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Differential Effects of 8-Anilino-1-naphthalenesulfonate upon Binding of Oxidized and Reduced Flavines by Bacterial Luciferase†

Shiao-chun Tu and J. Woodland Hastings*

ABSTRACT: Upon binding to bacterial luciferase, both the absorption and the fluorescence excitation maxima of 8-anilino-1-naphthalenesulfonate (ANS) shift from 353 to 370 nm while the fluorescence emission optimum shifts from 540 to 480 nm, and the fluorescence quantum yield increases from 0.003 to 0.39, indicating that the environment of the ANS binding site is hydrophobic. ANS binds to luciferase with dissociation constants of 1.9×10^{-5} and 2.3×10^{-5} *M* at 5 and 23°, respectively. As with both oxidized flavine mononucleotide (FMN) and reduced flavine mononucleotide ($FMNH_2$), ANS also binds to luciferase with a stoichiometry of 1 site per dimeric luciferase molecule. ANS acts as a luciferase inhibitor, competitive with $FMNH_2$, with an inhibitor dissociation constant of 2.3×10^{-5} *M* at 23°. However, the binding of ANS does not significantly displace FMN from binding to luciferase. Interactions of FMN and $FMNH_2$ with luciferase are thus differentially regulated by the ANS binding.

Bacterial luciferase catalyzes the bioluminescent mixed function oxidation of $FMNH_2$[1] and a long chain aldehyde to yield light (λ_{max} 490 nm), FMN, H_2O, and carboxylic acid (Hastings and Gibson, 1963; Eberhard and Hastings, 1972; Nealson and Hastings, 1972; Shimomura et al., 1972; McCapra and Hysert, 1973; Dunn et al., 1973; Hastings et al., 1973; Hastings and Balny, 1975). Luciferase has been shown to be highly specific for $FMNH_2$; a negative charge on the flavine side chain is necessary for both tight binding and good substrate activity while structural variations of the flavine ring system generally result in poor activity with luciferase (Meighen and MacKenzie, 1973; Mitchell and Hastings, 1969). The binding of $FMNH_2$ to luciferase was demonstrated by kinetic analysis to have a stoichiometry of

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received May 8, 1975.* This work was supported in part by grants from the National Science Foundation (BMS74-23651) and the National Institutes of Health (GM 19536).

[1] Abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized flavine mononucleotide; ANS, 8-anilino-1-naphthalenesulfonate.

one site per enzyme molecule (mol wt ~79,000) with a K_d of 1-8 × 10^{-7} M at room temperature and pH 7 (Meighen and Hastings, 1971; Nicoli et al., 1974; Watanabe et al., 1974). Using the method of continuous variation of Job (Job, 1928; Asmus, 1961), the 1:1 binding of $FMNH_2$ to luciferase has also been established by both activity and circular dichroism measurements (Becvar and Hastings, 1975). Recently, luciferase was shown to bind FMN also in a 1:1 molar ratio, with K_d of 1.2-2.4 × 10^{-4} M at 2-3° and an enthalpy of binding of −10 cal/mol at pH 7 (Baldwin, 1974; Baldwin et al., 1975).

The identity of the emitting species of the bacterial bioluminescence reaction is not yet established, but certainly involves an excited enzyme-bound flavine molecule generated at some point during the overall enzymatic conversion of $FMNH_2$ to FMN (Eberhard and Hastings, 1972; Hastings et al., 1973; Mitchell and Hastings, 1969; Eley et al., 1970). Knowledge concerning the relationship between and the consequences of the binding of $FMNH_2$ and FMN by luciferase is thus of fundamental importance for understanding the mechanism of the formation of such an electronically excited flavine species from which the emission of light occurs. Using ANS as a fluorescent probe to compare the properties of the $FMNH_2$ site and FMN site, we have found that interactions of $FMNH_2$ and FMN with luciferase are subjectable to different regulations by ANS binding.

Materials and Methods

Materials. Luciferase was purified from the bacterium *Beneckea harveyi* (Reichelt and Baumann, 1973), previously designated MAV (Hastings et al., 1969), according to the method of Gunsalus-Miguel et al. (1972) with modifications (Baldwin et al., 1975). The homogeneity of the luciferase preparation was evaluated by sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969). Luciferase concentrations were determined based on 79,000 molecular weight and an absorption coefficient of 0.94 (0.1%, 1 cm) at 280 nm (Hastings et al., 1969; Gunsalus-Miguel et al., 1972). Decanal and FMN were gifts from Aldrich and Sigma, respectively. 8-Anilino-1-naphthalenesulfonic acid (purity >98%) was purchased from Fisher.

