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Bioluminescence emission of bacterial luciferase with 1-deaza-FMN

Evidence for the noninvolvement of N(1)-protonated flavin species as emitters

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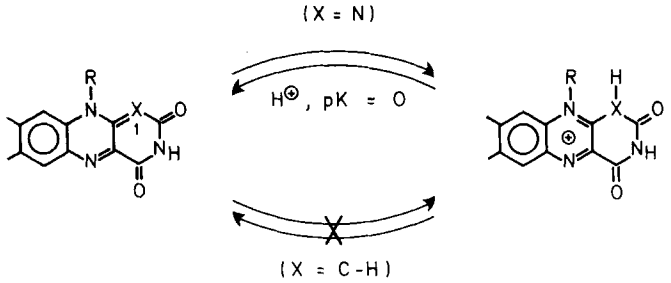
The reaction of reduced 1-d-FMN with oxygen and decanal results in bioluminescence with kinetic and spectral properties similar to those of the reaction with $FMNH_2$, even though the spectral (absorbance, fluorescence) and chemical properties of the oxidized forms differ greatly. This emission, which is about 10–15% as efficient as with $FMNH_2$, is postulated to involve the intermediacy of the corresponding 4a-hydroperoxide, the fluorescence of which occurred transiently. The N(1) protonated species had been proposed as the emitter in the reaction with $FMNH_2$, but the 1-deaza analog cannot be protonated at the corresponding position, thus excluding this possibility.

Bacterial luciferase catalyzes the emission of light with a characteristic maximum around 490 nm, in a reaction involving FMN, long-chain aldehyde and oxygen [1]. It has long been recognized that the singlet excited state of FMN itself cannot be the emitter, since its fluorescence emission, $\lambda_{max} \approx 525$ nm, does not coincide with that of bioluminescence ($\lambda_{max} \approx 490$ nm) [2, 3]. Among the several types of candidates for the emitter proposed over the years was that of a modified flavin formed in the course of the reaction, but finally regenerated as oxidized FMN. One such candidate was flavin protonated or substituted at position N(1) (Scheme 1). Its fluorescence properties in a rigid medium (possibly mimicking those existing at the active center of luciferase) were compatible with those of a putative emitter [4]. Based on this, several mechanistic proposals were put forward with the N(1)-protonated FMN cation as the emitter [4–7]. Although more compelling mechanistic alternatives have emerged in the meantime, one can exclude the possible involvement of the N(1)-protonated species in the light-emitting step if, as is reported here, the reduced 1-deaza-1-carba-FMN (1-d-FMN) is active in the luciferase reactions. Because of its chemical structure, this FMN analog cannot be protonated at position 1 (see Scheme 1).



Scheme 1. *Structure of 1-carba-1-deaza-FMN (1-d-FMN) and comparison with normal FMN.* Note that protonation of FMN occurs at position N(1), and that this is not feasible for 1-d-FMN. Protonation of the latter might occur at position O(2)

MATERIALS AND METHODS

Bacterial luciferase was isolated from the bioluminescent bacterium *Vibrio harveyi*, mutant strain M-17 (which requires exogenous aldehyde for maximum luminescence *in vivo*) [8] and purified according to the procedure of [9]. Based on SDS slab gel electrophoresis, the purity of the enzyme was estimated to be > 95%; its concentration was determined by using a molecular mass of 79 kDa for the heterodimer and an absorbance coefficient of 1.2 (0.1%, 1 cm) at 280 nm.

1-Deaza-riboflavin [10] was a kind gift from Prof. V. Massey; it was converted to 1-deaza-FAD by using FAD synthetase from *Brevibacterium ammoniagenes* according to the method of [11]. The hydrolysis of 1-deaza-FAD to 1-d-FMN was performed with *Naja naja* venom (Sigma). The purified 1-d-FMN contained no actual FMN, as judged by the lack of fluorescence detectable by visual inspection of either solutions, thin-layer plates (silica gel, Merck; solvent, butanol/acetic acid/water, 12:3:5) or after elution by high-pressure liquid chromatography (RP-18 column, 10–45% methanol gradient in 20 mM phosphate buffer, pH 6.0, in 30 min). FMN was obtained from Sigma (99% grade) and was not purified further, decanal (Aldrich) was prepared as a buffered 0.1% (vol/vol) suspension by sonification. All other reagents were of analytical grade.

