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STUDIES ON BIOLUMINESCENCE AND ENERGY TRANSFER MECHANISMS

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Introduction

The chemistry of firefly luminescence is gradually being resolved. After a number of years of intensive work by a large number of investigators, it is now possible to propose a reasonable organic mechanism for the overall reaction leading to light emission (1, 2, 3, 4). The most recent findings which are of considerable interest to all investigators in the field of bioluminescence concern the mechanism of the chemical processes leading to the excited state and the identification and synthesis of the product emitter (4, 5).

This final report on the firefly system will be limited to a capsule presentation of the facts which have been uncovered concerning the chemical and enzymatic mechanisms.

It should be kept in mind that most of the detailed chemistry and enzymology has been done primarily with the lymprid beetle, Photinus pyralis; however, enough work has been done on over twenty other species of true fireflies to suggest that the same proposed mechanisms are valid. In addition, the present evidence indicates that the luciferin structure and the action of ATP serve the same function in the Elateridae, the Phengodidae, and possibly the Drilidae as they do in the Lampyridae (6).

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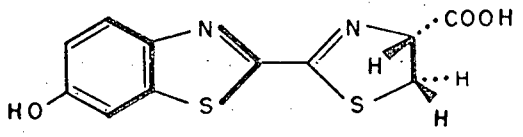
Structure of the Product Emitter, (Oxyluciferin), Luciferin (LH_2) and Dehydroluciferin (L)

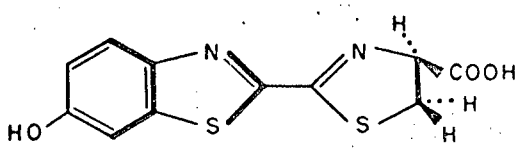
Before discussing the detailed mechanisms which have been proposed for firefly bioluminescence we would like to review briefly the structure of luciferin and point out some of the key features that are important for the light emitting process. The structure of D(-)LH_2 and L are shown in Figure 1. (7,8).

1. The carboxyl group of LH_2 is the important site for the formation of the anhydride with adenylic acid from ATP and as discussed later is the source of the CO_2 which is released in the chemical reaction leading to light emission.

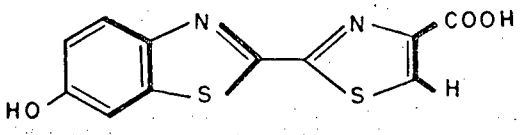
2. The hydrogens at the 4 and 5 carbon atoms are of great importance. The data indicate that the enzyme must abstract a proton from the 4 position prior to the addition of oxygen at that point. The fact that the substitution of deuterium at that position inhibits the rate of the light reaction by almost fifty percent supports this conclusion (9).



L (+) LUCIFERIN



D (−) LUCIFERIN



DEHYDROLUCIFERIN

Fig. 1. Structure of firefly luciferin and dehydroluciferin.

Proton abstraction at the 5 carbon atom is essential in determining the color of the light emitted, and will be discussed later.

3. The state of the hydroxyl group at the 6' position of the benzothiazole ring is important for both the luminescent and fluorescent properties. All evidence indicates however that it is the phenolate ion that is essential for both red and yellow-green light emission.

   The titration of LH_2 both potentiometrically and spectrophotometrically shows a single ionization between pH 4 and 11.5. This reflects the dissociation of the 6'-hydroxyl group to the phenolate ion with $pK_a = 8.7$. The ionization of this group has a large effect on the absorption spectrum. This shift in the spectrum on ionization is both necessary and sufficient to conclude that the pK_a of ionization of the excited state is different from the ground state. From the calculated pK_a, one would predict that, if equilibrium were established in the excited state, the predominate form between pH 2 and 12 would be the phenolate ion. The fluorescence emission spectrum of LH_2 at pH 4.5 shows that if the phenol form is excited, proton transfer to the solvent occurs and emission is from the excited state of the phenolate ion ($LH_2\text{-}O^{-*}$). The quantum yields of fluorescence due to absorption by the two ground-state forms are not the same; the phenolate ion is higher by a factor of 2.5, suggesting only partial equilibrium in the excited state is obtained. We will discuss this proton transfer to the enzyme later as well as the effect of substituting other groups at the 6' position on the color of light (10).

4. The binding of dehydroluciferin to the enzyme brings out a blue fluorescence property which is characteristic of the phenol excited state. This property can be used to study proton transfer and suggests the presence of a proton acceptor located in a highly hydrophobic environment of the enzyme. (See below). Dehydrolucerfin, when it reacts with ATP, can also form the adenylate anhydride. The high fluorescence of L essentially disappears when it forms the adenylate, a property that is very useful in studying the activation reaction.

5. The strength of binding of luciferin to luciferase has been determined indirectly by studying the binding of competitive analogues and calculating the corresponding K_i values.

The fact that the K_i values of all the luciferin analogues are at 10^{-5} M level suggest that these ring structures alone are responsible for most of the binding forces. Using the average K_i values, the free energies of binding of the two ring systems were calculated. These values are given below, together with those of the thiazoline ring system and the carboxylate group which were calculated from the difference in K_i values of the compounds with and without such groups (11, 12).

