Tryptophan 250 on the α Subunit Plays an Important Role in Flavin and Aldehyde Binding to Bacterial Luciferase. Effects of W→Y Mutations on Catalytic Function†

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ABSTRACT: Bacterial luciferase is a heterodimer ($\alpha\beta$) that catalyzes the oxidation of $FMNH_2$ and a fatty aldehyde, resulting in light emission. To explore the nature of the flavin binding site with respect to the role of tryptophan residues, the catalytic and binding properties of single-point mutants of *Xenorhabdus luminescens* luciferase with one of the eight tryptophans converted to a tyrosine residue were investigated by luminescence and fluorescence measurements. Conversion of tryptophans 194 and 250 on the α subunit to tyrosine had relatively large effects on the properties of luciferase with only minor changes in the properties on mutation of the other four tryptophans on α and the two on the β subunit. Mutation of αW250 decreased the binding to $FMNH_2$, FMN, aldehyde, and fatty acid, causing major changes in luminescence emission and decay. The results are consistent with αW250 interacting with flavin which in turn affects aldehyde binding. Mutation of αW194 did not affect the interaction with flavin or aldehyde but did change the relative rate of decay of light emission with aldehydes of different chain lengths as well as the activation energy for this process. Moreover, these results provide evidence for αW250, and to a lesser extent αW194, being in contact with the isoalloxazine ring of flavin, a proposal that has been recently made based on a model with flavin bound to the α subunit and anchored at a binding site for the phosphate moiety of $FMN(H_2)$ identified in the crystal structure of *Vibrio harveyi* luciferase [Fisher, A. J., Raushel, F. M., Baldwin, T. O., & Rayment, I. (1995) *Biochemistry 34*, 6581−6586].

Although bacterial luciferase has been investigated for over 3 decades, very little is known about the role of specific residues in the catalytic mechanism and in particular the nature of the active site (Baldwin & Ziegler, 1992; Meighen & Dunlap, 1993). Bacterial luciferase catalyzes the oxidation of $FMNH_2$ and a long-chain aldehyde with the concomitant emission of light. The enzyme is a heterodimer ($\alpha\beta$) composed of two subunits (α and β) of similar molecular mass (36 and 40 kDa, respectively) with approximately 30% identity in amino acid sequence. Most of the catalytic properties appear to be dictated by the α subunit based on mutation and hybridization experiments (Cline & Hastings, 1972; Ruby & Hastings, 1980; Meighen & Bartlett, 1980).

Recently, Fisher et al. (1995) have shown by X-ray crystallography that each of the luciferase subunits of *Vibrio harveyi* forms a $(\beta\alpha)_8$ barrel structure. This result was previously predicted by Moore and James (1994) based on primary and secondary structural comparisons with LuxF, which has a $(\beta\alpha)_7$ barrel and is related in sequence to the luciferase subunits. The crystal structure of luciferase also showed the presence of a strong peak of electron density in the α subunit that could accommodate a phosphate or sulfate ion. Assuming that this site corresponds to the phosphate moiety of $FMNH_2$, the flavin substrate could by modeling be positioned into the COOH-terminal portion of the α subunit, leading to the prediction that αW194 and αW250 interact with the isoalloxazine ring (Fisher et al., 1995). This assumption is supported by earlier work showing that the activity of *V. harveyi* luciferase with charged flavins (i.e., riboflavin 5′-phosphate or FMN) is competitively inhibited by phosphate. Moreover, luciferase activity with neutral flavins (e.g., riboflavin) was greatly stimulated by inorganic phosphate or sulfate, suggesting that these dianions could bind independently of the flavin and substitute for the phosphate moiety in $FMNH_2$ (Meighen & Mackenzie, 1973).

Fluorescence studies (Baldwin et al., 1975; Li & Meighen, 1992) have shown that luciferase forms a complex with FMN leading to the complete quenching of flavin as well as the intrinsic protein fluorescence. As revealed by X-ray crystallography of other flavin binding proteins (Ludwig et al., 1982; Burnett et al., 1974; Rao et al., 1992), interactions between flavin and aromatic side chains of amino acid residues are of primary importance, with static quenching of the flavin fluorescence being the largest with tryptophan side chains (MacKenzie et al., 1968; Wu & McCormick, 1971a). Interaction of flavin and tryptophan side chains in model compounds has also been shown to lead to complete quenching of the flavin fluorescence (MacKenzie et al., 1968) presumably due to formation of a ground-state charge-transfer complex (Weber, 1950; Bystra-Mieloszyk et al., 1985). These observations clearly support the involvement of tryptophan residues at the active site of bacterial luciferase, particularly with respect to interaction with the flavin ring.

