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ON THE CHEMICAL MECHANISMS OF BACTERIAL LUCIFERASE, AN UPDATING

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The purpose of this review is to focus on the chemical and physicochemical problems inherent to the mechanism of generation of excited states in the bacterial luminescence reaction. We refer to some excellent reviews by others for the discussion of biochemical/enzymological [1,2,3], bacteriological [4] and general problems including the different chemistry of bioluminescence in other organisms [5-8].
The net reaction can be reduced to the following equation:

$$2\ NAD(P)H + ATP + O_2 + 2\ H^+ \Longleftrightarrow 2\ NAD(P)^+ + ADP + 2\ H_2O + h\nu$$

Two molecules play particular roles in the process: Long chain aldehyde (C_{14}, myristic aldehyde) and FMN are cosubstrates of the enzyme luciferase. The former is oxidized to the corresponding carboxylic acid and then reconverted to the aldehyde by a reductase using NADPH and ATP (2 ATP may be required). FMN is reduced by FMN reductase, serves in the activation of O_2 and can play the role of the emitter:

$$FMNH_2 + RCH{=}O + O_2 \longrightarrow FMN + R\text{-}COOH + H_2O + h\nu\ (0.1 - 0.2)$$

The following properties, steps and intermediates, which have been disputed at one time or another, are now accepted in their essence, although discrepancies might still exist about details:

- Substrates [9-12] and stoichiometry of the reaction [9,10]
- Incorporation of 1/2 $*O_2$ in the products R-C*OOH and H_2*O [13]
- Physical characteristics of emitted radiation and quantum yield [1,2,8]
- Involvment of flavin in the emission [14]

- Turnover mechanism [15], and details of kinetic steps [3,16,17]
- Formation of flavin-4a-hydroperoxide prior to reaction with R-CHO [18-20].
- Formation of flavin-4a-hydroxide subsequent to light emisssion [21,22]
- Exclusion of N(1) protonated flavin (cation) as emitter [23]
- Flavin-4a-hydroxide as emitter [21]
- Blue- and Yellow-fluorescent proteins (BFP [8,24], and YFP [25-27]) as alternative emitters

In contrast to the above, the following questions are still unsolved:

- Mode of oxidation of aldehyde by the flavin-4a-hydroperoxide [28-31].
- Isotope effects on light emission using C(1)-H/D aldehydes [31,32]
- Mode of population of the excited state [33].
- Chemical structure of the primary (excited) state.
- Existence of more than one excited state (or emitter)
- Mechanism of excitation of, or mode of transfer of energy to "alternative emitters" such as blue- or yellow-fluorescent proteins.
- The question of light emission in the absence of aldehyde, i.e. the mechanism of the so called "endogenous light" [34]
- The nature of the product and of the fluorescence increase observed upon photoirradiation of luciferase flavin-4a-hydroperoxide [35].

The chemical reactivity of luciferase bound flavin-4a-hydroperoxide

For an elucidation of the mechanism of oxidation of the substrate aldehyde, an understanding of the chemical reactivity of luciferase flavin-4a-hydroperoxide is important. Bruice [28] has pointed out that the properties of this peroxide are quite different from those of aliphatic hydroperoxides, as deduced e.g. from the low pK of ~9.2 of the corresponding flavin-4a-hydroxide (dissociation 4a-OH $\leftrightharpoons$ 4a-O^- + H^+ [29]). The reaction of flavin-4a-hydroperoxide with thioxane is 10^5 faster than that of t-butyl hydroperoxide [30]. Enzyme bound flavin-4a-hydroperoxides exhibit typical reactivities like the oxygenation of sulfur-containing substrate analogs by the aromatic hydroxylases [36], by N,S-monooxygenase [37], and by cyclohexanone monooxygenase [38]. The latter performs catalysis by a Baeyer-Villiger mechanism including the monoxygenation of

boronic acids to the corresponding alcohols. The study of the reactions of luciferase flavin-4a-hydroperoxide with these two analogs would thus be of particular interest. The expected reactions would be the following:

$$\text{L-FlH-OOH} + \text{R-CH}_2\text{-SH} \longrightarrow \text{L-Fl}_{ox} + \text{R-CH}_2\text{-SOH} + \text{H}_2\text{O} + h\nu\ ?$$

