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Fluorescent Probes for Conformational States of Proteins. II. The Binding of 2-*p*-Toluidinylnaphthalene-6-sulfonate to α-Chymotrypsin*

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ABSTRACT: 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) fluoresces strongly when bound to hydrophobic regions of proteins, although alone it is virtually nonfluorescent in aqueous solution. TNS was used as a hydrophobic probe to study the conformation of chymotrypsin in the presence and absence of competitive inhibitors. Chymotrypsin and chymotrypsinogen each bind TNS with similar dissociation constants (2–5 × 10^{-4} M). The binding of substrate analogs to chymotrypsin inhibits the fluorescence of the TNS–chymotrypsin system without altering the apparent dissociation constant of the TNS–chymotrypsin complex. Similarly, the binding of TNS noncompetitively inhibits the hydrolysis of acetyl-L-tyrosine ethyl ester by chymotrypsin. These findings indicate that there is a hydrophobic binding site in chymotrypsin which is not part of the active site of the enzyme. Studies of the binding of TNS by chymotrypsin as a function of pH showed a peak of fluorescence intensity at pH 7.8. If chymotrypsin is replaced by either of the enzymatically inactive species, chymotrypsinogen or phenylmethanesulfonylchymotrypsin, this peak is no longer seen. This suggests that the binding of substrate analogs or protons to chymotrypsin may alter the enzymatic conformation.

Certain derivatives of aminonaphthalenesulfonic acids do not fluoresce in water, although they have relatively high quantum yields of visible fluorescence when dissolved in organic solvents or aqueous solutions of various proteins (Weber and Laurence, 1954). The mechanism of fluorescence of one of these compounds (TNS)[1] is described in the first communication of this series (McClure and Edelman, 1966). TNS fluorescence was enhanced by solvents of low dielectric constant or, to a lesser extent, by solvents of high viscosity.

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[1] Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ANS, anilinonaphthalenesulfonate; ATEE, acetyl-L-tyrosine ethyl ester.

It was proposed that the TNS fluorescence observed in protein solutions resulted from binding of the fluorophore to hydrophobic sites upon the protein surface. This interpretation is supported by several other lines of evidence. For example, ANS is bound firmly in the hydrophobic heme crevice of myoglobin (Stryer, 1965). Many proteins enhance ANS fluorescence with increased efficiency after hydrophobic amino acid side chains are exposed by thermal denaturation (Gally and Edelman, 1965).

These findings suggest that TNS, and structurally related compounds, could be used as probes of conformational changes in proteins. In order to test this hypothesis, we have examined the activation of chymotrypsinogen by following the variation in fluorescence of TNS bound to the zymogen (McClure and Edelman, 1967). Changes in TNS fluorescence were shown to be an accurate measure of the activation process. During these investigations, it became necessary to examine the TNS binding sites of chymotrypsin and chymotrypsinogen. In the present paper, we provide evidence for the existence of a site in chymotrypsin that has a high affinity for TNS. This site is not part of the enzyme active site, although binding of substrate analogs to the active site of chymotrypsin is accompanied by large changes in TNS fluorescence. We propose that the changes in TNS fluorescence result from conformational changes in the protein induced by the binding of substrate analogs.

Materials and Methods

The following commercially available compounds were used without further purification: β-phenylpropionic acid, Matheson Coleman and Bell, East Rutherford, N. J.; L-tryptophan, Eastman Organic Chemicals, Rochester, N. Y.; D-tryptophan, California Corp. for Biochemical Research, Los Angeles, Calif.; and acetyl-L-tyrosine ethyl ester and acetyl-L-tryptophan, Mann Biochemical Corp., New York, N. Y. TNS was synthesized according to McClure and Edelman (1966). *n*-Butyl alcohol and *n*-butylamine were obtained from Eastman Organic Chemicals (Rochester, N. Y.) and were redistilled before use. The purity of these two solvents was checked both by boiling range and refractive index (McClure and Edelman 1966).

α-Chymotrypsin (three times recrystallized, lots CDI 6113-4 and 6150-1) and chymotrypsinogen A (lot CG 763) were purchased from Worthington Biochemical Corp., Freehold, N. J., and used without further purification. An all-or-none assay using *p*-nitrophenyl acetate (Hartley and Kilby, 1954) indicated that the enzyme preparations were 85–87% active. Phenylmethanesulfonyl-chymotrypsin was prepared using the procedure of Fahrney and Gold (1963). After removal of excess reagents, phenylmethanesulfonyl-chymotrypsin possessed less than 1% of the original activity against ATEE.