Luciferases from the terrestrial bacterium *Xenorhabdus luminescens* and the marine bacterium *V. harveyi* contain

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eight tryptophan residues. Five of the tryptophans are conserved in all luciferases; three in the α subunit (194, 250,[1] and 277) and two in the β subunit (182 and 194) (Szittner & Meighen, 1990). Three other tryptophans (40, 131, 182) are present in the α subunit; two are conserved in all luciferases except that from a *P. leiognathi* strain isolated from the ponyfish (Lee, C. Y., et al., 1991), and one tryptophan (131) is not present in luciferases from *Photobacterium* species including *P. fischeri*, which was previously classified as *V. fischeri*. Phosphorescence and fluorescence studies of *X. luminescens* luciferase with each of the tryptophan residues replaced by tyrosine (W→Y), have demonstrated that the structural integrity of the enzyme is retained (Li & Meighen, 1994), although these studies did not investigate if the interactions with flavin had been altered.

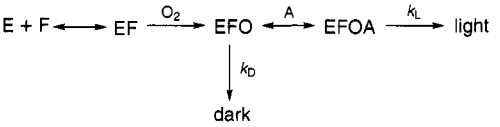
In the present work, the kinetic and binding properties of the W→Y luciferase mutants were characterized by luminescence and fluorescence measurements. These results have demonstrated that mutation of αW250 and αW194 clearly affects the catalytic properties of *X. luminescens* luciferase and that conversion of αW250 to αY250 has major effects on flavin, aldehyde, and fatty acid binding. The results provide strong support for the isoalloxazine ring of the flavin substrate interacting with αW250 as proposed by Fisher et al. (1995).

MATERIALS AND METHODS

Chemicals. Oxidized flavin mononucleotide (FMN) of >90% purity was purchased from Fluka Chemical Co. (Ronkonkoma, NY) and used without further purification. Reduced flavin mononucleotide ($FMNH_2$) was prepared by reducing FMN with H_2 using Pt as catalyst. Aliphatic aldehydes with different lengths of carbon skeleton were obtained from Aldrich. Mutants of *X. luminescens* luciferase with each of the tryptophan residues replaced by a tyrosine residue were expressed and purified to homogeneity as described previously (Li & Meighen, 1994). The purified wild-type and mutant enzymes had purities of 95% or higher based on SDS−polyacrylamide gel electrophoresis. Protein concentrations were determined from the absorbance at 280 nm using extinction coefficients of 7.72×10^4 M^{-1} cm^{-1} for the wild-type and 7.28×10^4 M^{-1} cm^{-1} for mutant enzymes. The enzymes were stored at −20 °C in the presence of 30% glycerol (v/v) and 10 mM β-mercaptoethanol until use.

Measurement of Luminescence Activity. Luciferase activity was determined with the standard flavin injection assay in which 1 mL of 50 mM reduced flavin mononucleotide was rapidly mixed with 1 mL of solution containing aldehyde (0.001%) and luciferase (pH 7.0, 50 mM phosphate, 0.2% bovine serum albumin). The rate of luminescence decay was determined by following the time course of luminescence intensity. The apparent affinity of luciferase for reduced flavin mononucleotide was measured in the dithionite assay described by Meighen and Hastings (1971). The stability of the hydroperoxyflavin intermediate (Hastings & Gibson, 1963) was determined by the dual-injection method (Meighen & MacKenzie 1973), in which the luminescence reaction was initiated by first injecting $FMNH_2$ into the enzymatic solution followed by injecting aldehyde at different time intervals. Assays were conducted at room temperature (24 °C) unless otherwise noted.

Scheme 1: Basic Intermediates in the Bacterial Bioluminescence Reaction



The specific activity of luciferase was expressed in light units per milligram of enzyme (LU/mg).[2] One light unit was converted to 5.5×10^9 quanta per second, based on the light standard of Hastings and Weber (1963). The light intensity was measured by a custom-built photometer, and the output was displayed by an XY recorder equipped with a built-in time base.