2-(2-benzothiazolyl)-^{2}-thiazoline . . .	-7.5 kcal
benzothiazole	-6.0 kcal
thiazoline	-1.5 kcal
carboxylate group	+1.1 kcal

The difference in the effect of a methyl group at the 5'- and 6'- position of the benzothiazole ring suggests that a benzothiazole derivative or a luciferin analogue is bound to its site only in one fixed position. If it could also be bound upside down, the 5'-position would be equivalent to the 6'-position. We do not have any evidence at this moment concerning the group or groups on the enzyme which are responsible for this binding. As to the groups on the benzothiazole molecule, the data are also suggestive that the OH group at the 6'-position is probably not needed for this specific binding. Replacement of the OH group with other groups did not impair the binding to any significant extent. It is likely, therefore, that the hetero-atoms in the ring, either N or S or both, are responsible in directing the compound to the set position.

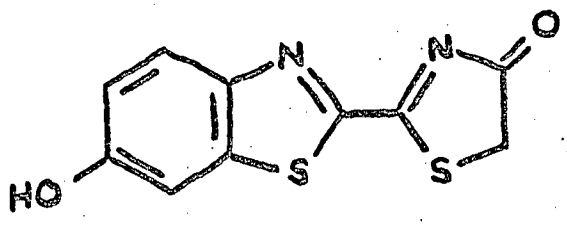


Fig. 2 Structure of Product-Emitter (Suzuki and Goto 1971)

6. Hydrophobic Nature of the Active Site

The use of dyes as probes for hydrophobic sites on proteins is now well documented (13, 14). The interaction between dye and protein may be followed by monitoring the change in the intensity or spectrum of fluorescence when the dye is bound in a hydrophobic environment. The binding of dyes to luciferase has been used in an attempt to obtain more information about the nature of the "active site" of this enzyme.

A surprising finding is that 2,6-TNS (toluidinonaphthalene sulfonate) binds much better than 1,5-ANS or the corresponding isomer, 2,6-ANS (anilinonaphthalene sulfonate). The only difference in the structure of these two classes of dyes is a methyl group, yet the K_A for TNS is tenfold greater than that for ANS. It is not obvious why such a small change in the dye should result in such an increase in affinity for the protein.

Calculations of the ΔF of binding of the dyes from the equilibrium constants shows for 1,5-ANS, $\Delta F = -6.3$ kcal/mole while for 2,6-TNS, $\Delta F = -8.2$ kcal/mole. Therefore, the addition of a methyl group to ANS results in a change of ΔF of binding of 1.9 kcal/mole. The large difference in ΔF observed between the binding of 1,5-ANS and 2,6-TNS to luciferase cannot be attributed entirely to the increased hydrophobic character of the latter molecule. The position of substituents on the naphthalene ring does not seem important for binding since all of the ANS isomers tested have similar binding constants. The results may mean a larger change in the conformation of the enzyme when 2,6-TNS combines with luciferase which results in an apparent tighter complex than observed for 1,5-ANS (14).

7. Structure of the Emitter Product

The emitter product of the luminescent reaction has been identified and shown to be identical for both the chemiluminescent and the enzyme catalyzed light reaction (5). In addition, Suzuki et al have been able to synthesize the product and to demonstrate fluorescent properties identical to the natural products. The structure of "oxyluciferin" is shown in Fig. 2.

The Enzyme Catalyzed Light Reaction

When one starts with free luciferin and luciferase, it is necessary to add ATP and magnesium or manganese ions in order to obtain light emission. In the initial reaction, there is an adenyl transfer from ATP to the carboxyl group of luciferin with the elimination of inorganic pyrophosphate. (15) The reaction is analogous to the fatty-acid- and amino-acid -activating reaction. The luciferyl-adenylate (LH_2-AMP) remains tightly bound to the enzyme and subsequently reacts with molecular oxygen to give light emission as indicated in the following reactions:

$$E + LH_2 + ATP \xrightarrow{Mg^{++}} E \cdot LH_2\text{-AMP} + PP \qquad (1)$$

$$E \cdot LH_2\text{-AMP} + O_2 \longrightarrow \text{Oxyluciferin} + CO_2 + AMP + \text{light} \qquad (2)$$

It is possible to eliminate the necessity of ATP for light emission if one synthetically makes LH_2-AMP from LH_2 and AMP. The addition of LH_2-AMP to an enzyme solution leads to a rapid production of light indicating that the activation step is the rate limiting reaction for the overall process.

In addition to reactions 1 and 2, luciferase will catalyze the formation of dehydroluciferyl-adenylate (L-AMP) as shown in the following reaction:

$$E + L + ATP \xrightarrow{Mg^{++}} E \cdot L\text{-AMP} + PP \qquad (3)$$

The quantative utilization of substrate and product production has been studied in great detail during light emission. It has been established that for each LH_2 molecule used one quantum of light is emitted (16). In addition one mole of oxygen is consumed per mole of LH_2 utilized (17), and one mole of CO_2 is released (18). In the activation of LH_2 to form LH_2-AMP one ATP is used and one PP is formed.

These facts eliminate a large number of possible mechanisms and suggest that at least one atom of oxygen is incorporated into the product emitter.

Oxidative Mechanism for Light Emission

Plant et, al, using ^{14}C- carboxyl labeled luciferin in the presence of excess enzyme, ATP and O_2 demonstrated the quantitative liberation of $^{14}CO_2$ from the luciferin

during the light reaction. Based on studies from chemiluminescent reactions by McCapra and associates (19,20,21) and White and associates (4) a mechanism of the organic reaction was proposed. The suggested scheme is presented in Fig. 3. Following the loss of a proton at carbon 4 oxygen addition occurs at that position which eventually led to the formation of a four-membered peroxide ring after the loss of AMP. Decarboxylation would lead to the expected products and theoretically calculations indicate that the final reaction yields more than enough energy to give rise to the excited state.

