Acknowledgments

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Hydrophobic Nature of the Active Site of Firefly Luciferase*

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ABSTRACT: Anilinonaphthalenesulfonates and toluidinonaphthalenesulfonates bind to firefly luciferase with an enhancement of fluorescence. Approximately 2 moles of dye is bound per mole of enzyme. The affinity of 2,6-toluidinonaphthalenesulfonate for the enzyme is much greater than 1,5-anilinonaphthalenesulfonate or the corresponding isomer 2,6-anilinonaphthalenesulfonate. Both 2,6-toluidinonaphthalenesulfonate and 1,5-anilinonaphthalenesulfonate are competitive inhibitors of luciferin. The addition of 2 moles of dehydroluciferyl adenylate/mole of enzyme completely removes the bound dye from the enzyme. Inhibition of the enzymatic activity by reaction of the two essential sulfhydry groups with *N*-ethylmaleimide decreases the affinity of the enzyme for 2,6-toluidinonaphthalenesulfonate but does not alter the number of dye molecules bound. The binding of 2,6-toluidinonaphthalenesulfonate is independent of pH between 6 and 9. The increase in fluorescent intensity and the shift in the emission maximum of the bound dye are indicative of hydrophobic binding sites on the enzyme.

The data support the conclusion that the dyes bind at the normal luciferin binding sites, thus inhibiting catalytic activity.

The use of dyes as probes for hydrophobic sites on proteins is now well documented (Weber and Young, 1964; Stryer, 1965; McClure and Edelman, 1966, 1967; Brand *et al.*, 1967). The interaction between dye and protein may be followed by the increase of fluorescence observed when the dye is bound in a hydrophobic environment. In the case of firefly luciferase the dyes used are competitive inhibitors of the enzyme indicating a specific interaction where either the dye or the substrate may be bound at the same hydrophobic site. These studies with luciferase as well as similar findings with other enzymes (Brand *et al.*, 1967; Stryer, 1965; Hymes *et al.*, 1965) suggest the role of such hydrophobic sites may be to dissolve the substrate in a specific region of the protein thus making it available in high concentration at the active site where catalysis takes place.

Methods

Five-times-crystallized firefly luciferase was prepared as described by Green and McElroy (1956). The enzyme was stored at 0° as a concentrated solution (10–20 mg of protein/ml) in 10% $(NH_4)_2SO_4$. This stock solution was diluted into 0.025 M glycylglycine (pH 7.5) just prior to use. Protein concentration was measured by

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absorbance at 278 mμ. One milligram of luciferase per milliliter in a 1-cm cell gives an optical density of 0.75. Enzymatic activity was assayed by measuring the intensity of the initial flash of light emitted upon injection of ATP in the presence of excess LH_2[1] (McElroy and Seliger, 1961). All assays were performed in triplicate.

Crystalline D-(−)-luciferin and dehydroluciferin were synthesized according to White *et al.* (1961). Dehydroluciferyl adenylate was synthesized as described by Rhodes and McElroy (1958) and purified by elution from a Sephadex G-25 column. The concentration was determined from the absorbance at 353 mμ using a molar extinction coefficient of 1.5×10^4 (R. Morton, 1967, personal communication). 1,5-Anilinonaphthalenesulfonate, 2,6-anilinonaphthalenesulfonate, and 2,6-toluidinonaphthalenesulfonate were synthesized by D. Turner in this department. The concentration of 2,6-toluidinonaphthalenesulfonate in aqueous buffer was determined by absorbance at 366 mμ using a molar extinction coefficient of 4.08×10^3 (McClure and Edelman, 1966). 2,6-Anilinonaphthalenesulfonate and 1,5-anilinonaphthalenesulfonate have molar extinction coefficients of 6.56×10^3 and 6.04×10^3, respectively, at 350 mμ (D. Turner, 1968, personal communication).

The corrected emission spectra for the bound dye was obtained with the spectrophotofluorometer constructed at the Johns Hopkins University (Brand *et al.* 1967; Witholt and Brand, 1968). The absorption spectra were measured on a Cary Model 14 recording spectrophotometer.

