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The mechanism of electronic excitation in the bacterial bioluminescent reaction

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Abstract. The current state of the problem of formation of the electron-excited product in the chemiluminescent reaction that underlies the bacterial luminescence is analysed. Various schemes of chemical transformations capable of producing a bacterial bioluminescence emitter are presented. The problem of excitation of secondary emitters is considered; two possible mechanisms of their excitation are analysed. The bibliography includes 76 references.

I. Introduction

The bioluminescence, *i.e*. glowing of living organisms, is based on chemiluminescent reactions catalysed by biological catalysts, *i.e*., enzymes that are called luciferases.[1,2] Luminescence is inherent in more than 700 animal species, the best studied of which are bacteria, fireflies, marine coelenterates (jelly-fishes, hydranths, *etc*.), fishes, and so on.[1] Among the luminescent organisms, only bacteria emit light continuously. Being developed on organic remainders formed, they make a substantial contribution to the ocean glow. It is believed that by luminous bacteria attract more advanced species (for example, fish or shrimp) and thus they can populate their organs as symbiotes or parasites.[1] However, the signal function of bioluminescence may be secondary. Many researchers suggest an important metabolic role of light-producing systems,[3] in particular, the relationship between the bioluminescent reactions and the antioxidant system of the organisms is considered.[4–6] Presumably, bioluminescence is due to the hereditary change in the mechanisms that protect cells specially releasing reactive oxygen species for particular purposes from self-destruction.[7] In addition, it has been suggested that luciferases and their substrates perform only metabolic functions in the cells, while emission of a light quantum is a 'random' process.[4–6] According to a hypothesis, the bioluminescent reaction serves for hydrogen peroxide deactivation by the cells under conditions of intensive energy metabolism.[8] The excess energy is emitted as a light quantum; otherwise, its dissipation as heat or side chemical reactions would threaten the cell viability.

The substrates of bioluminescent reactions (traditionally called luciferins) present in various organisms differ in chemical structure and belong to different classes of organic compounds.[1,9] The amino acid composition and structure of luciferases are also different even for related species of luminescent bacteria.[10] Currently, about 30 bioluminescent reactions, *i.e*., about 30 luciferin – luciferase combinations, are known.

Unlike most chemiluminescent processes in the inanimate nature, the luminescence of living organisms has a high intensity. This is due, first, to high rates of enzymatic chemiluminescent reactions, and second, to high quantum yields of the luminescence of the reaction products, *viz*., emitters. Despite the fact that emitter molecules of various bioluminescent organisms belong to different classes of chemical compounds, they bear similarity in the electronic structure: they belong to the same spectral luminescent group in the classification of molecules according to spectral luminescence properties.[11] The molecules of this group are characterised by high quantum yields of $\pi\pi^*$ fluorescence and high efficiency of intramolecular relaxation of energy from upper excited states.

In addition, certain contribution to the high bioluminescence yields (the number of quanta per reaction event) is made by the protein environment of the bioluminescence emitters, which protects them from energy transfer and transformation processes competing with light emission. In this case, the protein can act as a matrix that ensures rigid fixation of the fluorophore. In some bioluminescent reactions, the yields of bioluminescence are close to unity.[2,9]

High efficiency of formation of the light quantum distinguishes bioluminescence from another type of biological glow, namely, weak and ultraweak chemiluminescence, which always accompanies the vital activity of an organism. In the case of ultraweak luminescence of animal tissues, photons are usually formed as by-products of free radical autoxidation of unsaturated fatty acids.[12] In addition, bioluminescence usually performs a definite biological function (*e.g*., attracts a partner or deters beasts from prey) and in this sense, the light quantum is physiologically the main reaction product.

A common feature of all biological light emitting reactions is that molecular oxygen is always a reaction component.

The luminescence of bacteria has been attracting the attention of researchers for four decades; in some aspects, this is one of the best studied bioluminescent processes.[2,13] This, however, does not refer to the chemical mechanism of formation of the electron-

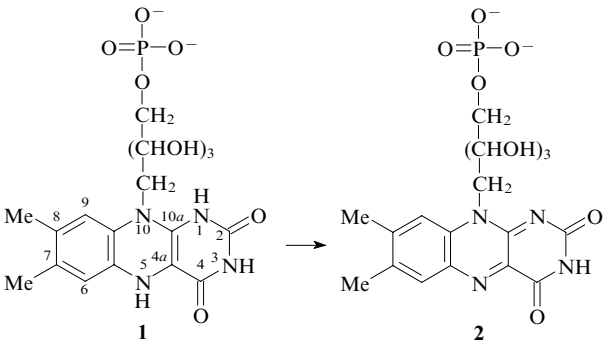
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excited product in the bioluminescent reaction; this question still remains open and arouses the interest of chemists and physicists who study chemiluminescence. Throughout the history of investigations of bacterial luminescence, a dozen of possible routes for the excitation of chemiluminescence in the bacterial luciferase-catalysed reaction have been proposed. This review gives comparative analysis of the main excitation mechanisms in the bioluminescent reaction of bacteria.

II. General characteristics of the bioluminescent reaction of bacteria

It has been found long ago [13] that the chemical grounds of bacterial luminescence include the enzymatic oxidation of the reduced flavin mononucleotide [$(FMN)H_2$, **1**] to flavin mononucleotide (FMN, **2**) and oxidation of long-chain aldehyde **3** to myristic acid (**4**) with atmospheric oxygen.



1 **2**

$$n\text{-}C_{13}H_{27}CHO \longrightarrow n\text{-}C_{13}H_{27}CO_2H$$

3 **4**

The overall reaction can be written as follows:

$$(FMN)H_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} \longrightarrow FMN + RCO_2H + H_2O + h\nu. \quad (1)$$

The bacterial luciferase is a heterodimer with a molecular mass of ~80 kDa. The components of the bioluminescent reaction can also react without an enzyme but the efficiency of chemiluminescence in this process is 10 orders of magnitude lower.[2] From this standpoint, bacterial luciferase is considered to be the most catalytically active known enzyme.

The heat of this reaction is 110 kcal mol^{-1}; this comprises the heats of oxidation of $(FMN)H_2$ (40 kcal mol^{-1}) and the aldehyde (70 kcal mol^{-1}).[7] The overall heat of the reaction is greater than the energy of the bioluminescence quantum equal to ~60 kcal mol^{-1}. However, it can be seen that the oxidation energy of only $(FMN)H_2$ does not suffice for the observed light quantum emission.