Steady-State Fluorescence Measurements. The steady-state fluorescence apparatus was a Hitachi fluorometer (Model F-3010) with a 150-W xenon short-arc lamp as excitation source. The sample chamber was equipped with a magnetic stirring unit, and the temperature was regulated by a circulating water bath and measured by a digital thermometer. The wavelength dispersion was 3 nm for excitation and 5 nm for emission in all cases. The background emission and scattered light were subtracted from the fluorescence signals of the samples. The FMN dissociation constant was derived from the intrinsic tryptophan fluorescence measurement in which the concentration of FMN in the protein sample was varied. The variations in fluorescence intensity due to changes of sample volume were corrected. The inner filter effect was corrected with the absorption spectrum of FMN as follows (Lakowicz, 1983):

$$F(\lambda)_{Corr} = F(\lambda)_{Obs}\ \text{antilog}[OD_{Ex}(\lambda)/2 + OD_{Em}(\lambda)/2]$$

where $OD_{Ex}(\lambda)$ is the optical density of the sample at the excitation wavelength and $OD_{Em}(\lambda)$ the optical density of FMN at the emission wavelength of the protein fluorescence.

RESULTS

Single-Turnover Assay. Bacterial luciferase catalyzes the oxidation of $FMNH_2$ and a long-chain fatty aldehyde to FMN and the corresponding fatty acid, resulting in the emission of a blue-green light at 490 nm. A reaction scheme (Scheme 1) formulated by Hastings and Gibson (1963) has provided the basic framework for our general understanding of the enzyme mechanism. A key intermediate in the pathway is a stable peroxyflavin enzyme complex (EFO), formed on reaction of $FMNH_2$ (F) and O_2 (Hastings et al., 1973). This intermediate interacts reversibly with the long-chain aldehyde (A) to form the EFOA complex, which decays at a rate given

[1] Although W250 (position 249 in *P. leoignathi* luciferases) on the α subunit appears to be absent in the luciferase of one *P. leoignathi* strain, *Pl*-741(Baldwin & Ziegler, 1992), it is present in a luciferase from a closely related *P. leoignathi* strain, *Pl*-554 (Illarionov et al., 1990), which differs in sequence by only four amino acids. As 3 of the amino acid differences and 7 of the 15 nucleotide differences in the two *P. leoignathi* sequences (1062 nucleotides) occur in a span of only 10 nucleotides (the region expected to encode a tryptophan residue), it strongly indicates that a frameshift error has been introduced into the sequence. Deletion and addition of a single nucleotide flanking this region in the gene for the *Pl*-741 α subunit result in complete identity for the nucleotide sequences in this region for the two *Pl* strains with the amino sequence in the *Pl*-741 α subunit being converted from IKL to that in the *Pl*-554 strain (i.e., SNW).

[2] Abbreviation: LU, light unit.

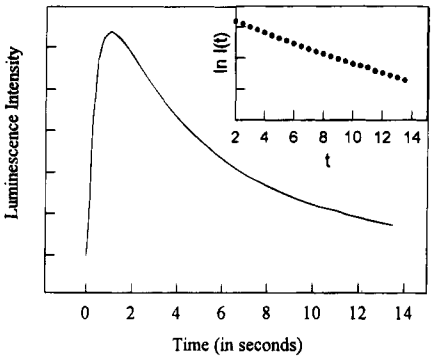


FIGURE 1: Bioluminescence of *X. luminescens* luciferase measured in the standard assay with decanal after initiation of the reaction by injection of $FMNH_2$. The light intensity [$I(t)$]on a semilogarithmic scale appears as a linear function of time (inset).

Table 1: Relative Specific Activities of Tryptophan Mutants Compared to Wild-Type Luciferase with Different Aldehydes[a]

	relative specific activities (% of wt)			
mutant enzyme	octanal	decanal	dodecanal	tetradecanal
αW40Y	50	50	60	80
αW131Y	90	90	100	90
αW182Y	30	30	60	80
αW194Y	11	7	20	20
αW250Y	3	1	6	6
αW277Y	100	100	100	110
βW182Y	110	110	100	110
βW194Y	30	40	60	40

[a] The specific activities of wt luciferase with octanal, decanal, dodecanal, and tetradecanal were 2.4×10^{13}, 20×10^{13}, 1.8×10^{13}, and 30×10^{13} quanta s^{-1} mg^{-1}, respectively, in the standard assay.

by k_L with the emission of light. In the absence of aldehyde, the peroxyflavin intermediate decays in a dark pathway at a rate given by k_D.

The standard bioluminescence assay, in contrast to most enzyme assays, is unique in that the decay of the EFOA intermediate is actually much slower than the rate of chemical oxidation of the substrate $FMNH_2$, which occurs in less than a second. Consequently, the enzyme turns over only once in the assay, and the luminescence intensity, after rising to a maximum, decays exponentially (Figure 1) with the rate constant (k_L) being highly dependent upon factors such as temperature and the choice of aldehyde substrate. The activity of luciferase is given by the maximum initial luminescence, I_o (in light units, LU), while the relative bioluminescence quantum yield for a single turnover of luciferase can be calculated from I_o and the rate of decay of the light intensity given by k_L.