The absolute quantum yield was calculated according to the method of Parker and Rees (1960). Quinine sulfate in 1 N H_2SO_4 was used as a reference with an assumed quantum yield of 0.55 (Melhuish, 1961).

Routine dye titrations were done with the Turner fluorometer, Model III, using a primary filter Corning No. 7-60, and an exit filter Turner No. 2A-12. The optical density of the final titration mixture was less than 0.1 at the exciting wavelength. The titrations were carried out in 0.025 M glycylglycine (pH 7.5) at 24°.

The stoichiometry and equilibrium constant of the dye–enzyme complex were obtained from experiments in which aliquots of the dye were titrated into samples of different concentrations of enzyme. X, the fraction of dye bound to the protein, was obtained from eq 1 (Laurence, 1952).

$$X = (F_{obsd} - F_{free})/(F_{bound} - F_{free}) \qquad (1)$$

F_{free} and F_{bound} are the fluorescent intensities of the free and completely bound dye, respectively. F_{obsd} is the fluorescence obtained upon adding various amounts of the dye during the titration.

$\bar{v}$, the average number of dye molecules bound, is calculated from eq 2

$$\bar{v} = X(A_t)/(P_t) \qquad (2)$$

[1] Abbreviations used in this paper are: LH_2, luciferin; L, dehydroluciferin; L-AMP, dehydroluciferyl adenylate; E·L-AMP, enzyme-bound dehydroluciferyl adenylate.

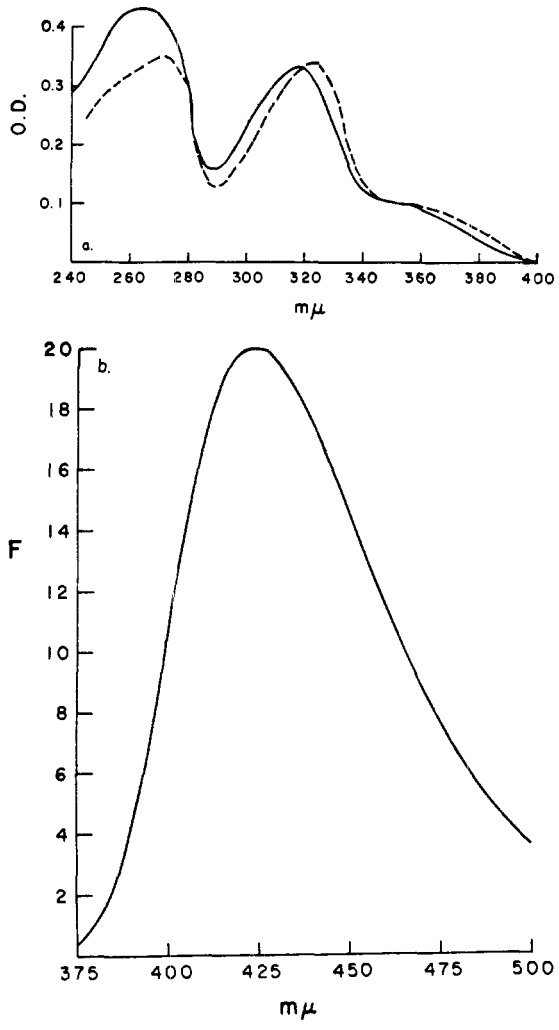


FIGURE 1: Spectral studies. (a) Absorption spectra of 2,6-toluidinonaphthalenesulfonate in 0.025 M glycylglycine (pH 7.5) (——). Difference spectrum of 2,6-toluidinonaphthalenesulfonate and luciferase against luciferase (---). (b) Fluorescence emission spectrum of bound 2,6-toluidinonaphthalenesulfonate. The fluorescence of the free dye under these conditions is negligible. The spectrum is corrected.

where A_t = total dye concentration and P_t = total protein concentration. The data are plotted according to the method of Scatchard (1949).

