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A PROPOSED MECHANISM FOR LIGHT EMISSION BY BACTERIAL LUCIFERASE INVOLVING DISSOCIATIVE ELECTRON TRANSFER

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A new mechanism that involves dissociative electron transfer in the energy transducing step is set forward for bacterial luciferase catalyzed light emission. The proposal involves (1) dissociation of the 4a-hydroperoxyflavin to a flavin radical and $\cdot O_2^-$,accounting for 570 and 620nm absorption, (2) $\cdot O_2^-$ addition to the aldehyde carbonyl to form a peroxyl radical, (3) abstraction of H from an enzyme thiol group to form RCH(OOH)OH, (4) thiyl radical abstraction of the H on C in RCH(OOH)OH, a step which can show a k_H/k_D of ca. 4, and (5) dissociative electron- transfer,a highly exothermic step that leads to a protonated flavin excited state,a carboxylic acid and water.

Bacterial luciferase catalyzes the production of blue-green light(λ_{max}, 490nm) in a reaction of reduced flavin mononucleotide(FH_2), molecular oxygen and a long chain aliphatic aldehyde(RCHO). This occurs via an enzyme:reduced flavin complex(intermediate I) and an enzyme:hydroperoxyflavin complex(intermediate II) (Eq.1)(1-3).#

$$(1)\quad E + FH_2 = \underset{(I)}{E,FH_2} \xrightarrow{O_2} \underset{(II)}{E,FHOOH} \xrightarrow{RCHO} E,FX^* \longrightarrow h\nu$$

None of the proposals(8,9,10) for the generation of the emitting state has been satisfactory, nor is the identity of the emitting

#Straight chain saturated aldehydes with chain lengths between 8 and 16 carbon atoms are functional in the in vitro reaction (4,5). Recent work suggests that tetradecanal is the naturally occurring aldehyde that is functional in the system (6,7).

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molecule established with certainty (3,10). We present here a mechanistic scheme involving dissociative electron transfer in the energy transducing step. The scheme also accounts for many details of our current knowledge of the system, including the recently reported long wavelength absorbing form of intermediate II (11,12).

We begin our consideration of the scheme with Intermediate II which has been established by uv and nmr spectroscopy as the enzyme complex of the 4a-hydroperoxy adduct of flavin mononucleotide(2,3,13,14); a model compound with similar spectroscopic properties has been described(15). In addition to the absorption band at 373 nm (2), intermediate II exhibits absorption maxima at 570 and 620 nm. This long wavelength absorption is now assigned to a flavin radical (16) arising from a reversible dissociation of the flavin hydroperoxide into the flavin radical and superoxide radical ion(the equilibrium form of superoxide ion at pH 7 (17)). The radical pair, illustrated in Eq.2, would not exhibit a readily detectable epr signal, consistent with earlier studies in which none was found (11). [Either (a) rapid recombination of the singlet caged pair formed by dissociation or (b) the low concentration of triplet,which normally exhibits a weak epr signal, could account for the failure to observe an epr signal]

(2) $FHOOH = FH\cdot,\cdot OOH = FH\cdot,\cdot O_2^- + H^+$

The fact that bioluminescence is not altered by added superoxide dismutase and free radical trapping agents (8) can be attributed to the inaccessibility of the radical pair in the lucerifase:flavin hydroperoxide complex.

In the next step of the mechanistic scheme, we propose that the radical pair combines with aldehyde to form a new complex, in which an electrophile(aldehyde) is in proximity to a nucleophile (superoxide ion). The nucleophilic character of superoxide ion towards alkyl halides is now well documented (18); addition to a carbonyl group, especially in the solvent-poor environment at the enzyme active site, could be expected, producing a peroxyl-hydroxyalkane (eq.3), a radical that has sufficient stability to serve as an intermediate in other reactions (19).

(3) RCHO + $E,FH\cdot,\cdot O_2^-$ ---> $E,RCHO,FH\cdot,\cdot O_2^-$ ---> $E,RCH(O^-)OO\cdot,FH\cdot$

Since it appears likely that the proton should be attached to N_{10} rather than N_5 in the emitting state, we postulate that the initially formed peroxyl,flavin radical pair (pair "A") undergoes a conformational rearrangement to an isomeric pair (pair "B"). (see the Scheme) Several observations are readily explained in terms of this conformational rearrangement, including the fluorescence behavior of intermediate II.

