Activity and Fluorescent Derivatives of Aminotyrosyl Trypsin and Trypsinogen*

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ABSTRACT: Bovine trypsin and trypsinogen, each containing an average of 1.0 nitrotyrosyl residue per molecule, were reduced to the corresponding aminotyrosyl proteins and then treated with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) at pH 5.0. Under the conditions used, the dansylation reaction was specific for aminotyrosyl residues. The activatability or activity and the fluorescence properties of the resulting dansyltyrosyl proteins were investigated. As judged by active site titrations, dansyltyrosyl trypsinogen could be activated to 40% of the value attained by activation of native trypsinogen. Dansyltyrosyl trypsin was found to be an active enzyme but with a reduced catalytic efficiency toward an ester and a protein substrate as compared with native trypsin. The spectral properties of dansyltyrosyl chromophores in both proteins were essentially those of the dansyltyrosine model compounds, ethyl α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinate (EADT) and α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinamide (ADTA). In both dansyltyrosyl proteins the dimethylamino groups were half-titrated at approximately pH 3, which is the same value observed for EADT and ADTA. During the tryptic activation of dansyltyrosyl trypsinogen there was a reciprocal decrease in the fluorescence quantum efficiency and increase in wavelength of fluorescence emission with increase in esterase activity.

These changes corresponded in kind to the changes in fluorescence properties of ADTA in going from a nonpolar to a polar solvent. No large changes during activation were detected in tryptophanyl fluorescence. These observations are interpreted in terms of a change in the local environment of the dansyltyrosyl residue during the conversion of the zymogen to the enzyme. The data are discussed in relation to a three-dimensional model of trypsin based on the assumption of "conformational homology" of trypsin and chymotrypsin.

In contrast to the detailed information on the three-dimensional structure of several enzymes in the crystalline state, our knowledge of the dynamic aspects of protein conformation is less complete and depends on more indirect experimental approaches. Such conformational changes in proteins as accompany enzyme–substrate interaction or, more generally, the interaction of proteins with large or small molecules, are essential aspects of the biological functions of proteins and enzymes. Reporter groups (Burr and Koshland, 1964) designed to signal information about the microenvironment of amino acid side chains are of utility in gaining an understanding of the motility of protein conformation. Fluorescent compounds conjugated in unique locations on enzymes provide probes of the local environment not easily obtained by other physicochemical methods.

Fluorescent conjugates of macromolecules were first extensively investigated by Weber and coworkers (Weber, 1952; Weber and Laurence, 1954; Weber and Young, 1964) and more recently by Stryer (1965, 1968), Laurence (1966), Brand *et al.* (1967), McClure and Edelman (1966, 1967a,b), Parker *et al.* (1967), Chen and Kernohan (1967), and Haugland and Stryer (1967). To be well suited as a probe of microenvironment, the fluorophore should be bound or covalently attached at a unique location on the protein and undergo large changes in one or more spectral parameters with changes in the microenvironment. Implied in the concept of fluorescent probes is the assumption that the placement of the chromophore in a unique location does not alter substantially the conformation of the macromolecule. Furthermore, such a chromophore must be positioned in an area of change, *e.g.*, near the active site, but in such a position that activity is not impaired or abolished. The introduction of a dansyl[1] fluorophore into nitrotyrosyl residues in bovine trypsin and trypsinogen satisfactorily meets most of these requirements.

Bovine trypsin and trypsinogen contain two sets of tyrosyl residues (Inada *et al.*, 1964; Smillie and Kay, 1961; Delaage and Lazdunski, 1965; Riordan *et al.*, 1965; Hachimori *et al.*, 1966; Kenner *et al.*, 1968; Holeysovsky *et al.*, 1969; Gorbunoff, 1969). Of the ten residues of tyrosine in trypsin (or trypsinogen), one set of five to six is "exposed," probably occupying surface positions, and the remainder are "buried" in the interior. It was shown (Kenner *et al.*, 1968) that in bovine trypsin and trypsinogen maximally six "exposed" tyrosyl residues can be nitrated with tetranitromethane (TNM), but two of the six residues show preferential kinetic reactivity. Nitration of an average of 1.0 tyrosyl residue results only in small changes in rate constants for esterase activity of trypsin or activatability of trypsinogen. Three nitrotyrosyl residues were isolated and identified (tyrosyl residues 137, 11, and 28) from trypsin and trypsinogen

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[1] The following abbreviations are used: TNM, tetranitromethane; dansyl chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate·HCl; EADT, ethyl α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinate; ADTA, α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinamide.