Results

A large enhancement of fluorescence accompanies the binding of either 1,5-anilinonaphthalenesulfonate or 2,6-toluidinonaphthalenesulfonate to firefly luciferase. The emission maximum shifts to shorter wavelengths when the dyes are bound to the enzyme. 1,5-Anilinonaphthalenesulfonate in buffer has an emission maximum at 525 mμ while the enzyme-bound dye emission has a maximum at 467 mμ. The maximum for 2,6-toluidinonaphthalenesulfonate shifts from 500 mμ in buffer to 425 mμ when it is bound to the enzyme. 2,6-Toluidinonaphthalenesulfonate has a quantum yield of 0.0008 in

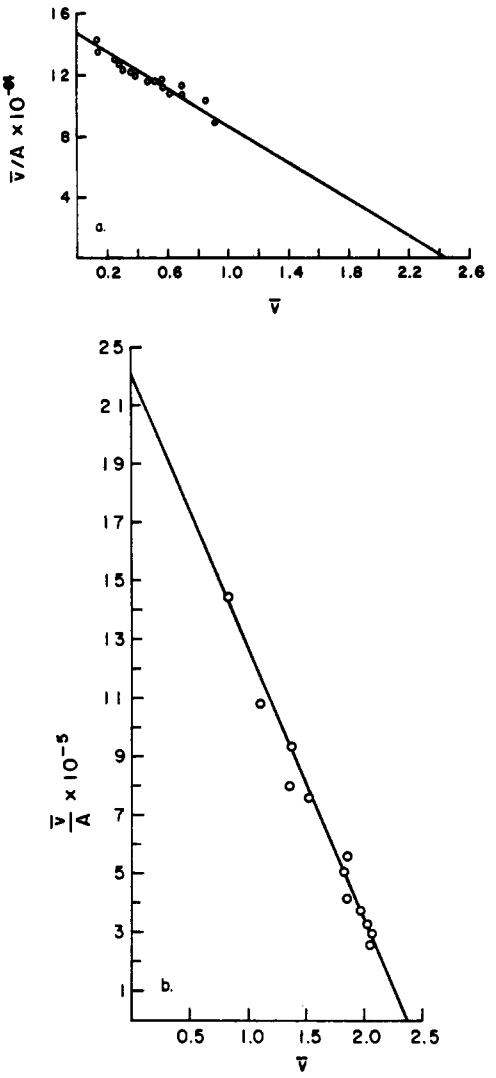


FIGURE 2: Data obtained from representative titrations of luciferase with 1,5-anilinonaphthalenesulfonate (a) or 2,6-toluidinonaphthalenesulfonate (b) plotted according to Scatchard. With 1,5-anilinonaphthalenesulfonate n is 2.4 and $K_A = 6 \times 10^4$ M^{-1}. With 2,6-toluidinonaphthalenesulfonate n is 2.4 and $K_A = 9.5 \times 10^5$ M^{-1}. For the titrations enzyme concentrations were varied from 6×10^{-6} to 9×10^{-6} M. The dye was added in 20-μl aliquots to a final concentration of 9×10^{-6} M. All titrations were carried out in 0.025 M glycylglycine buffer at pH 7.5 and at 25°.

water (McClure and Edelman, 1966). When bound to luciferase the quantum yield is 0.71, about 1000 times greater.

The absorption spectrum of 2,6-toluidinonaphthalenesulfonate also shows a marked change upon binding to the enzyme. The band at 263-mμ shifts to 272 mμ with a decrease in extinction. There is also a shift in the 317-mμ peak to 324 mμ. The absorption spectra of free and bound toluidinonaphthalenesulfonate and the emission spectra of bound toluidinonaphthalenesulfonate are shown in Figure 1a,b.

Figure 2a shows a representative experiment for the binding of 1,5-anilinonaphthalenesulfonate, 2b, the data for 2,6-toluidinonaphthalenesulfonate. The enzyme binds approximately 2.4 moles of anilinonaphthalenesulfonates/mole of enzyme with a K_A of 5–7 $\times$ 10^4 M^{-1}. Because of the relatively low binding constant it was difficult to obtain points at high $\bar{\nu}$. The direct determination of F_{bound} was also questionable since this required large amounts of protein. F_{bound} was therefore estimated by reciprocal plots of fluorescence *vs.* protein concentration (Weber and Young, 1964).

