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Structural Studies on Bacterial Luciferase Using Energy Transfer and Emission Anisotropy†

Shiao-Chun Tu,‡ Cheng-Wen Wu, and J. Woodland Hastings*

ABSTRACT: The distance between specific sites on bacterial luciferase was estimated by energy transfer. Luciferase was fluorescently labeled by reaction of an essential sulfhydryl group with *N*-(1-pyrene)maleimide and *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide. Both of the modified enzymes bind 8-anilino-1-naphthalenesulfonate (Ans) with affinities similar to that exhibited by the native luciferase. Using each of the two fluorescent probes as a donor and the bound Ans as an acceptor, the energy transfer efficiencies were determined by the resulting enhancement of fluorescence of the acceptor. The corresponding distance was calculated to be in the range of 21 to 37 Å. Energy-transfer studies were also carried out using fluorescence lifetime measurements of bound ANS, acting as a donor with bound FMN as an acceptor. The corresponding distance was calculated to be between 30 and 58 Å. Using samples of luciferase:Ans complex and luciferase modified with *N*-(1-pyrene)maleimide, the rotational correlation time of the enzyme–dye conjugate as a whole was found to be 47 ± 2 ns. The observed rotational correlation time is much longer than that calculated for luciferase assuming a spherical structure, thus indicating an elongated form for the luciferase–dye conjugate.

Bacterial luciferase functions as a monooxygenase in catalyzing the bioluminescent mixed function oxidation of $FMNH_2$[1] and a long-chain aldehyde by molecular oxygen (Hastings & Gibson, 1963; Becvar & Hastings, 1975; Nealson & Hastings, 1972). The final reaction products are light,

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 (S.-C.T. and J.W.H.), and the Department of Biophysics, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461 (C.-W.W.). *Received August 10, 1977.* This work was supported in part by National Institutes of Health Grants 5 F32 GM00348-02 and -03 to S.-C.T., National Science Foundation Grant BMS 74-23651 to J.W.H., and National Institutes of Health Grant GM-19062 and American Cancer Society Grant BC-94 to C.-W.W.

‡ Present address: Department of Biophysical Sciences, University of Houston, Houston, Texas 77004.

[1] Abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized riboflavin 5′-phosphate; Ans, 8-anilino-1-naphthalenesulfonate; NBPM, *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide; NPM, *N*-(1-pyrene)maleimide; NBPS and NPS, the *N*-[*p*-(2-benzoxazolyl)phenyl]succinimido group and the *N*-(1-pyrene)succinimido group, respectively (both are linked to the essential sulfhydryl group of luciferase); NBPS-E and NPS-E, the NBPS-luciferase adduct and the NPS-luciferase adduct, respectively; FMN_b and Ans_b, luciferase-bound FMN and Ans, respectively; *q*, quanta; *Q*, fluorescence quantum yield.

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FMN, carboxylic acid, and H_2O (Shimomura et al., 1972; McCapra & Hysert, 1973; Dunn et al., 1973; Hastings & Balny, 1975). An excited state of a flavin species is generated as an intermediate in the reaction (Mitchell & Hastings, 1969; Eley et al., 1970) which, in the enzyme-bound form (Cline & Hastings, 1974), falls to ground state with the production of light. Although there are many flavomonooxygenases, bacterial luciferase is the only enzyme of this type known to catalyze a reaction with significant light production. To elucidate the biochemical nature of the bacterial bioluminescence reaction, both mechanistic and structural aspects of luciferase have been extensively studied in recent years (Hastings & Wilson, 1976; Hastings & Nealson, 1977; and references therein). In the present work, we have carried out further structural studies on bacterial luciferase using techniques of steady-state and nanosecond fluorescence spectroscopy.

Bacterial luciferase is a heterodimer designated $\alpha\beta$ with molecular weights of 42 000 for α and 37 000 for β (Hastings et al., 1969; Meighen et al., 1970), the affinity between the two being high (Gunsalus-Miguel et al., 1972; Tu et al., 1977b). Results of chemical (Meighen et al., 1971a,b) and mutational (Cline & Hastings, 1972) modifications of luciferase have demonstrated that α is the catalytic subunit; α and β subunits are not individually active (Hastings et al., 1969; Gunsalus-Miguel et al., 1972), and the specific function of β remains unknown.

