Photoexcited Bacterial Luminescence. Spectral Properties and Mechanistic Implication of a Reduced Flavine-Like Prosthetic Group Associated with Photoexcitable Luciferase†

Shiao-chun Tu and J. Woodland Hastings*

ABSTRACT: A prosthetic group, designated B, has been isolated from bacterial photoexcitable luciferase and found to possess spectral and photochemical properties characteristic of substituted reduced flavines. Its fluorescence when bound to luciferase has an excitation maximum at 375 nm, correlating well with the absorption spectrum, and an emission peaking at 495 nm. However, free B is nonfluorescent in aqueous solution at ambient temperature. Both free and luciferase-bound B show similar negative circular dichroism in the region 330–475 nm with troughs at 375 and 380 nm, respectively. In the luciferase reaction initiated by $FMNH_2$, B is an inhibitor competitive with $FMNH_2$. Irradiation of photoexcitable luciferase converts B to FMN, the latter identified spectrally, enzymatically, and chromatographically. These findings lead to the suggestion that B is a substituted $FMNH_2$. The luciferase-bound B resembles but is not identical with the normal flavine intermediate obtainable by reacting luciferase with reduced flavine mononucleotide and oxygen. It is hypothesized that B is a false intermediate of the bacterial bioluminescence reaction, and a mechanism for the photoexcited bioluminescence reaction is suggested.

Extracts of luminous bacteria have been shown to contain two protein species capable of light emission. One is bacterial luciferase (L)[1] which catalyzes the bioluminescent mixed function oxidation of $FMNH_2$ and long chain aldehyde. The second is photoexcitable luciferase (PL), shown to be a luciferase molecule modified by a noncovalently bound flavine-like chromophore designated B (Mitchell and Hastings, 1970; Tu et al., 1975). In the present work, isolation and spectral characterization of B are described. It has been found that the chemical nature of B closely resembles substituted reduced flavines, and a reaction mechanism for photoexcited bioluminescence is proposed.

Experimental Procedures

Most of the materials and methods were the same as described previously (Tu et al., 1975). Florisil (100–200 mesh) was obtained from Fisher, washed once with 30% pyridine in water followed by extensive washing with water, and air dried. Sodium hydrosulfite (dithionite) and silica gel thin-layer chromatogram sheets (No. 6061) were from Mallinckrodt and Eastman, respectively. In addition to the standard ($FMNH_2$ initiated) luciferase assay, the method using dithionite as a reductant (Meighen and MacKenzie, 1973) was also used. Absorption spectra were measured with a Cary 15 and circular dichroism (CD) with a Jasco (J-20). Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with 1200 lines/in. gratings with slit arrangement No. 2 and were plotted uncorrected.

NAD(P)H:FMN oxidoreductase (flavine reductase) was purified from *Beneckea harveyi* by Dr. J. E. Becvar following a modification of the previous method (Gunsalus-Miguel et al., 1972). For the detection of flavine, a 25-μl aliquot of sample solution was added to 1 ml of 0.1 *M* phosphate (pH 7) containing 0.3 unit of flavine reductase and 2.6 m*M* NADH, and the activity was measured by following the decrease in absorbance at 340 nm. One unit of activity is defined as the oxidation of 1 μmol of NADH/min at 23°. Apoprotein of pyridoxine (pyridoxamine) 5′-phosphate oxidase was purified by M. N. Kazarinoff and used to assay FMN as previously described (Kazarinoff and McCormick, 1974). FMN was also assayed using bacterial luciferase itself, employing the dithionite assay described above.

Results

The isolation of B was carried out by denaturing the PL in urea followed by gel filtration to separate it from protein, and chromatography on Florisil to resolve the flavines. PL (500 mg) in 5 ml of 0.02 *M* phosphate (pH 7) containing 5 *M* urea was applied to a Sephadex G-50 column (2.5 × 25 cm) preequilibrated in 5 *M* urea, and eluted (~0.6 ml/min) with 5 *M* urea in water at 23°. Fractions containing B activity were pooled and chromatographed on a Florisil column (Figure 1).

Peak I was shown to be FMN both by its fluorescence excitation and emission spectra, and by its activity with bacterial luciferase using dithionite as a reductant. Examined by thin-layer chromatography (TLC) in 1-butanol–acetic acid–water (2:1:1; volume ratios), its migration corresponded to that of FMN; a trace amount of riboflavine was also detected. This flavine was well separated from the major B activity (III) and partially resolved from II. Fractions in III were pooled, lyophilized, redissolved in 1.5 ml of H_2O, and centrifuged to remove insoluble material. This is referred to as B stock solution. By ascending TLC in 1-butanol–acetic acid–water (2:1:1), the isolated B moves as a single nonflu-

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received November 20, 1974.* This work was supported in part by National Science Foundation Research Grant GB 31977X.