Protein concentrations were determined by measuring the absorbance at 282 mμ, using a molecular weight of 25,000 and an extinction coefficient of 2.06 (1 mg/ml, 1-cm light path) for both chymotrypsin (Dreyer *et al.*, 1955) and chymotrypsinogen (Schwert and Kaufman, 1951). The same values were assumed for solutions of phenylmethanesulfonyl-chymotrypsin. TNS concentrations were determined by absorbance at 317 mμ (ϵ 1.89×10^4; McClure and Edelman, 1966). Concentrations of other solutes were determined by weight using dried reagents.

Distilled water was used to prepare all solutions. The proteins were dissolved in 10^{-3} M HCl and were diluted into concentrated buffers to obtain the final buffer compositions and pH values given in the figure legends. No difference in the strength of binding of TNS to chymotrypsin was noticed when Tris buffers were replaced by phosphate buffers.

Initial rates of hydrolysis of ATEE by chymotrypsin were measured at 25.0° by potentiometric titration in 0.002 M Tris–HCl. The pH was maintained at 7.8 with NaOH added by means of automatic titration (Radiometer Model TTT1 titrator) or manual titration with a microburet and a Radiometer Model 4 pH meter.

Intensities of fluorescence were corrected for self-absorption of incident light using the relationship $I_{cor} = I_{obsd}[2.303\epsilon_{366}F_0/(1 - 10^{-\epsilon_{366}F_0})]$, where F_0 refers to the total TNS concentration, ϵ_{366} is the molar absorptivity of TNS at 366 mμ, and I_{cor} and I_{obsd} refer to the corrected and observed intensities, respectively. An ϵ_{366} value of 4.30×10^3 was used for TNS in aqueous solution. Control experiments indicated that self-absorption of fluorescent light was negligible at wavelengths equal to or greater than 460 mμ. Fluorescence emission spectra, quantum yields, and related data were obtained with the instrument described by Rosen and Edelman (1965) using the conditions of McClure and Edelman (1966).

Theory

Binding Phenomena. A general mechanism which satisfies the data presented below invokes noncompetitive interaction between an enzyme (E) and two small molecules, a fluorophore (F) and a modifier of fluorescence (M). The equations describing the system are

$$\begin{aligned} E + F &\overset{K_f}{\rightleftharpoons} C_f \\ E + M &\overset{K_m}{\rightleftharpoons} C_m \\ C_t + M &\overset{K_m'}{\rightleftharpoons} C_t \\ C_m + F &\overset{K_f'}{\rightleftharpoons} C_t \end{aligned} \qquad (1)$$

The various K values are the dissociation constants for the formation of the binary complexes, C_f and C_m, and the ternary complex, C_t. By the principle of mass

action

$$K_f K_m' = K_f' K_m \tag{2}$$

Under the conditions used in these experiments ($M_0 >> E_0 \simeq F_0$), conservation of mass requires that

$$\begin{aligned} M_0 &= M \\ E_0 &= E + C_f + C_m + C_t \\ F_0 &= F + C_f + C_t \end{aligned} \tag{3}$$

The subscript zero refers to the total amount of the relevant species and unsubscripted symbols refer to unbound species.

Equations 1 and 3 may be combined to yield

$$C_f = \frac{K_m' E_0 F_0}{(E_0 + F_0)(K_m' + M_0) + K_f' M_0 + K_m' K_f} + R_f \tag{4}$$

$$C_t = \frac{E_0 F_0 M_0}{(E_0 + F_0)(K_m' + M_0) + K_f' M_0 + K_m' K_f} + R_t$$

R_f and R_t are the residuals of a binomial expansion used to obtain eq 4. The details of similar derivations may be found in Reiner (1959) and McClure and Neurath (1966). Since in the systems discussed below $R_f \simeq R_t \simeq 0$, the specific forms of these residuals are unimportant.[2]

If only fluorophore–protein complexes possess significant fluorescence, we may write

$$I = \psi_f C_f + \psi_t C_t \tag{5}$$

in which I is the observed intensity of fluorescence, after correction for self-absorption, and ψ_f and ψ_t are proportionality constants relating the fluorescence intensity to the concentration of the appropriate complex.

Substituting eq 4 into eq 5 and simplifying, it may be shown that

$$I = \frac{\psi_f K_m' E_0 F_0 + \psi_t E_0 F_0 M_0}{(E_0 + F_0)(K_m' + M_0) + K_f' M_0 + K_m' K_f} \tag{6}$$

if, and only if, $R_f \simeq R_t \simeq 0$.

Three cases of interest may be obtained by a consideration of eq 6.