It is noteworthy that none of the starting reactants of the discussed reaction can exist in a bacterial cell in the free state, in particular, $(FMN)H_2$ (**1**) is rapidly autoxidised, while aldehyde **3** is a poison and is not produced by organisms. Therefore, bacteria have special enzyme systems that facilitate the reduction of FMN (**2**) and carboxylic acid **4** only for the purpose of bioluminescence. The reduction of FMN in bacteria is believed to be catalysed by NADH : FMN-oxidoreductase (NADH is the reduced form of nicotinamide adenine dinucleotide):

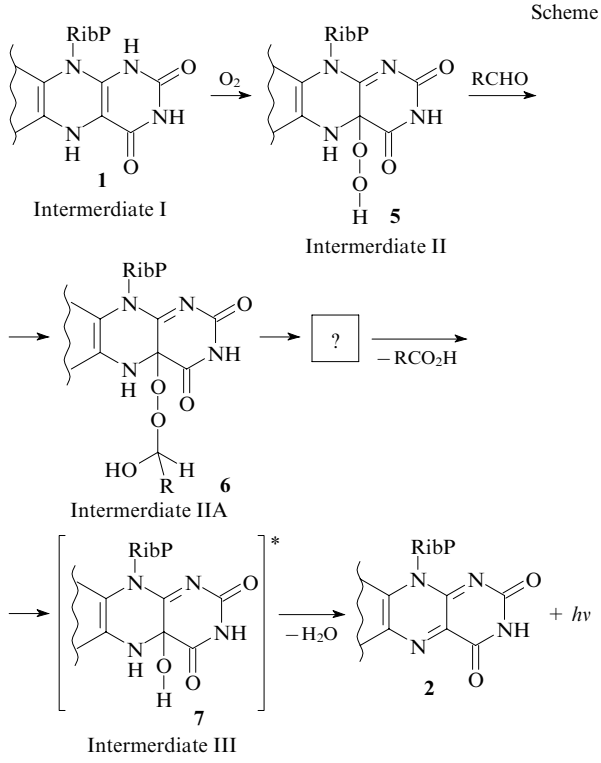
$$FMN + NADH + H^+ \xrightarrow{\text{NADH : FMN-oxidoreductase}} \longrightarrow NAD^+ + (FMN)H_2.$$

The question of the transfer mechanism of the reduced flavin mononucleotide between the oxidoreductase and bacterial luciferase still remains open. There is indirect evidence for both the independent operation of these enzymes [14] and the formation of a strong complex between them.[15,16] This problem has been studied in detail.[16–18]

Luciferases from various sources have different specificities with respect to the second substrate (aldehyde). The affinity of the aldehyde for luciferase is due to the hydrophobic interactions between the aliphatic fragment of the aldehyde and the hydrophobic groups of the enzyme. Therefore, as the length of the carbon chain in the aldehyde increases, the aldehyde becomes more strongly bound to luciferase.[1,13] In addition, the length of the aliphatic chain determines the position of the aldehyde carbonyl group involved in the chemical rearrangements in the luciferase active site. Perhaps, this is why aldehydes with chains shorter than the octanal chain are not involved in the luminescent reaction. Tetradecanal (**3**) is considered to be the natural substrate of bacterial luciferase,[19] because the enzymatic system that reduces a carboxylic acid for the purpose of bioluminescence is specific exactly toward myristic acid (**4**).[20]

The general scheme of reactant transformation in the reaction catalysed by bacterial luciferase was proposed more than forty years ago [21] and confirmed by subsequent investigations. According to this scheme, the reaction proceeds *via* three intermediate complexes (intermediates) formed by flavin derivatives with luciferase (Scheme 1). Complex I contains reduced flavin, $(FMN)H_2$, complex II, flavin hydroperoxide and complex IIA, flavin peroxyhemiacetal (the numbers **1**, **2**, and **5**–**7** refer to the proper substrates rather than to their complexes). The existence of intermediates I and II was proved by NMR spectroscopy.[22] The formation of intermediate IIA has not been proven experimentally as yet, but is recognised as a necessary step of the reaction.[9] It was shown by NMR spectroscopy that the major chemical transformations during the reaction affect the C(4*a*) atom of the FMN isoalloxazine fragment.[22] The results of these studies made it possible to compose the sequence of chemical transformations of the reduced flavin mononucleotide in the active site of bacterial luciferase (see Scheme 1) (this and subsequent schemes show only the part of flavin that is involved in transformations).

Scheme 1



Here and below, RibP = $CH_2(CHOH)_3CH_2OP(O)(OH)_2$.

There is still no unified opinion concerning the mechanism of transformation of intermediate IIA [5-hydroflavin mononucleotide 4*a*-peroxyhemiacetal (**6**) bonded to luciferase] into the excited emitter. This step, designated by the interrogation mark in Scheme 1, may comprise several steps. The problem of identification of the structure of the emitting chromophore (emitter) has not been completely solved either. It has been accepted by most researchers that luciferase-bound 4*a*-hydroxy-5-hydroflavin mononucleotide (**7**) acts as the bacterial bioluminescence emitter. We will call this complex intermediate III. The photoluminescence spectrum of compound **7** (in the bound state) is similar to the emission spectrum recorded during the bioluminescent reaction. The problem of formation of the emitter is considered in more detail in Section III.

The key steps of the bacterial bioluminescent reaction are as follows.[12, 23]

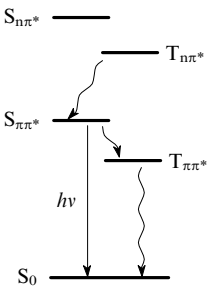
1. Formation of intermediates I, II and IIA [the energy range of the possible excited states of intermediates is approximately 260 – 390 kJ mol^{-1} (21 700 – 32 700 cm^{-1})].

2. Elementary excitation event in which the intermediate IIA is converted into emitter P* (intermediate III).

3. Light emission step $P^* \longrightarrow P + h\nu$.

The optical properties of the reactants deserve attention. The spectrum of bacterial bioluminescence is a nearly symmetrical Gaussian distribution over the frequency scale with a peak at a wavelength of ~490 nm. The position of the peak does not depend on the acidity or temperature of the reaction mixture. The bioluminescence yield in this reaction ranged from 0.1 to 0.3 under optimal conditions, depending on the luciferase and aldehyde types.[2] According to NMR studies,[22] the reduced flavin, $(FMN)H_2$ (**1**), is bound to luciferase as the $(FMN)H^-$ anion. The absorption spectra of intermediates I, II, IIA and III differ little from one another and have a peak at 380 nm characteristic of all hydroflavins.[2, 21] Among these intermediates, noticeable fluorescence (with a peak at ~490 nm) is observed only for luciferase-bound hydroxyflavin **7**.

The emitter of bacterial bioluminescence **7** refers to group V of the classification of molecules in terms of their spectral luminescence properties.[11] This group includes all aromatic heterocyclic compounds characterised by high quantum yields of fluorescence. The energy levels of the electron-excited states of the molecules of this group, taking into account the orbital nature and multiplicity, can be represented as follows:



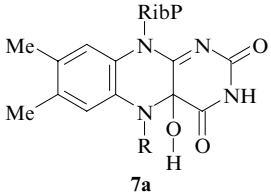
These molecules have upper excited states of the $n\pi^*$ type and undergo effective conversion of the excitation into the fluorescent $S_{\pi\pi^*}$ state from the $T_{n\pi^*}$ and $S_{n\pi^*}$ states.[24]

As noted above, an interesting fact is that the emitters of bioluminescent reactions of other organisms (for example, glowworms and jelly-fishes) also belong to group V of the spectral-luminescent classification, although the chemical structures of the emitters differ from that of the bacterial emitter.