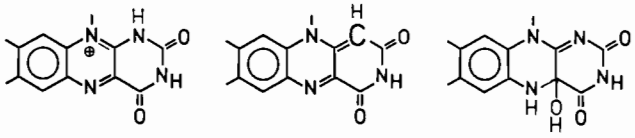
$$\text{L-FlH-OOH} + \text{R-CH}_2\text{-B(OH)}_2 \longrightarrow \text{L-Fl}_{ox} + \text{R-CH}_2\text{-OH} + \text{B(OH)}_3 + h\nu\ ?$$

Finding of oxidation with concomitant light emission with either analog would exclude the requirement of aldehyde C-H bond breaking, and also the involvment of an excited carboxylate in the light emission process [1]. The latter has been considered as a possible "primary" excited state with potential for transferring its excitation energy to different possible emitters, thus a "branched" type of pathway [1,39]. We found that 1-decanethiol actually stabilizes luciferase flavin-4a-hydroperoxide [40] in a way similar to that observed with various compounds including long chain alcohols [34,41]. Thus in the presence of a 5-fold excess of this analog, the decay rate of the hydroperoxide at 0^{o} was decreased by ~50%, and no further effect on the light emission was observed [40]. Although an analysis of the possible products was not done, we conclude that, in contrast to other peroxides, luciferase-flavin-4a-hydroperoxide is not particularly reactive with thiols.

The second analog, long chain boronic acid, has been thought to induce light emission and thus to be reactive [39]. We have reinvestigated this reaction paying particular attention to the purification of nonylboronic acid, and have found, that most preparations contain trace impurities, which induce light emission with luciferase [42]. They could be long chain aldehydes arising from autoxidation of the boronic acid. With the purest preparations no light emission above the level of "endogenous light" was obtained. Actually, nonylboronic acid is an inhibitor competitive with aldehyde in the normal luciferase reaction: Added to purified luciferase flavin-4a-hydroperoxide in the absence of aldehyde it slightly enhances the rate of decay (~ twofold effect on decay rate at 0^{o}, and at ~20 μM, equimolar concentrations) [42]. No significant amounts of nonanol and of boric acid, the two expected products, could be detected in the incubation mixtures. Since boronic acids typically react according to a Baeyer-Villiger mechanism, our results suggest that bacterial luciferase is not set up to promote this type of reaction.

On the nature of the emitter(s)

This has been a major point of controversy due to the lack of coincidence of the fluorescence emission spectrum of Fl_{ox} (λ_{max} ~520 nm), the product found in the spent luciferase reaction, with the luciferase luminescence (λ_{max} ~490 nm). Although earlier porposals envisaged different emitters, the involvment of flavin was clearly documented by the use of flavin analogs [14]. Among the proposals one was $1H\text{-}Fl_{ox}^{+}$ (1) [43], which, in spite of clear cut evidence to the contrary [23], is still encountered in newer text books and reviews. 1-Deaza-FMN (2) is competent in light emission, the quantum yield being ~15 % that of FMN, it forms a hydroperoxide which has a fluorescence emission λ_{max} ~485 nm. This FMN analog cannot be protonated at position 1; its cationic form (protonation occurs probably at N(5) with a $pK < 0$), has an absorption λ_{max} ~510 nm, which virtually excludes an emission below 500 nm [23].