Luciferase has been shown to have a single flavin site for either the substrate $FMNH_2$ (Meighen & Hastings, 1971; Becvar & Hastings, 1975) or the product FMN (Baldwin et al., 1975). It also has a single site for Ans, which is an inhibitor competitive with $FMNH_2$ in the activity assay, but the Ans binding and the FMN binding by luciferase are independent (Tu & Hastings, 1975). Nicoli et al. (1974) showed that luciferase has, among a total of 13 cysteinyl residues per $\alpha\beta$ dimer, an essential sulfhydryl group on the α subunit. Chemical modification of this sulfhydryl group results in complete inactivation of luciferase. However, the modified luciferase still binds FMN (Nicoli et al., 1976) and probably also $FMNH_2$ (Cousineau & Meighen, 1977). The mechanisms by which luciferase is inhibited by bound Ans and inactivated by sulfhydryl modification are still unclear.

In the present study, we have utilized dimeric luciferase, labeled covalently and noncovalently with fluorescent probes. Based on time-dependent fluorescence emission anisotropy measurements, the enzyme appears to possess an elongated shape.

The spatial relationships between the different specific sites on bacterial luciferase have also been examined.

Materials and Methods

Materials. Luciferase was purified to homogeneity by the previously described method (Gunsalus-Miguel et al., 1972; Baldwin et al., 1975) from cells of *Beneckea harveyi* strain MAV 392 (Reichelt & Baumann, 1973). Luciferase concentrations were determined based on 79 000 molecular weight and an absorption coefficient of $A_{1\,cm}^{0.1\%} = 1.2$ at 280 nm (Becvar, J. E., & Hastings, J. W., unpublished results). Decanal was a gift from Aldrich. FMN was synthesized enzymatically (Spencer et al., 1976). Ans and NBPM were purchased from Fisher and Eastman, respectively. NPM was synthesized by the method of Weltman et al. (1973). Extinction coefficients, in M^{-1} cm^{-1}, used in concentration determinations are 1.2×10^4 at 450 nm for FMN (Whitby, 1953), 6.24×10^3 at 351 nm for Ans (Ferguson et al., 1975), 3.2×10^4 at 308 nm for NBPM (Kanaoka et al., 1968), and 4×10^4 at 345 nm for NPM (Rawitch et al., 1969).

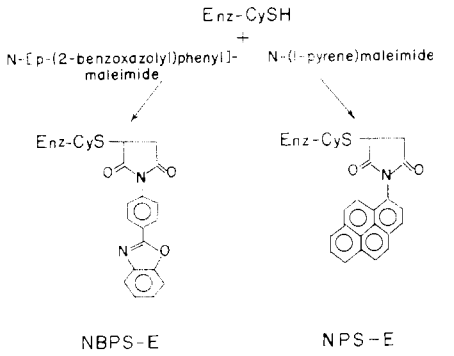

FIGURE 1: Fluorescent labeling of the essential sulfhydryl group on luciferase.

Enzyme Assay. Luciferase activity was determined at 23 °C by the standard assay (Gunsalus-Miguel et al., 1972), involving the injection of 1 mL of 5×10^{-5} M $FMNH_2$ (catalytically reduced with H_2) into 1 mL of 0.02 M phosphate (pH 7) containing luciferase, 0.2% bovine serum albumin, and 10 μL of 0.1% decanal suspension. The initial maximal intensity of each assay was measured in quanta s^{-1} (Hastings & Weber, 1963). The purified luciferase had a specific activity of 1.8×10^{14} q s^{-1} mg^{-1} as determined by the standard assay at 23 °C.

Preparation of NBPS-E and NPS-E. The essential sulfhydryl group of luciferase was stoichiometrically labeled by reacting with NBPM and NPM to form the corresponding succinimido-luciferase adducts, NBPS-E and NPS-E, respectively (Figure 1). Luciferase samples (1.7 to 4.8 mg) in 1 mL of 0.05 M phosphate (pH 7) were each reacted with equimolar quantity of NBPM or NPM at 23 °C. Stock solutions of NBPM (1 mM) and NPM (0.36 mM) were prepared in dimethyl sulfoxide and methanol:acetone (1:1; v/v), respectively. Reactions were monitored by measuring the remaining enzyme activity using aliquots withdrawn at different times. In all cases, >98% inactivation was achieved in less than 1 min using either NBPM or NPM. The use of less than stoichiometric amounts of either reagent results in partial inactivations of luciferase. The stoichiometrically labeled enzyme samples were each applied to a Sephadex G-25 column (1 × 16 cm) preequilibrated and eluted with 0.05 M phosphate (pH 7) at 4 °C. Fractions containing protein molecules (NBPS-E or NPS-E) were collected and used for subsequent analyses. In the case of NBPS-E, the stoichiometry of sulfhydryl labeling was further established to be 1.06 NBPS per luciferase molecule, using extinction coefficients of 3.02 and 1.91×10^4 M^{-1} cm^{-1} for NBPS at 308 and 280 nm, respectively (Kanaoka et al., 1968), and absorption coefficients, $A_{1cm}^{0.1\%}$, of 0.039 and 1.2 for luciferase at 308 and 280 nm, respectively. Unlike some cases in which NPS apparently reacts with amino groups and results in secondary spectral changes (Wu et al., 1976), both excitation and emission spectra of the NPS-E remained unchanged, indicating that the modification of the essential sulfhydryl group is indeed the cause of luciferase inactivation.