[1] Abbreviations used are: L, bacterial luciferase; PL, photoexcitable luciferase; LB, reconstituted L–B complex.

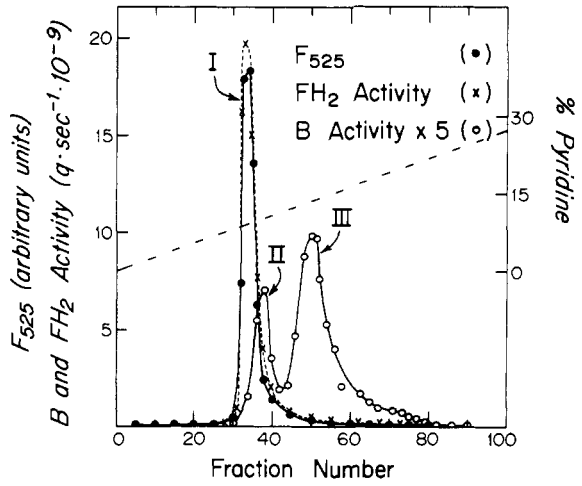


FIGURE 1: Isolation of B by Florisil chromatography. Approximately 50 ml of a solution 5 *M* in urea containing B activity and contaminating flavines obtained from 500 mg of PL was applied to a Florisil column (1 × 17 cm) preequilibrated with H_2O. The column was eluted, at 23°, with 50 ml of H_2O to remove urea followed by a gradient of 150 ml of H_2O–150 ml of 30% pyridine in H_2O with a flow rate of 0.15 ml/min, collecting 2.5-ml fractions. Fluorescence (●) was measured in arbitrary units at 525 nm using 380-nm excitation light. For determinations of B (○) and reduced flavine (FH_2;X) activities, 20-μl aliquots of selected fractions were each added to 30 μg of luciferase in 2 ml of 0.02 *M* phosphate (pH 7) and 1 ml of the same buffer containing 0.025 *M* 2-mercaptoethanol, respectively. After 1 min at 23°, photoexcited luminescence and FH_2-initiated luminescence activities were measured by the flash assay and the dithionite assay, respectively.

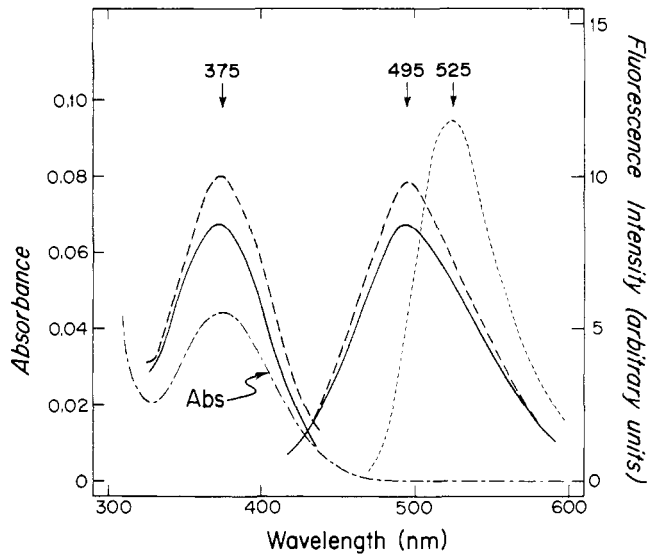


FIGURE 2: Absorption spectrum of luciferase-bound B and fluorescence spectra of PL and LB. Luciferase obtained from aldehyde-requiring mutant was complexed with B (26% saturation). Both the reconstituted LB (1.1 mg of protein/ml) and PL samples (1 mg of protein/ml) were thoroughly dialyzed as described in the text. Absorption spectrum of the luciferase-bound B (– • –) was taken with the LB sample using the same concentration of L in the reference cell. Excitation and emission spectra of LB (—) and PL (- - -) were measured with fluorescence monitored at 495 nm for the former measurements and using 375-nm excitation light for the latter. Fluorescence emission spectrum of FMN (• • •) in the same buffer is included for comparison.

orescent but iodine-stainable spot above a weakly fluorescent spot identified as a trace amount of FMN. Material from II contained much higher levels of oxidized flavines, and was not used for the present studies.