Thus, the bioluminescent reaction of bacteria is an efficient chemiluminescent process which passes, like most known liquid-phase chemiluminescent reactions, through the formation and decomposition of organic peroxides. However, this reaction is still an exception, because no other efficient chemiluminescent processes involving hydroperoxides (such as derivative **5**) have been found as yet.[9]

III. Formation of the emitter in the bioluminescent reaction

Kurfurst and Hastings [25] identified and characterised the emitting intermediate of reaction (1). While monitoring both the variation of the absorption and bioluminescence intensity during the reaction of intermediate II [luciferase complex with 4*a*-hydroperoxy-5-hydroflavin mononucleotide (**5**)] with decanal, the researchers detected the formation of two forms of enzyme-bound flavin. The greater part of bound flavin was FMN (**2**) responsible for characteristic absorption peaks at 370 and 440 nm and a fluorescence maximum at 530 nm. The second form of the enzyme complex with flavin exhibited an absorption maximum at 360 nm and a fluorescence maximum at 490 nm and was identified as the intermediate responsible for bioluminescence. Based on the spectral-luminescent characteristics, the researchers postulated that the fluorophore of this intermediate has the structure of 4*a*-hydroxyflavin **7**. It was found that the emitting intermediate has a relatively short lifetime (7 min at 9 °C) ant its fluorophore is readily oxidised to FMN (**2**) by dissolved oxygen (the activation energy for this process equals 83 kJ mol^{-1}).[26] The ultimate identification of 4*a*-hydroxyflavin **7** as an emitting fluorophore (*i.e.*, emitter) is hampered by the fact that this compound has not yet been synthesised in a stable form, which is required to describe comprehensively its spectral-luminescent properties. The studied stable analogues of this compound, even those having suitable absorption spectra, exhibit very weak luminescence and provide much lower yields in chemiluminescent reactions (10^{-4} – 10^{-7}).[27, 28] Only recently was a paper published describing a model derivative of flavin with acceptable luminescent characteristics.[29] The authors studied binding to bacterial luciferase and fluorescence quantum yields for 5-decyl-4*a*-hydroxyflavin mononucleotide (**7a**). It was found that this compound having weak fluorescence in the free state provided a fluorescence quantum yield of 0.08 (this makes about 50% of the yield of bacterial bioluminescence) and a maximum intensity at 440 nm when bound to bacterial luciferase. In the opinion of the researchers cited, this fact confirms the identity of 4*a*-hydroxyflavin **7** and the natural bacterial bioluminescence emitter.

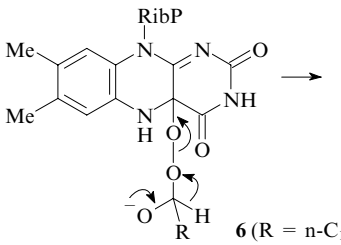


7a

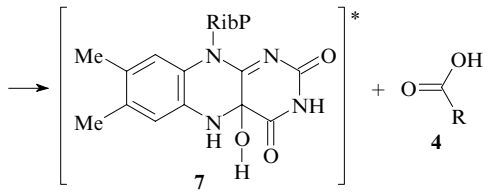
R = n-$C_{10}H_{21}$.

Of the multitude of mechanisms of excitation of the bacterial bioluminescence emitter proposed to date, six main mechanisms discussed below are most interesting from our standpoint.

The first mechanism was proposed rather long ago by Eberhard and Hastings.[30] Having postulated the key steps of reaction (1), they suggested that flavin peroxyhemiacetal **6** incorporated in intermediate IIA undergoes the Baeyer – Villiger rearrangement to give directly carboxylic acid **4** and excited hydroxyflavin **7**.



6 (R = n-$C_{13}H_{27}$)



7 **4**

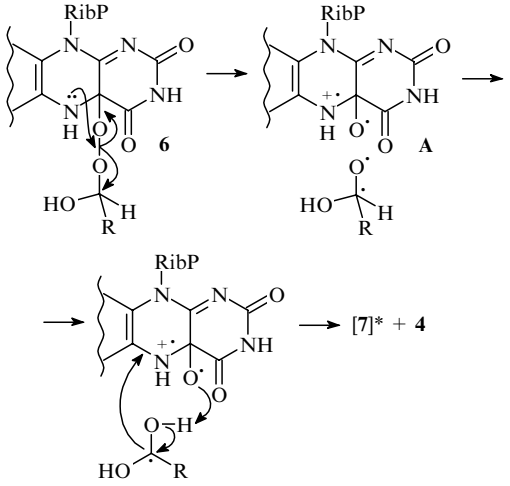
An objection to this hypothesis is the fact that in chemical reactions, the Baeyer–Villiger rearrangement does not give electron-excited products.[31]

Subsequently, Kosower proposed another route to the bioluminescence emitter.[32] It is worth noting that this mechanism is the first attempt to rationalise the excitation process based on analysis of elementary physicochemical processes. According to this hypothesis (Scheme 2), the excited state results from dissociative electron transfer from the flavin semiquinone radical **8a**. The peroxide radical anion $RCH(O_2)O^{-\bullet}$ formed after the superoxide attack abstracts a hydrogen atom from the luciferase SH group; this is followed by cleavage of the C–H bond in aldehyde to give protonated flavin mononucleotide **8b** in the excited state, which acts as the emitter.

Three critical remarks are pertinent as regards the above mechanism. First, this hypothesis is considered doubtful because it implies the occurrence of free radical steps in this reaction, which have not yet been detected by EPR.[33] (However, this may be due to limitations of the method.) Second, this mechanism implies involvement of the enzyme mercapto group in the process. Indeed, it has now been found[34] that the active site of the bacterial luciferase contains a cysteine residue Cys106, which could theoretically provide the SH group for the reaction with the peroxy radical anion $RCH(O_2)O^{-\bullet}$. However, the subsequent studies showed that this amino acid residue is not involved in the bioluminescent process, because its replacement by serine, alanine or valine does not result in the loss of bioluminescent activity of the enzyme.[35] It was found that the SH group of the Cys106 residue influences the aldehyde binding and the stability of intermediate IIA.[36] Third, an analogue of reduced flavin has been synthesised that cannot form the *N*(1)-protonated form [1-deaza-$(FMN)H_2$] but is nevertheless involved in the bacterial luciferase-catalysed reaction with emission of a light quantum.[37]