The luciferase-bound 1-d-FMN hydroperoxide was prepared based on the method of Becvar et al. [12]. To 150 µl luciferase (0.6 mM) previously dialyzed overnight at 2°C

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Abbreviations. FMN and $FMNH_2$, oxidized and reduced forms of riboflavin 5′-phosphate, respectively; 1-d-FMN, 1-deaza-1-carba-FMN.

Enzyme. Bacterial luciferase (EC 1.14.14.3).

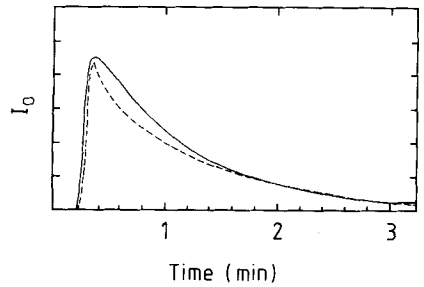


Fig. 1. *Kinetics of the luciferase reaction initiated with $FMNH^-$ and 1-d-$FMNH^-$.* Reactions were carried out at 20 °C in 0.01 M phosphate buffer pH 7.0 by injecting 1.0 ml 0.1% (vol/vol) decanal suspension saturated with O_2 into 200 µl of a solution which contained 10 µM luciferase and either 1 µM FMN (——) or 10 µM 1-d-FMN (-----) reduced by a minimal amount of dithionite; I_o, light intensity, arbitrary units

against 0.01 M phosphate buffer pH 7.0, containing 0.01% (vol/vol) decanol (Aldrich) [13], was added 150 µl 0.5 mM 1-d-FMN. After adding few grains of dithionite to reduce the 1-d-FMN, the mixture was applied to a Sephadex column (G-25 fine) and eluted under aerobic conditions at 2 °C. The protein fraction was collected and transferred either to the spectrophotometer (Kontron, Uvikon, model 820), with a thermostatted cuvette holder or to the fluorimeter (Perkin Elmer MFP-44), also temperature controlled. Fluorescence and bioluminescence spectra were corrected. Bioluminescence was measured with a photomultiplier photometer [14] calibrated with the standard of Hastings and Weber [15]. Luminescence assays were carried out with the dithionite method [9].

RESULTS AND DISCUSSION

1-d-FMN is competent in the light-emitting reaction. When 1-d-$FMNH_2$ is used, light emission equal to about 10–15% of that obtained with $FMNH_2$ is obtained [16]. Moreover, and contrary to the case with many flavin analogs that are active with luciferase [3], the kinetics and color of the luminescence initiated with 1-d-$FMNH_2$ are similar but not identical to those observed with $FMNH_2$ (Fig. 1).

This figure also illustrates that the kinetics of light-emission decay is slightly biphasic in the case of 1-d-FMN, compared to the monophasic reaction found with normal FMN. Also, the bioluminescent emission spectrum of the reaction with 1-d-$FMNH_2$, peaking at about 485 nm, is slightly blue-shifted, compared to that with $FMNH_2$ itself (see Fig. 6 below). In considering this it should be recalled that the oxidized analog has an absorption maximum at about 535 nm (Fig. 4 below), and is nonfluorescent. Also, it is crucial to know that this bioluminescence is not due to the presence of normal FMN as a contaminant in the 1-d-FMN preparation. This was judged not to be so by virtue of the absence of detectable fluorescence at 530 nm (see Materials and Methods). The amount of FMN required to give the luminescence observed would have been readily detectable by its fluorescence. Moreover, FMN would not be expected to be formed in the course of the synthesis of the 1-d-FMN [10].

The interaction of luciferase with reduced 1-d-FMN is substantially weaker than it is with $FMNH_2$. From the data of Fig. 2, a K_m of 40 µM can be estimated, which is some 50 times weaker than that reported for $FMNH_2$ by Meighen and Hastings [17]. The interaction is competitive with $FMNH_2$,