Spectral Measurements. Absorption spectra were measured with a Cary 15 spectrophotometer. Steady-state fluorescence excitation and emission spectra were recorded with a Perkin-Elmer MPF-44 fluorescence spectrophotometer using a microcuvette (0.3-cm path). Fluorescence spectra were corrected for wavelength-dependent variations in light-source output, phototube response, and monochromator efficiency. In certain experiments, inner filter effects were significant and fluorescence intensity measurements were corrected for such effects

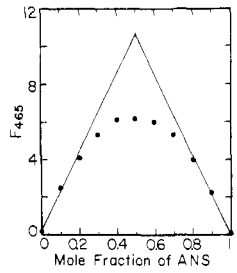

FIGURE 2: Determination of the stoichiometry of Ans binding to luciferase. The total concentration of enzyme and Ans was 8×10^{-5} M in 0.05 M phosphate (pH 7). Mole fraction of the ligand was continuously varied as indicated. An excitation light at 410 nm was used, and emission at 465 nm was measured at 23 °C.

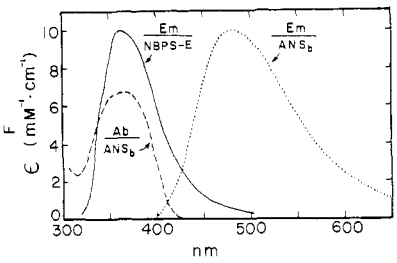

FIGURE 3: Corrected fluorescence emission spectra of NBPS-E and Ans$_b$, and absorption spectrum of Ans$_b$. The emission spectrum of NBPS attached to luciferase (—) was measured using 4 μM NBPS-E and an excitation light at 310 nm. Both absorption (- - -) and fluorescence emission (• • •) spectra of Ans$_b$ are taken from our earlier report (Tu & Hastings, 1975). All measurements were carried out at 23 °C in 0.05 M phosphate (pH 7).

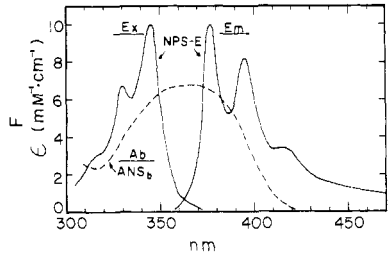

FIGURE 4: Corrected fluorescence excitation and emission spectra of NPS-E. NPS-E, at a concentration of 0.7 μM in 0.05 M phosphate (pH 7) was used for the measurements at 23 °C. Excitation and emission spectra were measured with fluorescence monitored at 386 nm for the former and using 320-nm excitation light for the latter. Absorption spectrum of Ans$_b$ (- - -) is also included to show the degrees of overlap with both excitation and emission spectra of NPS-E.

on excitation (Parker & Barnes, 1957) and/or emission (Wu et al., 1970; Tu & McCormick, 1973). The method of Azumi & McGlynn (1962) was used to calculate the values of steady-state fluorescence polarization. All spectral measurements were made at 23 ± 0.5 °C in 0.05 M phosphate (pH 7).