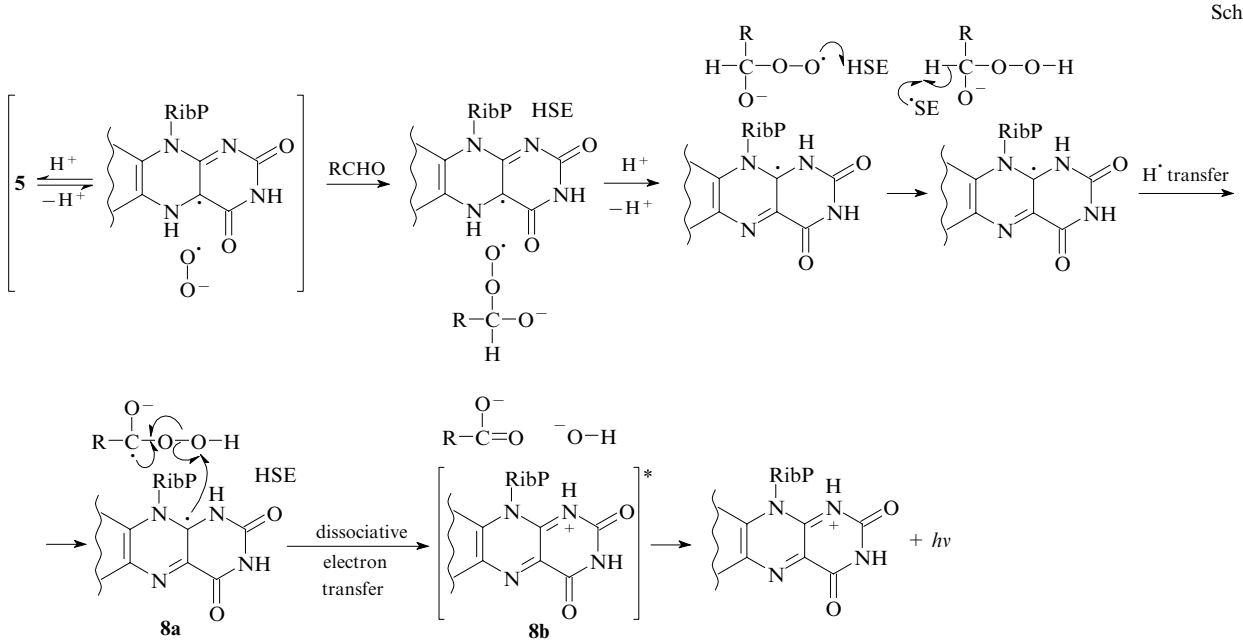
The mechanism proposed by Kosower can be criticised regarding several points; however, its main value is that the excitation of molecules during bioluminescence was related for the first time to dissociative electron transfer, *viz*., one of the key probable mechanisms of chemiluminescence.

After the disovery[38] of chemically initiated electron exchange luminescence (CIEEL) as a mechanism of chemiluminescent reactions, Mager and Addink attempted[39] to adopt this mechanism to bacterial bioluminescence. The CIEEL process typical of the chemiluminescence of some peroxides implies the following steps: (i) the reaction of peroxide with an electron donor and the formation of a radical ion pair, (ii) the transformation of the radical anion into the carboxyl radical and the reverse electron transfer to give the excited fluorophore. According to the Mager and Addink concept,[39] the formation of flavin peroxyhemiacetal **6** within intermediate IIA is followed by intramolecular single-electron rearrangement to give hydroxyflavin radical cation **A**. This rearrangement includes the transfer of an electron from the N(5) atom of flavin to the antibonding orbital of the peroxide fragment and the subsequent localisation of this electron on an oxygen atom accompanied by simultaneous homolytic cleavage of the peroxide bond.



Presumably, radical cation **A** is a key intermediate in the reverse electron transfer giving rise to either excited hydroxyflavin **7** or excited carboxylic acid **4**. The existence of 4*a*-hydroxyflavin

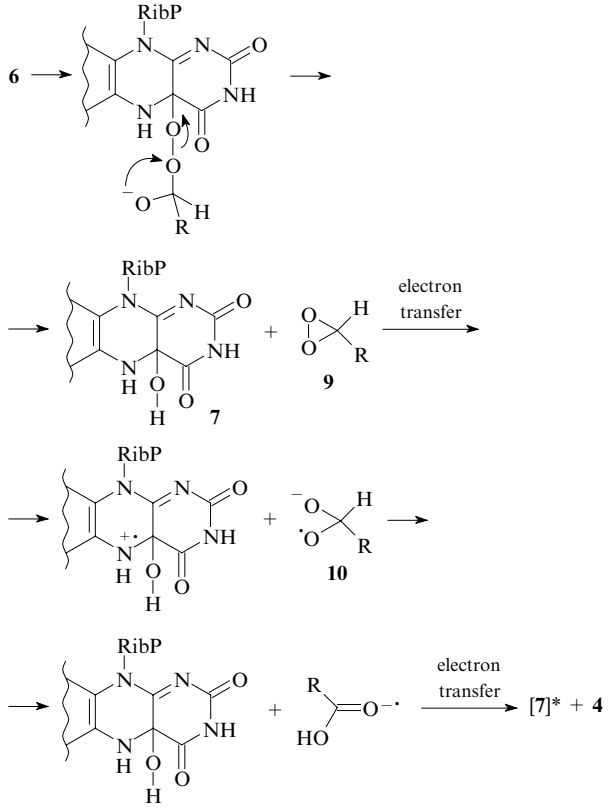
Scheme 2



HSE is luciferase.

radical cations structurally related to radical **A** has been shown using electrochemistry and radiolysis of model systems.[40] The efficiency of this reaction is controlled, first of all, by the single-electron transfer rate. On this basis, numerous attempts to verify this hypothesis using modified flavins with different redox properties have been undertaken. Eckstein *et al.*[41] generalised the results of these studies. The kinetic parameters of bioluminescence for the reaction with FMN analogues having different substituents at C(8) were measured. An inverse correlation has been found between the rate constant for the first-order reaction and the single-electron redox potential of FMN analogues. In the researchers' opinion, this means that in the rate-limiting step the flavin moiety of the complex loses the negative charge. This result is consistent with the intramolecular CIEEL mechanism for the bacterial luciferase-catalysed reaction. The Baeyer–Villiger type rearrangement of the intermediates implies an inverse dependence of the rate on the redox potential of flavins. Nevertheless, McCapra criticised[42] the study by Eckstein *et al.*[41] He noted that the obtained dependences were due to the shift of the equilibrium between flavin derivatives **6** and **7**; it was concluded that no evidence for preference of the CIEEL mechanism for the bacterial luciferase reaction is currently available.

Yet another line of research in the studies of the mechanism of bacterial bioluminescence has been pursued by Raushel and Baldwin.[43] They were the first to propose[43] the 'dioxirane' mechanism of the bioluminescent reaction catalysed by bacterial luciferase. According to their hypothesis, instead of elimination of the α-proton from the carbonyl carbon of the aldehyde (initiating the Baeyer–Villiger rearrangement), the formation of flavin peroxyhemiacetal **6** is followed by an attack by the aldehyde oxygen atom on the peroxide bond, resulting in its cleavage to give 4*a*-hydroxyflavin **7** and dioxirane **9**.

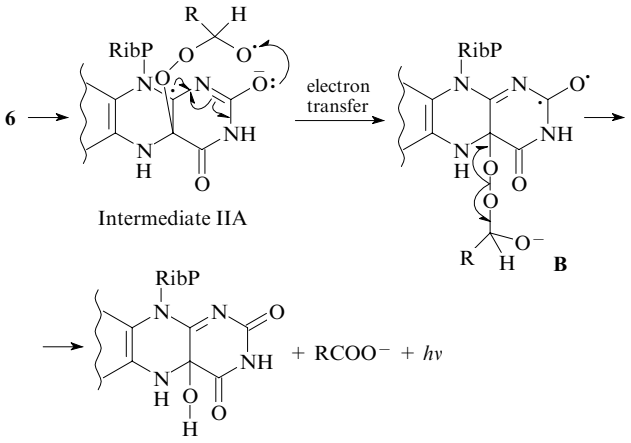


The formation of dioxirane is the key point of this hypothesis. There are two possible ways of dioxirane decomposition. First, dioxiranes can undergo homolytic cleavage of the peroxide bond to give radical anion **10**, which rearranges to give carboxylic acid (in either singlet or triplet excited state). This pathway bears direct analogy with decomposition of dioxetanes. Hydroxyflavin **7** is this type of a fluorophore, which can accept the electron excitation energy from both triplet and singlet states of the dioxirane decomposition product. Second, the excited fluorophore can be formed *via* CIEEL in which dioxirane accepts an electron from fluorophore thus forming a radical ion pair. The subsequent transformation of the dioxirane radical anion into the carboxyl radical and the reverse electron transfer give the electron-excited fluorophore. Both the first and second route of dioxirane decomposition release sufficient energy for occupation of the excited states capable of emitting a photon upon deactivation, even in the blue region.