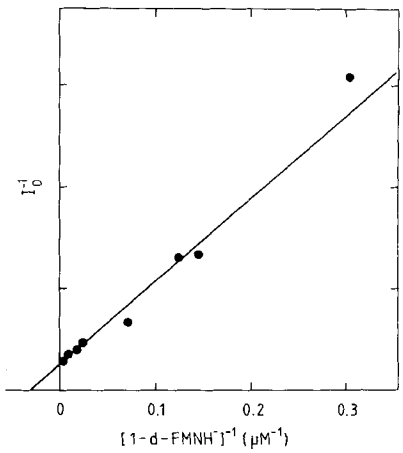


Fig. 2. *Dependence of initial maximum bioluminescence emission intensity upon the concentration of reduced 1-d-FMN.* Conditions were as described in the legend to Fig. 1, with luciferase at a concentration of 1.3 µM. The points represent the average of three measurements

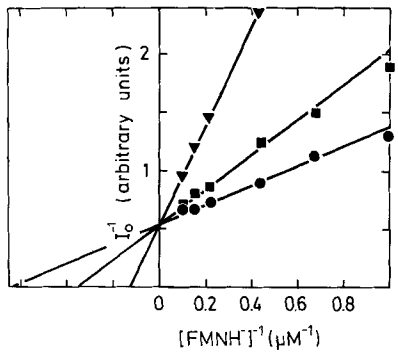


Fig. 3. *Competitive inhibition of the light emission in the presence of 1-d-FMN.* Double-reciprocal plot of the reduced flavin concentration (abscissa) versus the initial maximum light intensity (ordinate). Conditions were as described in the legend to Fig. 1 using 6 µM luciferase and the amounts of $FMNH_2$ shown. The concentrations of reduced 1-d-FMN were: (●) none; (■) 0.96 µM and (▲) 48 µM. Both flavins were reduced with a minimal amount of a concentrated dithionite solution

with an inhibition constant (K_i) of about 10 µM (Fig. 3). This supports the expectation that the analog and $FMNH_2$ share the same catalytic site, and that they thus might react by the same mechanism in the luciferase reaction.

Binding of oxidized 1-d-FMN to luciferase

Oxidized FMN binds to luciferase, but with a lower affinity than the reduced form, and with an altered absorbance [18]. Oxidized 1-d-FMN also binds to luciferase (K_d = 0.1 mM) and exhibits some small absorbance changes (Fig. 4); from the temperature dependency of this absorption change at 536 nm, an energy of activation for binding of about 38 kJ · mol^{-1} was estimated (data not shown). Within the limit of experimental error, this is the same as that found for the binding of oxidized FMN to the same luciferase [18].

Reaction of luciferase 1-d-$FMNH_2$ with oxygen

The reaction of luciferase-bound $FMNH_2$ with oxygen has been shown to result in the formation of an intermediate

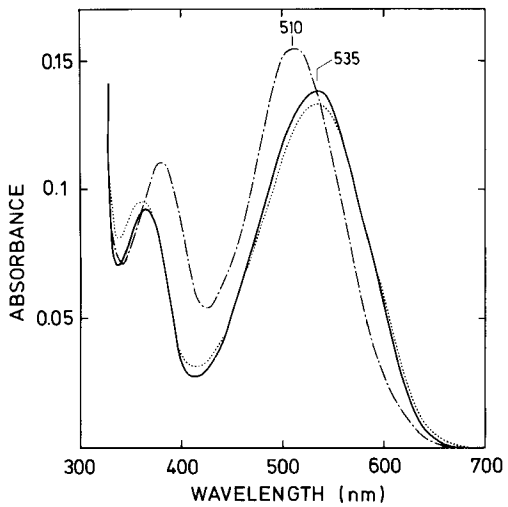


Fig. 4. *Comparison of the absorption spectra of free and luciferase-bound 1-d-FMN, along with that for the protonated form of 1-d-FMN.* Cuvettes contained 20 μM 1-d-FMN with or without luciferase (40 μM) in 0.01 M phosphate buffer, pH 7.0, at 4 °C. Protonated 1-d-FMN (same concentration) at $H_0 = -1$, measured at 20 °C. (——) Free 1-d-FMN; (-----) luciferase-bound 1-d-FMN; (— · —) protonated form of 1-d-FMN