Although this mechanism was very attractive for a number of reasons subsequent experiments indicate that an alternate pathway for oxygen utilization must be proposed. In addition the necessity for water (OH) is also suggested.

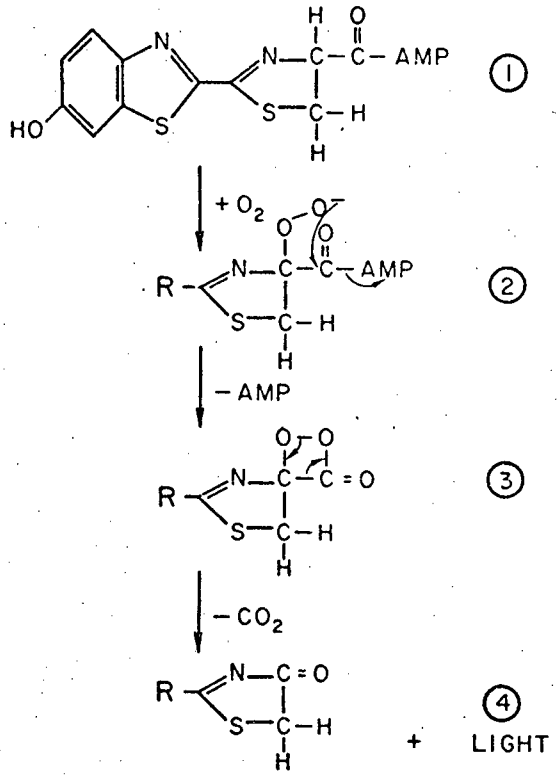


Fig. 3 Proposed Oxidative Mechanism for Light Emission (Plant, White and McElroy 1968)

For example, the proposed scheme indicates that one of the oxygens in the CO_2 must come from molecular oxygen. Using $^{18}O_2$ and $H^{18}OH$ DeLuca and Dempsey (22) have shown that one of the oxygens of the released CO_2 originates from water and that neither of the oxygens of CO_2 is derived from molecular oxygen. Similar results have been obtained for the chemiluminescence of LH_2-AMP in alkaline DSMO. (23); furthermore it has been shown recently that the addition of water to the DSMO solution greatly accelerates light emission. (23)

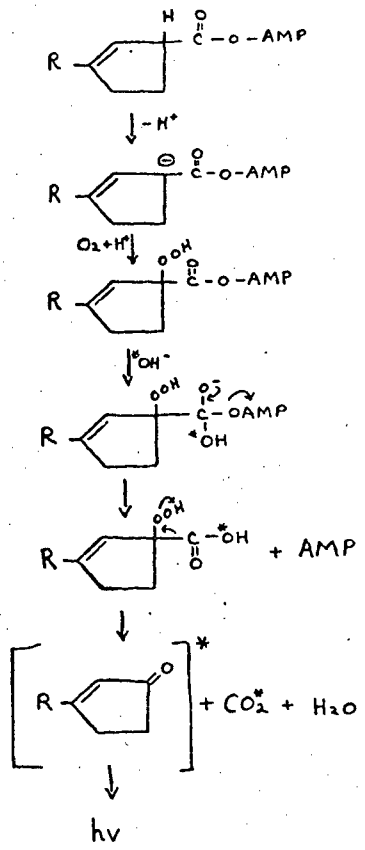


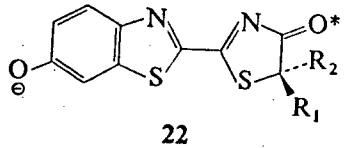
Fig. 4 Modified Oxidative Mechanism for Light Emission Based on the Results of DeLuca and Dempsey (1970).

A possible explanation of these results is presented in Fig. 4. Starting with luciferyl-adenylate, the first step is removal of a proton from the number 4 carbon atom of luciferin. The fact that the rate of the light emission starting with LH_2-AMP is slower if deuterium is substituted for hydrogen at the number 4 carbon supports this conclusion. The carbanion then adds oxygen at carbon number 4. The peroxide does not cyclize but OH is added at the carbonyl carbon. If the reaction medium is $H_2{}^{18}O$, this is the step where ^{18}OH is incorporated. In the next step AMP is assumed to be removed leaving a linear peroxide of luciferin bound to the enzyme. This is followed by a rapid dehydration and decarboxylation leading to the excited product.

If the reaction was carried out in the presence of $^{18}O_2$, it can be seen one of the oxygen atoms would appear in the keto group of the product, while the other oxygen would be released to the medium water, no ^{18}O would be incorporated into CO_2.

Factors Affecting the Color of Light Emission

The peak emission for bioluminescence of Photinus pyralis is 562 mμ (2). As an absolute minimum the energy requirement for the light reaction is estimated to be 57 Kcal/mole. Since the color of light emitted in in vitro reactions can be altered and the fact that other fireflies show different peak emissions studies of the chemiluminescent reaction under various conditions are useful in the interpretation of color changes.



