A POSTULATED MECHANISM FOR THE BIOLUMINESCENT OXIDATION OF REDUCED FLAVIN MONONUCLEOTIDE

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A mechanism is presented for the luciferase catalyzed oxidation of reduced flavin mononucleotide with oxygen in the presence of long-chain aldehyde. The mechanism involves the formation of a flavin peroxy anion which attacks aldehyde. A Baeyer-Villiger type shift leads to oxidation of aldehyde to acid, and to formation of hydroxide and excited protonated flavin which emits a photon. The mechanism is consistent with known details of the bioluminescent reaction and with known reactions of flavins and allows several verifiable predictions to be made.

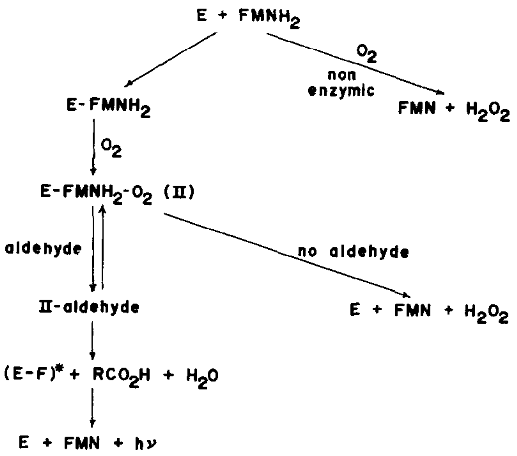
Bacterial luciferase catalyzes an oxidation of reduced flavin mononucleotide ($FMNH_2$) by molecular oxygen in which a product occurs in an electronically excited state, thereby resulting in light emission.[1,2] To obtain a high photon yield a long-chain aldehyde is required.[3] The mechanism has been postulated to involve its oxidation to the co-responding acid in a mixed-function oxidase type reaction:[4,5]

$$FMNH_2 + O_2 + RCHO \xrightarrow[\text{luciferase}]{} FMN^* + H_2O + RCOOH$$

Although pathways for the non-enzymic oxidation of reduced flavins have been proposed,[6,7] no specific detailed mechanism has been put forward for the bioluminescent pathway. We here postulate such a mechanism, based on evidence concerning both flavin reactions and bioluminescence.

An enzymic intermediate in the bacterial bioluminescence reaction is long lived. This can be easily demonstrated because the free substrate ($FMNH_2$) is lost by autoxidation in less than 1 sec (Scheme I),[7] yet light emission via the enzymic pathway continues for tens of seconds.[8,9] Our mechanism suggests the chemical nature of the long-lived intermediate.

Scheme I

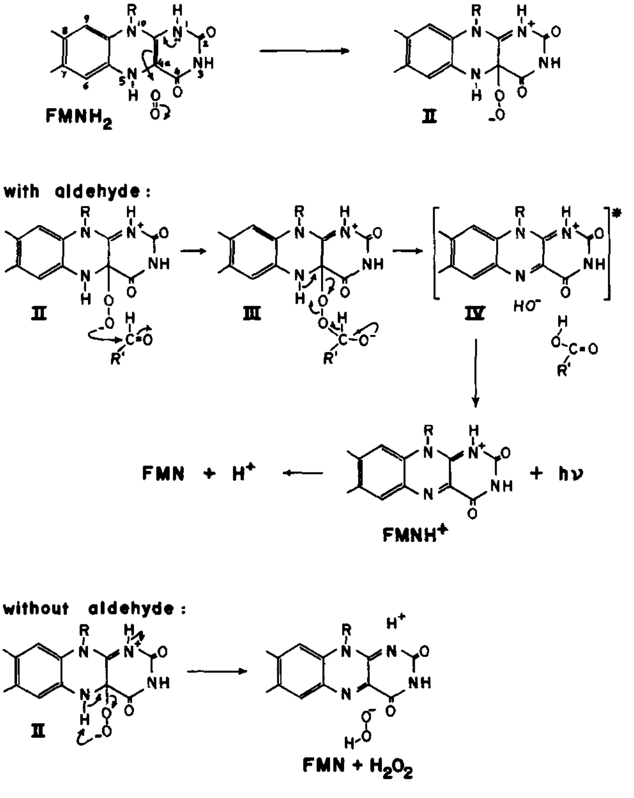


The enzymic oxidation of $FMNH_2$ involves at least two pathways, as shown. The first, in the presence of aldehyde, is assumed in our mechanism to involve acid formation,[4,5] and results in a high quantum yield emission. The second pathway involves the same long-lived intermediate (II) as before; in the absence of aldehyde, however, it breaks down with little or no light emission. In this second pathway luciferase may be viewed as an enzyme which functions to actually slow down a reaction.