CASE 1. TITRATION WITH MODIFIER. If F_0 and E_0 are maintained constant and I is measured at varying levels of M_0, eq 6 may be rearranged to

$$(I_0 - I) = (I_0 - I_\infty) - K_{app}\frac{(I_0 - I)}{M_0} \tag{7}$$

where I_0 and I_∞ are the limits of I observed as M_0 equals zero and approaches infinity, respectively. These values would correspond to the observed fluorescence of the binary enzyme–fluorophore complex, C_f, and the tertiary enzyme–fluorophore–modifier complex, C_t.

Under the conditions given in this case a plot of $(I_0 - I)$ *vs.* $(I_0 - I)/M_0$ will yield a straight line only if $R_f \simeq R_t \simeq 0$. If a straight line is obtained, I_∞ and K_{app} may be determined from the intercept and slope, respectively.

In the three cases discussed here, K_{app} is an apparent dissociation constant characterizing the reaction under study. The actual species involved in the dissociation must be determined from the experimental context. In eq 7, K_{app} is defined by

$$K_{app} = \frac{(E_0 + F_0 + K_f)}{(E_0 + F_0 + K_f')} K_m' \tag{8}$$

It is clear that K_{app} will equal K_m' in either of two experimentally accessible cases: if $K_f = K_f'$, or if $F_0 >> K_f$ and $F_0 >> K_f'$. If $K_f = K_f'$, K_{app} will give a valid measure of the affinity of a modifier for either free enzyme or complexes of fluorophore and enzyme (*cf.* eq 2).

CASE 2. TITRATION WITH FLUOROPHORE IN THE PRESENCE OF MODIFIER. If M_0 and E_0 are held constant and I is measured at varying levels of F_0, eq 6 may be modified to yield

$$I = I_{max} - K_{app}\frac{I}{F_0} \tag{9}$$

I_{max} will, in this and the following case, be treated as an empirical parameter giving the maximal fluorescence of which a given system is capable when saturated with titrant. In the case under consideration, I_{max} is the maximal intensity which can be attained by saturating with fluorophore a solution containing fixed concentrations of enzyme and inhibitor. I_{max} is given by

$$I_{max} = \frac{\psi_f K_m' E_0 + \psi_t E_0 M_0}{K_m' + M_0} \tag{10}$$

and must vary between the limits of $\psi_f E_0$ and $\psi_t E_0$ as M_0 increases from zero toward infinity.

A plot of I *vs.* I/F_0 will yield a straight line, if $R_f \simeq R_t \simeq 0$, from which both I_{max} and K_{app} can be evaluated. If $K_m = K_m'$, K_{app} can be shown to equal K_f and should provide a valid estimate of the affinity between the fluorophore and enzyme.

CASE 3. TITRATION WITH FLUOROPHORE IN THE ABSENCE OF MODIFIER. If $M_0 = 0$, eq 6 will also reduce to eq 9.

[2] Under the more common conditions of very low enzyme concentration ($M_0 \simeq F_0 >> E_0$) both R_f and R_t will vanish. A derivation of the form given here was preferred in this case because some of the titrations were carried out with only a small excess of dye over protein. The linearity of titrations (Figure 1) attests to the fact that, in these systems, the residuals are negligible.

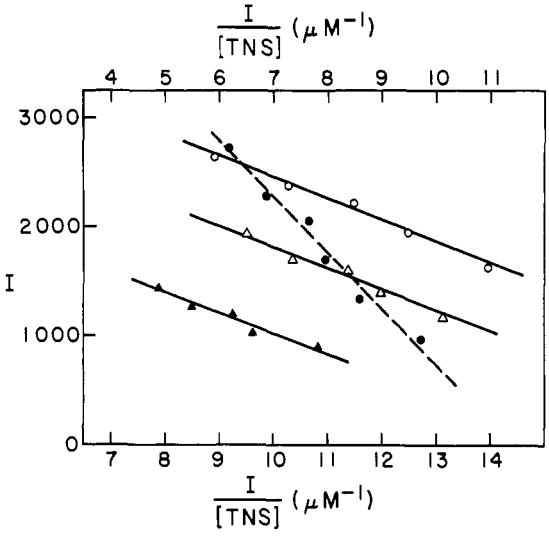

FIGURE 1: Fluorescence titrations of chymotrypsin and chymotrypsinogen with TNS in the absence and presence of β-phenylpropionate. Titration of chymotrypsinogen with TNS: ●, dashed line. Titrations of chymotrypsin with TNS: ○, no added β-phenylpropionate; △, β-phenylpropionate concentration = 1.65×10^{-3} M; ▲, β-phenylpropionate concentration = 5.15×10^{-3} M. The two titrations containing β-phenylpropionate are plotted with respect to the upper ordinate. Values of I in the TNS titration of chymotrypsinogen have been increased tenfold. All titrations were performed in 0.05 M Tris–HCl buffers, pH 7.8, at 25.0°. Irradiating wavelength, 366 mμ; analyzing wavelength, 460 mμ. Data are plotted according to eq 9. TNS concentrations were varied between 3×10^{-5} and 3×10^{-4} M, and in each case the protein concentration was maintained at 4.0×10^{-5} M.