Fluorescence Life-time and Time-Dependent Emission Anisotropy Measurements. Nanosecond fluorescence spectroscopic measurements were made by the single photon counting technique (Lami et al., 1966), using an Ortec 9200 nanosecond fluorescence spectrometer, an Ortec 6220 multichannel analyzer interfaced to a PDP-11 digital computer, and a spark-gap lamp filled with air as the light source. The excitation light was always polarized in the y direction using a Polaroid HNP'B sheet polarizer. Another polarizer was also used on the emission side, and was set at 54.7° to the y direction for measurements of the time-dependent total emission, $S(t) = Fy(t) + 2F_x(t)$. Fluorescence lifetime measurements were analyzed by deconvolution, for both single and double exponential decays, using the method of moments (Isenberg & Dyson, 1969). For emission anisotropy measurements, the emission polarizer was used to select the x and y components of the fluorescence, $F_x(t)$ and $F_y(t)$, which are normalized as previously described (Yguerabide et al., 1970). The time-dependent emission anisotropy, $A(t)$, was determined as (Jablonski, 1962)

$$A(t) = \frac{F_y(t) - F_x(t)}{F_y(t) + 2F_x(t)} \quad (1)$$

For a single exponential depolarization process, $A(t)$ is expressed as

$$A(t) = A_0 e^{-t/\phi} \quad (2)$$

where A_0 is the limiting emission anisotropy at time zero and ϕ is the rotational correlation time.

The following combinations of filters were used for selection of excitation (Ex) and emission (Em) light: Corning 7-37 (Ex; 310–390 nm) and Kodak Wratten 47-B plus 2A (Em; 400–490 nm) for luciferase-bound Ans (in the absence or presence of FMN); Kodak Wratten 12 (Ex; 300–340 nm) and 36 (Em; 340–360 nm) for NBPS-E; and Kodak Wratten 12 (Ex) and Oriel G-774-4000 (Em; 330–500 nm) for NPS-E.

Results

Binding of Ans to Native Luciferase, NBPS-E, and NPS-E. The binding stoichiometry of Ans to luciferase was examined using the method of continuous variation (Job, 1928). The fluorescence intensities of the enzyme-bound Ans were plotted as a function of the ligand mole fraction (Figure 2). Fluorescence of free Ans was negligible. Both the position of the maximal fluorescence intensity and the intersection of the extrapolated linear portions of the curve locate at 0.5 mol fraction of Ans, confirming our earlier report (Tu & Hastings, 1975) that one Ans binds per luciferase molecule.

Ans also binds to NBPS-E and NPS-E. Samples (3.2 μM) of native luciferase, NBPS-E, or NPS-E were each titrated with Ans. Increases in Ans fluorescence due to binding were measured and analyzed as a function of total Ans concentration in the form of double-reciprocal plots. From intercepts on the abscissa, values of K_d for Ans binding to native luciferase, NBPS-E, and NPS-E were determined to be 2.3, 2.5, and 5.0 $\times 10^{-5}$ M, respectively, at neutral pH and 23 °C. Based on the value of 0.39 for the fluorescence quantum yield (Q) of luciferase-bound Ans (Ans$_b$) (Tu & Hastings, 1975), from intercepts on the ordinate, values of Q for Ans bound to NBPS-E and NPS-E were calculated to be 0.30 and 0.22, respectively. The stoichiometry of Ans binding to NBPS-E, determined by fluorimetric titration experiments previously described (Tu & Hastings, 1975), was found to remain as one site per luciferase molecule.

Absorption and Fluorescent Spectra. The absorption of Ans$_b$ overlaps closely with the emission of NBPS, whereas the latter is well resolved from the emission of Ans$_b$ (Figure 3). These spectral properties allow NBPS and Ans$_b$ to be used as the donor and the acceptor, respectively. Reasonably good spectral overlaps between donor:acceptor pairs of NPS:Ans$_b$ and Ans$_b$:FMN$_b$ are also shown in Figures 4 and 5.

Steady-State and Time-Dependent Fluorescence Properties. Fluorescence quantum yield values (Q) for NBPS and NPS attached to luciferase were calculated to be 0.05 and 0.10, respectively, from sample absorbances at the excitation wavelength and areas enclosed by the corrected emission spectra (Parker & Rees, 1960). Both quinine sulfate (in 0.1

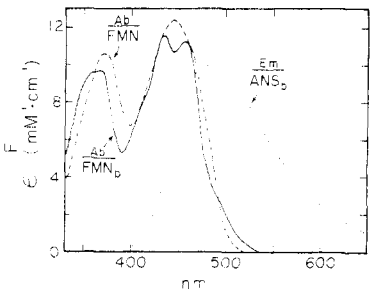

FIGURE 5: Overlap of the corrected fluorescence emission of Ans_b (• • •) with absorption spectra of free FMN (- - -) and FMN_b (—). The FMN_b absorption spectrum is taken from a report by Baldwin et al. (1975).

