THE BINDING AND SPECTRAL ALTERATIONS OF OXIDIZED FLAVIN MONONUCLEOTIDE BY BACTERIAL LUCIFERASE

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The binding of oxidized flavin mononucleotide (FMN) to bacterial luciferase was studied by equilibrium dialysis. A Scatchard plot of the data indicates a single FMN binding site per luciferase molecule, with a dissociation constant of 2.4×10^{-4} M at 2° in 0.05 M Bis-Tris, 0.2 M NaCl, pH 7.0. The visible absorbance spectrum of luciferase-bound FMN is altered considerably relative to the spectrum of free FMN. The spectrum of the bound flavin shows an apparent splitting of the 443-nm peak yielding well-defined maxima at 458 nm and 434 nm.

Bacterial luciferase catalyzes the oxidation of reduced flavin mononucleotide and a long-chain aliphatic aldehyde by molecular oxygen, yielding FMN, the corresponding aliphatic acid, and blue-green (λ_{max} 490 nm) light (1,2,3).

It was previously reported that binding of FMN to the luciferase could not be detected with 1.4×10^{-5} M enzyme (4). Those studies, with luciferase from Photobacterium fischeri (ATCC 7744), demonstrated that if FMN does bind, its dissociation constant must be greater than 1×10^{-4} M, while the substrate $FMNH_2$ binds approximately 1000 fold more tightly (4,5). The binding of oxidized flavin mononucleotide to bacterial luciferase was first clearly shown by Nicoli (6) who was studying the protection of a reactive sulfhydryl group in the active center from chemical modification (7). The studies reported here were undertaken to determine the dissociation constant for the complex and the number of flavin binding sites on the protein.

MATERIALS AND METHODS: FMN and Bis-Tris were products of Sigma. The purity of the FMN used in these experiments was assessed to be greater than 95% on the basis of flavin fluorescence quenching experiments performed at high protein and low FMN concentrations. All other chemicals were of reagent quality. Dialysis tubing was purchased from Fisher.

Luciferase from a strain of luminous bacteria designated MAV (8) was purified as described (9) with the addition of a second DEAE-Sephadex column step to reduce the amount of the light inducible form of luciferase (10). Molar concentrations of pure luciferase were based on a molecular weight of 79,000 and an absorptivity of 0.94 $ml \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm (9).

Absorbance spectra were taken with a Cary 15 recording spectrophotometer with both the sample and reference cells maintained at $2^{\circ} \pm 2$.

Equilibrium dialysis was performed using small volumes (about 100 µl) of protein in a thin film on the surface of a dialysis membrane stretched over the end of a small glass tube and held in place with an O-ring. The small tubes were suspended in larger tubes containing FMN and placed in an ice-water bath. The solutions were protected from light by a black cloth and stirred by small magnetic bars. In some experiments, larger volumes of protein (1.2 - 2 ml) were placed in individual bags, and dialyzed against FMN in 100 ml Erlenmeyer flasks. All solutions were prepared in 0.05 M Bis-Tris, 0.2 M NaCl, 5×10^{-4} M dithiothreitol, at pH 7.0.

RESULTS AND DISCUSSION: The visible absorbance spectra of both free and luciferase-bound flavin mononucleotide are given in Figure 1. The spectrum of the enzyme-bound flavin was measured in the presence of free flavin by placing an equal concentration of free flavin in the reference beam. These solutions were obtained by dialyzing a luciferase sample against the pH 7.0 buffer containing flavin so that, at equilibrium, the free flavin concentration should be the same on both sides of the membrane. The material inside the bag was then placed in compartment A (sample cell) and that outside the bag in compartment C (reference cell). A second luciferase sample was dialyzed against the pH 7.0 buffer without flavin, the material inside the bag being placed in compartment D and that outside in compartment B, thereby compensating for spectral contributions from protein alone. The observed spectrum is therefore that of the enzyme-bound flavin.

To determine the isosbestic point, the spectrum of concentrated luciferase containing 7×10^{-5} M FMN was first measured versus the apoenzyme. The luciferase with FMN was then diluted with buffer containing an equivalent total FMN concentration and the spectrum

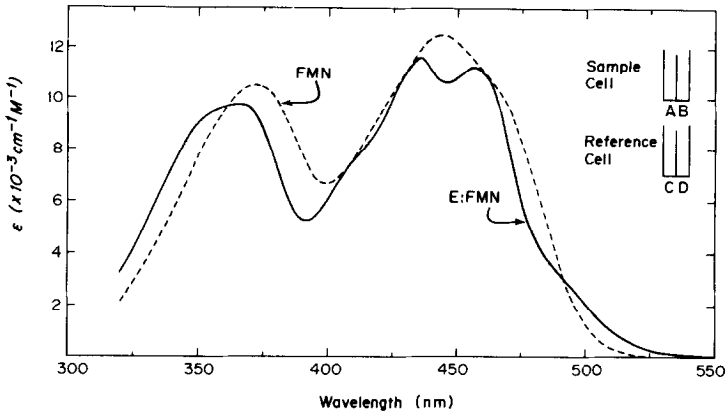

Figure 1. Visible absorbance spectra of free (---) and bacterial luciferase-bound (——) flavin mononucleotide were recorded at 2° with a Cary 15 spectrophotometer. Samples were prepared by equilibrium dialysis. Compartment A contained luciferase with flavin, compartment B contained buffer, compartment C contained luciferase without flavin, and compartment D contained the flavin-buffer solution against which the protein in compartment A had been dialyzed.

determined versus the reference sample of apoenzyme diluted in an equivalent fashion with buffer. Each dilution decreased the concentration of enzyme-bound flavin while maintaining a constant total flavin concentration, thereby revealing an isosbestic point at 492 nm. The spectrum of luciferase-bound flavin was then normalized to the 492 nm absorptivity of free FMN to determine the molar absorptivity of luciferase-bound FMN at all wavelengths.