The Raushel and Baldwin's research group have studied the isotope effect of deuterated aldehyde on the rate of the bioluminescent reaction under various conditions.[44] This gave a relatively low but reliable k_H/k_D value in the range of 1.5–1.9, which is consistent with the theoretical calculations of the same researchers carried out with the assumption of intermediate formation of dioxiranes.

However, there are some serious objections to the formation of dioxiranes in the bacterial luciferase reaction, in particular, those stated by McCapra.[9] First, no cases of dioxirane formation from an aldehyde are known to date.[45] Usually, the loss of a proton by an aldehyde results directly in carboxylic acid. Second, dioxiranes are highly reactive molecules,[45] and their formation would result in oxidation (injury) of the enzyme in the active site.

A distinctive feature of the next mechanism proposed by Kudryasheva *et al.*[46] is the occurrence of only intramolecular rearrangements during the formation of the excited structure of the bioluminescent emitter upon transition from structure **6** to **7**. The possibility of occupation of the upper excited states as the primary states of the bioluminescent emitter through the intramolecular electron transfer from the deprotonated ketoimide group to the peroxyhemiacetal fragment of intermediate IIA has been substantiated.[46]



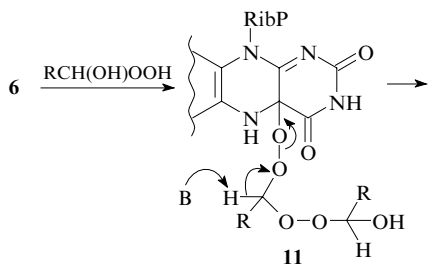
For flavins **5**–**7**, the $T_{n\pi^*}$ states are not the lowest excited states. The authors suggested that it is in this excited state (structure **B**) that flavin is formed in the bioluminescent reaction. According to this hypothesis, the formation of the excited carbonyl group is followed by intramolecular transfer of energy to the fluorescence state, $T_{n\pi^*} \longrightarrow S_{\pi\pi^*}$, which is allowed as a transition between the levels of different orbital nature and multiplicity (the El-Sayed rule[47]). The prohibition as regards multiplicity is eliminated due to spin–orbit coupling. This fact has been explained previously in a study by Plotnikov,[24] who calculated the rate constants for intramolecular transitions in this type of molecules; the resulting values were high, being equal to 10^{10}–10^{11} s^{-1}. The importance of spin–orbit couplings in the physicochemical processes has been emphasised by Minaev *et al.*[48–50] The efficiency of intramolecular transitions between the levels of different multiplicity was proved for many complex heteroaromatic compounds. Indeed, for a series of aromatic compounds including flavins, photoexcitation is known to be typically accompanied by reverse intersystem crossing from the

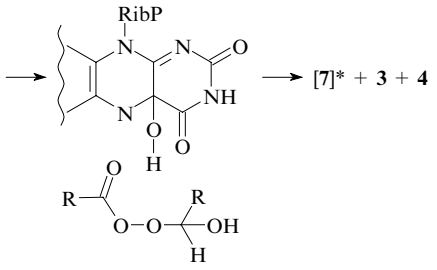
upper excited triplet states to the excited singlet states followed by fluorescence during transition to the ground state.[51]

The above-described relaxation process of the excitation in the bioluminescence emitter is associated with rearrangements of the electronic structure of the emitter molecule. The excitation located on the carbonyl group ($n\pi^*$ excitation) is delocalised throughout the molecule ($\pi\pi^*$ excitation). The process ends by the radiative transition $S_{\pi\pi^*} \longrightarrow S_0$. It was noted [24] that the upper triplet states of these structures have too short lifetimes ($10^{-10}-10^{-11}$ s) to be detected by EPR. It is noteworthy that this course of bioluminescent reaction also predicts an inverse dependence of the rate constant for the first-order reaction on the single-electron oxidation potential of FMN analogues, which was mentioned above in the discussion of the CIEEL mechanism.[39] The data obtained using the FMN derivative in which oxygen at the C(2) atom was replaced by sulfur (2-thio-1,5-dihydroflavin mononucleotide) as the substrate of the bioluminescent reaction are also in line with this hypothesis.[52] This is accompanied by a shift of the bioluminescence spectrum toward longer wavelengths (to 535 nm).

The fluorescence spectroscopy techniques have been used to carry out a number of studies aimed at detection of upper excited states of the bacterial bioluminescence emitter.[53–55] In particular, the energy transfer to exogenous molecules introduced in the bioluminescent reaction has been attempted.[53,54] Fluorescent aromatic molecules with energies of the first singlet excited state in the range of 22 000 – 32 000 cm^{-1} (*i.e.*, higher than the analogous energy of the emitter molecule) have been used as the exogenous molecules (potential acceptors of excitation energy). Note that the absorption spectra of the chosen molecules did not overlap with the bioluminescence spectrum. During the bioluminescent reaction in the presence of these compounds, their sensitised luminescence was observed as low-intensity bands in the short-wavelength region of the spectrum. This result was attributed to non-radiative energy transfer from the upper electron-excited states of the emitter to the levels of these exogenous molecules, probably, by the exchange-resonance (collision) mechanism. Since the sensitised luminescence was detected only for compounds with an energy of the fluorescent state of not more than 26 000 cm^{-1}, this energy was taken as the characteristics of the upper electron-excited state of the bacterial bioluminescence emitter.[54] A study of the fluorescence anisotropy decay of the acceptor molecules in the presence of bacterial luciferase proved the possibility of hydrophobic binding of acceptor molecules to luciferase, and, hence, these molecules can approach the emitter to distances appropriate for energy transfer by the exchange mechanism.[55]

The last mechanism of bacterial bioluminescence to be mentioned was proposed by McCapra.[9] He considered the following advantages of this mechanism over all other mechanisms: (i) this mechanism is consistent with the isotope effect data [44] and with experimental results obtained by variation of substituents;[41] (ii) this mechanism has a chemiluminescent analogy, namely, the reaction of peroxy esters with fluorescent compounds. McCapra postulated that the formation of intermediate IIA in the bacterial luciferase reaction is followed by the addition of hydrogen peroxide and second aldehyde molecule to the peroxyhemiacetal fragment of the complex.[9]





B is a base.