Dithionite Assay. Luciferase activity was determined, at 21 or 23°, by a modification of the previously described assay using dithionite (Meighen and MacKenzie, 1973). An anaerobic dithionite solution was prepared by adding 300 mg of sodium dithionite to 10 ml of O_2-free water, bubbled thereafter with argon. An appropriate volume (10-20 μl) of such a dithionite solution was added, with the use of a Hamilton syringe, to 1 ml of 0.02 M phosphate (pH 7) containing enzyme, 0.025 M 2-mercaptoethanol, and varying concentrations of FMN. The volume of dithionite added was determined as the minimum required for the optimal luminescence activity which was initiated by the rapid injection of 1 ml of 0.1% Triton X-100 containing 0.1% decanal. A fresh dithionite solution was prepared every 4 hr.

Spectral Measurements. Absorption spectra were measured with a Cary 15 spectrophotometer. Corrected fluorescence excitation and emission spectra were recorded with an Aminco-Bowman spectrophotofluorometer SPF-1000. With this instrument, fluorescence spectra were corrected for wavelength-dependent variations in light source output, monochromator efficiency, and phototube response. For fluorimetric titration experiments, measurements were obtained with either a Perkin-Elmer fluorescence spectrophotometer MPF-3 or a Farrand spectrofluorometer MK-1. Unless otherwise stated, all samples used for fluorescence studies had absorbances of less than 0.05 at the excitation wavelength to avoid the inner-filter effect.

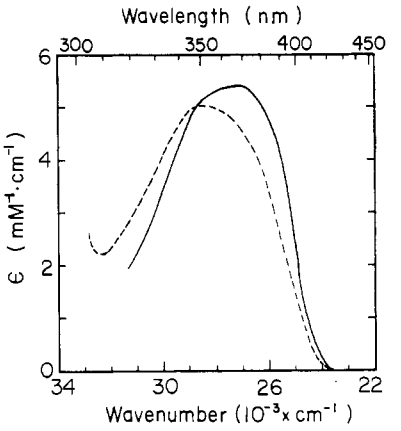


FIGURE 1: Absorption spectra of free and bound ANS. Spectral measurements were obtained at 23° with 4.5 × 10^{-5} M ANS in 0.02 M phosphate (pH 7) containing no (- - -) or 5.1 × 10^{-4} M luciferase (—). A buffer solution containing the same concentration of luciferase was used in the reference cell for the latter measurement.

Determination of FMN Concentration by Fluorescence. A standard curve was obtained by measuring the fluorescence intensity at 530 nm (10-nm band resolution) of a series of FMN solutions (at 5° in 0.05 M phosphate (pH 7)) of known concentration. A 510-nm excitation light (6-nm band resolution) was used because the low absorptivity of flavine at this wavelength enabled us to obtain a linear standard curve up to 10^{-4} M FMN. Since the luciferase-bound FMN is nonfluorescent (Baldwin et al., 1975), the observed flavine fluorescence of a FMN solution partially saturated with luciferase can thus be entirely attributed to that of free FMN. The exact concentration of free FMN can then be determined based on the standard curve obtained under identical experimental conditions. Since ANS, free or bound, does not absorb any light at 510 nm (Figure 1), determinations of FMN concentration with the above described method are not invalidated by the presence of this dye.

Results

Absorption Spectra of Free and Luciferase-Bound ANS. Free ANS in neutral aqueous solution exhibits an absorption maximum at 353 nm with a molar extinction coefficient of 5 × 10^3 M^{-1} cm^{-1} (Figure 1). Upon binding to luciferase, the absorption of the bound ANS is red shifted and exhibits an increased molar extinction coefficient (5.4 × 10^3 M^{-1} cm^{-1}) at the absorption maximum (370 nm).

Corrected Fluorescence Excitation and Emission Spectra of Free and Luciferase-Bound ANS. In good agreement with the absorption spectra, free and bound ANS exhibit fluorescence excitation peaks at 353 and 370 nm, respectively (Figure 2). The fluorescence emission maximum of bound ANS is blue shifted to 480 nm from 540 nm for the free species. The quantum yield of free ANS (ϕ_f) has been previously determined to be 0.003 (Turner and Brand, 1968). Compared with this value, the fluorescence quantum yield of bound ANS (ϕ_b) can thus be calculated from sample absorbances (A) at the excitation wavelength and areas enclosed by the corrected emission spectra using the equation (Parker and Rees, 1960)