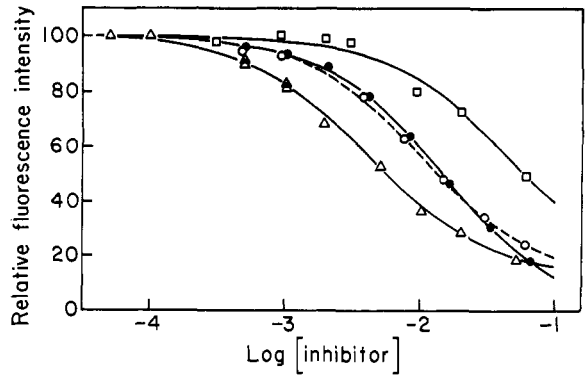

FIGURE 2: The effect of substrate analogs on the fluorescence of solutions containing TNS and chymotrypsin. Intensities were evaluated at 460 mμ and are expressed as percentage of the intensity observed in the absence of modifier. Irradiating wavelength, 366 mμ. The data given for β-phenylpropionate (△) represent I_{max} values, obtained from titrations with TNS of solutions containing chymotrypsin (4.0×10^{-5} M) and β-phenylpropionate. The remaining sets of data represent values of I observed with the following modifiers: ○, D-tryptophan, TNS concentration = 7.9×10^{-5} M, enzyme concentration = 4.1×10^{-5} M; ●, acetyl-L-tryptophan, TNS concentration = 1.2×10^{-4} M, enzyme concentration = 9.2×10^{-5} M; □, L-tryptophan, TNS concentration = 5.8×10^{-4} M, enzyme concentration = 3.4×10^{-5} M. All data were obtained in 0.05 N Tris–HCl, pH 8.0, at 25.0°. The curves are predicted from theory using the parameters of Table I in a rearranged form of eq 7.

In this case, however, I_{max} will represent the maximal fluorescence of the fluorophore–enzyme complex. K_{app} will equal K_i.

Results

Data obtained in a typical titration of chymotrypsin with TNS are given in Figure 1. It is clear that, within experimental error, the binding of TNS to chymotrypsin yields data which give a straight line when plotted according to eq 9. In a graph of this type, the intercept on the ordinate (I_{max}) represents the maximal fluorescence of the protein–TNS complex; *i.e.*, the fluorescence which would be observed if all the protein were complexed with TNS. The slope of the line is equal to $-K_{app}$, where K_{app} is an apparent dissociation constant characterizing the reaction under study. Using the data of Figure 1, the dissociation constant calculated for the binding of TNS by chymotrypsin was 2.05×10^{-4} M (pH 7.8).

For comparison, the data obtained in a similar titration of chymotrypsinogen with TNS are included in Figure 1. Because the intensity of fluorescence of the TNS–chymotrypsinogen system was much less than that of the TNS–chymotrypsin system (see below), it was necessary to multiply the intensities observed in the chymotrypsinogen titration by a factor of 10 in order to include them conveniently in Figure 1. The dissociation constant for the interaction of TNS with chymotrypsinogen (4.8×10^{-4} M) was similar to that observed with chymotrypsin, although the maximal fluorescence for the TNS–chymotrypsinogen complex was only 16% of that observed for the TNS–chymotrypsin complex. Apparently TNS is bound firmly to either chymotrypsin or chymotrypsinogen. The difference in maximal intensity may be explained by differences in the local environments of the TNS binding sites in the two proteins.

The effect of known inhibitors of chymotrypsin on the binding site for TNS was studied. β-Phenylpropionate is a simple competitive inhibitor of the activity of this enzyme (Kaufman and Neurath, 1949; Huang and Niemann, 1952; Canady and Laidler, 1958). We found that the addition of β-phenylpropionate to solutions of TNS and chymotrypsin markedly diminished the intensity of the observed fluorescence. The presence of β-phenylpropionate in solutions of TNS

and chymotrypsin did not alter the band width or the maximum of the emission spectrum. These results suggest that β-phenylpropionate might quench the fluorescence by absorbing incident light, without specific effects due to interaction with chymotrypsin. Control experiments showed, however, that β-phenylpropionate, even at the highest concentration tested, had no absorbance either at the exciting wavelength (366 mμ) or within the wavelength limits of the emission band (400–600 mμ). Titrations with TNS of enzyme solutions containing β-phenylpropionate showed that interaction of the inhibitor with the TNS–chymotrypsin complex was the cause of the quenching. Two experiments, carried out at different levels of β-phenylpropionate, are presented in Figure 1. The presence of β-phenylpropionate decreased I_{max} without significantly altering the apparent dissociation constant of the TNS–chymotrypsin reaction. This behavior is not consistent with competitive binding of TNS and β-phenylpropionate to chymotrypsin, but agrees well with the behavior predicted for a noncompetitive system (*cf.* Theory).