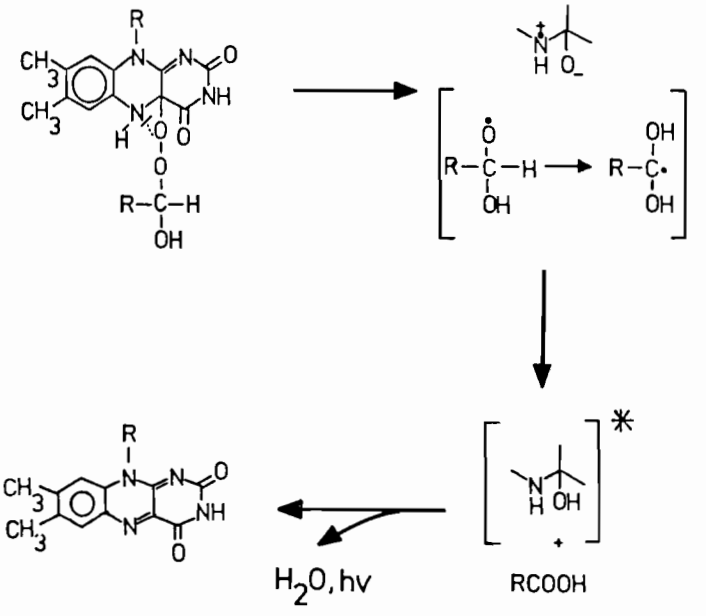
1 2 3

Very recently we succeeded in isolating and characterizing luciferase-flavin-4a-hydroxide (3). Its formation parallels emission of light, and its fluorescence emission spectrum is essentially identical to that of luciferase luminescence emission under the same conditions [21,22]. This, and the logic of the chemical reaction strongly suggest, that 3 is the emitter, at least under the specific experimental conditions used. In our opinion Lee's fluorescent transient [17], proposed as one of the emitters, is identical with 3. In contrast to this, the role as emitters of the so called sensitizer protein BFP and YFP, which contain 8-ribityllumazine [8,24] and FMN [25,27] as chromophores, is undisputed. Therefore the discussion shall be restricted to mechanistic aspects.

On the mechanism of generation of the excited state

Recently, based on the original proposal of Schuster and coworkers [44], a so called CIEEL Mechanism (Chemically Initiated Electron Exchange

Luminescence) has been put forward [2,39] as an alternative to previous proposals such as the Baeyer-Villiger fragmentation [45]. The shortcomings of the latter and of several other mechanisms, were the lack of a satisfactory explanations for the generation of the excited state, the postulation of the "wrong" emitter (cf. above), or the requirement of several chemical transformations involving excited molecules. The original CIEEL scheme can be adapted for an internal activation as shown in Scheme 1 [33,45].



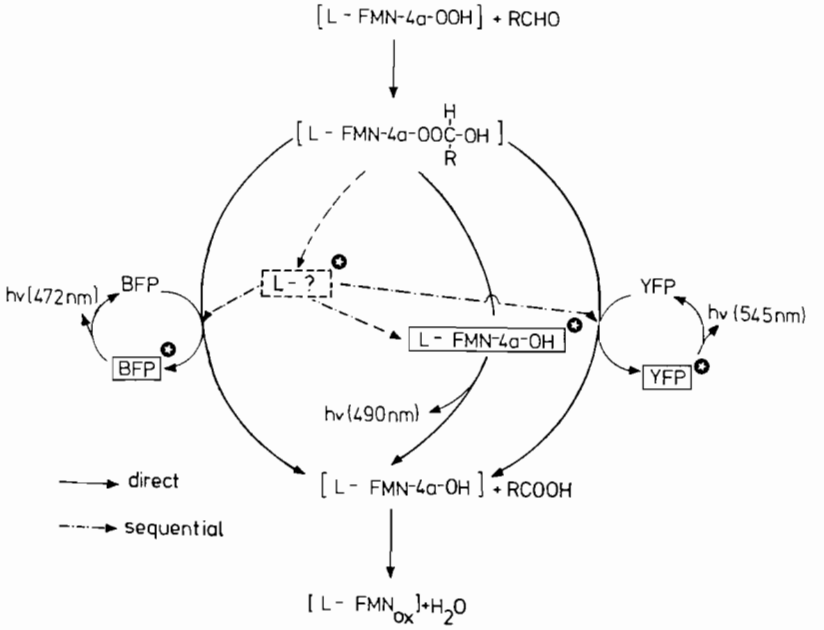
Scheme 1) Adaptation of the CIEEL mechanism to the bacterial luciferase reaction. In this case the 4a,5-dihydroflavin moiety acts as a donor and induces breaking of the peroxide bond. In the crucial step the aldehyde C(1)-H bond is broken via a radical mechanism and the resulting fragment back-donates $1e^-$ to the flavin to generate the excited state. Upon relaxation the flavin-4a-hydroxide eliminates water to reform FMN_{ox}