Upon titration of luciferase with increasing amounts of B, increasing amounts of photoexcited bioluminescence activity are generated, with a concomitant loss of luciferase activity. For the reconstituted LB samples, the degree of saturation of L with B is defined as the percent inhibition of the initial L activity. In similar experiments, it was previously noted that the binding of B to L did not restore the original PL fluorescence emission, λ_{max} near 490 nm (Mitchell and Hastings, 1970). Instead, an emission maximum near 525 nm was observed. This can now be attributed to contamination by small amounts of FMN arising, it is believed, from the photochemical conversion of B to FMN. The FMN fluorescence, being much greater than that of LB, masks that of the latter. Although our B stock solution contained only trace amounts of FMN, it was necessary to remove this strongly absorbing and fluorescent flavine in order to obtain accurate spectral measurements of enzyme-bound and free B. To do this, B was added to PL-free L to form LB which was then dialyzed in the dark at 0–4°, over 2 days against four changes (500 ml each) of 0.02 *M* phosphate (pH 7) containing 2 *M* NaCl, and then over a period of 1 day against another four changes (500 ml each) of the same buffer without NaCl. This dialysis successfully removes FMN but does not cause any decrease in photoexcited bioluminescence activity. All PL preparations were similarly dialyzed to eliminate any possible FMN contamination. Spectral measurements of luciferase-bound B were obtained with LB and/or PL so prepared while those of free B were determined with dialyzed LB samples immediately after the addition of 5 *M* urea.

As shown in Figure 2, PL and LB have the same fluorescence excitation and emission spectra, with λ_{max} at 375 and 495 nm, respectively. Free B is nonfluorescent at room temperature (Figure 1); this has also been found with B obtained from LB treated with 5 *M* urea. The dissociated B has been found to be nonfluorescent, with excitation ranging from 350 to 480 nm. This is in contrast to FMN which is itself highly fluorescent in aqueous solution (λ_{max} at 525 nm) but is nonfluorescent when bound to luciferase (Baldwin et al., 1975). The lack of oxidized flavine fluorescence of LB in 5 *M* urea clearly demonstrates that the dialysis treatment described above selectively and completely removed FMN from the LB sample. The absorption spectrum of luciferase-bound B is also shown in Figure 2, with the maximum at 375 nm correlating with the fluorescence excitation maximum. No significant changes in its absorption spectrum were detected after the addition of 5 *M* urea.

The specific activity of reconstituted light induced bioluminescence (LB) was determined to be about 4×10^{11} quanta (q) sec^{-1} mg^{-1} with decanal at 23°, or about threefold higher than the activity of the best preparation of native PL (1.4×10^{11} q sec^{-1} mg^{-1}). This is reasonable, since the purity of PL had been estimated to be 30–50% (Tu et al., 1975). To obtain the LB, purified B (Figure 1) was added to PL-free luciferase ($\sim 10^{-7}$ *M*) to the point of >99% inhibition, where all of the L was assumed to be in the LB form. Subjected to the flash assay in 0.02 *M* phosphate buffer (pH 7) values of 3.9 and 4.3×10^{11} q sec^{-1} mg^{-1} were obtained in two experiments. Using the same samples, the $FMNH_2$ initiated luciferase activities in the same buffer and otherwise identical conditions were 4.6 and 5.1×10^{13} q sec^{-1} mg^{-1} respectively. Under optimal assay conditions, a value of 1×10^{14} q sec^{-1} mg^{-1} was obtained for the $FMNH_2$-initiated activity, but the lower value obtained in the phosphate buffer alone is probably better to use for comparison. The optimal assay mixture contains bo-

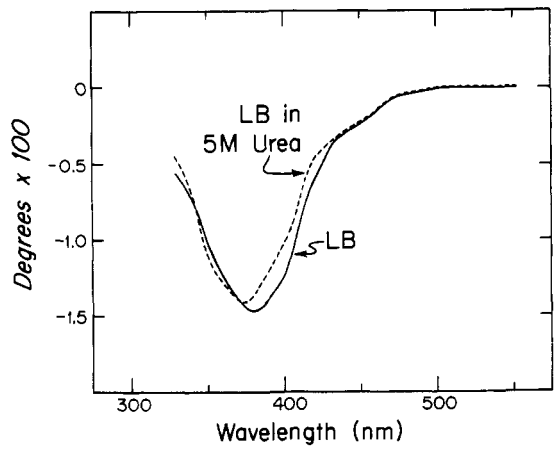


FIGURE 3: Circular dichroism spectra of free and luciferase-bound B. A reconstituted LB sample was subjected to dialysis treatment as described in the text. The final sample had a protein concentration of 22 mg/ml and an absorbance of 0.175 at 375 nm. CD spectra were taken using 1-cm light path, in the presence (···) and absence (—) of 5 *M* urea for free and luciferase-bound B, respectively. The observed signals, in degrees, were normalized to that of a sample with a unit absorbance at 375 nm. Since the molar extinction coefficients of free and luciferase-bound B are not known, molar ellipticities were not calculated.