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↓

red light

Fig. 5 Proposed structure of the Red Light Emitter (White et al 1971)

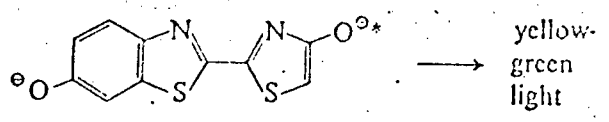

Fig. 6 Proposed Structure of the Yellow-Green Emitter (White et al 1971)

a. Chemiluminescence

Recent data from studies of White et al, indicates that the product emitter in the red chemiluminescence of luciferyl-adenylate in organic solvents is the monoanion of the decarboxylated, 4-keto derivative of LH_2 (4). The nature of the reaction is shown in Fig. 5.[2] The chemiluminescence of LH_2 and its derivatives in base-organic solvent was first studied by Seliger and McElroy (24). Since then, a number of hypotheses have been suggested for the mechanism of the enzyme-catalyzed emission. The fact that one can obtain red emission from enzyme-catalyzed reactions (acid pH, high temperature, etc.) suggests that the red chemiluminescence in organic solvents may be of some biological significance.

In the presence of excess base, the red chemiluminescence of esters or anhydrides of LH_2 shifts to a yellow-green emission. These results suggest that proton abstraction at carbon 5 is essential for obtaining the yellow-green emitter. The nature of the reaction proposed is shown in Fig. 6. By measuring fluorescence of spent reaction mixtures, it was possible to obtain data to support the hypothesis that the dianion is the light emitter in the yellow-green chemiluminescence.

b. Bioluminescence

It has been shown that the variations in color of eight emitted by various species of fireflies is due to a difference in the structure of the luciferase (1). Since the structure of LH_2 and the product emitter are identical for all species the shifts in color must be attributed to a change in the polarity or relative hydrophobicity of the binding site of luciferase. A charge change on the enzyme can affect the binding as well as abstraction of protons at both carbon 6 and carbon 5; in addition the relative hydrophobicity of the solvent is known to affect the fluorescence properties of LH_2 and the product.

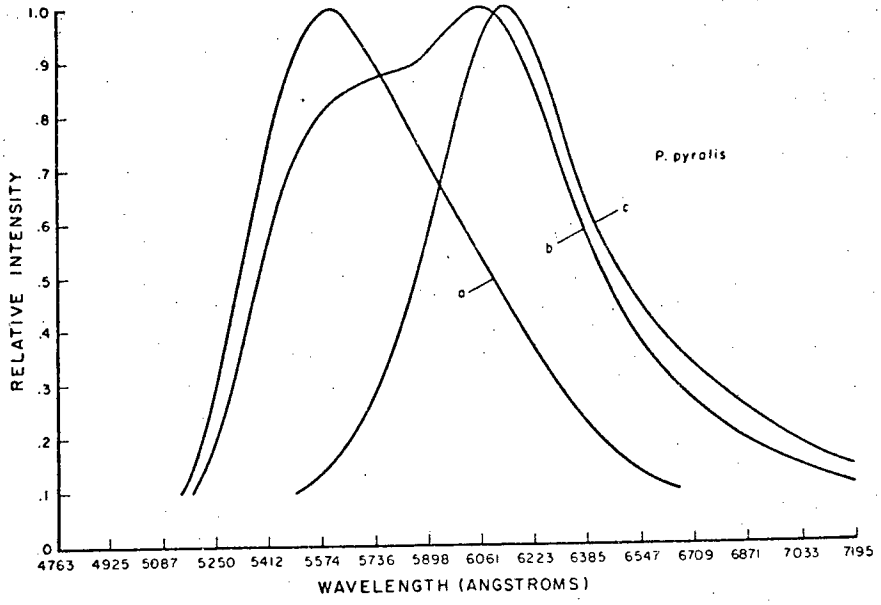

Fig. 7 Effect of pH on the in vitro emission spectrum. P. pyralis luciferase. a: pH 7.6; b: pH 6.5; c: pH 5.0. (Seliger and McElroy 1960)

(1) Temperature, pH and metal ions

As the pH of the P. pyralis extract is lowered, it can be observed that the intensity of the yellow-green bioluminescence decreases, leaving a dull brick-orange glow (25). This variation in bioluminescence emission with pH is shown in Fig. 7. As can be seen at neutral (and alkaline) pH, there is a single emission band in the yellow-green region. At intermediate pH, a red emission band appears at 616 mμ, and at pH value below 5.5, the yellow-green emission is completely suppressed and only the red band is evident: At acid pH, the number of light quanta emitted per luciferin molecule exidized is markedly lower than 1 and indicates a predominantly dark reaction. However, at alkaline pH, although the rate of light emission is reduced to a fraction of the rate of pH 7.6, the quantum yield is essentially unity. Since the red emission is known to be due to the monoanion form of the product emitter this suggests that the pH change must be affecting a group on the enzyme concerned with the abstraction of a proton from carbon 5. The pK for the appearance of red light is approximately 6.8. This suggests the strong possibility that a histidine residue in the enzyme is the active group concerned with proton abstraction.

Except for the partial denaturation of the enzyme in acidic buffer, the pH effect on the emission spectrum shift is completely reversible. A reversible red shifts in emission spectra can be observed by increasing and the decreasing the temperature of the reaction, by carrying out the reaction in 0.2 M urea at normal pH values (7.6) in glycylglycine buffer, or by adding small concentrations of Zn^{++}, Cd^{++} cations, as chlorides.