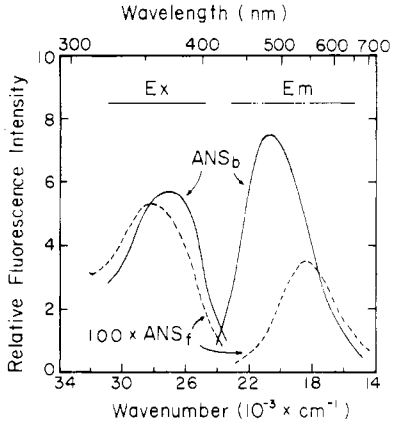


FIGURE 2: Corrected fluorescence excitation and emission spectra of free and bound ANS, at 23° with 10^{-5} M ANS in 0.02 M phosphate (pH 7) containing no (- - -) or 4.1 × 10^{-4} M luciferase (—). Excitation and emission spectra were measured with fluorescence monitored at 540 nm for the former and using 390-nm excitation light for the latter. A spectral resolution of 5 nm was selected for all measurements.

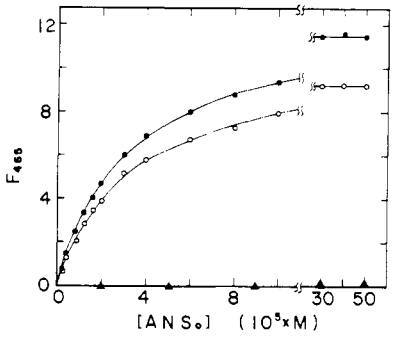


FIGURE 3: Fluorimetric titration of luciferase with increasing concentrations of ANS. A 1.2-ml phosphate solution (0.02 M, pH 7) containing 0 (▲), 3.5 (○), or 5.25 μM (●) luciferase was titrated with microliter increments of ANS solutions at 23°. Fluorescence intensities were measured at 465 nm with excitation light at 410 nm and are plotted vs. total ANS concentration, [ANS$_0$].

$$\phi_b = \phi_f \frac{(1 - 10^{-A})_f}{(\text{area})_f} \frac{(\text{area})_b}{(1 - 10^{-A})_b} \quad (1)$$

where the subscripts f and b refer to free and luciferase-bound ANS species. Using this method, ϕ_b for ANS was determined to be 0.39, a 130-fold enhancement of the corresponding ϕ_f. Both the blue shift in fluorescence emission maximum and the enhancement of fluorescence quantum yield observed upon the binding of ANS to luciferase indicate that the environment of the ANS binding site is hydrophobic, with the polarity resembling that of ethanol (Turner and Brand, 1968).

Dissociation Constants and Binding Stoichiometry of Luciferase-ANS Complex. The stoichiometry and K_d of the ANS binding by luciferase were determined by making use of the fluorescence enhancement observed upon the binding of ANS to the enzyme. Fluorimetric titrations of two different concentrations of luciferase with increasing amounts of ANS were carried out at 23° (Figure 3). For these measurements, an excitation light of 410 nm was chosen to avoid the inner-filter effect and to ensure that the fluorescence of up to 5 × 10^{-4} M free ANS be negligible. The observed fluorescence can then be attributed entirely to the bound ANS species. Thus, the micromolar concentration of bound ANS, x, can be determined according to

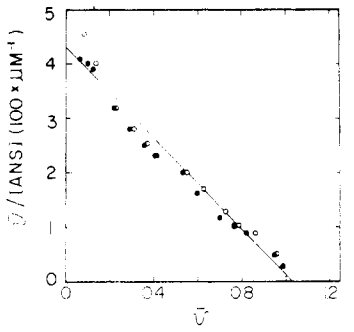


FIGURE 4: A graphical determination of the dissociation constant and number of ANS-binding sites by the Scatchard plot. Data were taken from Figure 3. $\bar{\nu}$ is the average number of ANS bound per enzyme molecule and [ANS] is the free ANS concentration. Data were fitted by least-squares analysis.

$$x = F/F_b \quad (2)$$

where F is the observed fluorescence intensity (at 465 nm) at each tested ANS concentration (Figure 3) and F_b is the micromolar fluorescence activity (at 465 nm) of bound ANS determined by measuring the fluorescence intensity of a 1 μM ANS solution in the presence of a saturating concentration of luciferase using the same instrumental settings. From the calculated values of x, the average number of ANS molecules bound per enzyme molecule, $\bar{\nu}$, can then be determined as

$$\bar{\nu} = x/[E_0] \quad (3)$$

where [E$_0$] is the total μM enzyme concentration. Results of fluorimetric titrations (Figure 3) were analyzed as described above and presented in the form of a Scatchard plot (Scatchard, 1949) as shown in Figure 4. From this, a K_d of 2.3 × 10^{-5} M and 1.03 binding sites per enzyme molecule were determined for the binding of ANS to luciferase at 23°. Fluorimetric titrations were also carried out at 5° (data not shown). Following the same analysis method, a K_d of 1.9 × 10^{-5} M and 1.02 ANS binding sites per luciferase molecule were obtained at this temperature.