Specific Activities of Luciferase Mutants with Tryptophan Replaced by Tyrosine. Table 1 compares the specific activities of the W→Y luciferase mutants with different aldehydes. Three of the mutants (αW131Y, αW277Y, and βW182Y) had about the same specific activity (90−110%) as the wild-type luciferase with all aldehydes. Three of the mutants (αW40Y, αW182Y, and βW194Y) had lower but still rather high specific activities (30−80% of wild-type luciferase) while mutants αW194Y and αW250Y had the lowest specific activities (1−20% of wild-type luciferase). The specificity for chain length of aldehyde has also changed to the largest extent with mutants αW250Y and αW194Y.

Luminescence Decay. As differences in specific activity may sometimes reflect the instability of a particular mutant

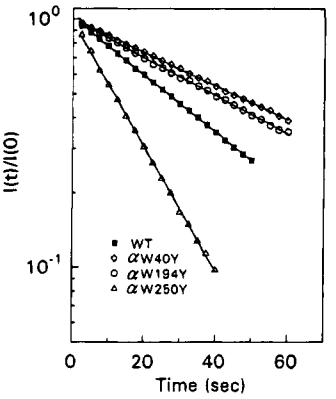


FIGURE 2: Decay of luminescence of wild-type and mutant luciferases in the standard assay at 24 °C with dodecanal (0.001%) as substrate. The light intensity at different times [$I(t)$] has been divided by the initial maximum light intensity [$I(o)$] for each luciferase and plotted on a logarithmic scale versus time.

Table 2: Rates of Luminescence Decay of the Tryptophan Mutants and Wild-Type Luciferase with Different Aldehydes

	$k_L(min^{-1})$[a]			
luciferase	octanal	decanal	dodecanal	tetradecanal
wild type	1.7 ± 0.1	9.2 ± 0.4	1.5 ± 0.1	10.0 ± 0.3
αW40Y	1.0 ± 0.1	6.1 ± 0.1	0.9 ± 0.1	10.4 ± 0.5
αW131Y	1.6 ± 0.1	10.9 ± 0.3	1.7 ± 0.1	10.0 ± 0.5
αW182Y	1.7 ± 0.1	10.0 ± 0.3	1.6 ± 0.1	10.0 ± 0.5
αW194Y	1.4 ± 0.1	2.5 ± 0.1	1.0 ± 0.1	4.0 ± 0.2
αW250Y	3.3 ± 0.1	3.5 ± 0.1	3.3 ± 0.1	4.3 ± 0.2
αW277Y	1.6 ± 0.1	10.4 ± 0.5	1.7 ± 0.1	10.0 ± 0.3
βW182Y	1.6 ± 0.1	10.0 ± 0.1	1.7 ± 0.1	10.6 ± 0.3
βW194Y	1.6 ± 0.1	10.0 ± 0.3	1.8 ± 0.1	11.2 ± 0.6

[a] All assays were conducted with the same stock aldehyde solutions prepared on the same day. Standard deviations are given for six assays of the same sample. A larger standard deviation will be observed if different stock aldehyde solutions are used. The decay rate($k_L = 0.69/t_{1/2}$) was calculated from the time interval for luminescence to decay from 50% to 25% of maximum light intensity.

and/or enzyme preparation, analysis of the decay of luminescence during the reaction is extremely advantageous as it can clearly show if mutation of a tryptophan residue caused changes in the catalytic properties. Figure 2 shows that the rate of decay of luminescence with dodecanal as substrate is slower with αW40Y or αW194Y and more rapid with αW250Y than the decay rate for wt luciferase. Analyses of the luminescence decay for all mutants with octanal, decanal, dodecanal, and tetradecanal showed that only these mutants had decay rates significantly different from those with wt luciferase (Table 2). For αW194Y, the rates for luminescence decay were less than wt luciferase with all aldehydes, decreasing by 20−40% with octanal and dodecanal and by a remarkable 3.5−2.5−fold with decanal, and tetradecanal. For αW40Y, the decay rate decreased by about 40% with octanal, decanal, and dodecanal. Surprisingly, the rates of luminescence decay for mutant αW250Y showed relatively little dependence on the aldehyde chain length with the rate constant being in the range of 3−4 min^{-1} for all aldehydes. In contrast, the rate constants for decay for wt luciferase are 9−10 min^{-1} for decanal and tetradecanal and 1.5−1.7 min^{-1} for octanal and dodecanal.