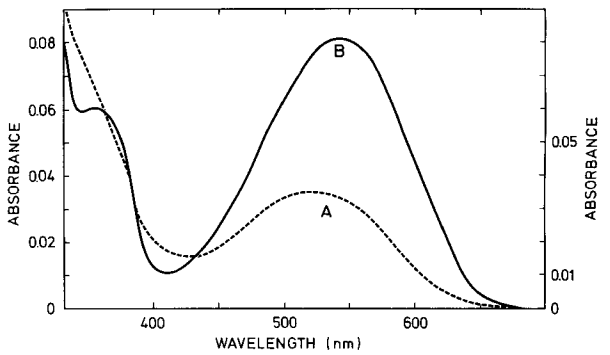


Fig. 5. *Attempted preparation of luciferase-1-d-FMN 4a-hydroperoxide, and spectral changes accompanying the formation of oxidized 1-d-FMN.* A solution of 150 μl luciferase (0.6 mM, dialyzed overnight at 2 °C against the elution buffer) with 150 μl 1-d-FMN (0.5 mM) was reduced with a few grains of dithionite and put on a Sephadex G-25 column (1.5 × 10 cm), which had been equilibrated with a 0.01% (vol/vol) dodecanol solution in 0.01 M phosphate buffer, pH 7.0 at a temperature of 2 °C. The elution of the protein was carried out under aerobic conditions at a rate of approximately 1 ml/min. After the void volume (8 ml), the protein fraction (0.8 ml) was collected in a 1-ml cuvette and its absorption spectrum determined (spectrum A) at a temperature of 7 °C. Spectrum B shows the final absorption spectrum which was taken 30 min later after the reaction was complete

identified as the 4a-hydroperoxide [19, 20]. Its fluorescent properties were found to correspond to those expected of the emitter [21], suggesting that a similarly substituted flavin, i.e., the luciferase-bound 4a-hydroxy-FMN, might have the same fluorescence and thus be the emitter [22]. Both the hydroperoxide and the hydroxide were viewed as derivatives of 4a,5-dihydroflavin, which has fluorescence in the range of 470 – 510 nm in a rigid frozen state or when bound to proteins, including luciferase [23].

The mode of reaction of reduced 1,5-dihydro-1-deaza-flavins with dioxygen is basically similar to that of $FMNH_2$ [11]. The reaction of 1-deaza-5-ethyl-1,5-dihydrolumiflavin models with oxygen has been studied extensively by Ball and Bruice [24] and shown to yield the 4a-hydroperoxide quantitatively, e.g. in dimethylformamide. Accordingly, when reduced 1-deaza-flavin bound to *p*-hydroxybenzoate hydroxylase was reacted with O_2, an intermediate was formed, which was deduced from its spectral (absorption, $\lambda_{max} = 385$ nm and fluorescence emission, $\lambda_{max} \approx 530$ nm), and chemical properties to have the structure of a 1-deaza-flavin 4a-hydroperoxide [25]; the enzyme also stabilizes the flavin 4a-hydroperoxide.

Since a similar reaction may be expected for 1-d-$FMNH_2$ bound to luciferase, we attempted the preparation of luciferase-bound 1-deaza-flavin 4a-hydroperoxide, using the method described earlier for the isolation of the luciferase-FMN hydroperoxide [26]. This consists in the application of a mixture of luciferase and FMN reduced with dithionite to a short gel-filtration column (Sephadex G-25) equilibrated with aerated buffer. Under these conditions luciferase-bound, reduced 1-d-FMN should react on the column with O_2 to form the 4a-hydroperoxide, which would then separate from small molecules during elution.

The absorption of the eluted protein fraction is shown in Fig. 5 (curve A); it peaks at about 535 nm, indicating the presence of oxidized 1-d-FMN. Upon standing, the absorbance at 535 nm increases to that shown by curve B, indicating completion of the oxidative process. Trace B represents a mixture of free and luciferase-bound 1-d-FMN (see Scheme 2). In trace A, however, there is no apparent band in the 380-nm region, as is expected for the 4a-hydroperoxide species [24, 25]. On the other hand, the preparation does exhibit readily measurable fluorescence (Fig. 6). The excitation maximum corresponds well with the absorption maximum for the 1-deaza-4a,5-dihydroflavin chromophore [24], and also with that of the same hydroperoxide bound to *p*-hydroxybenzoate hydroxylase [25]. The fluorescence emission spectrum ($\lambda_{max} \approx 490$ nm) also corresponds well to the bioluminescence emission spectrum ($\lambda_{max} \approx 485$ nm), as also shown in Fig. 6.