ANS as an Inhibitor Competitive with $FMNH_2$. The relationship between ANS binding and $FMNH_2$ binding was investigated in two sets of experiments by using ANS as an inhibitor in the $FMNH_2$-dependent luciferase activity assay. Luciferase activity was first determined at 23° by the dithionite assay using constant levels of ANS and varying concentrations of $FMNH_2$. Results so obtained are shown in Figure 5A in the form of double reciprocal plots. ANS appears to be an inhibitor competitive with $FMNH_2$; the inhibitor dissociation constant, K_i, is 2.3 × 10^{-5} M. In a second set of experiments, the inhibition of luciferase activity by ANS was studied at 21° as a function of varying concentrations of ANS in the presence of four constant concentrations of $FMNH_2$. Results obtained in these experiments were plotted according to both the method of Dixon (1953) (Figure 5B) and the method of Cornish-Bowden (1974) (Figure 5C). Both analyses show that ANS competitively inhibits the binding of $FMNH_2$ to luciferase (K_i = 1.9 × 10^{-5} M).

The inhibitory effect of ANS on luciferase activity is not affected by varying the decanal concentration indicating that no significant interaction exists between the ANS site and the aldehyde site.

Relationship between ANS Binding and FMN Binding. According to the previously described method (Baldwin et

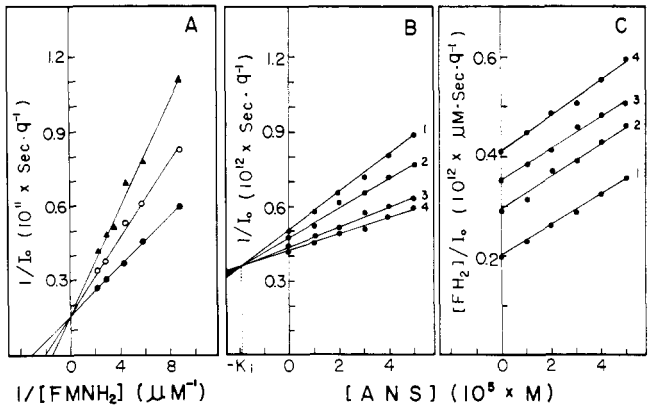


FIGURE 5: Inhibition of luciferase activity by ANS. (A) The initial light intensities (I_0), using 6 μg of luciferase/assay, were determined at 23° by the dithionite assay over a range of 0.11–0.46 μM $FMNH_2$ in the absence (●) and presence of 12.8 μM (○) or 25.6 μM (▲) ANS. Data are presented as double reciprocal plots. (B) The initial light intensities (I_0), using 10 μg of luciferase/assay, were determined at 21° by the dithionite assay over a range of 0–5 × 10^{-5} M ANS in the presence of 0.4, 0.6, 0.8, and 1 μM $FMNH_2$ (shown as lines 1, 2, 3, and 4, respectively). Data are plotted according to the method of Dixon (1953). (C) The same data from Figure 5B are plotted by the method of Cornish-Bowden (1974). Least-squares analysis was used for data fitting.

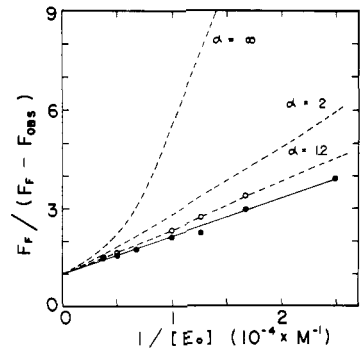


FIGURE 6: Fluorescence quenching of FMN by luciferase in the absence or presence of ANS. Samples in 0.05 M phosphate (pH 7) containing 10^{-5} M FMN and zero (●) or 1.3 × 10^{-4} M ANS (○) were titrated with 0.6–2.7 × 10^{-4} M luciferase at 5°. Flavine fluorescence, F_F, and F_{obsd} in the absence and presence of luciferase, respectively, was measured at 530 nm (10-nm resolution) using 510-nm excitation light (6-nm resolution). K_d for the luciferase–FMN interaction was calculated to be 1.13 × 10^{-4} M from the solid line according to eq 4. (- - -) Computer-generated curves according to mechanism 5, using a K_d of 1.13 × 10^{-4} M for FMN binding and 1.9 × 10^{-5} M for ANS binding, and adopting different α values as indicated. $[E_0]$ is the total luciferase concentration.