In the subsequent step, we postulate that an enzyme thiol group donates an H atom to the reactive peroxyl radical,$RCH(O^-)OO\cdot$. The importance of the thiol group for the activity of bacterial luciferase has been demonstrated by experiments involving reaction with an alkylating agent such as N-ethylmaleimide (20,21). Hydrogen atom transfer may be direct or may occur via proton transfer followed by electron transfer from the thiolate anion formed by proton transfer. The products of the reaction of the peroxyl-hydroxyalkane with the thiol group are a hydroperoxy- hydroxyalkane and a thiyl radical (Eqs. 4 and 4a; flavin not shown) .

(4) $E,RCH(O^-)OO^\bullet + ESH \longrightarrow E,RCH(O^-)OOH + ES^\bullet$

(4a) $E,RCH(OH)OO^\bullet + ES^- \longrightarrow E,RCH(OH)OO^- + ES^\bullet$

We now consider the transfer of the H that is derived from the CHO group of the aldehyde to the thiyl radical. The hydroperoxy-hydroxyalkane carries this hydrogen as part of a carbon-hydrogen bond which should be highly susceptible to attack by free radicals, since it is in the alpha position with respect to two oxygen substituents. The analogous hydrogens of acetals, $ROCH_2OR$, are very sensitive to attack by free radicals (22). The attack of the thiyl radical on the hydrogen of the CH would be subject to a primary hydrogen isotope effect, and we interpret the observed hydrogen isotope effect on bioluminescence (12) in terms of this hydrogen abstraction reaction (Eq.5). Although the thiyl radical-hydrogen abstraction reaction is apparently unprecedented among biochemical reactions, it seems to be quite reasonable from the chemical point of view (23).

(5) $E,RCH(O^-)OOH + ES^\bullet \longrightarrow E,RC^\bullet(O^-)OOH + ESH$

(flavin not shown)

Finally, we take up the crucial energy transduction step responsible for populating the excited (flavin) state. The carbon-centered aldehyde-derived radical formed by hydrogen abstraction is located near the flavin radical that was formed in Equation 2. We propose that dissociative electron transfer from the highest doubly occupied pi-orbital of the flavin radical to the carbon-centered radical would lead to a protonated excited flavin, a carboxylate anion and a hydroxide ion, as shown in Eq.6. The orbital configuration changes of the flavin in the transformation are indicated below the equation.

(6) $E,FH\cdot,RC\cdot(O^-)OOH \longrightarrow E,FH^{*+},RCOO^-,OH^-$

$(pi)^2(pi^*)^1 \qquad (pi)^1(pi^*)^1$

The high electron affinity of the hydroxyl radical (17) and the formation of a second carbon-oxygen bond (24) can be identified as the chief thermodynamic contributions to the exothermicity of the electron transfer reaction. [$-\Delta H$ = 83 kcal/mole, composed of the following contributions: O-O bond cleavage, 51 kcal/mole; second C-O bond from an alcohol-like C-O single bond, 83 kcal/mole (165 kcal/mole - 82 kcal/mole); $\cdot OH$ electron affinity (formation of OH^-), 46 kcal/mole; oxidation potential of $FH\cdot$, 5 kcal/mole]

The final step in the luciferase sequence is the emission of light from the protonated flavin. This species, with the emission peaking at about 490 nm (emission energy, 59 kcal/einstein), was proposed as the bacterial emitter on the basis of its spectroscopic properties (25). The overall mechanistic scheme is summarized in Fig.1.

A non-light-emitting reaction (a "dark reaction") can occur in which reduced flavin reacts with oxygen to form intermediate II, and the latter decomposes to oxidized flavin (FMN) and hydrogen peroxide (26,27). A simple mechanism for the formation of hydrogen peroxide involves escape of hydrogen peroxide from the ion pair produced by dissociation of the 4a-hydroperoxy adduct, intermediate II, as shown in Eq.7.