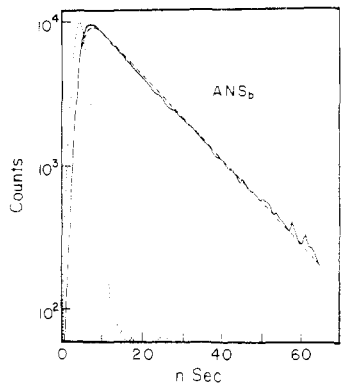

FIGURE 6: Nanosecond emission kinetics of Ans_b. The sample solution contained 1 and 4.7×10^{-5} M of luciferase and Ans, respectively, in 0.05 M phosphate (pH 7), and measurements were made at 23 °C. Traces shown are light pulse (• • •), observed Ans_b fluorescence decay (—), and calculated single exponential decay with $\tau = 14.1$ ns (- - -).

N H_2SO_4; $Q = 0.55$) and Ans_b ($Q = 0.39$) were used as standards. The steady-state polarizations (p) were determined at 23 °C in 0.05 M phosphate (pH 7) at different sucrose concentrations and analyzed using plots of $1/p$ vs. $1/\eta$ (Perrin, 1926) where η is the viscosity. The limiting polarization (p_0) is obtained by an extrapolation of $1/p$ to infinite viscosity. The values of Q, p, and p_0 for chromophores Ans_b, NBPS, and NPS, along with lifetime measurements, are summarized in Table I.

The time-dependent emission decay of both NBPS and Ans_b can be satisfactorily fitted by single exponentials with lifetimes of 1.6 and 14.1 ns, respectively. Although analysis of the latter shows, as expected (Figure 6; Gafni et al., 1977), that the fit is somewhat imperfect, the approximation is adequate for the present study. The NPS attached to luciferase exhibits two distinct lifetimes of 11.1 and 69.8 ns. Similar observations with other NPS-protein complexes have been reported (Weltman et al., 1973).

The emission anisotropy decay of both the enzyme:Ans complex and the NPS-E exhibits single exponential kinetics, which reflect the rotational relaxation of the luciferase-dye conjugate as a whole. From the slopes, the rotational correlation time, ϕ, was calculated to be 48.8 and 45.1 ns for E:ANS complex and NPS-E, respectively. Values of the limiting dynamic emission anisotropy, A_0, for Ans_b and NPS were also determined, by an extrapolation to time zero, to be 0.27 and 0.18, respectively. The NPS exhibited significantly a lower A_0 and p_0 than that of Ans_b, and also a lower p_0 than that of NBPS (Table I). This may indicate the NPS on luciferase has a higher degree of orientation freedom than the other two chromophores. However, it is not apparent why this is so.

Energy Transfer from Ans_b to FMN_b. For any given donor:acceptor pair the Förster distance, R_0, for 50% energy transfer (Förster, 1966) is described as

$$R_0 = (9.79 \times 10^3)\,(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \qquad (3)$$

where K^2 is a transition moment orientation factor, Q_D is the fluorescence quantum yield of the donor in the absence of acceptor, and n is the refractive index of the medium, taken to be 1.4 in the present work. The term J (in $cm^3\ M^{-1}$) is an overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor and can be expressed as

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda)d\lambda\ \text{cm}^3\ \text{M}^{-1} \qquad (4)$$

where $F_D(\lambda)$ is the corrected fluorescence emission of the donor, and $\epsilon_A(\lambda)$ is the molar absorptivity (in $cm^{-1}\ M^{-1}$) of the acceptor at wavelength λ (in cm). The actual distance separating the donor from the acceptor (R) can be calculated by the equation

$$R = R_0(E^{-1} - 1)^{1/6} \qquad (5)$$

The efficiency of energy transfer (E) is commonly determined either from the shortening of donor fluorescence lifetime (τ) or the quenching of donor fluorescence quantum yield (Q) in the presence of acceptor:

$$E = 1 - (\tau_{DA}/\tau_D) = 1 - (Q_{DA}/Q_D) \qquad (6)$$

where the subscripts D and DA refer to donor alone and donor in the presence of acceptor, respectively.