Here the activator is the o-phenylenediamine moiety of the 4a,5-dihydro-4a-hydroperoxy-flavin itself, and it would initiate the reaction by transiently transferring an electron to the peroxide of the peroxy-

hemiacetal, and resulting in the breakage of the O-O bond, as shown in Scheme 1. The occurrence of the peroxyhemiacetal is perhaps somewhat speculative, although kinetic evidence obtained with luciferase [32], as well as with flavin models [46,47] is compatible with its formation prior to the light generating step. The (co)involvement of a sulfhydryl residue analogous to the proposal of Kosower [48] can also be formulated. In order to test this CIEEL hypothesis, we have attempted the correlation of the rate of k_{obs} (light emission rise or decay) to the redox poten- tial or Hammett parameters of a series of 8-substituted flavin analogs. The results [49] are clearly compatible with Schuster's mechanism and with a radical type reaction, but they argue against a Baeyer-Villiger formu- lation. A few considerations might add weight to this interpretation: Aliphatic aldehydes are known to be prone to radical oxidation by oxygen/peroxides [50]. The flavin-4a-hydroperoxide is a molecule containing an oxidizing (peroxide) combined with a reducing moiety (phenylene diamine). That the latter type is a good donor and easily autoxidable does not need particular documentation. A further example of excellent $1e^-$ oxidability is the close relative 1,5-dihydro-flavin. A CIEEL formulation is also consistent with model work from Bruice's lab, who studied the luminescence accompanying the decay of N(5)-blocked flavin-4a-peroxihemiacetals [46,47].

On the mechanismus of population of excited states of "alternative emitters"

A central issue is whether the so called "fluorescent proteins" are excited directly as a consequence of the energizing chemical step, or whether they accept the excitation from a higher energy "primary" excited chromophore. Emission by a number of alternative emitters demands that there be branching; an unsolved question is whether this branching occurs before or after the formation of an electronically excited state, i.e. whether excitation is direct or not [2,8,39]. These differences are are summarized in Scheme 2. A strict requirement of the indirect mechanism is the occurrence of an excited chromophore of still unknown nature [L-?]*, scheme 2, which possesses enough energy for efficient transfer to all emitters, in particular to BFP. This excited species must precede the flavin-4a-



Scheme 2) Different modes for the population of the emitter excited states in the bacterial luciferase reaction. In the so-called "direct" mechanism (——) the external emitters compete with the 4a,5-dihydroflavin itself in the activation of the peroxide fragmentation e.g. via a CIEEL mechanism. The "indirect" mechanism foresees primary formation of an excited state of still unknown chemical constitution [L-?], the energy of which must be sufficient for transfer to the secondary emitters (BFP or YFP) e.g. via a Förster transfer (-.-.-). Alternatively [L-?] relaxes to excited flavin-4a-hydroxide.

