Flavoenzymes catalyze a great variety of biological redox reactions. The diversity is marked by not only the types of substrates utilized but also the underlying reaction mechanisms (1,2). Therefore, flavoenzymes provide one of the most challenging systems for the elucidation of enzyme structure-function relationships. For flavin-dependent oxidases and external monooxygenases, the initial oxidation of a substrate is coupled with the reduction of the enzyme-bound flavin (E-F). The subsequent oxidation of the bound reduced flavin (E-FH₂) by O₂ is associated with the formation of H₂O₂ for oxidases (3) (Equation 1) or the insertion of one oxygen atom into a second substrate (SH) and the reduction of the other (M, 40,100) (10) and an additional six on p (M, 36,400) (11). The cysteinyl residue at position 106 on the α subunit (12, 13), which is particularly reactive, can be selectively modified by a number of chemical reagents leading to luciferase inactivation (14–17). Recently, we have selectively methylated the V. harveyi luciferase αCys²⁰⁶ by methyl p-nitrobenzenesulfonate and have found that the modified enzyme retains the ability to bind aldehyde and FMNH₂, but the formation of the peroxyflavin intermediate II is impaired (13). Therefore, the intriguing question as to whether or not the αCys²⁰⁶ modified luciferase is active in catalyzing the oxidation of FMNH₂ or aldehyde in a dark reaction should be addressed. In this work, we have conducted site-directed mutagenesis to replace the αCys²⁰⁶ with either an alanine (αC106A) or a valine (αC106V) to gain further insights into the luciferase structure-function relationship.

**EXPERIMENTAL PROCEDURES**

Luciferase Purification and Activity Assay.—Wild-type luciferase was purified from V. harveyi cells, and mutated luciferases were each isolated from Escherichia coli HB101 cells harboring the desired recombinant plasmid following the literature procedure (18). In all cases, the purified enzymes appeared >95% pure on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bioluminescence activities were determined in 0.1 M KP₂, pH 7.0, at 23 °C by the dithionite assay (19). Activities were measured at various concentrations of FMNH₂ or aldehyde at a saturating level of the co-substrate. The maximal activity and Kₐ were determined by double reciprocal plots. The former values are expressed as either the peak light intensity (Lp) in quanta per s or the total light output (Q) in quanta, both normalized to per A₅₆₅ per mi of the enzyme sample. The decay rate of intermediate II (kₙ) was determined in the absence of oxygen atom to water for monooxygenases (hydroxylase activity) E·FHz + O₂ + SH → E·F·HOOH·SH (1) (2a) (Equation 2b)
Kinetics of FMNH₂ Oxidation—Kinetics of autooxidation and luciferase-catalyzed oxidation of FMNH₂ were measured in 0.05 M KP₂, pH 7.0, at 23 °C using a stopped flow spectrophotometer (Dionex D-110). FMNH₂, in the presence or absence of luciferase, was prepared from FMN by photochemical reduction in the presence of 1 mM EDTA using an apparatus similar to that described by Williams et al. (21). Flavin solutions were first made anaerobic by seven cycles of evacuation and reequilibration with nitrogen and the flavin subsequently reduced by irradiation. After transfer to a stopped flow syringe, the sample was kept under irradiation to photoreduce any FMN formed during the transfer. Upon mixing with an O₂-containing solution in the second syringe, changes in A₄₅₀ were recorded as a function of time.

Molecular Cloning and Site-directed Mutagenesis of Luciferase Genes—A recombinant pBR322 plasmid (designated pTH2) containing the luxA (10) and luxB (11) genes which encode the α and β subunits, respectively, of V. harveyi luciferase was obtained following literature procedures (22) with minor modifications. The luxAB genes in a Sall-BglII fragment of pTH2 were subcloned into the Sall-BamHI sites of M13mp19. One of such recombinant phages (MTX1) was selected by the positive bioluminescence activity (in the presence of exogenously added decanal) of Escherichia coli JM 109 transfected by this phage. The identity of the DNA insert in MTX1 was confirmed by DNA sequencing using the dideoxy-sequencing method (23-25).

Site-directed mutagenesis (26) of luxA was carried out using synthetic 21-base oligonucleotide primers (Genetic Design Inc.) which contain the necessary AGC and AAC replacing ACA for the substitution of cysteine, but this mutant enzyme is highly active in bioluminescence. Such an observation unambiguously indicates that the αC106S enzyme does not necessarily rule out a catalytic function for the cyCys'OG residue. Consistent with this argument, activity retention has been observed for subtilisin (30, 31) and trypsin (32) with their active site essential serine converted to a cysteine. In this connection, the αC106A and αC106V variants of V. harveyi luciferase have also been constructed, following a different cloning and expression strategy, and reported to be active in a recent independent study (33). Values of Kₘ (indicated as Kₚ) for FMNH₂ for luciferase, the decay rate of the intermediate II (kₙ) formed with the αC106A enzyme is 10-fold faster whereas that for the αC106V luciferase is about 100-fold faster. Similar to the wild-type luciferase (20), dodecanol significantly stabilizes the intermediate II formed with the two mutant enzymes but to different degrees. The wild-type and the two mutant enzymes all show significantly different patterns of I, Q, Kₘ, and kₙ indicating further that the activities observed for the mutant enzymes are not due to any contamination of wild-type luciferase.