In a more extensive series of titrations, the observed binding constant remained independent of the β-phenylpropionate concentration; however, the values of I_{max} were strongly dependent upon the concentration of the inhibitor (Figure 2). Using eq 7, the data given in Figure 2 on the effect of β-phenylpropionate were replotted (Figure 3) and values of K_{app} and I_∞ were calculated (Table I). A value of I_∞ greater than zero was obtained.

TABLE I: The Effect of Substrate Analogs on the Fluorescence of Complexes between Chymotrypsin and TNS.[a]

Analog	K_{app} (mM)[b]	I_∞/I_0[c]
β-Phenylpropionate	4.2	0.13
Acetyl-L-tryptophan	14.7	0.00
D-Tryptophan	10.7	0.11
L-Tryptophan	44.7	0.12

[a] All values were obtained at 25.0° in pH 8.0 Tris–HCl buffers of 0.05 M ionic strength. [b] Apparent dissociation constant for the reaction. [c] Ratio of the fluorescence intensities at saturating and zero concentrations of analog, respectively.

To determine whether other substrate analogs of chymotrypsin acted in a manner similar to that of β-phenylpropionate, acetyl-L-tryptophan was added to solutions of chymotrypsin and the mixtures were titrated with TNS. It was found that the I_{max} values decreased when acetyltryptophan was present, although the apparent dissociation constant describing the binding of TNS to chymotrypsin was unaffected. The dissociation constant of acetyltryptophan and chymotrypsin was determined by measuring the intensity of fluorescence observed in solutions of TNS and chymotrypsin containing varying concentrations of inhibitor (Figure 2). The inhibition of fluorescence caused by acetyltryptophan followed a simple sigmoid dependence upon the logarithm of the concentration. Values of K_{app} and I_∞ were calculated with the aid of eq 7 and are presented in Table I. In contrast to β-phenylpropionate, acetyltryptophan completely suppressed the fluorescence of TNS–chymotrypsin.

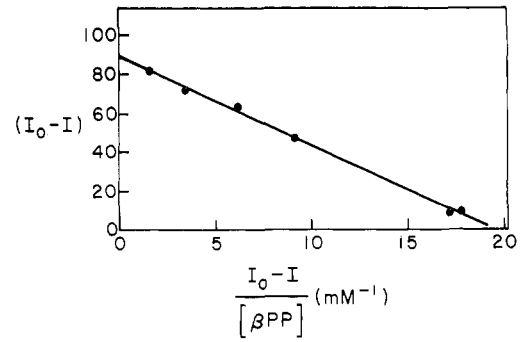

FIGURE 3: The effect of different concentrations of β-phenylpropionate on the fluorescence of TNS–chymotrypsin complexes. Data taken from Figure 2, plotted according to eq 7. I_0, intensity observed in the absence of β-phenylpropionate; I, intensity observed in the presence of β-phenylpropionate.

The two enantiomorphs of tryptophan were also examined as inhibitors of the fluorescence of solutions containing both TNS and chymotrypsin. Both enantiomorphs affected the titrations in the same way as acetyltryptophan and β-phenylpropionate. Treatment of the data according to eq 7 yielded the values of I_∞ and K_{app} shown in Table I. D-Tryptophan binds more strongly to chymotrypsin than does the L isomer of this amino acid.

Enzyme–substrate interactions involve at least two distinguishable steps: binding of the substrate to the enzyme and a subsequent reaction of the enzyme–substrate complex to form products and regenerate free enzyme. Studies on the binding of competitive inhibitors can only yield information on the first of these two steps. To study the effect of TNS upon the second step, the initial rates of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester were measured in the absence and presence of TNS (Figure 4). Even at the high levels of substrate utilized in this experiment (0.010 M), TNS inhibits the reaction. The appearance of a limiting activity at high concentrations of TNS suggests the possibility of an enzymatically active ternary complex consisting of TNS, ATEE, and chymotrypsin.