hydroxide as excited state. It has been suggested that this could be the excited carboxylic acid product [1]. Its formation by a CIEEL mechanism is, however, difficult to envision. Another candidate might be a flavin-4a-hydroxide in a higher energy conformation. We do not believe in the existence of mysterious chromophores, which might be present in luciferase. Since this enzyme can be prepared in gram quantities (0.1 mMoles), if such chromophores would exist it is unlikely they could have escaped detection with modern analytic techniques. The choice of such a putative chromophore should thus be restricted to different forms of the flavin itself, to species derived from the substrate or to amino acid residues present at the enzyme active center. In this context it is tempting to speculate about an alternative, which has not yet been

considered: 4a,5-Dihydroflavins are nonfluorescent in solution, but can be highly fluorescent when bound to proteins (examples: luciferase flavin-4a-hydroxide [21] and luciferase flavin-4a-hydroperoxide [35]) or in frozen solution [51]. The stokes shift in the case of the 4a,5-dihydroflavin chromophore is unusually large suggesting the presence of energetically different conformers. The absorption spectra of 4a,5-dihydroflavins have maxima ranging from 330 to 390 nm depending on the substituents at positions N(5) or C(4a), and are also strongly dependent on the temperature [51,52]. This has been interpreted in terms of a different degree of bending of the flavin depending on (sterical interactions of) the substituents. NMR investigations of 1,5-dihydro-FMN bound to luciferase indicate a planar structure [52], while the indroduction of a substituent at C(4a) induces bending to some degree. From this we think it is worth considering that conformers of 4a,5-dihydroflavin are stabilized by the enzyme, and play the role of the primary excited state.

In the indirect mechanism any primary excited state is required to be much less fluorescent than 4a-hydroxy-flavin, but to have a life time sufficiently long for efficient transfer of energy to a secondary emitter. It is interesting that Shepherd and Bruice have postulated a primary, nonfluorescent excited state X* to be formed from the decomposition of 4a-peroxyhemiacetals of N(5) substituted flavins, which transfers excitation to several (secondary) emitters [47]. Clearly also other species which can be formed from a flavin-4a-hydroperoxide and which can be converted to flavin-4a-hydroxide could play the same role.

In contrast to the above, the so called "direct" mechanism [2,39] envisages several external chromophores as well as the 4a,5-dihydroflavin nucleus itself to act as alternative reactants, e.g. according to the CIEEL mechanism. Preliminary experiments from our laboratory designed to give an answer to this problem, involved the use of purified luciferase flavin-4a-hydroperoxides formed using different flavin analogs (iso-FMN, FMN, 2-thio-FMN), which have emission spectra ranging from 478 to 540 nm, and YFP. The results [53,54] are compatible with a direct mechanism; they cannot, however exclude an indirect one (Scheme 2). We will pursue this lead, expanding the range of FMN analogs used, in particular varying the type of FMN substitution when bound to YFP. A prediction for this mechanism is that a correlation similar to that presented in the accompanying paper

[49] for luciferase itself, should be found when k_{obs} is plotted against the redox potential of the FMN bound to YFP (of BFP). Note that in this case an "oxidized" species (FMN, and 8-ribityllumazine in the case of YFP and BFP respectively) must serve as donor in spite of the fact that it is considered an "acceptor", and that the redox potential would be that for the oxidation of the species. Since information of the last point is not available an assessment of the role of these molecules as "donors" in a putative CIEEL mechanism is difficult.

Conclusions

From the experiments discussed in this and in the accompanying paper, and from the chemical considerations discussed above the following points emerge:

- A Baeyer-Villiger mechanism appears improbable.
- No experimental result contradicts a radical (or a CIEEL) mechanism. This also would be in agreement with most recent findings from Lee's group [55], showing production of light from luciferase-flavin-4a-hydroperoxide and free radicals.
- A differentiation between a "direct" or an "indirect" type mechanism (Scheme 2) is not (yet) possible.
- The chromophore of a putative primary excited state has to be found among the species and intermediates directly involved in the reaction.

Finally we would like to stress the point, that some ideas and speculations put forward above should be taken as such; they are intended to promote discussion and hopefully to help find a solution to the fascinating puzzle of flavin mediated luminescence.

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