The resulting adduct **11** decomposes to hydroxyflavin and peroxy ester. The next step involves decomposition of the ester in the presence of fluorescent compound (hydroxyflavin) to give the excited molecules of the latter. This reaction route is confirmed by the detected enhancement of bioluminescence upon the addition of hydrogen peroxide.[56] The spontaneous formation of H_2O_2 adduct with two aldehyde molecules is also a proven fact.[56] The McCapra scheme proposed for bacterial bioluminescence is rather original and substantiated; however, as the author himself admits, this does not explain the key step, *viz.*, the formation of the emitter, because the mechanism of chemiluminescence of peroxy esters has not been ultimately established as yet.

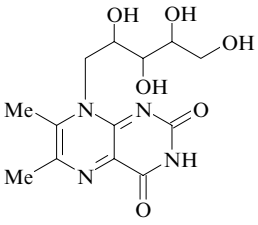
Thus, the question of the excitation mechanism of the molecules in the reaction catalysed by bacterial luciferase is still debated. The proposed schemes are diverse and cover almost all mechanisms currently applied to liquid-phase chemiluminescent reactions. However, as some researchers remark, none of the proposed reaction mechanisms is consistent with the whole set of experimental data accumulated throughout a five-decade history of the studies of bioluminescence.

IV. Excitation of secondary emitters of bacterial bioluminescence

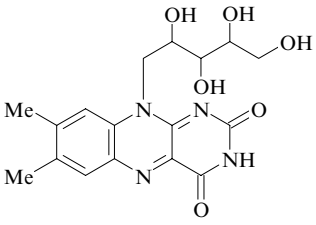
1. Characteristics of the fluorescent proteins, secondary emitters of luminescent bacteria

The fact that bacterial luminescence is based on reaction (1) was established by 1965. However, the difference between the spectral characteristics of bioluminescence of the bacteria themselves (bioluminescence *in vivo*) and luminescence in the reactions of the reagents isolated from them (bioluminescence *in vitro*) has remained uninterpreted for many years. The position of the spectral peaks of bioluminescent reactions depends on the luciferase type but is confined within the range of 487 – 505 nm. Meanwhile, the bioluminescence maxima of living bacteria differ much more appreciably. Indeed, some bright strains of luminescent bacteria of the *Photobacterium* genus emit light with a maximum at 475 nm,[13] while the bioluminescence of the Y1-strain of *Vibrio fischeri* discovered in the mid-1970s has a maximum at 545 nm.[57] It has now been proven that the reason for the change in the spectral composition of the above-mentioned bacterial emission is the presence of specific fluorescent proteins functioning as bioluminescence emitters, *i.e.*, in these cases, indirect or sensitised chemiluminescence takes place.

The fluorescent proteins of luminescent bacteria are protein complexes with low-molecular fluorophores capable of fluorescing in the visible spectral region. A fluorescent protein was isolated from the bacteria *Photobacterium*; this protein was called the lumazine protein (LumP), as its fluorophore is represented by 6,7-dimethyl-8-(1-D-ribityl)lumazine (**12a**).[58] The yellow green strain of *V. fischeri* was found to contain [59,60] the so-called yellow fluorescent protein (YFP) with a similar mass and, as has been found later,[61,62] with a primary structure also similar to that of LumP. The YFP fluorophore is represented by FMN (**2**) or riboflavin (**12b**).[63]



12a



12b

The key characteristics of these fluorescent proteins are given in Table 1.

To avoid confusion, we will designate the protein parts of LumP and YFP, which remain after removal of the fluorophore (this is called apoprotein), by $LumP_A$ and YFP_A, respectively. The proteins $LumP_A$ and YFP_A are notable by the fact that, unlike most other proteins, they do not quench the fluorescence of flavin bound to them.[64] On binding to $LumP_A$ and YFP_A, the fluorescence maxima of their natural fluorophores shift to opposite directions (see Table 1): a hypsochromic shift is observed for lumazine **12a** and a bathochromic shift is observed for flavin **2**. However, both these facts indicate a high polarity of the microenvironment of the above fluorophores in the binding site of the protein (since it is known that with a decrease in the solvent polarity, the peak of flavin fluorescence shifts to shorter wavelengths and the peak of lumazine luminescence shifts to longer wavelengths).[2, 64]

It was found that in the presence of micromolar concentrations of LumP or YFP, the following effects can be observed depending on the type of luciferase, the length of the aldehyde carbon chain and the source of the fluorescent protein: (i) a shift of the emission peak (hypsochromic in the presence of LumP or bathochromic in the presence of YFP); (ii) an increase in the emission intensity (up to a threefold in the presence of LumP and up to tenfold in the presence of YFP).[2] The mechanisms that underlie these effects are considered in more detail below.

Since the effect of fluorescent proteins on the characteristics of the bacterial bioluminescent reaction starts to be observed at low effective concentrations, they probably (or even obviously) form complexes with the reaction intermediates. It was shown by fluorescent spectroscopy and chromatography that LumP and YFP form 1 : 1 complexes with intermediates II and III (but not with free luciferase).[65, 66] The lack of interaction with luciferase looks reasonable, because otherwise, the addition of the fluorescent protein would prevent the substrates of the bioluminescent reaction (mainly reduced flavin **1**) from accessing the active site of this enzyme. The binding constant of intermediate III with the YFP determined by fluorescent titration is equal to 7×10^{-7} mol litre^{-1} (0 °C), which is comparable with the effective concentration at which the effect of this protein on the bioluminescent reaction starts to be observed.[66]

A study of the kinetics of bioluminescent reactions involving LumP and YFP has shown that an important role in the formation of protein complexes is played by aliphatic components (aldehydes, carboxylic acids, alcohols).[67] In the absence of aliphatic components, no reaction was detected between the proteins.

2. The mechanism of the change in the spectral composition of bioluminescence by fluorescent proteins

Comparison of the changed patterns of the bioluminescence spectra with the fluorescence spectra of LumP and YFP on photoexcitation indicates unambiguously that these proteins are involved in the light emission event resulting from the reaction. The problem of the excitation mechanism of the fluorescent proteins upon their introduction into reaction (1) is still debated. A non-radiative inductive resonance energy transfer (IRET) from the primary emitter due to weak dipole–dipole interaction could be the simplest and most attractive mechanism responsible for the excitation of secondary emitters. The rate constant for this transition k_{ET} is determined by the Förster equation:

$$k_{ET} = AQ_D k^2 \tau^{-1} R^{-6} J,$$

Table 1. Comparative characteristics of fluorescent proteins of luminescent bacteria.