The fluorescence intensity of solutions containing TNS and chymotrypsin was found to depend strongly upon the pH of the medium. As shown in Figure 5, there was a marked increase in fluorescence intensity

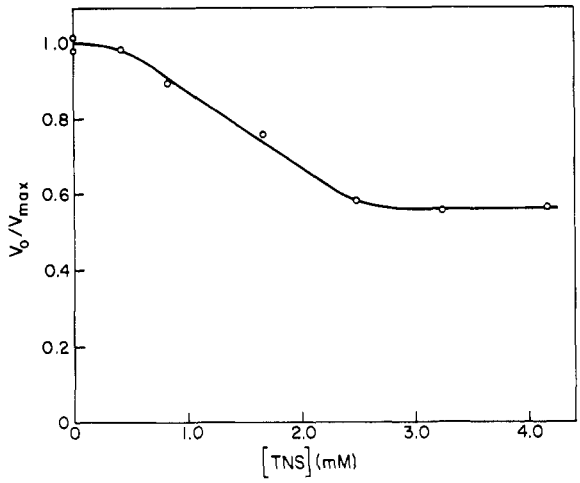

FIGURE 4: The effect of TNS on the initial velocity of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester. V_0, initial velocity in the presence of TNS; V_{max}, initial velocity in control reactions lacking TNS. All reactions were carried out in 0.002 M Tris–HCl, pH 7.8, at 25.0°. Concentration of enzyme, 5.5×10^{-8} M; concentration of ATEE, 0.010 M.

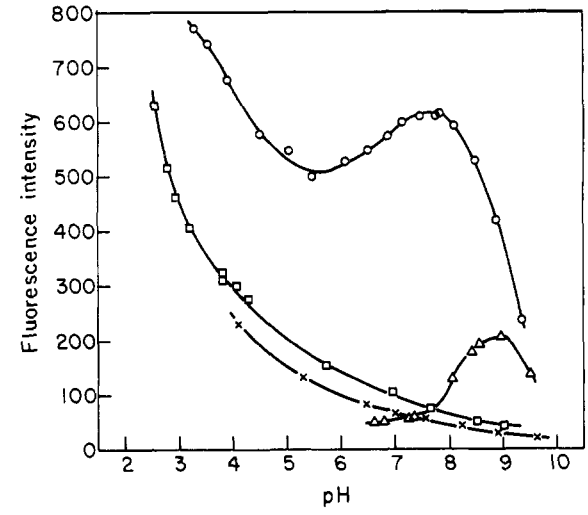

FIGURE 5: The variation with pH of the fluorescence intensity of TNS in solutions of chymotrypsin and related proteins. Titrations were performed in unbuffered aqueous solutions, using an irradiating wavelength of 366 mμ and an analyzing wavelength of 460 mμ. Concentration of protein = 2.0×10^{-4} M; concentration of TNS = 2.0×10^{-5} M. Systems being titrated: O, chymotrypsin; □, chymotrypsinogen; ×, phenylmethanesulfonyl-chymotrypsin; Δ, chymotrypsin + 0.05 M β-phenylpropionate.

at pH values less than 5.5. This phenomenon has been observed in similar titrations of a number of proteins in addition to chymotrypsin. In the range between pH 7 and 9, the fluorescence of TNS in chymotrypsin solutions differs markedly from that seen in solutions of the enzymatically inactive species, chymotrypsinogen and phenylmethanesulfonyl-chymotrypsin (Figure 5). The most striking difference was a peak at pH 7.8 which was present in the fluorescence intensity–pH profile of TNS in chymotrypsin solutions, but was absent in solutions of chymotrypsinogen and phenylmethanesulfonyl-chymotrypsin. The effect of pH on the emission spectra of TNS in chymotrypsin solutions (not shown) also differed from that found for TNS in solutions containing chymotrypsinogen or phenylmethanesulfonyl-chymotrypsin. The fluorescence in solutions of the two inactive proteins merely changed the intensity as the pH was varied, without changing the band width or wavelength maximum of the emission band. In contrast, the fluorescence of TNS in chymotrypsin solutions underwent a change in intensity and also showed a 15-mμ shift to longer wavelengths as the pH was increased from pH 2.5 to 7.8. The presence of competitive inhibitors might be expected to alter the observed titration curves. Such an effect was found when protein solutions containing β-phenylpropionate and TNS were titrated as a function of pH (Figure 5). In the presence of β-phenylpropionate, the peak is shifted from pH 7.8 to 9.0 with a marked reduction in fluorescence intensity.