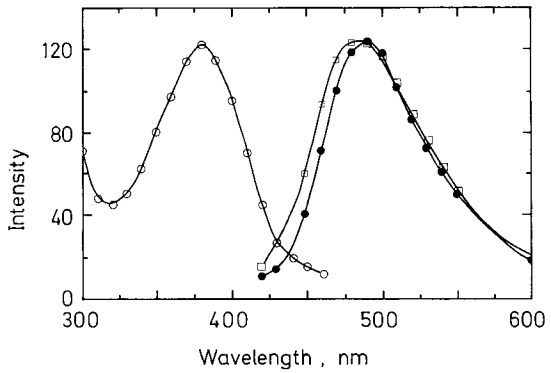


Fig. 6. *Fluorescence excitation and emission spectra of luciferase-bound 1-d-FMNH hydroperoxide and comparison of the latter with the bioluminescence spectrum.* The fluorescence excitation (○; λ_{max} emission = 490 nm) and emission spectra (●; λ_{max} excitation = 380 nm) were recorded at a temperature of 8 °C using a sample obtained as described in the legend to Fig. 5. The bioluminescence emission spectrum (□) was recorded at 4 °C starting with 1 ml 50 μM 1-d-FMN, which was reduced with a minimal amount of 0.2 M dithionite in 0.35 M phosphate buffer, pH 7.0 in the presence of 7.5 μM luciferase. Light emission was initiated by addition of 0.1 ml of a 0.1% solution of dodecanal in the same buffer. The spectra are corrected for lamp intensity and photomultiplier response

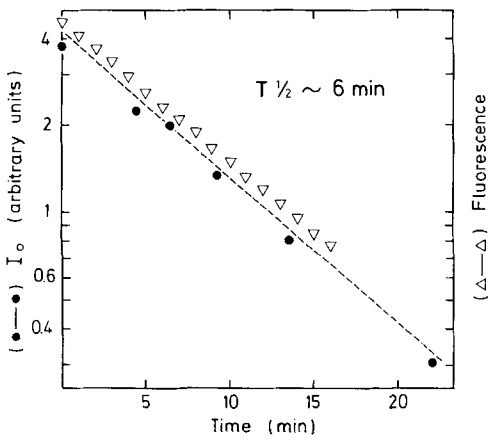
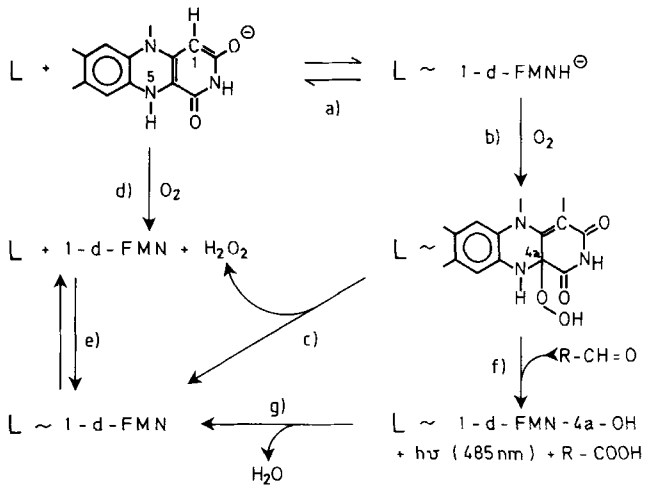


Fig. 7. *Kinetics of decay of fluorescence of luciferase-1-d-FMN 4a-hydroperoxide compared with loss of bioluminescence capacity.* A sample prepared as described in the legend to Fig. 5, except in 0.1 M phosphate buffer, pH 8.0 containing 0.3 M NaCl and 0.1% dodecanol, was incubated at 8 °C. At the times indicated, the fluorescence emission intensity at 490 nm (▽) was determined (λ_{max} excitation = 380 nm) while bioluminescence (●) was measured by injecting 20-µl aliquots into 1.0 ml of a 0.1% decanal solution