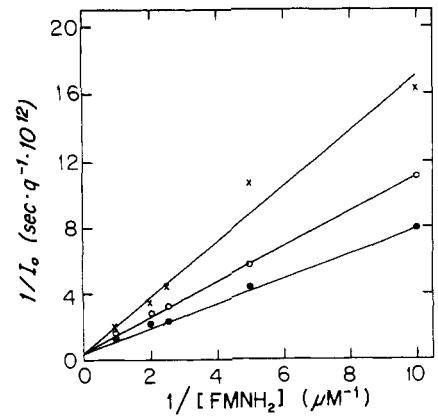


FIGURE 4: Substrate ($FMNH_2$) competitive inhibition of bacterial luciferase by B. The initial light intensities (I_0), using 6 μg of luciferase/assay, were measured by the dithionite assay over a range of 0.1–1 μM $FMNH_2$ in the absence (●) and presence of 0.4 μl (○) or 0.8 μl (X) of B stock solution/ml. Data, in double reciprocal plots, were fitted by least-squares analysis.

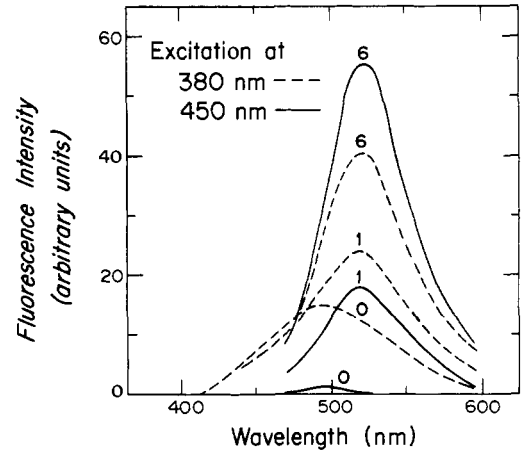


FIGURE 5: Fluorescence emission spectra of LB before and after flash irradiations. Luciferase from aldehyde-requiring mutant was complexed with B to 99% saturation, and subjected to dialysis treatment as described in the text. A 2-ml 0.02 *M* phosphate solution (pH 7) containing 0.55 mg of LB was flash irradiated repeatedly. Emission spectra were determined after each flash using excitation light at either 380 nm (- - -) or 450 nm (—). For simplification, only the spectra of samples irradiated once and six times are shown and compared with those of the starting LB sample. The number of flashes is indicated above each spectrum.

vine serum albumin which was not used in the flash assay due to its absorption of light. Flash initiation thus gives a yield of about 1% compared to $FMNH_2$. This can be attributed in part to the fact that the intensity of the flash is limiting; not all B molecules are converted to FMN in a single flash (Figure 6).

CD spectra of B, free and luciferase-bound, are shown in Figure 3. The LB sample shows a negative CD peak near 380 nm, in good agreement with the absorption maximum. The dissociation of B from LB, by the addition of urea to 5 *M*, blue shifts the CD trough to 375 nm with little reduction in the CD intensity.

As described before, the binding of B to L results in a loss of luciferase activity. Luciferase activity has been determined in the absence and presence of B at different $FMNH_2$ concentrations. From the double reciprocal plots, shown in Figure 4, it is clear that B inhibits the reaction, being competitive with $FMNH_2$, and indicating that B reversibly binds to the $FMNH_2$-binding site of luciferase. Aldehyde does not reverse or otherwise affect the inhibition by B, indicating that the aldehyde binding site is distinct from the site for B.

Photoexcited and $FMNH_2$-induced bioluminescence reactions are qualitatively similar in many respects but are different in the mode of reaction initiation. Thus, it was particularly important to examine further the chemical nature and consequences of the photoexcitation of luciferase-bound B. As shown in Figure 5, a fresh dialyzed LB sample is nearly nonfluorescent in 450-nm excitation light but emits fluorescence, λ_{max} at 495 nm, upon excitation by 380-nm light. When LB is repeatedly irradiated in the absence of aldehyde, a red shift of the emission maximum to 525 nm and progressive increases in fluorescence intensity were observed. Since free B is nonfluorescent at 23°, the observed changes in fluorescence intensity and emission spectrum of flash-irradiated LB cannot simply be due to dissociation of B from luciferase. It seems most likely that the changes are the result of photochemical modification of B to form a product (or products) having a higher fluorescence intensity and an altered fluorescence emission spectrum. The product spectrum closely resembles that of an oxidized flavine such as FMN. Furthermore, when the total fluorescence quantum output of a LB sample with excitation light at 450 nm is compared with that using 380-nm excitation light, where both luciferase-bound B and its photochemically derived product(s) absorb light, the ratio increases following each consecutive flash (Figure 5). This indicates that B is progressively converted to its product(s) by each photoexcitation treatment. As shown in Figure 6, successive flashes result in decreases in the remaining B activity in the LB sample accompanied by parallel increases in fluorescence at 525 nm (using 450-nm excitation light), reflecting the amount of product generated.