For the determination of energy transfer efficiency between Ans_b (donor) and FMN_b on luciferase, a sample containing 5×10^{-5} M luciferase and 6.1×10^{-5} M Ans was used for Ans_b lifetime measurements at 23 °C in the presence of zero, 2, or 3×10^{-4} M of FMN. Although free FMN was fluorescent under our experimental conditions, the amount of this flavin emission detected in the Ans_b lifetime measurements was negligible after passing through the filters used. The Ans_b exhibits an apparent single lifetime of 14.1 ns in the absence of FMN as described before. However, the total Ans_b fluorescence decay kinetics of the luciferase:Ans complex samples partially saturated with FMN showed double-exponential patterns. This is because some Ans_b will have an unchanged lifetime while others will have a shortened lifetime due to energy transfer to FMN_b. Such double exponential decay processes were resolved to obtain τ_{DA} by the method described by Langlois et al. (1976)

$$S(t) = I[(1 - F_b)e^{-t/\tau_D} + (F_b)e^{-t/\tau_{DA}}] \simeq I'e^{-t/\tau'} \qquad (7)$$

where τ_D and τ_{DA} are the same as described above, F_b is the fraction of enzyme complexed with an acceptor, and τ' is the effective lifetime obtained by a least-squares fit of $S(t)$ vs. t.

TABLE I: Fluorescence Quantum Yield, Polarization, and Lifetime Determinations of Luciferase-Bound Chromophores.

Chromophore	Q	p_0	p^a	τ_1 (ns)	τ_2 (ns)
Ans_b	0.39[b]	0.396[c]	0.319	14.1	
NBPS	0.05	0.351[d]	0.301	1.6	
NPS	0.10	0.228	0.137	11.1	69.8
				(A[e]: 0.59	0.41)

[a] Steady-state polarization measured in 0.05 M phosphate (pH 7), at 23 °C. [b] From Tu & Hastings (1975). [c] Reported by Stryer (1965). [d] Measured by Cantley & Hames (1976). [e] Relative amplitude factors for the emissions with lifetimes τ_1 and τ_2, respectively.

This method was particularly developed to determine τ_{DA} when the energy transfer efficiency is small (Langlois et al., 1976). Using this method, and known values of K_d for FMN binding to luciferase and luciferase:ANS complex (Tu & Hastings, 1975) for the calculation of F_b, the τ_{DA} was determined to be 12.3 ns, corresponding to a transfer efficiency of 0.13.

Energy Transfer from NBPS and NPS to ANS$_b$. Efficiencies of energy transfer from NBPS and NPS to Ans$_b$ were determined based on the fluorescence enhancement of Ans$_b$. For such measurements, ideally the wavelength of excitation should be where only the donor absorbs and that of the emission should be where only the acceptor emits. In practice, this may not always be achievable. For example, the absorption spectrum of Ans$_b$ overlaps considerably with that of NBPS and NPS. This problem can be resolved by using excitation at two wavelengths; both the donor and the acceptor absorb at one wavelength whereas only the acceptor absorbs at the other wavelength. The procedures used are described below using NBPS-E partially saturated with Ans as a sample. Excitation light was set at 310 (both NBPS and Ans$_b$ absorb) and 400 nm (only the Ans$_b$ absorbs), and emission was measured at 470 nm. The apparent efficiency of energy transfer can be expressed as

$$E = \frac{(F_{DA,310}{}^{470})(f_{A,400})(I_{400})}{(F_{A,400}{}^{470})(f_{D,310})(I_{310})} \quad (8)$$

where $F_{DA,310}{}^{470}$ is the enhancement of fluorescence emission, in arbitrary units, of the acceptor (Ans$_b$) at 470 nm due to energy transfer from the donor excited at 310 nm; $F_{A,400}{}^{470}$ is the fluorescence intensity (also in arbitrary units) of the acceptor at 470 nm using 400-nm excitation light; $f_{A,400}$ and $f_{D,310}$ are the fractions of light absorbed at 400 nm by the bound acceptor and at 310 nm by the donor, respectively; I_{400} and I_{310} are the actual quanta output available for sample excitation at 400 and 310 nm, respectively, from the light source after passing the monochromator. Using a sample containing NBPS-E partially saturated with Ans, $f_{D,310}$ and $f_{A,400}$ can be determined from known values of concentrations and extinction coefficients of donor and acceptor, the fraction of acceptor bound to the enzyme, and the cuvette light path. The ratio I_{400}/I_{310} can be obtained by the same procedures used for determining corrected excitation spectrum; if an instrument is equipped for direct measurements of corrected excitation spectra, the term I_{400}/I_{310} will be unity. The $F_{A,400}{}^{470}$ is determined by direct measurement, whereas

$$F_{DA,310}{}^{470} = F_{total,310}{}^{470} - F_{D,310}{}^{470} - F_{A,310}{}^{470} \quad (9)$$