(2) Effect of substrate structures

The above facts support the idea that the color of the emitted light depends upon the nature of the binding of the intermediate to the enzyme. It seemed likely, therefore, that a change in the structure of the substrate molecules (luciferin or ATP) may alter the binding and in turn affect the color of the light. Unfortunately, it is not possible to change greatly the luciferin structure and still obtain an active light-emitting substrate. It turns out that the 6'-aminobenzthiazole compound is an active substrate, and in this case, a red emission instead of the yellow-green is observed even at neutral pH. The emission at neutral and alkaline pH is red, peaking at 605 mμ, very close to the bioluminescence emission of firefly luciferin at acid pH. More significantly, the color of the 6'-aminoluciferin bioluminescence is entirely independent of pH, from below 6 to above 10 in exactly the range where native firefly luciferin shows the remarkable color shifts outlined above. Since phenols are stronger acids than anilines, this observation supports the idea that it is the phenolate ion of firefly luciferin that is involved in the normal yellow-green bioluminescence. The results also suggest that the amino group has a strong effect on the ability of the enzyme to abstract a proton from carbon 5.

Until recently, only adenosine triphosphate (ATP) was shown to be active for the enzymatic reaction leading to light emission. UTP, CTP, GTP, and ADP, and other pyrophosphate-containing nucleotides were inactive. Recently, Leonard and associates prepared an ATP with the ribose attached to the 3 position of the adenine ring (3-isoATP) and made a sample available to us. This compound appears to be about 10 - 15% as effective in the light reaction as normal ATP. The additional interesting observation, however, is that at pH 7.5, a significant fraction of the light emitted is red when 3-isoATP is used. Thus, the nature (stereochemistry) of the nucleotide attachment to the enzyme is also of importance in determining the color of the light.

A second modified ATP has been prepared by Leonard and associates known as ε-ATP (26). The structural alterations are due to the addition of an aldehyde group which couples the 6-amino group of adenine to the number 2 nitrogen thus making a four membered ring at that position on the purine moiety. The ε ATP is completely inactive for initiating the light reaction. However, when ε AMP is used as a substrate to make LH_2-εAMP the latter is active for light emission and the emission is red instead of the usual yellow-green. The results indicate that the 6-amino group on the purine ring is essential for the activation reaction; a result in agreement with the observation that ITP is ineffective as a substitute for ATP in the light reaction. In addition, the nature of the binding of the 6-amino group to the enzyme also influences the structure of the excited product in a manner which determines whether red or yellow-green light is emitted.

The results from the ε ATP, ε-AMP and iso-ATP indicates that the binding of the adenylate to the enzyme induces changes in the enzyme structure that must be sustained during the subsequent decarboxylation that leads to the enzyme-product complex excited state. If this were not true then the structure of the AMP should not affect the color of light since it must be removed from LH_2 before the final creation of the excited state according to all proposed mechanisms.

Mechanism of Enzyme Action

a. Number of Binding Sites and the Activation of L.

Studies on the substrate binding properties of firefly luciferase (11, 12) have shown that there are two binding sites each for luciferin and ATP per 100,000 molecular weight of enzyme. These results are consistent with the model that luciferase is a dimer of identical 50,000 molecular weight subunits (27), each with one binding site for each substrate. However, the enzymatically active substrate, the MgATP complex, is bound to only one site per 100,000 molecular weight of enzyme. Similarly, only one site is found for dehydroluciferyl adenylate (L-AMP) formed from the following reaction:

$$E + L + MgATP \rightleftharpoons E \cdot L\text{-}AMP + MgPPi$$

Dehydroluciferin (L) is activated by the enzyme to form dehydroluciferyl adenylate which remains tightly bound to the enzyme with no production of light. In order to have only one binding site per dimer of identical subunits, there must be asymmetry in the system. Therefore, the physical properties of firefly luciferase were examined in greater detail. The results indicate that the minimum molecular weight of enzymatically active protein is 50,000 and that only one of the subunits in the 100,000 molecular weight aggregate is enzymatically active (see below).

b. Binding Sites of Dehydroluciferyl Adenylate.

By adding small amounts of L to luciferase in the presence of MgATP it is possible to determine the number of L-AMP formed per enzyme molecule by following the decrease in fluorescence. Such experiments demonstrate that one L-AMP is formed per 100,000 molecular weight of protein. The enzyme concentration was varied from 0.4 mg/ml to 2.0 mg/ml in different experiments. Under all these conditions, there was only one L-AMP site per 98,000 ± 4000 molecular weight of enzyme.

Isolation of the E·L-AMP complex by Sephadex G-25 chromatography produced similar results. Measurements by fluorescence of the material bound to the protein gave 1.01 ± 0.05 molecules of L-AMP per 100,000 molecular weight. Counts of the radioactivity incorporated into the adenylate from ^{14}C ATP yielded 1.1 ± 0.1 molecules of L-AMP per 100,000 molecular weight.

c. Binding of Dehydroluciferin to E·L-AMP.