Scheme 2. *Reaction sequences and steps proposed to account for the interaction of 1-d-FMN with luciferase and for the bioluminescence reaction.* Reduced 1-d-FMN binds reversibly to luciferase (step a). The resulting complex can react with O_2 to form the 4a-hydroperoxide (step b), which can subsequently decay via step c to oxidized 1-d-FMN and hydrogen peroxide. Concurrently, free 1,5-dihydro-1-d-FMNH$^-$ can autoxidize to 1-d-FMN, which also can be bound reversibly by luciferase (step e). In the presence of long-chain aldehyde the 1-d-FMN hydroperoxide can undergo the bioluminescent reaction (step f) and produce light, long-chain carboxylic acid and the presumed luciferase-bound 1-d-FMN 4a-hydroxide. The latter then decays via steps g and e to oxidized 1-d-FMN. Note that in this scheme the concentration of the transient hydroperoxide will depend on the relative rates of steps b, c and f. In the present case these appear to be different from those observed with normal FMN [12]

As expected, this fluorescent species is metastable and decays at 8 °C with a half time of about 6 min. This decay was monitored by following the decrease of the fluorescence of the hydroperoxide excited at 380 nm and also by the decay of the light-emission capacity, as measured by the addition of decanal to aliquots. The coincidence of the kinetics of the two processes (Fig. 7) is compatible with the assumption that the metastable fluorescent species is identical with an active species in the light-emitting pathway. The observed fluorescence excitation and emission spectra shown in Fig. 6 can thus be attributed with confidence to the 1-deaza-4a-hydroperoxyflavin chromophore (see structure, Scheme 2).

The absence of absorbance corresponding to the luciferase-bound 1-deaza-flavin 4a-hydroperoxide might be understood if the reactivity of the luciferase-bound 1-d-FMNH$_2$ with dioxygen is low while the breakdown of the peroxide formed is relatively rapid, such that the steady-state level of the peroxide is low, albeit easily detectable by its fluorescence. Consistent with this is the fact that more light is obtained in the analog reaction with 100% oxygen (data not shown), and the fact that the spectrum of the enzyme fraction obtained immediately after Sephadex G-25 elution shows not only absorbance at 535 nm (oxidized 1-d-FMN) but there is also considerable absorbance in the region below 400 nm, characteristic of the presence of 1,5-dihydro-1-d-FMN [24]. Upon standing, the spectrum exhibits an absorbance which can be attributed to complete conversion to oxidized 1-d-FMN (Fig. 5), this species existing in an equilibrium between free and luciferase-bound forms. The overall rate of the decay would then be governed by the oxygen reaction and the conversion could proceed via several competing pathways, as summarized in Scheme 2.

The activity of 1-d-FMNH$_2$ with luciferase requires rather high concentrations, which may account for earlier reports [25, 27] that it was inactive. Its activity definitely excludes the flavin N-1 cation as the emitter, as had been proposed [4]; the possibility that a 1-deaza-flavin protonated at some other position is the emitter can also be excluded, since the absorption spectrum of the latter has a maximum at 510 nm (Fig. 4), while the bioluminescence peaks at about 485 nm. Similarly, the involvement of N(1)-acylated intermediates [28] can be excluded.

The replacement of the N(1) carbon in 1-d-FMN is expected to alter the chemistry of the system, in particular with respect to ring opening and closure processes involving splitting and formation of the bond between positions C(10) and N(1). Such rearrangements have been proposed to occur during the bioluminescent reaction [28]. In our opinion, the present results definitely exclude them.

The properties of the light emitted during luminescence are compatible with a 1-deaza-4a,5-dihydroflavin chromophore, notably the 4a-hydroxide, being the emitter (Scheme 2). This is in agreement with the proposal that 4a-hydroxy-4a,5-dihydro-FMN is the emitter in the normal bacterial luciferase reaction [1, 29].

The interactions of (oxidized) FMN and 1-d-FMN with luciferase do not differ substantially. This contrasts with the substantial differences encountered at the reduced level (approximately 50-fold difference in K_m), which might involve a weaker binding of reduced 1-d-FMN and/or lower oxygen reactivity. Conceivably, in (reduced) 1-d-FMNH$^-$, the negative charge is located at a different position compared to FMNH$^-$ (cf. Fig. 1), and a very precise interaction is required for optimal catalysis. Interestingly, with flavin-dependent hydroxylases reconstituted with 1-deaza-flavin coenzyme analogs, the hydroperoxide is formed, but it is unable to carry out hydroxylation of substrate [25, 30], suggesting that the N(1) locus plays a particular role in modulating the reactivity of flavin enzymes.

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