Although the most obvious candidate for the emitter is oxidized flavin, there is a large discrepancy between the fluorescence emission spectrum of FMN (λ_{max} 530 nm) and that of the bioluminescence (λ_{max} 490 nm).[1,10] This might be attributed to binding of the enzyme, for flavin in less polar solvents exhibits a substantial blue shift.[11] However, measurement of flavin fluorescence polarization showed that no binding of ground state

FMN occurs with bacterial luciferase. Recent studies demonstrated that the N-1 protonated form of flavin ($FMNH^+$) exhibits a fluorescence emission at 490 nm, suggesting $FMNH^+$ as the emitter in bacterial bioluminescence.[12] The mechanism we propose makes use of this hypothesis (Scheme II), though an essentially identical mechanism can be written with loss of the N-1 proton in the first step.

Scheme II



We have initiated the reaction with addition of oxygen at carbon atom 4a of the flavin ring.[13,14,15,16] Intermediate II results from this reaction and is common to the two enzymic pathways and to the non-enzymic pathway.[1] The luciferase presumably functions to stabilize it and to prevent the non-enzymic reaction, which leads via radical intermediates to the formation of the flavin semiquinone and superoxide ion.[6]

In the presence of aldehyde, the peroxy anion II is assumed to attack the aldehyde, giving the anion of a peroxy hemi-acetal III. Binding of the aldehyde by enzyme is reversible,[9] and must partly block the non-aldehyde pathway, since with some aldehydes the reaction is much slower than with no aldehyde and yet photons in high yield are produced.[18] The aldehyde is then oxidized to an acid in a step similar to a Baeyer-Villiger shift,[19,20] hydroxide is lost and an excited state of protonated flavin, IV, remains. We estimate that the gain in aromatic character in forming IV from III provides about 20 kcal, while bond energy contributions, calculated from standard tables, amount to about 75 kcal. The total of 95 kcal should be sufficient to leave IV in an excited state, since emission at 490 nm corresponds to about 60 kcal per einstein.

Whether the reaction without aldehyde is truly non-luminescent[21,22] or simply of low quantum yield[1,23] has not been completely resolved. If excited states can be populated from intermediate II without the intervention of aldehyde, the low quantum yield should be viewed as a low yield in the actual formation of excited states, not in emission, as was (incorrectly) indicated in a recent publication (24, Fig. 1). However, bond energy calculations, similar to those mentioned above, indicate that in the absence of aldehyde only 7 kcal would be liberated. Assuming the same 20 kcal yield from resonance energy, a total of only 27 kcal would be available, not enough to result in an excited state of flavin.

The mechanism accounts for several known features of the bacterial bioluminescence reaction: (1) Luciferase catalyzed oxidation of different reduced flavins results in different emission spectra.[11] (2) Different aldehydes affect the overall kinetics of light production,[18] as expected if the rate determining step is II→III. (3) There is no isotope effect with α deutero aldehydes,[9] consistent with the postulate that reaction II to III is the rate determining step. An isotope effect would be expected in the conversion of III to IV. (4) There is only a single binding site for $FMNH_2$ on luciferase,[24] consistent with a reaction mechanism involving only one $FMNH_2$ molecule. (5) We have recently found that both erythrocuprein, which leads to dismutation of superoxide anion radicals,[25,26] and many free-radical trapping agents, have no effect upon the luciferase reaction. (6) The various intermediates can be identified with those previously postulated.[1]

The mechanism we have presented also makes some predictions, though verification may prove to be experimentally difficult. It involves the oxidation of aldehyde to the corresponding acid: attempts to demonstrate acid production should be intensified. It predicts that one atom of the oxygen molecule should be incorporated into the acid and the other into water. It suggests that III might be chemiluminescent. It predicts that there should be no isotope effect with $FMND_2$ in the presence of aldehyde, though without aldehyde the mechanism as written would predict an isotope effect. Finally, it predicts that there should be no electron spin resonance signal.

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