Changes in the intensity of fluorescence of the kind portrayed in Figure 5 can result from changes in either, or both, of two parameters: the apparent dissociation constant (K_{app}), or the maximal intensity of fluorescence (I_{max}). To separate these parameters, titrations of chymotrypsin with TNS were carried out at different pH values. Data obtained in each titration were plotted using the method depicted in Figure 1, and I_{max} and K_{app} were obtained as a function of pH (Figure 6). In the range between pH 7 and 9 the variation in the observed fluorescence resulted from a change of I_{max}, with little or no change in the affinity of the enzyme for TNS. I_{max} and K_{app} both increased at low pH values, with a resulting net increase in the observed intensity of fluorescence. The variation in K_{app} as the pH is lowered from 8 to 5 can be described by a theoretical titration curve assuming values of $K_{app} = 2.0 \times 10^{-4}$ M at high pH, $K_{app} = 3.1 \times 10^{-4}$ M at low pH, and an apparent pK of 6.65.

Discussion

The experiments reported above deal with the nature of the interactions between TNS and chymotrypsin and closely related proteins. The results indicate that TNS acts as a fluorescent probe for conformational changes in chymotrypsin. A detailed understanding of these changes is required for meaningful interpretation of changes in fluorescence accompanying the conversion of chymotrypsinogen to chymotrypsin in the presence of TNS (McClure and Edelman, 1967).

The data on binding of TNS to chymotrypsin are in agreement with a formal model which assumes

an interaction between one molecule of TNS and one molecule of chymotrypsin to produce a fluorescent complex (*cf.* Theory). This interaction is characterized by a dissociation constant of 2.0×10^{-4} M, a value of the same magnitude as that observed for many inhibitors of chymotrypsin (Foster and Niemann, 1955; Wallace *et al.*, 1963).

Several lines of evidence support the suggestion that TNS is bound to chymotrypsin at some site other than the hydrophobic portion of the active site. The apparent binding constant of TNS and chymotrypsin is not affected by the presence of β-phenylpropionate, a known competitive inhibitor of this enzyme. β-Phenylpropionate does, however, cause a marked decrease in the maximal fluorescence intensity. This decrease in intensity can be used to evaluate a dissociation constant of 4.2×10^{-3} M for the interaction between β-phenylpropionate and TNS–chymotrypsin complexes. Both chymotrypsin and TNS–chymotrypsin complexes must exhibit equal affinities for β-phenylpropionate (*cf.* eq 2) and, therefore, the dissociation constant of 4.2×10^{-3} M should describe the binding of β-phenylpropionate to free chymotrypsin. This value compares favorably with the value of 4.5×10^{-3} M obtained by Kaufman and Neurath (1949), who studied β-phenylpropionate as a competitive inhibitor of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide. The agreement of the two values is compatible with the idea that β-phenylpropionate is bound to the active site of the TNS–chymotrypsin complex, and that TNS is bound at a second, spatially distinct, site.

Data obtained in the study of other substrate analogs also fit the noncompetitive mechanism elaborated above. Measurements using a series of competitive inhibitors of chymotrypsin yielded values of dissociation constants which could be compared with literature values determined by kinetic means. The ratio of the dissociation constants of the L and D forms of tryptophan was 4.1, which agrees well with the value of 3.7 (Huang and Niemann, 1951) obtained using tryptophan derivatives as competitive inhibitors of chymotrypsin. The dissociation constant observed for the binding of acetyltryptophan to chymotrypsin (1.5×10^{-2} M) is in good agreement with the value of 1.4×10^{-2} M found by Johnson and Knowles (1966) at pH 8.0. These results support the conclusion that inhibitors exert their effect on TNS fluorescence *via* a direct interaction with the active site of the enzyme.

With the exception of acetyltryptophan, saturating concentrations of the substrate analogs examined in this study were not capable of completely suppressing the fluorescence of TNS in solutions of chymotrypsin. The presence of stable ternary complexes may be responsible for this phenomenon (*cf.* Theory). It should be recognized, however, that other mechanisms are possible. For example, the data could be satisfied if two species of chymotrypsin were present in the enzyme preparation. The limiting fluorescence at high concentrations of β-phenylpropionate could be explained if about 85% of the protein were capable of binding β-phenylpropionate; this value agrees with the extent to which the enzyme preparation can be acetylated by *p*-nitrophenyl acetate. It is possible that the limiting fluorescence is almost entirely due to the 15% of the enzyme which does not bind β-phenylpropionate. Thus high concentrations of ternary complexes of chymotrypsin, TNS, and β-phenylpropionate need not be invoked to explain the results. The noncompetitive interaction between TNS and β-phenylpropionate would still suggest that a ternary complex undergoes dissociation to form free TNS and a binary complex of competitive inhibitor and chymotrypsin.