The data for binding of 2,6-toluidinonaphthalenesulfonate still showed some scatter but were more consistent. Four titrations such as that shown in Figure 2b gave n = 2.0–2.5 with $K_A = 9.5 \times 10^5$ M^{-1}. The fact that the data are represented by a linear Scatchard plot indicates the binding of the dye molecules are equivalent and independent over the concentration range studied.

Some preliminary binding experiments were carried out with 2,6-anilinonaphthalenesulfonate and it was found the enzyme binds approximately 2 moles of this dye with a $K_A = 6 \times 10^4$ M^{-1} which is similar to that for 1,5-anilinonaphthalenesulfonate.

Inhibition Experiments. In order to determine if the binding of these dyes occurs at the "active site" of luciferase the enzymatic activity was measured in the presence of several concentrations of 1,5-anilinonaphthalenesulfonate and 2,6-toluidinonaphthalenesulfonate. Figure 3 shows the data from kinetic studies with anilinonaphthalenesulfonate. The anilinonaphthalenesulfonate is a competitive inhibitor of luciferin with a K_i of 2.0–3.5 $\times$ 10^{-4} M. It is noncompetitive with respect to ATP.

Similar experiments were carried out with 2,6-anilinonaphthalenesulfonate. Toluidinonaphthalenesulfonate is also a competitive inhibitor of luciferin with a K_i of 5.9 $\times$ 10^{-6} M. The inhibition with respect to ATP is not either simply competitive or noncompetitive.

It is not possible to determine the effect of luciferin on the binding of the dyes fluorimetrically since the fluorescence of luciferin interferes with such measurements. It was possible to determine the effect of synthetic L-AMP on the binding of 2,6-toluidinonaphthalene. When the adenylate of L is made, the fluorescent intensity of the L-AMP is only about 2% that of free L (Rhodes and McElroy, 1958) and thus is negligible with respect to the fluorescence of the bound toluidinonaphthalenesulfonate. The enzyme was mixed with an excess of 2,6-toluidinonaphthalenesulfonate which resulted in an enhancement of fluorescence. Aliquots of L-AMP were then added which resulted in an immediate decrease of fluorescence presumably due to the release of the bound dye. Complete dissociation of the dye required a twofold molar excess of L-AMP over the enzyme. These results are shown in Figure 4. There was no effect of the L-AMP on the fluorescence of the free dye. If L-AMP was added to the enzyme before the dye, there was no increase in fluorescence, indicating complete inhibition of dye binding.

The observed decrease in fluorescence of bound dye in the presence of L-AMP can be interpreted in several ways. The L-AMP could displace the dye from the enzyme, L-AMP might bring about a conformational change of the enzyme which quenches the fluorescence of bound dye, or the L-AMP binding near the dye