in which the three terms with subscripts of total, D, and A refer to the total fluorescence observed, the remaining donor emission, and the acceptor emission due to direct excitation, respectively, all with excitation at 310 nm and emission measured at 470 nm. The term $F_{D,310}{}^{470}$ can be estimated using a NBPS-E sample in the absence of the acceptor, and $F_{A,310}{}^{470}$ can be expressed as

$$F_{A,310}{}^{470} = \frac{(F_{A,400}{}^{470})(I_{310})(f_{A,310})}{(I_{400})(f_{A,400})} \quad (10)$$

where $f_{A,310}$ has the same definition as that of $f_{A,400}$ but at a wavelength of 310 nm. The method described above provides determinations of all the parameters needed for the calculation of apparent efficiency of energy transfer. Finally, the apparent transfer efficiencies obtained with samples partially saturated with the acceptor must be normalized to actual transfer efficiency per acceptor.

Following these procedures, described above, energy transfer measurements were carried out at 23 °C, in 0.05 M phosphate (pH 7), using samples of 1.1 and 4.2×10^{-6} M NBPS-E, each containing zero, 5, 7, and 9×10^{-5} M Ans. The transfer efficiency per acceptor was calculated to be 13 ± 1%. Similar experiments were also carried out using 2.5 and 5×10^{-6} M NPS-E, each containing zero, 4.8, 7.2, and 9.6×10^{-5} M Ans. Excitation wavelengths were set at 320 (both NPS and Ans$_b$ absorb) and 400 nm (only Ans$_b$ absorbs), and emission was measured at 460 nm. The transfer efficiency per Ans$_b$ was found to be 37 ± 4%.

Discussion

The nanosecond emission anisotropy measurements of luciferase:ANS complex and NPS-E as a whole exhibit single exponential kinetics with the corresponding rotational correlation time, ϕ, of 48.8 and 45.1 ns, respectively. Based on a partial specific volume of 0.73 mL/g (Hastings et al., 1965; Meighen et al., 1971a) and assuming a spherical structure for luciferase with 0.2 mL/g hydration, the radius of the dimeric enzyme molecule and the rotational correlation times ϕ were calculated to be 30.9 Å and 28.6 ns, respectively. The latter value is much shorter than the 45 to 49 ns observed, suggesting that the luciferase–dye conjugates are not spherical. The two conjugates examined are quite different both with respect to the site and nature of dye attachment, and it seems unlikely that a native spherical luciferase is converted to an elongated form as a consequence of conjugation with NPS and Ans. Although this, as well as the formation of aggregates, was not excluded, the results can be readily accommodated by assuming that the dimeric ($\alpha\beta$) native luciferase is itself elongated, as might be expected for a dimer formed from two spherical subunits.

The efficiency of energy transfer is generally determined either based on the donor life-time shortening or on the donor emission quenching in the presence of acceptor as expressed in eq 6. However, in applying either of these two methods to systems involving a protein or other biological macromolecules, it is necessary to assume that the measured changes are not due to some change in the environment of the donor site resulting from the acceptor binding. This crucial assumption, in fact, cannot always be validated. Therefore, determinations of transfer efficiency should, whenever possible, be based on the resulting enhancement of emission by the acceptor. In the present work, we have adopted such a method in determining the transfer efficiency from NBPS and NPS to Ans$_b$. However, in the case of using Ans$_b$ as the donor and FMN$_b$ as the acceptor, the determination was based on the Ans$_b$ lifetime shortening. This is because the FMN$_b$ is nonfluorescent (Baldwin et al., 1975). Furthermore, the binding affinity of FMN to luciferase is weak. Even partial saturations of luciferase, or luciferase:Ans complex, require relatively high concentrations of FMN. Consequently, large inner filter effects were experienced and donor (Ans$_b$) fluorescence quenching due to energy transfer could not be accurately determined.

The distance between the donor and the acceptor is a function of the transfer efficiency and the Förster distance, R_0, as shown in eq 5. The calculation of R_0 involves the parameter K^2, the orientation factor for dipole–dipole interaction, which for dynamic random orientation is often assumed to be $\frac{2}{3}$, although the theoretical limits range from 0 to 4. Recently, Dale & Eisinger (1974, 1975) have described a method to estimate the upper and lower limits of K^2 involving determinations of the dynamic depolarization factor, $\langle d' \rangle_d$, of both the donor and the acceptor. If the emission transition moment vector

TABLE II: Energy Transfer Parameters.