Denburg et al (12) has shown that there are two binding sites for dehydroluciferin per 100,000 molecular weight. Since only one L-AMP is bound to the enzyme an attempt was made to demonstrate further binding of dehydroluciferin to the E·L-AMP complex. The E·L-AMP complex was isolated by chromatography on a G-25 column in 0.1 M Tris, pH 8.0. The increase in fluorescence at 440 mμ when dehydroluciferin is bound to the enzyme was used to measure binding. When dehydroluciferin was titrated into a solution of the E·L-AMP complex no increase in the 440 mμ fluorescence was observed. This technique is sensitive enough to detect a tenfold increase in the affinity of the enzyme for dehydroluciferin. Therefore, when one L-AMP molecule was bound to the enzyme two dehydroluciferin sites were no longer accessible. The possibility that dehydroluciferin can still bind to E·L-AMP but without the increase in

fluorescence at 440 mμ seemed unlikely since this fluorescence change arises from putting the molecule in a hydrophobic environment. Most of the energy for binding comes from such hydrophobic interactions and without them binding could not take place.

d. Equilibrium Dialyses.

Using equilibrium dialysis, the average number of molecules of dehydroluciferin bound per mole of enzyme (100,000 m.w.) was calculated. Using these parameters, a Scatchard plot was made, from which was obtained n, the number of binding sites per enzyme molecule and K_A, the association constant for this binding. In 0.05 M phosphate buffer at pH 7.8 at 25°, n = 1.8 and $K_A = 1.7 \times 10^5$. Equilibrium dialyses could not be performed with luciferin because of its instability under the conditions in which the enzyme maintains its activity.

Other evidence which indicates two sites for L and LH_2 come from luminescence studies. Hopkins and Seliger (unpublished) have demonstrated that starting with LH_2-AMP, two molecules are consumed per 100,000 molecular weight before the luciferase is completely product inhibited. Recently, Denburg and McElroy (27) have shown that there is a strong tendency of this protein to aggregate under certain conditions which was probably responsible for the previously reported value of 100,000 for the molecular weight. The aggregation was observed in solvents of relatively low ionic strength as the solubility limit of the protein was reached. The data were not sufficient to decide whether there was a monomer-dimer or monomer-dimer-trimer equilibrium occurring or to calculate the equilibrium constants. The single, symmetrical peak observed in the schlieren patterns of experiments in which the sedimentation coefficient was increasing as the protein concentration was increased suggested a rapidly reversible monomer-dimer system. However, the asymmetry observed in the schlieren pattern at saturating concentrations of enzyme indicated the presence of polymers greater than dimer. Since no change in the specific activity of the protein was observed as the molecular weight of the enzyme increased, this aggregation plays no physiological role in the regulation of the enzymatic activity and may be fairly nonspecific.

The tendency to aggregate and the poor solubility properties of luciferase may be expected in light of the very high percentage of nonpolar amino acids found in the enzyme. The average hydrophobicity of luciferase was calculated to be 1240 cal/residue from the amino acid composition as reported by DeLuca et al (28). This makes luciferase one of the most hydrophobic proteins ever reported. The high percentage of nonpolar amino acids necessitates that some of them be on the external surface of luciferase. The possibility of hydrophobic intermolecular interactions in luciferase helps to explain its physical properties.

Heterogeneity in the purified luciferase preparations of maximal specific activity was indicated by the results of the binding studies. One L-AMP binding site per 100,000 molecular weight of protein was observed under conditions in which only a single size species of 50,000 molecular weight was present. In addition, the active substrate, MgATP, had only one binding site per 100,000 molecular weight, while there were two sites for ATP.

e. ATP Binding Site.

Kinetically, the MgATP complex has been shown to be the substrate in the light reaction catalyzed by the firefly luciferase (29). Kinetic as well as inhibition studies showed that uncomplexed ATP is also bound to the luciferase, and is a competitive inhibitor with respect to MgATP. Similar studies for Mg^{2+} indicated that it is not bound to the luciferase by itself.

Kinetic inhibition studies with ATP analogs showed that the 6-amino group of adenine is important for the binding of bases and nucleosides at the MgATP site. Energetically, adenine and negative charges on phosphate groups contribute 57% and 43%, respectively, toward binding (total binding energy = 4.8 kcal).

dATP can also serve as a substrate in the formation of adenylate of both luciferin and dehydroluciferin. It is a poor substrate, having maximal velocity of light reaction only 5% that of ATP and equilibrium constant of dehydroluciferyladenylate formation five times smaller than that with ATP.

f. Effects of AMP.

Under the normal assay conditions (at pH 8.0), where only yellow-green light is emitted, 5'-adenylic acid (AMP) is a competitive inhibitor of luciferase with respect to one of its substrates, MgATP (30). However, AMP serves as a competitive activator of the yellow-green light emission and a noncompetitive inhibitor of the red light emission at pH 6.5 or below. The activation appears to be specific for AMP. Other nucleotides tested were all ineffective.

Luciferase has one MgATP site and one ATP site per molecular weight of 100,000. The MgATP site can also bind ATP, but the ATP site cannot bind MgATP. This latter site, the ATP site, seems to be the site involved in the AMP activation.

The competitive nature of activation suggests that a conformational change occurs in luciferase at pH 6.5 in the presence of AMP. This was demonstrated by comparing optical rotatory dispersion curves of luciferase in the presence and absence of AMP at pH 6.5. Since luciferase is in the monomeric state (molecular weight 50,000) under these experimental conditions, it is suggested that both the MgATP site and the ATP site are located on one of the monomers.

The differences in the subunits composition must be very small. A single N-terminal serine was found, and the number of peptides from a tryptic digest agreed with the theoretical number predicted on the basis of identical subunits of 50,000 molecular weight (31). However, recent evidence for heterogeneity was the observation of two different C-terminal amino acids, leucine and serine (32).