The identity of the product has been determined enzymatically and chromatographically. A LB sample was flashed six times at 23° as in Figure 5. Approximately 1 ml of the sample solution was then heated at 100° in the dark for 3 min, and the denatured protein was removed by centrifuga-

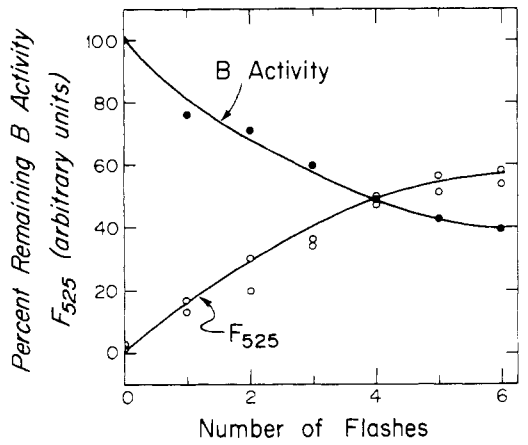


FIGURE 6: Correlation of the decrease in B activity and increase in fluorescence at 525 nm of a LB sample subjected to repeated flashing. The LB sample was the same as that described in Figure 5. Fluorescence intensities at 525 nm were measured using 450-nm excitation light. For the determination of the remaining quantity of B after each consecutive flash, 10 μl of sample solution was added to 1.2 ml of 0.02 M phosphate (pH 7) containing 0.15 mg of pure PL-free luciferase, and incubated at 23° for 3 min. The photoexcited bioluminescence activity of each sample, which reflects the remaining quantity of B, was subsequently assayed.

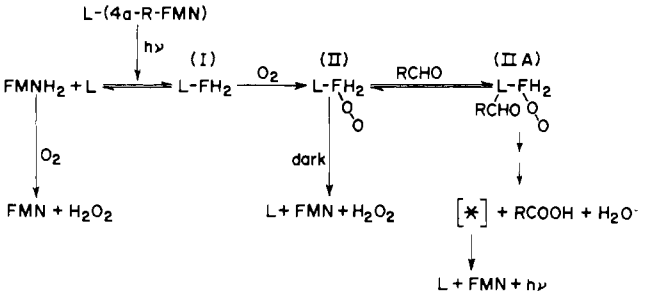


FIGURE 7: Hypothetical scheme depicting the pathways and intermediates in the luciferase-catalyzed ($FMNH_2$-initiated) and the photoexcited bioluminescence reactions. The structure of the chromophore B associated with the photoexcitable luciferase is proposed to be as 4a-R-FMN.

tion. A fresh LB sample, not flashed with light, was treated the same way and was taken as the control. Flavine (FMN) was found to be present in the flash-treated sample, but not in the control, determined by assays with flavine reductase, pyridoxine 5′-phosphate apooxidase, and bacterial luciferase as described in Experimental Procedures. Although flavine reductase is not completely specific for FMN (Duane and Hastings, 1975), both bacterial luciferase (Mitchell and Hastings, 1969; Meighen and MacKenzie, 1973) and pyridoxine 5′-phosphate apooxidase (Kazarinoff and McCormick, 1974) are. A quantitative estimation of the photochemical yield of FMN from LB gives a value of one FMN for every 3.2 luciferase molecules present in the original mixture, based on the assumption that the L used to make the LB was 100% pure, and that all B was converted to FMN. The luciferase used for this experiment was estimated to be 70% pure (based on its specific activity) and about 60% of the B was converted (Figure 6). After correcting for these, a ratio of 1 FMN per 1.3 luciferases is indicated, not too far, perhaps, from the expected 1:1 ratio.

For TLC chromatography the control and flash-treated LB samples were used directly without the above described heat treatment. Small aliquots, 50 μl, were applied to thin-layer chromatogram sheets and air dried. Ascending chromatography was carried out at 23° in darkness using solvent systems of 1-butanol–2 N NH_4OH–ethanol (3:1:1; volume ratios) and 1-butanol–pyridine–water (5:3:2; volume ratios). In both systems, only a single fluorescent spot was observed with the flash-treated sample under ultraviolet light. This was identified as FMN by comparison with flavine standards. No fluorescent compound was detected with the control.

Referring back to Figure 1, along with the experiments of Figures 5 and 6, it might be concluded that the FMN in Figure 1 occurs not as an adventitious contaminant, but arises via photochemical conversion from PL or free B. If so, it should be present only as a minor component: this appears to be so. The amount of FMN in peak I has been determined to be about 10% of the amount of B in peak III. This determination was based on binding and activity measurements of FMN and B to luciferase, assuming that one B is bound per luciferase as is known for both $FMNH_2$ and FMN (Meighen and Hastings, 1971; Baldwin et al., 1975).