Characteristics	Values for proteins [a]	
	LumP	YFP [b]
Molecular mass /kDa	21	24
Fluorophore	Lumazine derivative **12a**	FMN
Binding constant of the fluorophore with the protein /mol litre^{-1}	1.6×10^{-8} (20 °C, pH 7)	1×10^{-8} (4 °C, pH 7)
Long-wavelength absorption maximum of the fluorophore in the free/bound state /nm	406/417	445/470
Molar extinction coefficient of the fluorophore in the free/bound state /litre mol^{-1} cm^{-1}	10 300 (406 nm)/10 300 (414 nm)	12 500 (445 nm)/12 500 (460 nm)
Fluorescence spectral maximum of the fluorophore in the free/bound state /nm	490/475 (water)	525/538 (water)
Fluorescence quantum yield of the fluorophore in the free/bound state	0.45 (water, 22 °C)/0.58 (water, 20 °C)	0.26 (water, 22 °C)/–
Lifetime of the fluorescent state of the fluorophore in the free/bound state /ns	8.9 (20 °C)/1.7 (2 °C)	4.7 (20 °C)/5.5 (2 °C)
Rotational correlation time [c] of the fluorophore in the free/bound state /ns	0.15 (2 °C)/20 (0 °C)	0.2 (0 °C)/20 (0 °C)
Overlap integral of the absorption spectrum of the fluorescent protein with the bioluminescence spectrum /cm^3 litre mol^{-1}	2×10^{-15}	1.54×10^{-14}

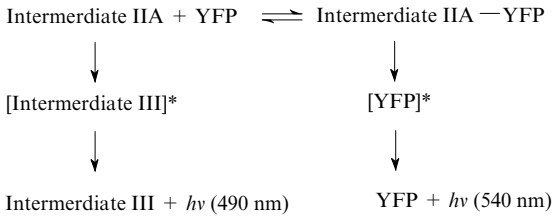
[a] The conditions of determination of the parameters are given in parentheses. [b] Characteristics are given for the best studied YFP form with FMN (**2**) as the fluorophore. [c] The rotational correlation time is the characteristic time of the exponential decay of the fluorescence anisotropy of the molecule upon pulse excitation.

where A is a constant, Q_D is the quantum yield of the donor fluorescence, k^2 is the orientation factor, τ is the lifetime of the fluorescent state of the donor in the absence of the acceptor, R is the distance between the energy donor and acceptor, J is the overlap integral of the absorption spectrum of the acceptor and the emission spectrum of the donor. The acceptor properties are characterised by three quantities in this formula: the distance R, the orientation factor k^2 and overlap integral J. The values of the first two can be such that they would favour a high rate of the IRET from hydroxyflavin **7** to fluorescent proteins: the proven existence of complexes formed by intermediate III with LumP and YFP can ensure a proximate arrangement and a favourable orientation of the fluorophores. The last factor, *viz*., the overlap integral of the spectra, is high for YFP and low for LumP (see Table 1).

It is the small overlap integral of the absorption spectrum of LumP with the bioluminescence spectrum that draws objections against effective accepting of energy by this protein according to the inductive resonance mechanism. In addition, the fluorescence maximum of hydroxyflavin **7** is at 495 nm, while the maximum of the absorption spectrum of LumP is at $\sim$420 nm, and the hypothetical energy transfer should be followed by an increase in the emitted quantum energy, which is not observed in the photoluminescence of dyes in solutions. A great contribution to the investigation of this problem was made by Lee's research group.[2,67–71] Initially, Lee and co-workers attributed [69] the effect of the lumazine protein to the existence of a high-energy precursor of the bacterial bioluminescence emitter with an excited state energy of $\sim$25 000 cm^{-1}. They assumed that this precursor transmits the electron excitation energy to either hydroxyflavin or the lumazine protein. However, subsequently, Lee refuted the high-energy precursor concept and attributed [2,64] the efficiency of IRET to a special orientation of the donor and the acceptor with a small overlap integral of the spectra. Fluorescence spectroscopy studies [64,68] revealed the existence of a short-time component in the fluorescence anisotropy decay of intermediates II and III complexed with LumP (on excitation to the flavin absorption band at 370 nm). This fast decay of the anisotropy was attributed to the occurrence of the IRET from flavin, incorporated in the intermediates, to lumazine **12a**. The results were adequately described by a model in which the rate constant for the IRET varied from 4×10^8 to 10^9 s^{-1} (depending on the luciferase type and LumP source).[65,68] Since the rate constant for the radiative transition of the primary emitter containing fluorophore **7** is 10^8 s^{-1}, it was concluded that the change of the spectral characteristics in the bioluminescent reaction in the presence of LumP can occur *via* the IRET. Lee's calculations [64] showed that a fixed distance of 1.5 nm and a favourable mutual orientation of flavin **7** and the lumazine fluorophore **12a** can make up for the small overlap integral of the spectra and account for the observed high efficiency of the 'anti-Stokes' migration of the excitation energy to LumP. Note that the spectral properties of the intermediate III – LumP donor – acceptor pair, namely, the close arrangement of the emission spectral peaks of the donor and the acceptor (490 and 475 nm, respectively), do not allow one to study the energy transfer by classical fluorescence spectroscopy methods. Usually, the energy transfer rate is determined from the change in the quantum yield of the donor in the presence of the acceptor (upon excitation to the donor absorption band).

Study of the energy transfer processes in the bioluminescent emitter – YFP system is easier than that for the LumP-containing system due to the large bathochromic shift between the emission peaks of the presumptive energy donor and acceptor. Hence, the emission bands of the donor and the acceptor can be effectively separated and the energy transfer rate can be measured more precisely from the change in the lifetime of the fluorescent state of the donor. This study has been carried out.[67] The rate constant for the IRET to the YFP (4×10^9 s^{-1}) was found from the change in the rate constant for the fluorescence decay of intermediate III. Thus, it was found that the IRET rate for the YFP system is about ten times as high as that for the LumP system.[60] The overlap integrals of the absorption spectrum with the bioluminescence spectra of LumP and YFP differ by a factor of 7.7. The authors [67] consider this to confirm the assumption that the secondary emitters are excited through weak dipole – dipole interaction according to the Förster (inductive resonance) mechanism.

The bioluminescence spectra in the presence of YFP consist of two bands: a strong yellow-green band with a peak at 540 nm and a weak blue band with a peak at 490 nm. For more detailed determination of the mechanism of formation of this spectrum, Eckstein *et al*.[72] studied the kinetics of reaction (1) in the presence of YFP. They found a complex and, what is more important, different patterns of variation of the time decay rates for the 'blue' and 'yellow-green' bioluminescence components. In the researchers' opinion, this indicates that the classical non-radiative energy transfer alone cannot account for the effect of YFP on the bacterial luciferase-catalysed reaction. A scheme was proposed according to which YFP forms a complex with intermediate IIA, thus decreasing its lifetime. This complex decomposes to give excited YFP, while the 'blue' component of the bioluminescence is emitted only by non-bonded intermediate III.



This mechanism resembles the intermolecular activated chemiluminescence where the fluorophore catalyses decomposition of the peroxide through charge or electron transfer, thus passing to the excited state. The mathematical model constructed according to the proposed scheme described adequately the experimental data obtained for the kinetics of two bioluminescence components (for low YFP concentrations). As has already been noted, subsequent studies showed that, upon photoexcitation, the IRET from intermediate III to YFP in the complex is a very efficient process.[67] Its rate is more than an order of magnitude greater than the rate of the radiative transition for hydroxyflavin **7** in intermediate III. Therefore, this excitation pattern for the yellow-green bioluminescence in a system involving YFP should not be underestimated.

3. Change in the bioluminescence intensity in the presence of fluorescent proteins

As noted above, both natural bacterial fluorescent proteins (LumP and YFP) increase the bioluminescence intensity of reaction (1). However, these effects were found to be due to different mechanisms.