(7) $FHOOH \longrightarrow FH^+,OOH^- \longrightarrow F + HOOH$

Intermediate II exhibits fluorescence (28). Irradiation of intermediate II at its absorption maximum, 373 nm, results in a

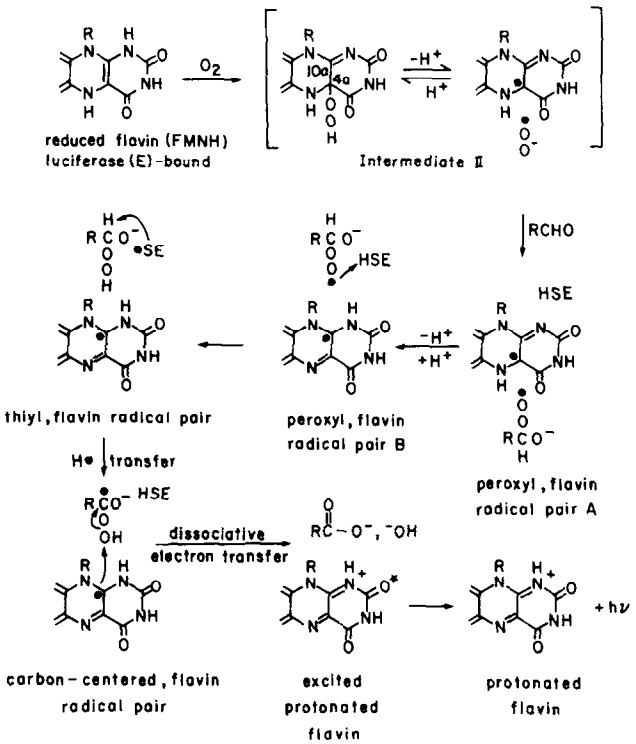

Fig.1.
The postulated molecular mechanism for the luciferase-mediated bacterial bioluminescence reaction.

considerable increase in fluorescence intensity, the final fluorescence spectrum being identical to the bioluminescence emission spectrum. We believe that the simplest way to explain these two phenomena ((a) identity of intermediate II emission spectrum and bioluminescence spectrum and (b) increase in intermediate II fluorescence quantum yield on irradiation) is as follows: On excitation, intermediate II undergoes photodissociation to a protonated flavin,hydroperoxide anion ion pair, which might be termed pair "A'" since its structure resembles that of the pair "A" shown in the Scheme (Fig.1). The protonated flavin in pair "A'" will be characterized by an emission spectrum and a quantum yield and will require another photon for excitation to the emitting state. The ion pair "A'"

can undergo conformational rearrangement to an isomeric pair,"B'" (again, analogous to the pair "B" shown in the scheme) . Pair "B'" will be characterized by an emission spectrum and a quantum yield and will also require another photon for excitation. The emission spectra of both pairs, "A'" and "B'" will be very similar to that of the bioluminescence emission for which the emitting species is a protonated flavin, carboxylate anion pair. It is reasonable that the quantum yield of fluorescence from pair "B'" will be higher than that from pair "A'" since the thiol group (an electron donor, and therefore a likely quenching agent) will be closer to the excited flavin in pair "A'" than in pair "B'". The fact that a subtle point like that of the fluorescence increase on irradiation of intermediate II can be explained in such a straightforward way on the basis of the Scheme lends credence to its validity. It may be supposed that that pair "B'" is in equilibrium with the 10a-hydroperoxide adduct of the flavin, and that photodissociation to the ion pair precedes excitation to a fluorescent excited state. Photodissociation may proceed by a single step heterolytic dissociation, or by a two-step process, homolytic dissociation to a radical pair followed by electron transfer (29).

The interesting difference between the aldehyde deuterium isotope effect found for the enzyme catalyzed reaction in low phosphate buffer (0.01 M) (high k_H/k_D) and that in high phosphate buffer (0.35 M) (low k_H/k_D) can be interpreted in terms of a shift in the rate-limiting step from that given in Eq.5 to an earlier one.

In addition to the new scheme being able to account for the currently known phenomena exhibited by bacterial luciferase, further work utilizing magnetic field effects on the

bioluminescence kinetics and yield might prove instructive with respect to the way in which the radical pairs participate in the overall reaction, effects being expected if the triplet pair recombination is important in any of the equilibria shown in the Scheme (For discussion of magnetic field effects on biochemical reactions, see ref. 30)

Electron transfer is clearly an attractive mechanism for the production of excited states via chemical (or biochemical) pathways (31,32); however, dissociative electron transfer has not been proposed previously for the production of electronically excited states.

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