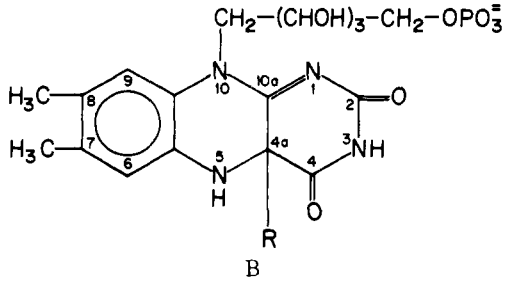
It has been previously reported that luciferase with B bound is inactive in the $FMNH_2$ initiated bioluminescence reaction, but that the activity can be recovered by removal of B, as for example by gel filtration in the presence of 5 M guanidine hydrochloride (Mitchell and Hastings, 1970). From the experiments of Figures 5 and 6, it might thus be expected that the photochemical reaction in which B is converted to FMN would involve the concomitant release of free luciferase. However, no increase in luciferase activity was observed upon flash irradiation. Flash irradiation was found to inactivate free luciferase, and thus might similarly destroy the L component of LB.

Discussion

A mechanism for photoexcitable bioluminescence can be proposed (Figure 7) based on the reaction sequence for bacterial luciferase first postulated by Hastings and Gibson (1963). B is postulated to be a substituted reduced FMN bound to luciferase in the $FMNH_2$ site, but incapable of reacting further via the normal pathway. Photochemically, however, the chromophore B can be converted to $FMNH_2$ so that the reaction can proceed via the conventional pathway and result in bioluminescence. This mechanism is consistent with the fact that light-induced bioluminescence is similar to $FMNH_2$-initiated bioluminescence in all respects except for the requirement for flavine and the mode of initiation (Gibson et al., 1965). It is also consistent with the fact that oxygen is not required during photoexcitation, but O_2 must be added immediately thereafter, within a half-time of less than 1 sec (Hastings and Gibson, 1967). The loss of activity, under anaerobic conditions, of the photochemically generated intermediate (I) requires explanation, since chemically formed luciferase-$FMNH_2$ is stable in the absence of oxygen. Since the concentration of the photochemically generated intermediate in the experiments of Hastings and Gibson (1967) was well below the K_D for the binding of $FMNH_2$ to luciferase ($\sim 2 \times t\ 10^{-7}$ M), the half-time of about 1 sec may be a measure of the off constant for the dissociation of $FMNH_2$ from luciferase. Base on a second-order rate constant of 10^7 M^{-1} sec^{-1} (Gibson et al., 1966) and a K_D of 2×10^{-7} M (Nicoli et al., 1974), the expected first-order rate constant for the dissociation of the $FMNH_2$–luciferase complex would be 2 sec^{-1}, in reasonable agreement with the value of 0.9 sec^{-1} in the experiment of Hastings and Gibson (1967).

Intermediates formed in several flavoprotein-catalyzed

reactions have been shown or suggested to be substituted reduced flavines (Spector and Massey, 1972a,b; Strickland and Massey, 1973; Porter et al., 1973; Hastings et al., 1973). The spectral and photochemical properties of the chromophore B also resemble those of reduced flavines, and those of 4a-substituted $FMNH_2$ in particular. The experimental findings which suggest this can be summarized as follows. (1) The luciferase-bound B shows a single absorption maximum at 375 nm in the near-ultraviolet to visible region. This is in accord with the absorption spectra of reduced flavine model compounds, both free and enzyme-bound, known to have substitutions at position 4a (Hemmerich et al., 1971; Ghisla et al., 1973, 1974). The 5-substituted flavines exhibit absorption optima in the region 300–345 nm (Hemmerich et al., 1967; Müller and Massey, 1969; Hemmerich et al., 1971; Hevesi and Bruice, 1973; Porter et al., 1973). An absorption optimum close to 445 nm has been predicted by P.-S. Song for the 10a oxygen adduct of flavine based on molecular orbital calculations (Strickland and Massey, 1973). In fact, a 10a-methoxy-1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine has been shown to exhibit an absorption maximum at 422 nm and a fluorescence emission maximum at 550 nm (Müller, 1971). Spectra for both 5 and 10a are thus quite different from those of 4a-substituted flavines. (2) Recently Ghisla et al. (1974) reported that reduced flavines with substitutions at the 5 and/or 4a positions do not fluoresce at room temperature but are fluorescent, λ_{max} in the region 476–520 nm, either at 77°K in rigid media or enzyme-bound at room temperature. In the present case, B bound to luciferase is fluorescent ($\lambda_{max\ em}$ 495 nm) whereas it is nonfluorescent in the dissociated state in aqueous solutions at 23°. (3) Hemmerich et al. (1967) have reported that oxidized flavines can be formed from 5-substituted reduced flavines by autooxidation at acidic condition, and from 4a-substituted reduced flavines by illumination in the presence of oxygen. With luciferase-bound B we have demonstrated that FMN is formed by flash irradiation under aerobic conditions. (4) The 4a- and 5-substituted reduced flavines have been isolated in the presence of dithionite as an antioxidant (Hemmerich et al., 1967). The fact that the addition of dithionite to B in the presence of luciferase and decanal does not initiate any $FMNH_2$-dependent bioluminescence activity (cf. Figure 1) is consistent with the suggestion that B is a substituted reduced flavine. (5) Luciferase-bound $FMNH_2$ exhibits a single negative CD optimum near 371 nm, while free $FMNH_2$ exhibits much weaker CD signal[2] due to the high degree of overall electronic symmetry associated with the flavine ring structure. The CD spectrum of the luciferase–B complex was found to closely resemble that of the luciferase-bound $FMNH_2$. Free B, however, exhibits a CD spectrum with structure and intensity similar to that of the luciferase-bound species (cf. Figure 3). The attachment of an additional group to the flavine isoalloxazine may conceivably perturb the ring electronic symmetry and thus result in an enhancement in the Cd intensity.