The addition of LumP to the bioluminescent reaction was found to increase the bioluminescence yield. The reason for this increase (trivial, in the opinion of some researchers [2,67]) is as follows: the quantum yield of LumP fluorescence (0.58) is almost twice as high as that of luciferase-bound hydroxyflavin **7** (0.28 – 0.33). Therefore, the emission event is more efficient with LumP. We would like to note that this explanation is reasonable if the rate of energy transfer to LumP actually exceeds the rate of the intrinsic radiative transition in hydroxyflavin **7** by a large factor (4 – 10), as it was estimated in the works cited.[65,68] Unfortunately, the spectroscopic properties of the intermediate III – LumP system preclude precise measurement of the energy transfer rate; hence, the explanation for the high efficiency of this transfer casts doubts due to the small overlap integral of the spectra.

The fluorescence quantum yields of YFP and hydroxyflavin **7** differ slightly; therefore, despite the known [67] high rate constant for the energy transfer in the YFP system, no increase in the total

number of the emitted quanta takes place in the reaction involving this secondary emitter.

The emission intensity of the bioluminescent reaction in the presence of YFP is controlled in a somewhat different way. It was found [73] that in the presence of this protein the rate of reaction (1) substantially increases. A unique feature of this system is that this effect is enhanced with a decrease in temperature. The highest efficiency of YFP defined as the ratio of the emission intensity at 540 nm (I_{540}) to the intensity at 490 nm (I_{490}) is observed at 4 °C. Some researchers argue [73] that this system is perhaps the only enzyme system having a negative temperature coefficient. The loss of YFP activity with the temperature rise cannot be attributed to dissociation of flavin and YFP_A alone (for example, on heating of the reaction mixture from 4 to 15 °C in the presence of 4×10^{-6} mol litre^{-1} of YFP, only 15% of YFP molecules dissociate, while the I_{540}/I_{490} value decreases threefold). A change in the conformation of this protein on heating could be an additional factor contributing to YFP inactivation with the temperature rise, but this phenomenon has not found experimental proof or disproof. It is also of interest that not only the optimal reaction temperature but also the optimal length of the aldehyde carbon chain change in the presence of YFP.[74] The highest efficiency of YFP is attained in the reaction involving octanal, whereas in the absence of YFP, the highest intensity was observed for aliphatic aldehydes containing no less than 12 carbon atoms. This was attributed to the fact that the longest lifetime of intermediate IIA in reaction (1) is observed with octanal (compared with reactions involving other aldehydes). As a consequence, YFP has more opportunities to react with this intermediate and to 'switch' the reaction to the pathway leading to excitation of the 'yellow-green' chromophore.[74]

The biological role of the secondary emitters of luminescent bacteria is not entirely clear. Most assumptions concerning this issue imply that the main function of bioluminescence is signalling. From this standpoint, the involvement of LumP in the light emission provides at least a gain in the luminescence intensity (due to the increase in the bioluminescence yield). And YFP allows bacteria to glow over a broad temperature range (from 5 to 25 °C). It is also assumed that the high luminescence intensity of 'yellow-green' bacteria at 4 °C (*i.e.*, at a temperature where their metabolic processes are retarded) makes them attractive symbiotes for fishes or other organisms.[73]

If the hypothesis of 'non-thermal energy discharge' in a bioluminescent process is taken as the basis,[8] the possibility of changing the energy of the 'discharged' quantum in the presence of secondary emitters can be considered as an important adaptation feature of bacteria under conditions of variable amounts of energy entering the cell (for example, in the presence of different amounts of hydrogen peroxide).

To conclude this Section, we would like to note that any proposed scheme of the bioluminescent reaction should be consistent with the above-described effects produced by fluorescent proteins. This is especially true for the lumazine protein, as it gives rise to a hypsochromic shift of the bioluminescence spectrum. Of the six schemes presented in the previous Section, the last four schemes imply occupation of the electron-excited states of the secondary emitter, which can be represented by LumP. If the reaction follows the CIEEL mechanism, this may give excited carboxylic acid, which then functions as the energy donor for fluorescent molecules. Similarly, dioxirane decomposition can involve LumP and result in the excitation of the lumazine chromophore. According to the scheme proposed by Kudryasheva *et al.*,[46] LumP is capable of accepting energy from highly excited levels of the hydroxyflavin intermediate. The reaction proposed by McCapra may also involve another fluorophore (apart from hydroxyflavin) in the step of peroxy ester decomposition followed by excitation of this fluorophore.

Thus, although the secondary emitters of bacterial bioluminescence (the lumazine and yellow fluorescent proteins) induce different changes in the spectral characteristics of the bioluminescent reaction, they still resemble each other in their properties: their proteins have similar structures and interact efficiently with the bioluminescent reaction intermediates, thus creating conditions for the excitation of the secondary fluorophore. The highly effective change in the spectra and bioluminescence intensity upon the addition of fluorescent proteins is due, first of all, to the formation of protein complexes. Therefore, their fluorophores occur in the vicinity of the luciferase active site, almost at the centre of events, and can be involved in some chemical steps of the process and the physical transfer of the excitation energy. To date, it can be stated that neither 'physical' nor 'chemical' way of excitation of secondary emitters can alone explain the whole set of effects of fluorescent proteins on the bacterial bioluminescence parameters. It is noteworthy that the uncertainty in the mechanism of energy transfer to secondary emitters is partly due to the uncertainty of the mechanism of the proper reaction catalysed by bacterial luciferase. Subsequent studies of the pathways leading to the primary emitter of bacterial bioluminescence would apparently make corrections in the proposed schemes of excitation of fluorescent proteins. Conversely, investigation of the interactions of the secondary bioluminescence emitters with the components of the main chemical process can, in the future, shed light on the mechanism of the bioluminescent reaction.

V. Conclusion

Bioluminescence is a unique natural phenomenon and an exceptionally convenient tool that can be used in various fields of human activities ranging from environmental monitoring and clinical analysis to investigations of gene expression and regulation.[3, 75] The transformation of the chemical reaction energy into light with a high quantum efficiency in bioluminescent reactions has always attracted the interest of scientists. By now, the sequences of formation of chemical intermediates have been established and the spectral and kinetic characteristics of bioluminescent reactions involving enzymes (luciferases) have been described. Genetic engineering works aimed at modification of luciferase are in progress.[76] However, since bioluminescence is traditionally a subject studied by biologists (physiologists, microbiologists and biochemists), the formation of excitation is the most debated issue. Solving this problem requires the use of results obtained in structural, luminescence and chemiluminescence studies based on analysis of elementary physicochemical processes. Thus classification of molecules in terms of their spectral luminescent properties developed in the 1970–1980s shows that the emitters of all bioluminescent organisms belong to the group of molecules characterised by high ππ*-fluorescence quantum yields. The belonging of bioluminescence emitters to this group of compounds determines (together with enzymatic catalysis) the high bioluminescence intensity. In our opinion, study of the intramolecular energy transfer, the use of the theory of intermolecular migration of the excitation energy developed for solutions of fluorescent dyes and data for chemiluminescent oxidation reactions would allow researchers to achieve considerable insight in the processes giving electron-excited states of molecules upon bioluminescence.

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