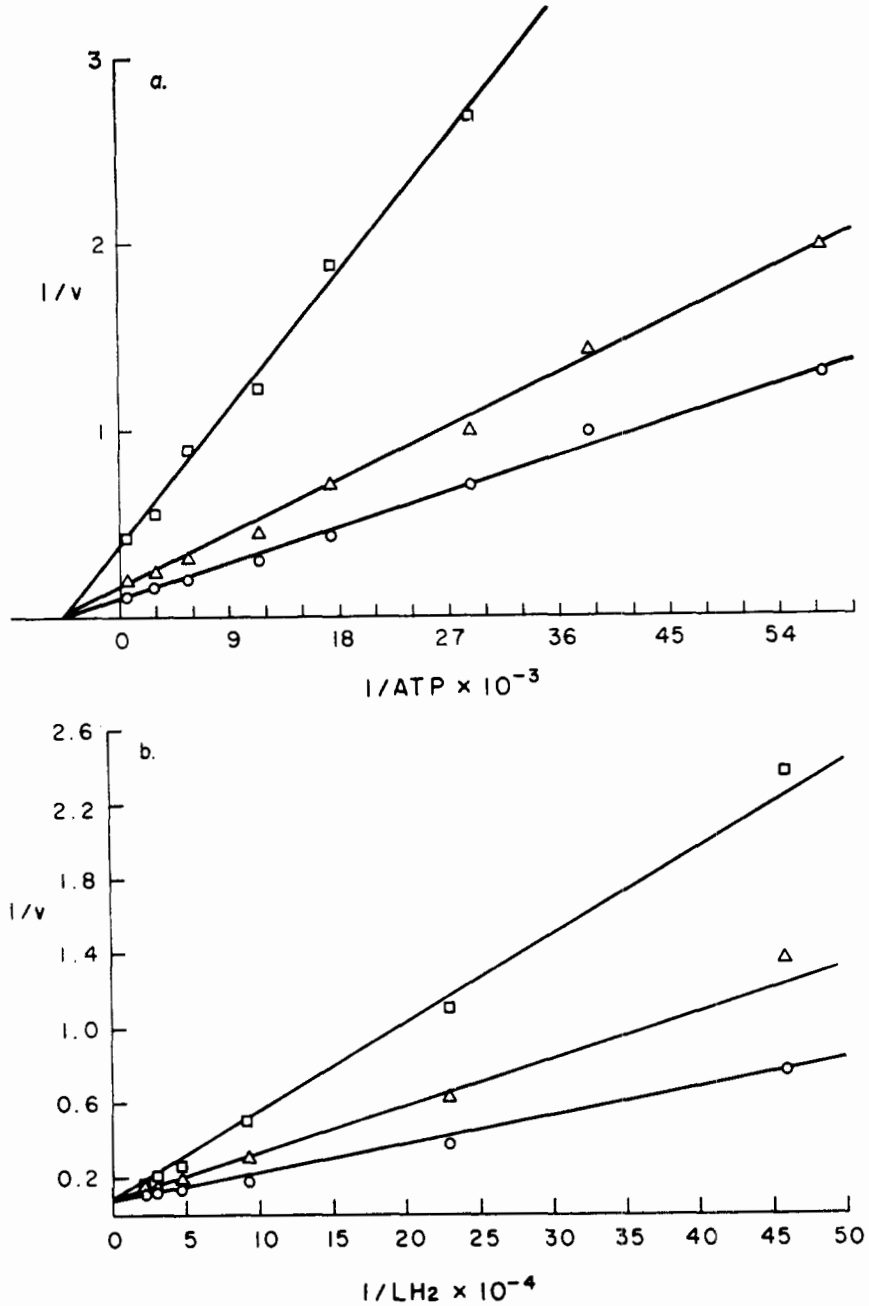


FIGURE 3: Inhibition of luciferase by 1,5-anilinonaphthalenesulfonate. (a) Inhibition as a function of ATP concentration. (b) Inhibition as a function of LH_2 concentration. (a) The reaction mixture contained 1×10^{-7} M enzyme in 0.025 M glycylglycine buffer (pH 7.5), 7×10^{-5} M LH_2, and varying amounts of an ATP plus $MgCl_2$ mixture so that $MgCl_2$ was always present in a two times molar excess over ATP. (○—○) No inhibitor: (△—△) 1.11×10^{-4} M anilinonaphthalenesulfonate; (□—□) 4.17×10^{-4} M anilinonaphthalenesulfonate. (b) The reaction mixture contained 1×10^{-7} M enzyme, 2×10^{-3} M ATP, and 4×10^{-3} M $MgCl_2$ in 0.025 M glycylglycine (pH 7.5). The LH_2 concentration was varied as shown. (○—○) No anilinonaphthalenesulfonate; (△—△) 1.53×10^{-4} M anilinonaphthalenesulfonate; (□—□) 2.88×10^{-4} M anilinosulfonelesulfonate. All assays were done in triplicate and an average value was reported.

might quench the fluorescence directly. To determine which of these interpretations is correct, absorption difference spectra were obtained with E·L-AMP and toluidinonaphthalenesulfonate against E·L-AMP.

The binding of toluidinonaphthalenesulfonate to the enzyme results in shifts of the 263- and 272-mμ bands to longer wavelengths. In the presence of L-AMP the absorption spectrum of the dye and enzyme mixture returns to that of free dye. It was also shown that when dye was added to E·L-AMP the resulting spectrum was identical with that of the free dye. These experiments are consistent with the interpretation that L-AMP is quantitatively removing the dye from the enzyme.