B

Although the properties of B appear to resemble those of 4a-substituted reduced flavine, its exact molecular structure cannot yet be specified. The luciferase intermediate in the normal reaction, designated as II (Figure 7), has recently been isolated by gel filtration at low temperature (−20 to −30°), and shown to possess a bound modified flavine having spectral properties which also resemble those of a 4a-substituted reduced flavine (Hastings et al., 1973). This intermediate has therefore been postulated to be the 4a-peroxy-FMN anion bound to luciferase. But II cannot be identical with PL or LB, since II rapidly breaks down in the dark at 20° to give FMN, H_2O_2, and free luciferase, or with aldehyde present, to give bioluminescence, FMN, free luciferase, but no H_2O_2 (Hastings and Balny, 1975).

Aldehyde-requiring mutants which are nonluminescent contain normal amounts of luciferase but no PL (Mitchell and Hastings, 1970). They thus differ in that the final light emitting steps (Figure 7, right) are blocked for want of the "natural" aldehyde (Shimomura et al., 1974). However, PL activity was found in the mutant if the in vivo bioluminescence was stimulated by the addition of aldehyde to the culture during growth. These findings indicate that the generation of B is not explicable as an artifact of the isolation procedures. They also make unlikely the possibility that B is synthesized as a 4a-substituted flavine product of other flavoprotein systems and is subsequently trapped by binding to luciferase. Instead, B can be best considered as a false intermediate of the bacterial bioluminescence reaction whose synthesis appears to be a direct consequence of the luciferase-catalyzed bioluminescence reaction, most likely formed in vivo by some side reaction(s), possibly with endogenous aldehyde.

Acknowledgments

We are grateful to Drs. T. O. Baldwin, M. Z. Nicoli, J. E. Becvar, and, especially, S. Ghisla for many stimulating discussions. We express our appreciation to Dr. V. D. Gooch for programming the least-squares analysis, to Patricia Dobson for purifying luciferase from the aldehyde-requiring mutant, and to Tufts University School of Medicine for the use of the Jasco spectropolarimeter. We also thank Mr. M. N. Kazarinoff and Dr. J. E. Becvar for carrying out the pyridoxine 5′-phosphate apooxidase and flavine reductase assays, respectively, with their own enzyme preparations.

References

Baldwin, T. O., Nicoli, M. Z., Becvar, J. E., and Hastings, J. W. (1975), *J. Biol. Chem.* (in press).

Duane, W., and Hastings, J. W. (1975), *Mol. Cell. Biochem.* (in press).

Ghisla, S., Hartmann, U., Hemmerich, P., and Müller, F. (1973), *Justus Leibigs Ann. Chem.*, 1338.

Ghisla, S., Massey, V., Lhoste, J.-M., and Mayhew, S. G. (1974), *Biochemistry 13*, 589.

Gibson, Q. H., Hastings, J. W., and Greenwood, C. (1965), *Proc. Natl. Acad. Sci. U.S.A. 53*, 187.

Gibson, Q. H., Hastings, J. W., Weber, G., Duane, W., and Massa, J. (1966), in Flavins and Flavoproteins, Slater, E. D., Ed., Amsterdam, Elsevier, p 341.

