kinetics, apparently the bioluminescence-active species was minute in quantity, so it was invisible by absorption measurement, whereas the major species detectable by absorption was inactive in bioluminescence. Importantly, the intermediate **II** species active in the imidazole-assisted bioluminescence appeared to be the same as the major and originally inactive species on the basis of their identical decay rates (Figure 3). Therefore, the lack of bioluminescence activity for the major intermediate **II** species formed with αH44A was apparently due to the removal of an enzyme catalytic base required for aldehyde oxidation in step(s) subsequent to the formation of intermediate **II**. Moreover, the lost catalytic group in αH44A could be replaced by the exogenous imidazole or amine to assist the luminescent oxidation of aldehyde by intermediate **II**. The exact difference between this major intermediate **II** and the trace amount of the imidazole-independent, bioluminescence-active intermediate **II** species formed by αH44A, however, still remains unresolved.

A number of critical residues of bacterial luciferase have been identified previously. Among the better characterized ones, the *Xenorhabdus luminescens* (reclassified as *Photorhabdus luminescens*) luciferase αTrp250 residue was shown to be important to flavin and aldehyde binding (*22*). For the *V. harveyi* luciferase, the "critical" αCys106 residue was later shown to be neither essential to $FMNH_2$ or aldehyde binding (*16*) nor directly involved in catalysis (*18*, *19*). The αAsp113 residue was proposed to be near the flavin site (*36*). Mutations of the αHis45 greatly reduced the stability and/or yield of the intermediate **II,** whereas mutations of the αHis44 residue was known, prior to this work, to render the major intermediate **II** species inactive (*20*). Chemical

modifications of a primary amine, identity and position undetermined, on either the α or the β subunit led to luciferase inactivation (*15*). Mutations of the βHis82 also resulted in marked reductions of the luciferase activity (*21*). However, none of these studies have identified any catalytic role for these critical residues. On the basis of chemical rescue of the αH44A activity by imidazole and amines, the functional role as a catalytic base at the *V. harveyi* luciferase active site has been assigned to the αHis44 residue. This is the first characterization of a catalytic residue for bacterial luciferase. In addition, the αHis44 residue may potentially serve as a reference point to facilitate the delineation of the exact location of the luciferase active site. To our knowledge, this work is also the first chemical rescue study for enzymes in the families of luciferases and flavoproteins and the second example for oxygenases (see ref 37 for the other case).

The drastically reduced activity of a subtilisin mutant lacking a catalytic histidine was shown a decade ago to be enhanced by "substrate-assisted catalysis" using substrates containing a histidine residue at an appropriate position (*38*). Toney and Kirsch (*23*) subsequently developed a more general approach in which restoration of activity to a mutant enzyme lacking a catalytic group could be achieved by the addition of small molecule catalysts functionally equivalent to the missing enzyme catalytic group. This latter chemical rescue approach has already been successfully applied to a number of enzymes. The rescue molecules were added exogenously in most cases, but could also be linked to the mutation site covalently. These substrate-independent chemical rescue studies include the activity restoration of lysine-mutated enzymes by amines (*23*, *37*, *39*−*42*), arginine-mutated enzymes by guanidines (*43*−*46*), and a tyrosine-mutated enzyme by phenols (*47*). Possible roles of fatty acids in chemical rescue of an aspartate-mutated enzyme have also been implicated (*48*). The present study provides the first demonstration of the rescue of the activity of a histidine-mutated enzyme by exogenously added imidazole. Moreover, ammonia and (derivatized) alkylamines, known for their rescue effects on lysine-mutated enzymes, were also shown for the first time to be effective rescue agents for a histidine-mutated enzyme. These simple amines may also exhibit similar rescue effects on other histidine-mutated enzymes to facilitate structure−activity studies in general and Brønsted analysis in particular.

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