Due to the instability of L-AMP at neutral pH and

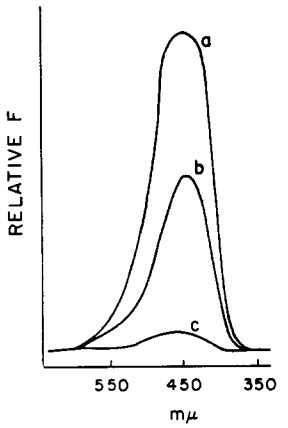


FIGURE 4: Fluorescence emission of toluidinonaphthalenesulfonate–luciferase mixtures in the presence and absence of L-AMP. (a) The reaction mixture contained, in 2 ml of 0.025 M glycylglycine buffer (pH 7.5), 1.8×10^{-6} M enzyme and 2.5×10^{-5} M 2,6-toluidinonaphthalenesulfonate. (b) 1.75×10^{-6} M L-AMP was added. (c) An additional aliquot of L-AMP was added to bring the final concentration to 3.5×10^{-6} M. Activation was at 350 mμ. Spectra were obtained on the Aminco-Bowman spectrophotofluorometer and are uncorrected.

the instability of luciferase in dilute solutions of low salt, it was not possible to do equilibrium dialysis experiments.

Effect of SH Reagents. It has been shown (DeLuca *et al.*, 1964) that luciferase requires two of the eight SH groups for enzymatic activity and L and ATP protect these two SH groups from reaction with SH reagents. Since the dyes are presumably binding at or near the substrate binding site it might be possible to prevent the binding by treating these sulfhydryls with *p*-mercuribenzoate.

Luciferase was incubated with an excess of *p*-mercuribenzoate until all enzymatic activity was lost. The inactive enzyme still bound the same amount of 1,5-anilinonaphthalenesulfonate as the native enzyme. If luciferase is incubated with a twofold molar excess of *N*-ethylmaleimide for several hours, 90% of the enzymatic activity is lost. Figure 5 shows the effect of *N*-ethylmaleimide on the binding of 1,5-anilinonaphthalenesulfonate and 2,6-toluidinonaphthalenesulfonate. The inhibited enzyme shows an apparent decreased ability to bind toluidinonaphthalenesulfonate while the anilinonaphthalenesulfonate binding is unaffected.

Figure 6 shows a Scatchard plot of the binding of 2,6-toluidinonaphthalenesulfonate to the *N*-ethylmaleimide-treated enzyme. The number of dye molecules bound remains the same but the K_A is 2.5×10^5 M^{-1} while that of the native enzyme was 9×10^5 M^{-1}. The effect of *N*-ethylmaleimide is to decrease the affinity of the dye for the protein.

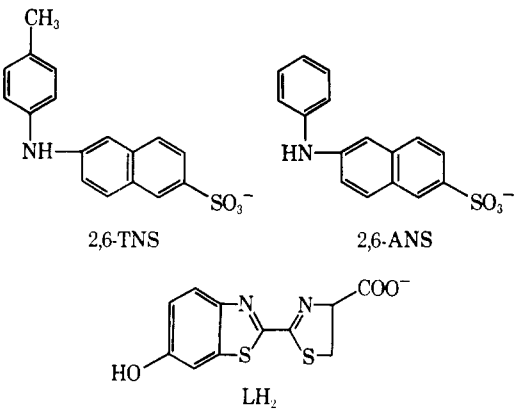
If the sulfhydryls are in the vicinity of the dye binding site, there might be an effect of pH on binding as one goes through the p*K* range of these groups. Titrations were carried out over the pH range of 6–9. There was no change of *n* or K_A at any of these pH values.

The isoleucyl-tRNA ligase is similar to luciferase in that it catalyzes the formation of enzyme-bound isoleucyl adenylate. It seemed possible that the binding sites for adenylates might be similar for many enzymes (McElroy *et al.*, 1967). However, all attempts to demonstrate binding to this enzyme of various anilinonaphthalenesulfonate isomers or toluidinonaphthalenesulfonate were completely negative.