A second isosbestic point may occur at 463 nm, but as the spectra do not cross, but approach tangentially, there may be a very slight decrease in the absorptivity at this wavelength upon binding to the protein. Nevertheless, the molar absorptivity of bound FMN at 463 nm is essentially the same as that of the free flavin, and is therefore a convenient wavelength for measurement of total flavin concentration.

Equilibrium dialysis experiments were carried out to elucidate the number of FMN binding sites on bacterial luciferase (Figure 2). Enzyme concentrations were varied from about 1×10^{-4} M to 6×10^{-4} M and FMN from about 2.5×10^{-5} M to 2.5×10^{-3} M in order to maintain

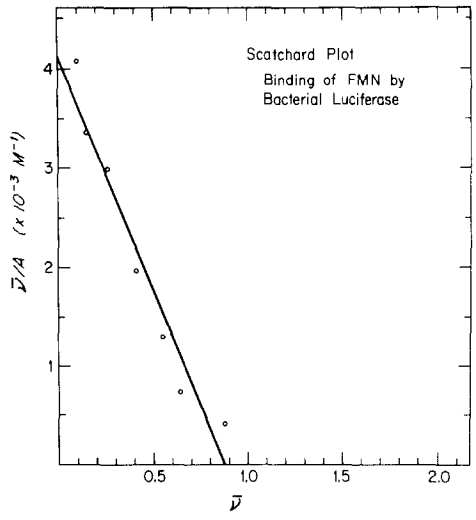

Figure 2. A Scatchard plot of binding of FMN to bacterial luciferase. The $\bar{\nu}/A$ intercept gives a dissociation constant for the FMN-luciferase complex of 2.4×10^{-4} M; the $\bar{\nu}$ intercept gives a value of 0.88 for the average number of moles of FMN bound per 79,000 gm luciferase.

the ratio of bound:free FMN near unity. Free flavin concentrations were measured from the absorbance of the solution at 450 nm assuming a molar absorptivity of 12,200 $M^{-1} \cdot cm^{-1}$ (11). Bound flavin concentrations were determined as the difference between the flavin concentration in the presence of the protein and the free flavin concentration. The protein concentrations in the presence of flavin were determined by the Lowry procedure (12) using pure luciferase as a standard. The data, plotted by the Scatchard procedure (13), intersect the $\bar{\nu}$ axis at 0.88, indicative of a single binding site for oxidized flavin mononucleotide; the $\bar{\nu}/A$ intercept of this plot gives a dissociation constant of 2.4×10^{-4} M.

Meighen and Hastings (5), using a kinetic technique, reported a single binding site for reduced flavin mononucleotide for luciferase from two different strains of luminous bacteria. The dissociation constant for enzyme-bound $FMNH_2$ was 9.7×10^{-7} M for P. fischeri luciferase and 8.0×10^{-7} M for MAV luciferase. The experiments reported here show the existence of a single binding site on the protein for oxidized FMN with a dissociation constant of 2.4×10^{-4} M.

Earlier studies, in which the lower limit for the dissociation constant of FMN to bacterial luciferase was estimated to be 1×10^{-4} M, utilized the technique of fluorescence polarization and the enzyme from P. fischeri (4). I have reinvestigated the binding of FMN to the P. fischeri luciferase, and find that indeed, it binds FMN more weakly than does the MAV enzyme: the dissociation constant is between 5×10^{-4} and 5×10^{-3} M. Also, the change in the spectrum of flavin bound to the P. fischeri enzyme does not appear to be so great. At enzyme concentrations of about 5×10^{-4} M with 7×10^{-5} M FMN, no shift was observed in the absorbance spectrum of the flavin, though the fluorescence of the flavin solution was decreased (Baldwin et al., manuscript in preparation) by the P. fischeri luciferase, indicative of binding.

The sharpness of the structure in the absorbance spectrum of luciferase-bound FMN is of significance. Structure develops in the spectra of flavins in non-polar solvents (14,15), and it is known that the active center of bacterial luciferase is hydrophobic (16). Binding of flavins to flavoproteins often results in significant structure development (17). With luciferase, the splitting of the peak and the narrowing of the absorbance bands indicate a restrictive structural interaction of the flavin with the protein. The rotational degrees of freedom of the flavin are probably decreased substantially on binding, resulting in more distinct expression of vibrational and electronic transitions.

Though the failure of luciferase from P. fischeri to substantially alter the spectrum of FMN may be due to weak binding, there is evidence that this enzyme differs considerably in numerous other physico-chemical and functional properties from the luciferase from strain MAV (8). If the structures of the active centers of luciferases from various luminous bacteria are conservative to evolutionary change, as has been observed for numerous other proteins (18), one must conclude that the observed structure in the absorbance spectrum of FMN bound to MAV

luciferase must be due to interaction with a small number of amino acids which are different in the P. fischeri enzyme. Studies are therefore in progress to determine the spectral properties of FMN bound to mutant MAV luciferases whose lesions result in altered catalytic properties and presumably are caused by amino acid changes in the active center (19).

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