Analysis of the temperature dependence from 5 to 25 °C of the luminescence decay rates with decanal shows that the activation energies (E_a) from Arrhenius plots are similar (67 ± 5 kJ/mol) for all the mutants except αW194Y (Table 3).

Table 3: Activation Energies of the Tryptophan Mutants from Arrhenius Plots of the Decay Rates of Luminescence[a]

luciferase	E_a (kJ/mol)	luciferase	E_a (kJ/mol)
wild type	67 ± 5	αW250Y	67 ± 5
αW40Y	69 ± 6	αW277Y	65 ± 3
αW131Y	67 ± 4	βW182Y	62 ± 2
αW182Y	68 ± 4	βW194Y	68 ± 4
αW194Y	53 ± 3		

[a] The decay rates were measured with decanal in the standard assay buffer at 5 °C intervals between 5 and 25 °C.

Table 4: Relative Bioluminescence Quantum Yields (%) of Luciferase Tryptophan Mutants[a]

	aldehyde			
luciferase mutant	octanal	decanal	dodecanal	tetradecanal
αW40Y	80	80	100	80
αW131Y	90	80	90	100
αW182Y	30	30	60	80
αW194Y	12	24	30	50
αW250Y	1	2	3	14
αW277Y	100	90	90	110
βW182Y	120	100	90	110
βW194Y	30	30	50	40

[a] Relative quantum yields compared to wt luciferase were calculated from the relative specific activities (Table 1) and the rates of luminescence decay (Table 2).

This result raises the possibility that αW194 interacts with the EFOA complex, affecting the rate-limiting step for conversion of EFOA into the excited state.

Quantum Efficiencies. From the measurements of luminescence decay (Table 2) and maximum intensity (Table 1), the total emission of light in a single turnover (relative quantum yields) for the tryptophan mutants can be determined relative to wt luciferase (Table 4). Quantum yields similar to the native luciferase were obtained for αW40Y, αW131Y, αW277Y, and βW182Y, the lower activity observed for αW40Y (Table 1) arising from a decrease in the rate of decay of luminescence (k_L) and not a decrease in quantum yield. The lowest quantum yield (1−14%) occurs with αW250Y with the relative quantum efficiency increasing with aldehyde chain length compared to wt luciferase. The relative quantum yields for αW194Y and αW182Y increase with aldehyde chain length but to a much lower degree. The dependence of the quantum yield for mutant βW194Y on aldehyde chain length is the same as wt luciferase, raising the possibility that the lower quantum yields (and activities) may be due to partial inactivation during preparation.

Decay of the EFO Intermediate. In the absence of aldehyde, the peroxyflavin intermediate (EFO) decays via a dark pathway at a rate determined by k_D (Scheme 1). By injection of aldehyde at different time intervals after formation of the peroxyflavin intermediate, the amount of EFO and hence the rate constant, k_D, can be determined. The rate constants for decay of the peroxyflavin intermediates were identical to wt luciferase (~4 min^{-1}) for all the tryptophan mutants except the αW40Y mutant. Figure 3 shows the decay of the EFO intermediates for wt luciferase as well as for αW40Y and αW250Y. The 40% decrease in decay rate of the αW40Y mutant (2.8 min^{-1}) appears to correspond to a similar decrease in the rate constants for decay (k_L) with octanal, decanal, or dodecanal (see Table 2).

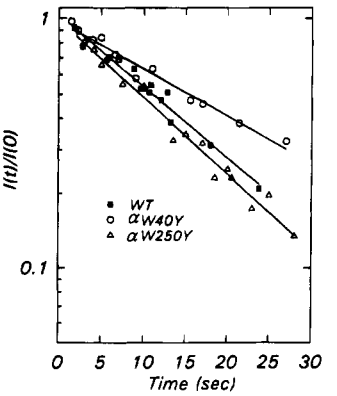


FIGURE 3: Luminescence capacity of the EFO intermediate as a function of time determined for the wild-type and mutant luciferases. The plotted times were the intervals between the injections of the $FMNH_2$ (5×10^{-5} M) and decanal (0.001%, v/v) solutions. The light intensity [$I(t)$] for each mutant has been divided by the light intensity obtained on extrapolation back to time zero (~5% higher than I_0).