Discussion

The binding of dyes to luciferase has been used in an attempt to obtain more information about the nature of the "active site" of this enzyme. The increase in fluoresence intensity and blue shift of the emission of the bound dye are similar to that observed with other proteins, and may be interpreted in terms of a hydrophobic binding site on the enzyme.

A surprising finding is that 2,6-toluidinonaphthalenesulfonate binds much better than 2,6-anilinonaphthalenesulfonate. The only difference in the structure of these dyes is a methyl group, yet the K_A for toluidinonaphthalenesulfonate is tenfold greater than that for anilinonaphthalenesulfonate. It is not obvious why such a small change in the dye should result in such an increase in affinity for the protein.



Calculation of the ΔF of binding of the dyes from the equilibrium constants shows for 1,5-anilinonaphthalenesulfonate $\Delta F = -6.3$ kcal/mole, while for 2,6-toluidinonaphthalenesulfonate $\Delta F = -8.2$ kcal/mole. Therefore the addition of a methyl group to anilinonaphthalenesulfonate results in a change of ΔF of binding of 1.9 kcal/mole. Data taken from Hymes *et al.* (1965) on the complex formation of α-chymotrypsin with benzene or toluene show a change of only 0.8 kcal/mole upon the addition of a methyl group. This is also true for the ΔF of solution of benzene and toluene.

Therefore the much larger difference in ΔF observed for binding of 1,5-anilinonaphthalenesulfonate and 2,6-toluidinonaphthalenesulfonate to luciferase cannot be attributed entirely to the increased hydrophobic character of the molecule.

The position of substituents on the naphthalene ring does not seem important for binding since all of the anilinonaphthalenesulfonate isomers tested have similar binding constants.

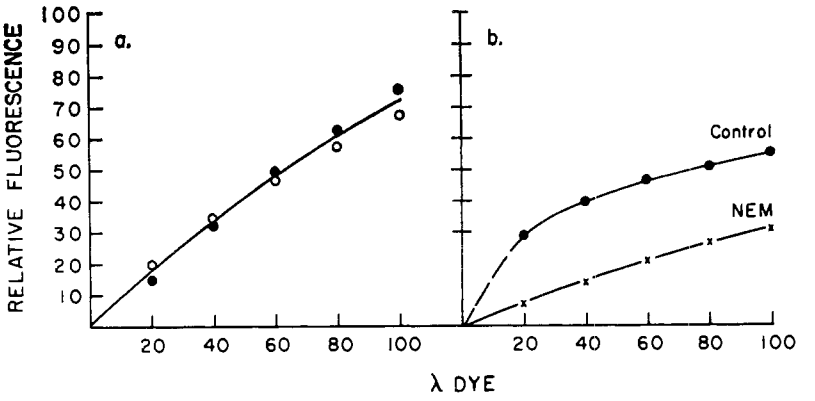


FIGURE 5: Titration of native luciferase and *N*-ethylmaleimide-inhibited luciferase with (a) 1,5-anilinonaphthalenesulfonate and (b) 2,6-toluidinonaphthalenesulfonate. Enzyme is 1×10^{-7} M in all samples. Toluidinonaphthalenesulfonate concentration from 0 to 1.4×10^{-6} M; anilinonaphthalenesulfonate concentration was varied from 0 to 10^{-4} M. Reaction was carried out in 0.025 M glycylglycine at pH 7.5.

Another interesting observation is that luciferase binds approximately 2 moles of dye/mole of enzyme. Previous experiments have indicated only one catalytic site on luciferase (Rhodes and McElroy, 1958). However, recent experiments suggest there may be two sites for light production on each enzyme (Hopkins, 1968). This is consistent with the dye binding stoichiometry and with the fact that 2 moles of L-AMP are required to remove all of the bound dye. Since the K_D for L-AMP is 1×10^{-9} M, if two molecules of dye were bound at a single "active site" then 1 mole of L-AMP should completely remove the dye. The observation that two sulfhydryls are essential for catalytic activity, and these appear in identical peptides (Travis and McElroy, 1966), is also consistent with two active sites per mole.