From the facts cited above, one concludes that only one of the 50,000 molecular weight species is catalytically active. Furthermore, it appears that both the L binding sites and the Mg-ATP and ATP (AMP) binding sites are all on this subunit.

The only data that do not completely support this conclusion is the appearance of two active bands on electrofocusing (27). However, these experiments should be interpreted with caution because of the close similarity of the two species and their strong tendency to aggregate.

g. Role and Reactivity of Sulfhydryl Groups.

The number of sulfhydryl groups of firefly luciferase has been determined by spectrophotometric titration with p-mercuribenzoate in the presence and absence of a competitive inhibitor (28). Between six and seven sufhydryls are titrated with p-mercuribenzoate in the native enzyme. In the presence of the inhibitor only four to five sulfhydryls will react with the p-mercuribenzoate. Four or five moles of p-mercuribenzoate can be reacted with the enzyme-inhibitor complex and subsequent removal of the inhibitor results in recovery of 90% of the original enzymatic activity. Addition of 4 moles of p-mercuribenzoate to the enzyme in the absence of inhibitor results in complete loss of activity. The enzyme is also inhibited by dithiol reagents such as arsenite-2.3-dimercaptopropanol, $CdCl_2$, and γ-(p-arsenosophenyl)-n-butyric acid. The data show that four or five of the enzyme sulfhydryls have no effect on the catalytic activity, but the two sulfhydryl groups which are "covered" by the inhibitor are essential in some way for the enzymatic reactions leading to light emission.

Recently, it has been found that TPCK (chloromethyl ketone derivative of N-tosyl-L-phenylalanine), an aromatic inhibitor of chymotrypsin, also inhibits luciferase activity (33). The inhibition of enzymatic activity is accompanied by a loss of approximately two sulfhydryl groups. TPCK is a competitive inhibitor for luciferin and a non-competitive inhibitor with respect to ATP. The aromatic character of TPCK appears to be the major factor for its binding to the active site of luciferase. The observation that N-tosyl-L-phenylalanine alone is a competitive inhibitor also with a K_i of the same order of magnitude of TPCK supports this conclusion. Inactivation by TPCK is pH dependent and it is of interest to note that the inactivation - pH curve corresponds very closely to the luciferase activity - pH curve.

The fact that TPCK competes with LH_2 and not with ATP suggests that the inhibitor is most likely reacting at the catalytic site. If the inhibitor were reacting at a different site thus preventing conformational changes, then one might expect TPCK to be competitive with both LH_2 and ATP rather than just for the one (LH_2).

In the studies with luciferase, TPCK also appears to react only with SH groups. This conclusion is supported by (i) the stoichiometry between number of SH groups lost and the number of TPCK groups incorporated into luciferase; (ii) decrease in cysteic acid content in oxidized TPCK-luciferase equivalent to the amount of SH groups lost; and (iii) absence of either 1-carboxymethyl or 3-carboxymethyl histidine in the hydrolysate of performic acid-oxidized TPCK-luciferase.

Sulfhydryl groups are apparently reactive enough so that almost all the SH groups of luciferase react with TPCK in an excess of TPCK. However, the two essential SH groups of luciferase react more rapidly than do the other SH groups.

A comparison of the inactivation of luciferase by several reagents of different reactivity shows that the ability of TPCK to inactivate luciferase far exceeds that expected from its chemical reactivity, suggesting that something more than chemical reactivity is operating in the TPCK inactivation of luciferase. Since N-tosylphenylalanine itself can inhibit luciferase competitively, and TLCK does not inhibit luciferase under the comparable conditions used for TPCK, it appears that the hydrophobic nature of TPCK brings this compound to the binding site of LH_2 (or L), thus achieving an effect of affinity labeling.[2] As might be expected, the tight binding of L-AMP to the active site protects the enzyme from TPCK inactivation. Luciferin alone in high concentrations also retards the TPCK inactivation rate whereas ATP-Mg^{2+} had no effect. Since neither of the substrates alone has any measurable effect on the conformational changes in luciferase, the above results must mean that LH_2 (or L) interferes with TPCK inactivation by competing for the same site.

Since the carboxylic acid group of LH_2 (or L) must react with the AMP-PP bond of ATP, the two substrates of luciferase must be in close proximity to each other on the luciferase surface. However, since TPCK is strictly noncompetitive with respect to ATP, it suggests that the inhibitor is specifically bound to the LH_2 (or L) site.

Because the titration of all of the sulfhydryl groups in luciferase results in total inhibition, it was of interest to determine the amino acid sequence in the vicinity of the protected sulfhydryl groups.

Labeling of the essential sulfhydryl groups with (1 - ^{14}C)N-ethyl-maleimide followed by tryptic digestion resulted in the isolation of a single radioactive decapeptide whose sequence was determined.

The amino acid analysis and partial acid hydrolysis of the radioactive peptides proves, unambiguously, that the two SH groups covered by dehydroluciferyl adenylate and the reactive pair in the native enzyme are the same. This is further substantiated by the inhibition studies performed on the native enzyme with NEM where the addition of only 1 mole of NEM results in approximately 50% inactivation.