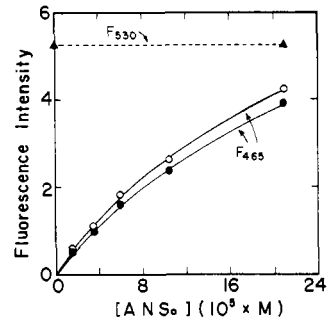


FIGURE 7: Fluorimetric titration of luciferase, FMN-free and partially saturated, with varying concentrations of ANS. Samples in 0.05 M phosphate (pH 7) containing 2 × 10^{-4} M luciferase and zero (○) or 6 × 10^{-5} M (▲, ●) FMN were titrated at 23° with ANS up to a total concentration, $[ANS_0]$, of 2.1 × 10^{-4} M. The front face measurement accessory from Perkin-Elmer was used to allow an angle of 53° between the surface of a regular (1 × 1 cm cross section) fluorescence cuvet and the excitation light. Flavine fluorescence, F_{530}, and bound ANS fluorescence, F_{465}, were measured using excitation light at 510 and 400 nm, respectively. Slits were set at 8-nm resolution for excitation and 5-nm resolution for emission.

al., 1975), the dissociation constant, K_d, and the intrinsic flavine fluorescence for the luciferase–FMN complex, f_{EF}, were determined at 5° in 0.05 M phosphate (pH 7) by following the changes in observed flavine fluorescence, F_{obsd}, upon titration of a 10^{-5} M FMN solution with increasing concentrations of luciferase (0.6–2.7 × 10^{-4} M). Results were analyzed according to the equation

$$\frac{F_F}{F_F - F_{obsd}} = \frac{f_F}{f_F - f_{EF}} \frac{K_d}{[E]} + \frac{f_F}{f_F - f_{EF}} \qquad (4)$$

where F_F is the observed flavine fluorescence in the absence of enzyme, f_F is the intrinsic fluorescence of free FMN, and [E] is the concentration of free luciferase. When the total enzyme concentration, $[E_0]$, is much greater than that of FMN, a linear plot of $F_F/(F_F - F_{obsd})$ vs. $1/[E_0]$ ($\simeq 1/[E]$) is obtained (● in Figure 6). A value of 1.0 was obtained for the ordinate intercept, $f_F/(f_F - f_{EF})$, indicating that the luciferase-bound FMN is nonfluorescent ($f_{EF} = 0$), in agreement with earlier findings (Baldwin et al., 1975). From such a plot, a K_d of 1.13 × 10^{-4} M was determined for the luciferase–FMN interaction at 5°. This graphic analysis, though not originally used for this purpose, also provides a sensitive test for changes in FMN binding affinity to luciferase in the presence of a second ligand such as ANS. When the above described titration experiment was repeated in the presence of 1.3 × 10^{-4} M ANS, slightly higher free flavine fluorescence, F_{obsd} (shown as $F_F/(F_F - F_{obsd})$ in Figure 6), was observed at every luciferase concentration tested indicating that the binding of FMN to luciferase was slightly depressed by the presence of ANS. Based on the square mechanism 5 (see Discussion) and experimentally determined dissociation constants for ANS and FMN binding (at 5°), the free FMN concentration, [FMN], at every $[E_0]$ tested was computer calculated by assuming different α values. Knowing the total FMN concentration, $[FMN_0]$, theoretical curves of $F_F/(F_F - F_{obsd})$ (= $[FMN_0]/\{[FMN_0] - [FMN]\}$) vs. $1/[E_0]$ were subsequently constructed as shown in Figure 6. Observed results are consistent with the theoretical curve obtained using an α value of 1.2.

The relationship between FMN binding and ANS binding was further examined at 23° by titrating a 0.05 M phosphate solution (pH 7) containing 2 × 10^{-4} M luciferase and 6 × 10^{-5} M FMN with increasing concentrations of ANS. The fluorescence of free FMN, measured at 530 nm, and of bound ANS, measured at 465 nm, was monitored by the method of front face measurement (Duysens and Amesz, 1957) using excitation light at 510 and 400 nm, respectively (Figure 7). The presence of up to 2.1 × 10^{-4} M ANS (capable of saturating about 70% of the luciferase molecules in the absence of FMN) did not appreciably displace FMN from binding to luciferase as evidenced by the constant fluorescence at 530 nm throughout the ANS titration range. The binding of ANS to luciferase was measured by following the enhancement of fluorescence at 465 nm. As a control, 2 × 10^{-4} M luciferase was first titrated with ANS in the absence of FMN. Results of this experiment