Table 5: Flavin Dissociation Constants for Wild-Type and Mutant Luciferases

protein	K_d(FMN)[a] ($\times 10^{-5}$ M)	K_d($FMNH_2$)[b] ($\times 10^{-7}$ M)
wild-type	6.9 ± 0.5	7 ± 2
αW40Y	2.6 ± 0.2	5 ± 1
αW131Y	2.4 ± 0.3	8 ± 2
αW182Y	8.2 ± 0.3	6 ± 2
αW194Y	5.5 ± 0.5	6 ± 2
αW250Y	32 ± 2	20 ± 3
αW277Y	4.7 ± 0.5	8 ± 1
βW182Y	2.1 ± 0.3	6 ± 2
βW194Y	11 ± 1	13 ± 3

[a] FMN dissociation constants were obtained from the quenching of the intrinsic fluorescence of the mutant luciferases by FMN (see Figure 5). [b] $FMNH_2$ dissociation constants were measured in the dithionite assay and reflect the average of six measurements.

Binding of $FMNH_2$ and Aldehyde. The possibility that low quantum yields arise due to weaker interactions with the substrates and inefficient production of the EFOA intermediate was also investigated. An apparent dissociation constant for $FMNH_2$ binding to luciferase can be measured using the dithionite assay. Analyses of all the mutants showed that αW250Y had the highest K_d (2×10^{-6} M) for $FMNH_2$ compared to 7×10^{-7} M for the wt enzyme (Table 5).

Analyses of the dependence of the luminescence response on aldehyde concentration demonstrated that the activity of only one of the mutants (αW250Y) could be stimulated at higher concentrations of aldehyde. Figure 4 shows a plot of luminescence response versus decanal concentration in the standard assay for the wild-type luciferase and the αW250Y and αW194Y mutants. Maximum response occurs for the native luciferase and αW194Y at $(2-5) \times 10^{-5}$ M with inhibition at higher aldehyde concentrations. In contrast, the activity of αW250Y can be stimulated by higher decanal concentrations, clearly showing that mutation of tryptophan 250 has greatly affected the ability to interact with the fatty aldehyde. This result can also account, in part, for the lower quantum efficiency under standard conditions for αW250Y; however, it should be noted that even at the aldehyde concentration (3×10^{-4} M) that gives the maximum response, the luminescence response is still far below (~3%) that for wt luciferase.

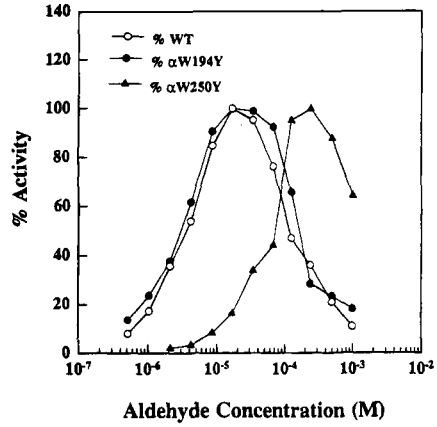


FIGURE 4: Bioluminescence intensity of wild-type luciferase, αW194Y, and αW250Y as a function of the molar concentration of decanal. The activity is given as a percentage of the maximum response for each luciferase.

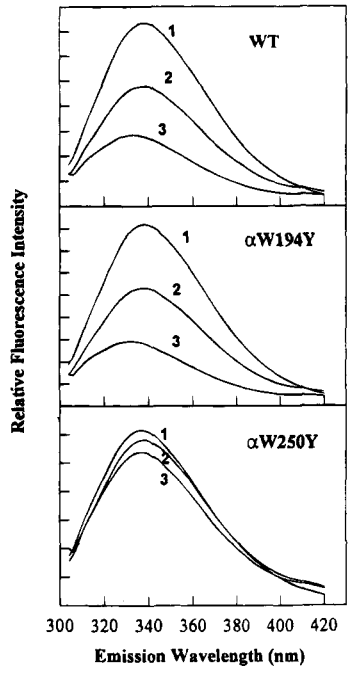


FIGURE 5: Tryptophan fluorescence spectra of wild-type *X. luminescens* luciferase (top panel) and the mutants of W194 (middle panel) and W250 (bottom panel) in the absence and presence of FMN and myristic acid. The spectra in each panel denote free luciferase (1); luciferase + FMN (2); and luciferase + FMN + myristic acid (3). The enzymes were in 50 mM phosphate buffer, pH 7.0. The concentrations of FMN, myristic acid, and luciferase were 5×10^{-5} M, 1×10^{-4} M, and 1×10^{-7} M, respectively. All the spectra were excited at 296 nm and corrected for the competitive absorption by FMN (Lakowicz, 1983).