Luciferase binds approximately 2 moles of dye per mole of enzyme suggesting that there are two catalytic sites on luciferase. This is consistent not only with the dye-binding stoichiometry but also with the fact that 2 moles of L-AMP are required to remove all of the bound dye. If two molecules of dye were bound at a single 'active site' then 1 mole of L-AMP should completely remove the dye. The observation that two SH groups are essential for catalytic activity and that one each of these appear in identical peptides is also consistent with two active sites per molecule.

Since 2,6-TNS, unlike TPCK, is competitive with both LH_2 and ATP, it suggests that the dye is binding at or near the normal substrate binding sites. However, the complete lack of pH effect on binding over the range pH 6-9 indicates that the groups which ionize in this region: imidazole; lysine; sulfhydryl; must not alter the binding site for the dye. There is a large change of enzymatic activity in this pH range with an optimum pH of 7.8. Functional groups essential for catalysis, however, appear to have no effect on binding of the dye.

Dehydroluciferin when bound to luciferase shows a slow rate of proton transfer, suggesting that the binding site is hydrophobic (34). Fluorescence lifetime measurements have been used to obtain nanosecond time-resolved emission spectra of dehydroluciferin in various solvents and when bound to luciferase. The blue fluorescence caused by the phenol decreases with decay time relative to the green emission caused by the phenolate. The time course of excited state ionization may thus be measured directly. The rate of proton transfer is very fast in aqueous solution but slower in 80% ethanol. Addition of imidazole increases the rate of proton transfer.

h. Effect of NEM and Its Analogues.

It was previously shown that NEM preferentially attacks the two SH-groups that are essential for luciferase activity (31). Incubation of luciferase with a concentration of NEM twice that of the enzyme in 0.05 M phosphate, pH 7.8 at 0^{o}, results in essentially complete loss of activity. Luciferase inactivated in such a manner by NEM did not have the ability to bind dehydroluciferin as measured by fluorescence and equilibrium dialysis. The apparent competition of NEM and luciferin for the same site on the enzyme is seen by the protection given by luciferin against NEM inactivation. The half-life of inactivation is three (3) hours without and eleven (11) hours with luciferin. These experiments implicate the position of the essential SH group to be near the luciferin-binding site. Detailed studies have been made using various analogues of luciferin and K_i values were determined (12).

i. Peptide Derived from Luciferin-Binding Site.

2-Cyano-6-chlorobenzothiazole (CCB), a substrate analog of firefly luciferase, inactivates this enzyme slowly at pH 8 to about 20% of original activity without affecting free sulfhydryl groups (32). The inactivated luciferase contained 1.5 - 2.0 moles of CCB per 100,000 daltons of luciferase. It is believed that the benzothiazole derivatives are incorporated at the luciferin-binding sites. Tryptic digest of the inactivated luciferase and subsequent electrophoresis yielded a fluorescent peptide containing benzothiazole derivative. The peptide was found to contain pyro-glutamic acid at the N-terminal end. The sequence of the peptide was established tentatively to be: pyroglu-X-gly-ala-val-(asp)-ile-leu where X is an amino acid (possibly tyr) to which the benzothiazole derivative is attached. There is little resemblance in the composition of the peptide isolated here and that of the peptide containing the reactive SH groups. This is not unreasonable, however, when one considers the distance between these two binding points. It is interesting to note that while the SH-peptide contained many hydrophilic amino acids (composition: 2 ser, 2 gly, 1/2-cys, glu, gln, asn, ala, lys), CCB-peptide has a high proportion of hydrophobic amino acids. This is in accord with the earlier observations that the SH groups are not located in a very hydrophobic environment, perhaps near the entrance of the LH_2

binding site, while the interior of the LH_2 binding site, including the 6'-position of the benzothiazole ring, is in a very hydrophobic environment.

Since the C-terminal residue of CCB-peptide is leucine, it is thought that this might actually be the C-terminal peptide of the luciferase. Preliminary studies of the carboxypeptidase digestion of the luciferase in native form and in 6 M urea indicated that there are one mole each of leucine and serine per 100,000 daltons. Isoleucine was not produced during such digestions (unpublished results). Therefore, it seems that CCB-peptide is not derived from the C-terminal end.

j. Concluding Summary on Luciferase Action.

From the results presented in the last section we can make the following conclusions concerning the properties of luciferase:

1. The enzyme at relative low concentrations of protein readily associates into a dimer of 100,000 molecular weight. However, the catalytically active unit is 50,000 m.w.

2. There are two binding sites for LH_2 and L per 100,000 molecular weight while there is only one Mg_2ATP binding site. There is a second site which binds ATP as well as AMP.

3. There are two binding sites for L-AMP per 100,000 molecular weight while only one L-AMP is formed from L and Mg_2ATP.

4. Dye binding indicates a very hydrophobic site for LH_2 binding. Two dye molecules are bound per 100,000 m.w. and two L-AMP molecules are required to displace the two dye molecules.

5. Starting with LH_2-AMP it is possible to show that only two molecules of the substrate are used for light production at which time the enzyme is 100 percent inhibited.

From this data we conclude that the 100,000 molecular weight is composed of two subunits of which only one is enzymatically active. If this is correct, the active subunit must contain two sites for LH_2 (LH_2-AMP) binding but only one Mg_2 ATP binding site. The second binding site for free ATP (AMP) appears to be concerned with regulatory activity. Unfortunately it has not been possible to separate the two different subunits which must be almost identical in amino acid composition.

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