Gunsalus-Miguel, A., Meighen, E. A., Nicoli, M. Z., Neal-

[2] J. E. Becvar and J. W. Hastings, unpublished results.

son, K. H., and Hastings, J. W. (1972), *J. Biol. Chem. 247,* 398.
Hastings, J. W., and Balny, C. (1975), *J. Biol. Chem.,* (in press).
Hastings, J. W., Balny, C., Le Peuch, C., and Douzou, P. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3468.
Hastings, J. W., and Gibson, Q. H. (1963), *J. Biol. Chem. 238,* 2537.
Hastings, J. W., and Gibson, Q. H. (1967), *J. Biol. Chem. 242,* 720.
Hemmerich, P., Ghisla, S., Hartmann, U., and Müller, F. (1971), in Flavins and Flavoproteins, Third International Symposium, Kamin, H., Ed., Baltimore, Md., University Park Press, p 83.
Hemmerich, P., Massey, V., and Weber, G. (1967), *Nature (London) 213,* 728.
Hevesi, L., and Bruice, T. C. (1973), *Biochemistry 12,* 290.
Kazarinoff, M. N., and McCormick, D. B. (1974), *Biochim. Biophys. Acta 359,* 282.
Meighen, E. A., and Hastings, J. W. (1971), *J. Biol. Chem. 246,* 7666.
Meighen, E. A., and MacKenzie, R. E. (1973), *Biochemistry 12,* 1482.
Mitchell, G., and Hastings, J. W. (1969), *J. Biol. Chem. 244,* 2572.
Mitchell, G. W., and Hastings, J. W. (1970), *Biochemistry 9,* 2699.
Müller, F. (1971), in Flavins and Flavoproteins, Third International Symposium, Kamin, H., Ed., Baltimore, Md., University Park Press, p 363.
Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244,* 4007.
Nicoli, M. Z., Meighen, E. A., and Hastings, J. W. (1974), *J. Biol. Chem. 249,* 2385.
Porter, D. J. T., Voet, J. G., and Bright, H. J. (1973), *J. Biol. Chem. 248,* 4400.
Shimomura, O., Johnson, F. H., and Morise, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4666.
Spector, T., and Massey, V. (1972a), *J. Biol. Chem. 247,* 5632.
Spector, T., and Massey, V. (1972b), *J. Biol. Chem. 247,* 7123.
Strickland, S., and Massey, V. (1973), *J. Biol. Chem. 248,* 2953.
Tu, S.-C., Waters, C. A., and Hastings, J. W. (1975), *Biochemistry,* preceding paper in this issue.

Regulation of *Escherichia coli* Glutamine Synthetase. Evidence for the Action of Some Feedback Modifiers at the Active Site of the Unadenylylated Enzyme†

F. W. Dahlquist* and D. L. Purich

ABSTRACT: The interaction of unadenylylated form of *Escherichia coli* glutamine synthetase with several substrates and effectors has been examined by magnetic resonance techniques. These studies show that two manganese ions bind per enzyme subunit. From the dramatic line broadening observed in the alanine spectra in the presence of manganese and enzyme, it is concluded that the binding of alanine occurs at a site nearer one of the two manganese sites. Electron spin resonance (ESR) titration experiments suggest apparent dissociation constants of 20 and 120 μM for manganese to these sites in the presence of 1.0 mM magnesium ion. The manganese concentration dependence of the broadening of alanine suggests an affinity of 30 μM for the manganese closest to the alanine binding site. This suggests that alanine binds closer to the more tightly bound manganese ion. Glutamate appears to displace the alanine and also appears to bind close to the strongly bound manganese ion. It is proposed that alanine and glutamine bind competitively and in the same site. The binding of alanine and ATP is shown to thermodynamically interact such that the presence of one ligand increases the affinity of the enzyme for the other ligand. The presence of ATP dramatically sharpens the alanine line width when manganese and glutamine synthetase are present. Addition of ADP or phosphate alone has little effect on the alanine line width but the addition of both ADP and phosphate shows the same dramatic sharpening as the addition of ATP alone, suggesting an induced fit conformational change in the enzyme induced by ATP or by both ADP and phosphate. A binding scheme is proposed in which all feedback inhibitors of the enzyme bind in a competitive fashion with substrates.

The structure and regulation of the catalytic activity of the enzyme glutamine synthetase isolated from *Escherichia coli* have been studied rather extensively in recent years (Stadtman and Ginsburg, 1974). The enzyme has been shown to consist of 12 polypeptide chains of identical or nearly identical primary sequence (Woolfolk et al., 1966), which are apparently arranged in two hexagonal rings (Valentine et al., 1968). Each polypeptide chain can exist in either of two chemically distinct forms depending upon the specific covalent modification of a single tyrosine residue by reaction with ATP to form *O*-adenylyltyrosine. Interesting-

† From the Molecular Biology Institute, University of Oregon, Eugene, Oregon 97403, and the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received August 2, 1974.* This research was supported in part by BioMed Support Grant RR-07099-06 and Research Grants GM-18994 and GM-47874 from the National Institutes of Health, U.S. Public Health Service.