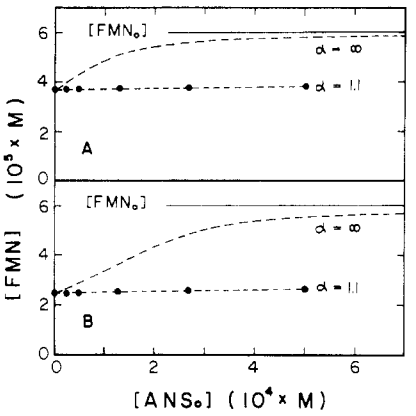


FIGURE 8: Free FMN concentration, [FMN], at equilibrium as a function of total ANS concentration, [ANS_0], determined at two luciferase concentrations. Samples in 0.05 M phosphate (pH 7) containing a total FMN concentration, [FMN_0], of 6×10^{-5} M and 1 (A) or 2×10^{-4} M (B) luciferase were titrated at 5° with ANS. Fluorescence at 530 nm (10-nm resolution) was measured using 510-nm excitation light (6-nm resolution). Free FMN concentrations at equilibrium (●) were then determined from the observed F_{530} as described in the text. (- - -) Computer-generated curves obtained as described in Figure 6.

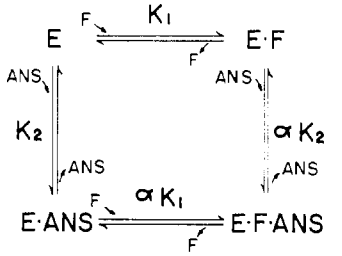
were quantitatively different from those presented in Figure 3 due to differences in enzyme concentration. Compared with this control, the binding of ANS to luciferase was only slightly depressed in the presence of 6×10^{-5} M FMN. The observed apparent decreases in fluorescence at 465 nm may be, in part, attributed to an inner-filter effect arising from the absorption of the 400-nm excitation light by FMN ($\epsilon_{400} = 6.7 \times 10^3$ M^{-1} cm^{-1}), even when the method of front face measurement was used (Udenfriend, 1962).

These findings indicate that luciferase is capable of interacting with FMN and ANS simultaneously with dissociation constants for the ternary complex formation similar to those of the respective binary complex formation. For quantitative analysis, similar experiments were also carried out at 5° over a range of $0.25\text{–}5 \times 10^{-4}$ M ANS with samples containing 6×10^{-5} M FMN and 1 or 2×10^{-4} M luciferase. The FMN fluorescence at 530 nm was measured at 90° from the 510-nm excitation light which was not absorbed by ANS. Nevertheless, we have noted that, in the absence of luciferase, FMN fluorescence is slightly quenched (1–4% quenching) at high ANS concentrations ($2\text{–}5 \times 10^{-4}$ M), probably due to dark complex formation. Therefore, observed flavine fluorescence intensities were corrected for this effect and free FMN concentrations were then determined as described in Materials and Methods. Results shown in Figure 8 are consistent with the theoretical curve constructed according to mechanism 5 (see Discussion) using an α value of 1.1.

Discussion

A direct analysis of interactions of FMN and $FMNH_2$ with luciferase is complicated by the very rapid nonenzymatic interaction between the two species themselves to generate the semiquinone, and also the FMN–$FMNH_2$ complex (Barman and Tollin, 1972). This, as well as the absorption of the bioluminescence emission by FMN, has made it impossible to evaluate the possibility that FMN inhibits the luminescence reaction, competitive with $FMNH_2$.[2] ANS, which has been used as a spectroscopic probe to investigate physical and chemical properties of a wide variety of biological macromolecules (Brand and Gohlke, 1972), is particularly suitable for the study of the relationship between FMN binding and $FMNH_2$ binding to luciferase. The binding of this dye to luciferase is accompanied by changes in the spectral properties and fluorescence quantum yield, and the 1:1 stoichiometry of binding simplifies data analysis. The absorption spectra of free and bound ANS are well resolved from the 445-nm absorption peak of FMN, allowing one to accurately monitor the flavine fluorescence in the presence of ANS by using an excitation light within the region of 430–515 nm.