Binding of FMN and Fatty Acid. Since FMN is a potent quencher of the intrinsic fluorescence of luciferase, binding of flavin to luciferase can be readily monitored by steady-state fluorescence. The spectrum of wild-type *X. luminescens* luciferase excited at 296 nm shows a broad fluorescence at 340 nm (Figure 5). In the presence of FMN, the intrinsic tryptophan fluorescence is significantly decreased. A further reduction in the intensity is observed on addition of myristic acid due to the enhancement of the affinity of luciferase for FMN by fatty acids (Li & Meighen, 1992). Quenching of the tryptophan fluorescence of seven of the eight tryptophan mutants in the presence and absence of fatty acid was identical to that of wild-type luciferase as shown in Figure 5 for the mutant αW194Y. The only exception was mutant αW250Y whose fluorescence was quenched much less than wt luciferase even in the presence of fatty acid (Figure 5).

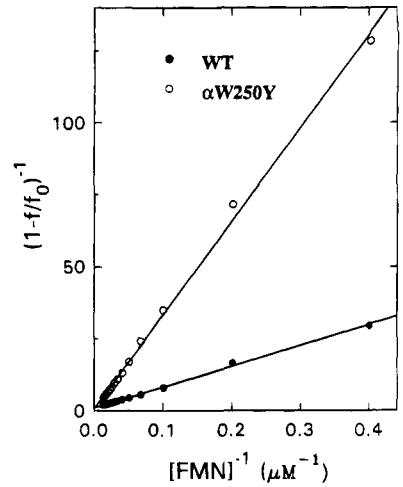


FIGURE 6: Changes of intrinsic fluorescence as a function of the reciprocal concentration of FMN measured for the wild-type luciferase and mutant αW250Y in 50 mM phosphate, pH 7. The excitation and emission wavelengths were 296 nm and 340 nm, respectively. The fluorescence intensities were corrected for both competitive absorption by FMN and variations in the volume of the samples.

To determine the affinity of the luciferase mutants for FMN, the reciprocal of $1 - f/f_0$, was plotted versus the reciprocal of the FMN concentration as given below (Li & Meighen, 1992). In this equation, f and f_0 are the fluorescence of luciferase in the presence and absence of flavin, respectively, and ϕ denotes the fluorescence quantum efficiency of the luciferase–FMN complex.

$$(1 - f/f_0)^{-1} = (1 - \phi)^{-1} + K_d(1 - \phi)^{-1}[FMN]^{-1}$$

From the slope of the plot, the dissociation constant (K_d) can be calculated. Figure 6 shows that the dissociation constant for FMN bound to αW250Y (3.2×10^{-4} M) has increased about 5-fold compared to FMN bound to luciferase (7×10^{-5} M). The αW250Y mutant had the largest dissociation constant for FMN of any of the luciferase mutants. Table 5 shows that conversion of a tryptophan to a tyrosine residue had a similar effect (if any) on the dissociation constants for FMN and $FMNH_2$ for most of the mutants although a 2-fold decrease in K_d for FMN without any apparent effect on the K_d for $FMNH_2$ occurs with some of the mutants. The simultaneous increase in K_d for FMN and $FMNH_2$ with αW250Y is particularly notable.

DISCUSSION

The presence of aromatic side chains of amino acid residues at the flavin binding site is a common feature of many flavin binding proteins (Ludwig et. al., 1982; Burnett et al., 1974; Porter, 1991; Rao et al., 1992) and is considered essential for interaction with the flavin ring. On formation of a complex between flavin and the aromatic amino acid residues, both the fluorescence of FMN and the intrinsic fluorescence of protein are effectively quenched as the result of formation of a weak ground-state complex (Weber, 1950, 1966). The quenching of the protein fluorescence by FMN is the result of efficient electronic energy transfer from the

aromatic residue to FMN as the extensive spectral overlap of tryptophan fluorescence and flavin absorption results in a large critical transfer distance of 35 Å (Li et al., 1976). The quenching of the fluorescence of FMN, on the other hand, occurs by direct contact with the aromatic side chain (Weber, 1950, 1966). Efficient quenching of the fluorescence of FMN and the intrinsic tryptophan fluorescence of protein have provided strong evidence for interaction of FMN and aromatic residues with luciferase (Baldwin et al., 1975; Li & Meighen, 1992) as well as other flavin binding proteins (Li et al., 1976).

Interactions of flavin and aromatic residues have been studied extensively with intramolecularly linked flavinyl aromatic amino acid peptides by fluorescence quenching (Wu & McCormick, 1971a; MacKenzie et al., 1968) and flavin-sensitized photooxidation (Wu & McCormick, 1971b). In all cases, the flavinyl moiety forms a stronger complex with tryptophan than with tyrosine. Substitution of a tryptophan by a tyrosine at a flavin binding site in luciferase should weaken the binding with FMN but maintain the aromatic nature of the interaction.