The interpretation of the experiments with the *N*-ethylmaleimide-inhibited enzyme is somewhat ambiguous. Since the 2,6-toluidinonaphthalenesulfonate is competitive with respect to LH_2, it is tempting to speculate that the dye is binding at or near the normal substrate binding sites. However, we know that formation of L-AMP on the enzyme completely protects these two sulfhydryls groups. It seemed reasonable to expect the *N*-ethylmaleimide-inhibited enzyme to lose its dye binding capacity along with catalytic activity. This was not found to be true. In the case of anilinonaphthalenesulfonate there was no difference in binding between native and *N*-ethylmaleimide-inhibited enzyme. The inhibited enzyme did show a decreased affinity for toluidinonaphthalenesulfonate but 2 moles was still bound per mole of enzyme.

One possible explanation is that the sulfhydryls are brought into the active site region only when the L-AMP is formed on the enzyme. This is consistent with the large conformational changes that accompany formation of L-AMP (DeLuca and Marsh, 1967). If this is true, then the *N*-ethylmaleimide enzyme should be able to bind L and ATP even though no catalytic activity remains.

The complete lack of pH effect on binding over the range 6–9 indicates that the groups which ionize in this region, imidazole, lysine, SH, must not affect the binding site for the dye. There is a large change of enzymatic activity in this pH range with an optimum pH of 7.8. Functional groups essential for catalysis, however, appear to have no effect on binding of the dye.

The binding of the substrates in a hydrophobic region is consistent with the observation that L-AMP, when bound to the enzyme, is much less reactive to solvent or to nucleophiles in the solvent than when it is free. Other experiments comparing the solvent dependence of the chemiluminescent emission of luciferyl adenylate support the conclusion that the environment of the excited product, bound to the enzyme, is nonpolar (Hopkins, 1968).

Hymes *et al.* (1965) and Wildnauer and Canady (1966) have developed a model for enzyme–inhibitor complexes with α-chymotrypsin and various hydrophobic molecules. They have suggested with this enzyme almost any substance with considerable hydrocarbon character would be expected to be a competitive inhibitor. The advantage to the enzyme in carrying out

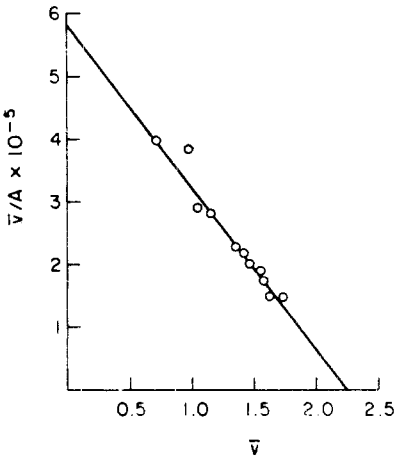


FIGURE 6: Data obtained from titration of *N*-ethylmaleimide-inhibited luciferase with 2,6-toluidinenaphthalenesulfonate, $n = 2.2$, $K_A = 2.58 \times 10^5$ M^{-1}. Enzyme concentration was varied from 6×10^{-7} to 3×10^{-5} M. Toluidinonaphthalenesulfonate was added in 20-μl aliquots to a final concentration of 1.5×10^{-5} M. All titrations were carried out in 0.025 M glycylglycine (pH 7.5).

catalysis in a hydrophobic environment will depend upon the relative contribution of lowered dielectric constant which results in increased attraction of charges and the loss of solvation effects on intermediate complexes. The rate of polar reactions is generally very susceptible to changes in the ion-solvating ability of the medium.

It is possible that the ability of the enzyme to dissolve the substrates in such hydrophobic regions, thus effecting an increased local concentration of substrates at the catalytic site, may be an important factor in enzyme catalyses. Further experiments with the sulfonyl chloride of toluidinonaphthalenesulfonate will be carried out in an attempt to obtain a covalently bound enzyme–dye complex. A study of the properties of such a complex will give more information on the nature of the binding sites.

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