In the present study, the effect of ANS binding on the binding of FMN and of $FMNH_2$ to luciferase was examined according to the square mechanism shown in eq 5,



where E refers to luciferase, F refers to FMN or $FMNH_2$, K_1 and K_2 are dissociation constants for enzyme–flavine complex and enzyme–ANS complex, respectively, and α is a constant representing the change in binding affinity. If the equilibrium E·F + ANS $\rightleftharpoons$ E·F·ANS is characterized by the dissociation constant αK_2 then the equilibrium E·ANS + F $\rightleftharpoons$ E·F·ANS must be described by αK_1. This is so because the free energy change for the formation of the ternary complex must be the same by both pathways (Webb, 1963). In this scheme, the independent binding and the exclusive (or competitive) binding of two ligands to a protein are characterized by α values of 1 and ∞, respectively.

It is known that the double reciprocal plot does not distinguish competitive from partially competitive inhibition (Webb, 1963) and the Dixon plot is incapable of differentiating competitive from mixed inhibition (Cornish-Bowden, 1974; Purich and Fromm, 1972). The kinetic data obtained using ANS as an inhibitor in the $FMNH_2$-dependent luciferase activity assay were thus analyzed by three graphic methods (Figure 5) for an unambiguous determination of the type of inhibition and for reliable measurements of the K_i values. As shown by all three graphic analyses, ANS functions as a competitive inhibitor for the binding of $FMNH_2$ to luciferase. The K_i values obtained by such kinetic measurements are in good accord with the spectrally determined dissociation constant of luciferase–ANS complex. Based on the square mechanism (eq 5), these findings strongly suggest that the ternary complex E·$FMNH_2$·ANS was not formed from either E·$FMNH_2$ or E·ANS under the experimental conditions. It should be pointed out, however, that an identification of absolute competitive inhibition ($\alpha = \infty$) is often difficult using such graphical analyses of kinetic data. If the binding of ANS were to decrease the luciferase affinity for $FMNH_2$ by a factor of 100 ($\alpha = 100$), differences between this case and that of absolute competitive inhibition would not be easily detectable in these graphical plots. On the other hand, the present study shows clearly that the ANS binding site is nearly independent from the FMN binding site. The α value was estimated to be approximately 1.1–1.2 indicating that dissociation constants

[2] T. O. Baldwin, M. Z. Nicoli, and J. W. Hastings, unpublished results.

for the E·FMN·ANS complex formation are similar to those of the respective luciferase binary complex formation.

The observation that ANS competitively inhibits $FMNH_2$ but not FMN binding is interesting. Strong binding of dyes to globular proteins has been noted to take place predominantly in hydrophobic areas often overlapping with the binding sites for substrates, coenzymes, and prosthetic groups (Glazer, 1970). The environment of the bacterial luciferase active center has been shown to be hydrophobic (Nicoli and Hastings, 1974), and we have found that the ANS binding site is also hydrophobic. One interpretation of our finding is that ANS binds near and overlaps with a single flavine binding site, but that oxidized and reduced flavines bind in different stereochemical configurations, such that bound ANS has an overlap with the $FMNH_2$ site but nearly none with the FMN site. An alternative, that the FMN and $FMNH_2$ sites are different, cannot be ruled out, but this means that there would be two sites for FMN binding, the site which also binds $FMNH_2$ having a much lower affinity for FMN than the second distinct site. The possibility of two FMN sites does not support and should be distinguished from the proposal that two $FMNH_2$ are required for activity in the bacterial bioluminescence reaction (Lee, 1972). Also, quantum yield measurements have now demonstrated that only one $FMNH_2$ per luciferase molecule is required for the bioluminescence reaction (Becvar and Hastings, 1975).

An alternative interpretation is that the binding of ANS may occur at a site remote from the flavine site(s) and cause a conformational change in the luciferase. Such a conformational change would have to selectively block the binding of $FMNH_2$ to luciferase with only small changes in the FMN binding affinity. To examine the possible ANS-induced conformational change, we have measured the luciferase fluorescence emission in the absence and presence of ANS. After correction for the uneven absorption of protein fluorescence by ANS using the method of Tu and McCormick (1974), we find that the binding of ANS to luciferase does not significantly shift the protein fluorescence emission maximum. Other techniques, including circular dichroism spectroscopy, are under current use to investigate the conformation of luciferase–ANS complex. With other enzyme systems, alterations of enzyme kinetic properties as a consequence of the binding of dyes to a site different from the catalytic site have been observed with chymotrypsin (McClure and Edelman, 1967), ficin (Hollaway, 1968), and ribonucleic acid polymerase (Wu and Wu, 1973).