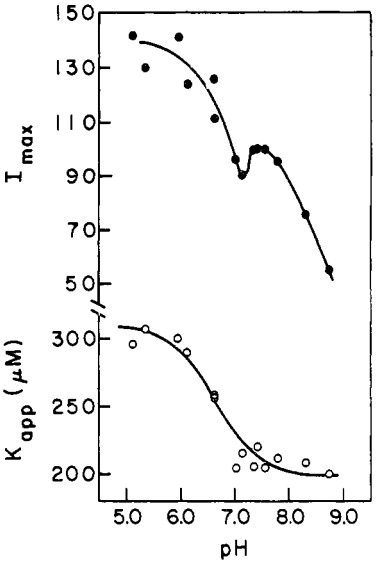

FIGURE 6: The effect of pH on the apparent dissociation constant and I_{max} values for the TNS–chymotrypsin system. Data were taken from TNS titrations carried out at the given pH values in a buffer containing 0.4 M NaCl and total concentrations of 0.02 M each of Tris, NaH_2PO_4, and sodium acetate. Irradiation wavelength, 366 mμ; analyzing wavelength, 460 mμ. I_{max} values (●) are expressed as percentage of the peak values observed at pH 7.8. ○, apparent dissociation constant (K_{app}). The line through the values of K_{app} was calculated as described in the Results. Temperature, 25.0°.

The arguments advanced above indicate that TNS does not affect the binding of substrate analogs. It was noticed, however, that TNS caused a marked inhibition of the initial velocity of the chymotrypsin-catalyzed hydrolysis of acetyltyrosine ethyl ester. Although insufficient data have been obtained to allow complete characterization of the TNS–chymotrypsin–ATEE system, the limiting value of the enzyme activity at saturating levels of TNS suggests the formation of a ternary complex and makes it likely that the interaction between TNS and ATEE is noncompetitive.

It may be concluded that chymotrypsin possesses two sites capable of binding hydrophobic structures. One site, responsible for the binding of the aromatic

portions of specific substrates, must lie in the catalytic center of the enzyme (Niemann, 1964). A second site, responsible for the binding of TNS, must lie outside the catalytic center; at present the distance between the two sites cannot be estimated.

The local environment of TNS bound to chymotrypsin can be altered by changes in the pH of the solution as well as by binding of substrate analogs. It is tempting to suggest that in the range between pH 7 and 9 the ionizing groups which influence the fluorescence may be the same groups which control the enzymic activity. The apparent dissociation constant describing the binding of TNS to chymotrypsin is governed by an ionizing group with an observed pK of 6.65. This value is in good agreement with the pK of a group on chymotrypsin which controls the rate of acylation of the enzyme (Bender *et al.*, 1964). The curves describing the effect of pH on both fluorescence and enzymic activity (Bender *et al.*, 1964) are similar in peak position and shape. Moreover, the emission maximum of TNS fluorescence is dependent upon the pH when the fluorophore is dissolved in chymotrypsin solutions, but not when it is dissolved in solutions of chymotrypsinogen or phenylmethanesulfonyl-chymotrypsin. The peak observed in the pH–fluorescence profile of TNS and chymotrypsin vanishes when chymotrypsin is replaced by either of the enzymatically inactive proteins, chymotrypsinogen or phenylmethanesulfonyl-chymotrypsin. Finally, between pH 7.5 and 9.0, the binding of protons to the amino acid side chains involved changing the fluorescence of the TNS–chymotrypsin complex without changing the apparent dissociation constant, in a manner similar to that observed for the binding of substrate analogs.

If the ionizing groups implicated in the pH–fluorescence profile are in fact the groups which govern the enzymic activity of chymotrypsin, it is likely that these groups exert their action on fluorescence or activity by pH-mediated changes in enzyme conformation. This would imply that the observed inflections in pH activity curves for chymotrypsin need not reflect groups involved in proton transfer to or from the substrate, but may reflect groups which are involved solely in altering the conformation of the protein between states of different enzymic activity. A similar conclusion has been reached by Oppenheimer *et al.* (1966), who studied the properties of acetylated derivatives of chymotrypsin and chymotrypsinogen.

At the present time, it is not possible to associate the phenomena observed in this study with conformational changes in chymotrypsin that may accompany enzymic activity. Substrate-induced changes in the conformation of chymotrypsin have been described by Sturtevant (1962), who studied small changes in the intrinsic fluorescence of the protein. Stopped-flow spectrofluorometric studies by Sturtevant (1962), however, showed that the conformational changes which he observed were too slow to be involved in the catalytic activity of the enzyme. It is possible that this will also be found to hold in the present work.

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