Previously, the V. harveyi luciferase αCys¹⁰⁶ has been converted to a serine residue with the resulting luciferase variant (αC106S) found active in bioluminescence (28, 29). Since serine and cysteine side chains are similar in size and are homologous nucleophiles, we have argued (13) that the retention of bioluminescence activity by the αC106S enzyme does not necessarily rule out a catalytic function for the αCys¹⁰⁶ residue. Consistent with this argument, activity retention has been observed for subtilisin (30, 31) and trypsin (32) with their active site essential serine converted to a cysteine. In the present case of αC106A luciferase, the chemical reactivity of alanine side chain is drastically different from that of cysteine, but this mutant enzyme is highly active in bioluminescence. Such an observation unambiguously indicates that the αCys¹⁰⁶ of V. harveyi luciferase is not directly involved in chemical catalysis. In this connection, the αC106A and αC106V variants of V. harveyi luciferase have also been constructed, following a different cloning and expression strategy, and reported to be active in a recent independent study (33). Values of Kₘ (indicated as Kₚ) for FMNH₂ for

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**TABLE I**

|           | Wild type | αC106A | αC106V |
|-----------|-----------|--------|--------|
| Iₙ (10⁻¹³ x quanta/s/µmol/µl) |           |        |        |
| Decanal   | 71.0      | 27.0   | 2.2    |
| Dodecanol | 9.7       | 4.4    | 1.0    |
| Q (10⁻¹⁵ x quanta/µmol/µl) |           |        |        |
| Decanal   | 14.0      | 8.4    | 0.3    |
| Dodecanol | 9.6       | 5.4    | 0.2    |
| Kₘ (µM)   |           |        |        |
| Decanal   | 5.7       | 5.7    | 21.6   |
| Dodecanol | 4.6       | 4.2    | 23.5   |
| FMNH₂⁺   | 0.6       | 3.9    | 1.6    |
| kₙ (min⁻¹) | 2.7      | 27.0   | 258.0  |
| + Dodecanol | 0.1 | 0.3    | 33.6   |

* Using decanal as a co-substrate.
phase is associated with a "direct" pathway of FMN formation and the minor phase representing a peroxoflavin-dependent pathway of FMN formation. The major phase is about twice as fast as the autoxidation of free FMNH₂, thus indicating the former process being enzyme-mediated. Moreover, the major phase is about six times as fast as the formation of FMN via the decay of aC106V peroxyflavin intermediate II as determined by activity assays (Table I). Therefore, the major phase of FMN formation from aC106V bound FMNH₂ does not involve 4a-hydroperoxy FMN as an intermediate. On the other hand, the minor phase is attributed to the slower dark decay of a small quantity of aC106V intermediate II formed in a different pathway.

Although the oxidation of the aC106V-bound FMNH₂ is only twice as fast as the nonenzymatic oxidation, one should note that autoxidation is itself remarkably fast and also that luciferase is an unusually slow enzyme. For the wild-type luciferase under similar conditions, the rate constant for the formation of FMN via the dark decay of II was 0.05 s⁻¹ (Table I) and that via the light pathway was 0.29 s⁻¹ (with decanal) and 0.04 s⁻¹ (with dodecanal) (20). In comparison, 90–630-fold rate enhancements are exhibited by the aC106V luciferase for the oxidation of FMNH₂.

It has been shown that the luciferase emission is coupled to the hydroxylase pathway (Scheme 1, A) in which the aldehyde is converted to acid (36) by reacting with the 4a-hydroperoxy FMN intermediate. We have examined the aC106V enzyme for any aldehyde oxidation activity. As a control, tetradecanal (0.2 mM) was mixed with dichloro- reduced FMNH₂ (10 μM) in 0.1 M KPi, pH 7.0, and then subjected to nonenzymatic oxidation. The recovery of tetradecanal was determined by bioluminescence assays using excess Photobacterium phosphoreum luciferase and aliquots of the sample solution as the aldehyde supply. When tetradecanal was similarly reacted with FMNH₂ and oxygen in the presence of aC106V (4.8 μM), 89 ± 2% of the expected aldehyde recovery was obtained. Therefore, the aC106V enzyme has little or no activity to oxidize aldehyde. This finding is consistent with the observation that the major pathway for the oxidation of aC106V-bound FMNH₂ does not involve 4a-hydroperoxy FMN as an intermediate.

It is important to note that the major route of FMNH₂ oxidation by aC106V, depicted as pathway C in Scheme 1, mimics the activity of a typical flavin-dependent oxidase (Equation 1) in contrast to that for the pseudooxidase activities of flavomonooxygenases (Equation 2b; Scheme 1, pathway B). Furthermore, this new oxidase activity for aC106V luciferase was elicited by a single mutation of a residue not directly involved in chemical catalysis. We propose that the positioning of oxygen and the enzyme-bound flavin has a crucial role in dictating the chemical mechanism of FMNH₂ oxidation. For flavomonooxygenases, the microenvironment of the active site is such that it favors the attack of the flavin 4a position by the molecular oxygen to form the key 4a-hydroperoxyflavin intermediate. However, for flavin-dependent oxidases the reduced flavin cofactor and oxygen are positioned in ways that disallow the accessibility of the flavin 4a site to O₂. In the case of luciferase, a point mutation of the catalytically nonessential aCyto65 is apparently a sufficient structural perturbation leading to the induction of a new oxidase-like pathway of flavin oxidation. The subtlety of enzyme active center microenvironment in relation to the expression of catalytic mechanism and the versatility of enzyme evolution are thus indicated.
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