Although a decision between these possibilities cannot be made at this time, there is evidence that FMN and $FMNH_2$ bind the same enzyme center. Nicoli et al. (1975) have shown that when FMN is complexed with luciferase from mutants selected as having altered $FMNH_2$ binding properties (Cline and Hastings, 1972), the absorption spectrum is considerably altered relative to that obtained for the wild-type luciferase–FMN complex (Baldwin, 1974). Thus, either the $FMNH_2$ and FMN sites are within close proximity, or, if spatially distinct, both are affected by a conformational change in luciferase caused by lesions. Nicoli et al. (1974) have also shown that binding of FMN to luciferase protects a sulfhydryl residue from reaction with alkylating reagents, whereas prior chemical modification of this sulfhydryl residue inhibits $FMNH_2$ binding (Nicoli et al., 1974). These results suggest that both FMN and $FMNH_2$ bind near this sulfhydryl residue, but the possibility remains that conformational changes occur upon flavine binding at different sites, both resulting in protection of the sulfhydryl group.

Acknowledgments

We are most grateful to Dr. V. D. Gooch for his help in computer programming, to Dr. J. E. Becvar for providing some of the luciferase preparation, and to Drs. T. O. Baldwin, M. Z. Nicoli, and J. E. Becvar for critical reading of the manuscript. We also express our appreciation to Tufts University, School of Medicine, Dr. E. R. Blout, and to Farrand Optical Co., Inc. for the use of their spectrofluorometers.

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Sites of Biological Methylation of Proteins in Cultured Chick Muscle Cells†

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ABSTRACT: The methylation of myosin and other proteins has been studied using primary cultures of 12-day-old embryonic chick leg muscle. The methyl group of [*Me*-^{3}H]methionine is incorporated into basic amino acid residues with the formation of N^{ϵ}-monomethyllysine, N^{ϵ}-dimethyllysine, N^{ϵ}-trimethyllysine, 3-methylhistidine, N^{G}-monomethylarginine, and N^{G}-dimethylarginine which are isolated from acid hydrolysates of purified myosin, and of proteins from polysomes and from the cytosol of the cultured muscle cells. In the presence of 0.1 m*M* cycloheximide, incorporation of [*Me*-^{3}H]methionine into the polysome-bound proteins was decreased to 16.3% of control levels with no change in the pattern of incorporation into the basic amino acid residues, although protein synthesis was inhibited 97.5%. When protein synthesis was allowed to resume in such cultures by the removal of cycloheximide, polypeptides containing labeled N-methylated residues were released from polysomes into the soluble fraction. Polypeptides containing N-methylated amino acids were also released from polysomes following treatment with 2 m*M* puromycin. Peptidyl-tRNA, isolated from ribosomes after exposure of cultures to [*Me*-^{3}H]methionine, contained labeled N-methylated amino acids. When [*Me*-^{3}H]methionine was incorporated in the presence of cycloheximide, the isolated peptidyl-tRNA still contained N-methylated amino acids although the amount of methylation was 22.4% of control levels. These experiments demonstrate that N-methylation of basic amino acid residues in proteins may occur while the polypeptide is still being synthesized on the ribosome. In addition, N-methylation can occur on the nascent polypeptides in the absence of protein synthesis.

Basic amino acids which are modified by N-methylation are present in proteins from many sources (Paik and Kim, 1971). Among the sources which have been investigated are the histones (Allfrey, 1971), ribosomal proteins (Comb et al., 1966; Terhorst et al., 1972; Reporter, 1973a; Alix and Hayes, 1974; Chang and Chang, 1974; Chang et al., 1974), plant and fungal cytochrome *c* (Lemberg and Barrett, 1973), rhodopsin (Reporter and Reed, 1972), and skeletal muscle actin and myosin from rabbit (Johnson and Perry, 1970), chicken (Krzysik et al., 1971), bovine (Asatoor and Armstrong, 1967), and cat (Kuehl and Adelstein, 1970), as well as cultured muscle from rat (Reporter, 1969) and chicken (Morse and McManus, 1973).

N-Methylation of arginine, lysine, and histidine occurs after incorporation of the unmodified amino acids into protein (Paik and Kim, 1971). This post-translational modification of amino acids is catalyzed by methyltransferase(s) which use *S*-adenosylmethionine as the methyl donor (Paik and Kim, 1971; Hardy et al., 1970; Krzysik et al., 1971; Reporter, 1973b). Attempts to find aminoacyl-tRNAs charged with N-methylated amino acids have been unsuccessful (Kim and Paik, 1965; Young et al., 1970, 1972; Reporter, 1973b).

This paper is concerned with the question of whether the post-translational methylation of amino acids takes place while the polypeptide is still being synthesized on the ribo-

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