Donor:acceptor	J^a ($10^{15} \times cm^3\ M^{-1}$)	K^2	R_0 (Å)	E/acceptor (%)	R (Å)	$R_{2/3}$ (Å)
NBPS:Ans_b	8.7	0.14–2.9	16–27	13	22–37	29
NPS:Ans_b	7.1	0.22–2.4	19–29	37	21–31	25
Ans_b:FMN_b	17.2	0.06–3.5	21–42	13	30–58	44

[a] Calculated using results shown in Figures 3, 4, and 5.

reorients rapidly and is constrained to the surface of a cone of half-angle ψ, the term $\langle d' \rangle_d$ can be related to ψ by

$$\langle d' \rangle_d = \left(\frac{3}{2}\cos^2\psi - \frac{1}{2}\right)^2 \qquad (11)$$

Once the values of ψ for both donor and acceptor are determined, the upper and lower limits of K^2 can be subsequently estimated. The value of $\langle d' \rangle$ can be best determined using dynamic emission anisotropy measurements. For Ans_b, $\langle d' \rangle_d = A_0/A_f = 0.27/0.4 = 0.68$ where A_0 is determined as described before and A_f is the fundamental emission anisotropy in rigid medium. The corresponding value of ψ is then 20°. The depolarization factors and the corresponding half-angle ψ for NBPS and NPS were either determined as described above or from steady-state emission anisotropy measurements (Dale & Eisinger, 1974, 1975) using emission polarization (p) measurements presented in Table I. Subsequently, the ranges of K^2 for donor:acceptor pairs of NBPS:Ans_b and NPS:Ans_b were obtained. The ψ value of FMN_b cannot be determined by this method because FMN_b is nonfluorescent. For the most conservative estimate of upper and lower limits of K^2 for the Ans_b:FMN_b pair, different values of ψ from 0 to 180° were assigned to FMN_b. Finally, the values of R_0 and R were calculated and are summarized along with other energy transfer parameters in Table II. For comparison, values of $R_{2/3}$, the distance calculated using $^2/_3$ for K^2, are also shown.

The findings that both NBPS-E and NPS-E still bind Ans with relatively little changes in affinity indicate that the essential sulfhydryl group and the ANS site do not occupy the same site on luciferase. This is supported by the energy transfer measurements which indicate that these two sites are at least 21 Å apart. But a modification at either site can nevertheless affect the other. The fluorescence quantum yields of ANS bound to NBPS-E and NPS-E are both lower than that of Ans bound to native luciferase, indicating that the immediate environment surrounding Ans is changed due to the sulfhydryl group modification. Conversely, the binding of Ans to luciferase apparently induces some conformational change, as evidenced by the fact that it protects the enzyme from both sulfhydryl modification by *N*-ethylmaleimide and proteolysis by trypsin.[2]

Since luciferase with the essential sulfhydryl group chemically modified still binds FMN (Nicoli et al., 1976), it is clear that this sulfhydryl group and the flavin site are also not overlapping. Measurements of efficiencies of energy transfer from NBPS-E and NPS-E to FMN_b were attempted, but could not be determined either by donor fluorescence quenching due to inner filter effects introduced by FMN or by acceptor fluorescence enhancement because FMN_b is quenched. Donor fluorescence lifetime shortening would have been a useful method, but was complicated by the fact that the τ for NBPS is too short to begin with and NPS exhibits two lifetimes. Other sulfhydryl-labeling fluorescent probes suitable for this purpose are being sought.

The ANS and FMN sites on luciferase are at least 30 Å apart. Previously, it has been shown, by separate experiments, that luciferase has a single site for FMN (Baldwin et al., 1975) and a single site for $FMNH_2$ (Meighen & Hastings, 1971; Becvar & Hastings, 1975). However, it is not absolutely certain that the FMN and $FMNH_2$ sites are indeed the same, although recent spectroscopic studies suggest that they are (Becvar et al., 1978). Irrespective of the location of the sites, the observations that (a) the Ans site is separated from the FMN site by ≥30 Å (Table II) and (b) Ans is an inhibitor competitive with $FMNH_2$ but not with FMN (Tu & Hastings, 1975) can be interpreted to mean that the $FMNH_2$ binding is much more sensitive to perturbations than the FMN binding. Consistent with this are the recent observations that luciferase is highly specific for $FMNH_2$ binding but can tolerate a wide range of flavin structural variations in complexing with oxidized flavins (Tu et al., 1977a).

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