The present study has shown that relatively large changes occurred in the catalytic properties of luciferase on mutation of αW250 or αW194 even though a conservative replacement of tryptophan to tyrosine was made to retain interactions with flavin. Mutation of αW250 resulted in a large decrease in activity and quantum yield, changes in specificity and decay rates with different aldehydes, and a decrease in the binding of substrates and products. The results are consistent with mutant αW250Y having altered flavin binding with the different interactions affecting fatty acid as well as fatty aldehyde binding, which, in turn, affects formation of the EFOA complex and the reactivity and efficiency of its conversion into the excited state. As aldehyde cannot interact effectively with the EFO complex of αW250Y, the primary decay of the EFOA complex would occur via EFO with a rate constant, k_D, of 4 min^{-1} (see Scheme 1), providing that the binding and dissociation of aldehyde are more rapid than the low rates of decay of EFO and EFOA. This would explain why a relatively constant decay rate of luminescence of 3−4 min^{-1} was observed for all aldehydes with αW250Y and, at least, partially account for the lower activity, and quantum yield. A similar effect on the decay rate of luminescence, activity and quantum yield occurs with wt luciferase if the aldehyde concentration is lowered below the optimum level.

The mutation of tryptophan to tyrosine in αW194Y had strikingly different effects on the catalytic properties of luciferase than did the conversion of αW250 to αY250. In particular, the rate of decay of luminescence with all aldehydes was decreased for αW194Y along with a decrease in activation energy for this process while interactions with $FMNH_2$, FMN, fatty acid, or aldehyde were unaffected. Consequently, conversion of αW194 to αY194 primarily affects the rate by which the EFOA intermediate is converted into the excited state, suggesting that specific stacking interactions at position 194 may only be important for facilitating the conversion of substrates to products. A similar conclusion has been reached for W574 at the FMN binding site of cytochrome $P450_{BM-3}$, a monooxygenase from *Bacillus megaterium* (Klein & Fulco, 1993). Conversion of the tryptophan to tyrosine or phenylalanine maintained the binding to FMN but caused a 80% decrease in myristate hydroxylation.

As the properties of the αW194 mutant are different than the wt luciferase only after binding of the aldehyde substrate, it agrees with the proposal of Lee et al. (1988) that luciferase undergoes a conformational change on binding of aliphatic compounds to the hydroperoxyflavin intermediate. Addition of myristic acid to luciferase and FMN not only greatly enhances the binding of flavin but a blue shift occurs in the intrinsic fluorescence along with a decrease in the ability of bound flavin to quench the intrinsic fluorescence (Li & Meighen, 1992), supporting a conformational change. A strong fluorescence of the intermediate flavin complex in the presence of long-chain aliphatic compounds also suggests that a change in the stacking interactions has occurred (Lee, J., et al., 1988, 1991). In this regard, the possibility that the mutation of the tryptophan residues causes a small conformational change at another locus affecting interactions with substrates cannot be excluded although differences in the intrinsic fluorescence or phosphorescence between the native and mutant luciferases in the absence of flavin have not been detected (Li & Meighen, 1994).

The identification of a site on the α subunit in the crystal structure of luciferase believed to correspond to the phosphate moiety of FMN (H_2) (Fisher et al., 1995) is strongly supported by the present experiments. Modeling of the flavin into the luciferase structure by anchoring phosphate at this site has led to the proposal that flavin is bound to the carboxyl region of the α subunit with the isoalloxazine ring being next to two tryptophans, αW250 and αW194 (Fisher et al., 1995). The presence of a flavin site near the carboxyl terminal sandwiched between two aromatic residues is also supported by the recent elucidation of the structure of a complex of flavin and the LuxF flavoprotein (Moore et al., 1993), which is a homodimer with subunits related in sequence to the α and β luciferase subunits (16% and 30% identity, respectively). Two flavin sites were detected per LuxF subunit; one site was at the subunit interface while the second site was in the carboxyl-terminal region with the flavin ring flanked by two aromatic side chains.

The results with LuxF also raise the possibility that the luciferase heterodimer may contain more than one flavin site with perhaps one site for FMN and a different site for $FMNH_2$. Although the present experiments certainly do not exclude the existence of two different sites, mutation of αW250 to αY250 affects the interaction with both the substrates ($FMNH_2$, aldehyde) and the products (FMN, fatty acid), suggesting that FMN and $FMNH_2$ bind to a common site. The present data are consistent with the proposal that luciferase undergoes a major conformational change during the bioluminescence reaction (Lee et al., 1988) and that the flavin moiety binds to the α subunit of luciferase with αW250 and αW194 being in contact with the isoalloxazine ring (Fisher et al., 1995).

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