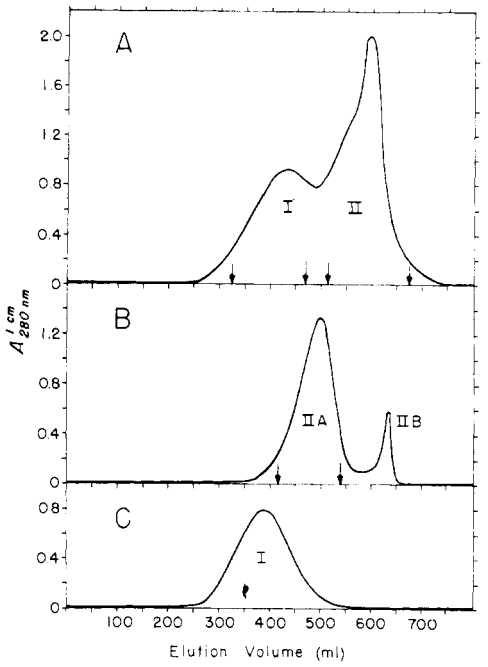

FIGURE 1: Chromatography of dansyltyrosyl trypsin. (A) Chromatography of dansyltyrosyl trypsin (one dansyltyrosyl residue per molecule) on a 2.5 × 115 cm column of SE-Sephadex C-50. Dansyltyrosyl trypsin was eluted with 0.5 M NaCl buffered at pH 3.1 with 5 mM Na citrate at 4°. The flow rate was 20 ml/hr. (B) Rechromatography of dansyltyrosyl trypsin corresponding to peak II of A. (C) Rechromatography of dansyltyrosyl trypsin corresponding to peak I of A. The column and conditions of chromatography in B and C were identical with those in A.

containing on the average 1.0 nitro group per molecule. Recovery data suggested, however, that in both the enzyme and the zymogen, tyrosyl residue 137 was the major residue nitrated by TNM.

A nitrotyrosyl residue is easily reduced to the corresponding aromatic amine with a pK estimated to be below 5.0 (Sokolovsky *et al.*, 1967). Thus, a convenient means of specifically locating fluorescent reporter groups in trypsin and trypsinogen is available. Trypsin and trypsinogen containing an average of 1.0 nitrotyrosyl residue per molecule were reduced to the corresponding aminotyrosyl proteins and then treated with dansyl chloride. Dansylation resulted in modification of only aminotyrosyl residues. Thus a covalently attached fluorophore was introduced into the proteins on specific tyrosyl residues.

Dansyltyrosyl residues in the two proteins investigated proved to be sensitive fluorescent probes of their microenvironment. The fluorescent properties of the dansyltyrosyl proteins were essentially those observed for the model dansyltyrosine compounds discussed in the preceding paper (Kenner, 1971). With the assumption that chymotrypsinogen and trypsinogen are homologous both in primary sequence (Walsh and Neurath, 1964) and in three-dimensional structure (Sigler *et al.*, 1968), conclusions regarding the positions of dansyltyrosyl residues and their environment in trypsinogen and trypsin have been drawn.

Experimental Procedure

Materials. All solvents were reagent grade. Bovine trypsin twice crystallized (lot TR 7JA), trypsinogen once crystallized from dilute ethanol (lot TG 6423), and five times crystallized chymotrypsinogen (lot CGC 8HA) were purchased from Worthington Biochemical Corp. α-*N*-Benzoyl-L-arginine ethyl ester (BAEE) was purchased from Fox Chemical Co. *p*-Nitrophenyl *p'*-guanidinobenzoate·HCl (NPGB) was synthesized and used as an active site titrant for trypsin as described by Chase and Shaw (1967).

Methods. PREPARATION AND PURIFICATION OF DANSYLTYROSYL TRYPSIN AND TRYPSINOGEN. Nitrotyrosyl trypsin and nitrotyrosyl trypsinogen, each containing an average of 1.0 nitrotyrosyl residue, were synthesized according to methods published earlier (Kenner *et al.*, 1968). A fourfold molar excess of TNM over protein was used and the reaction terminated after 15 min. The trypsinogen and trypsin derivatives were separated from inert nitrated protein on SE-Sephadex C-50 to obtain single species of fully active material, *i.e.*, a nitrated enzyme containing one NPGB active site and a nitrated zymogen with one potential NPGB active site.

Nitrated trypsinogen and trypsin were reduced with a tenfold molar excess of $Na_2S_2O_4$ over nitrotyrosine in 0.05 M pyridine–acetate, pH 5.0, at room temperature for 30 min. The aminotyrosyl derivatives (*ca.* 10 mg of trypsin or trypsinogen per ml) were reacted with a 20-fold molar excess of freshly prepared dansyl chloride over aminotyrosine (Horton and Koshland, 1965). The dansylation reaction was carried out at 22° in 0.05 M pyridine–acetate at pH 5.0 for 5 hr in the dark.[2] At pH 5 the reaction of dansyl chloride with water was negligible (Gros and Labouesse, 1969). To effect dispersion of the dansyl chloride, the reagent was added in 8% acetone. The resulting dansyl proteins were exhaustively dialyzed at 5° against changes of 1 mM HCl. Following dialysis, the proteins were forced under nitrogen pressure through a 0.5 × 10 cm column of Dowex 1-X4 200–400 mesh, equilibrated with 0.1 M KCl in 0.05 M pyridine–acetate, pH 5.0. Owing to the hydrophobic nature of the resin, the small amount of reagent tightly bound to the proteins was effectively removed. Under the conditions of pH and salt concentration used, neither dansyltyrosyl trypsin nor dansyltyrosyl trypsinogen was retained on Dowex. The dansyl proteins were once more dialyzed against 1 mM HCl at 5°. Dansyltyrosyl trypsinogen was concentrated in a Diaflo cell (Amicon UM10 membrane) to approximately 7 mg/ml and stored at −15° in 1 mM HCl. Dansyltyrosyl trypsin was similarly concentrated to approximately 20 mg/ml and adjusted to 0.5 M NaCl and 5 mM sodium citrate at pH 3.1 for further purification on SE-Sephadex C-50.

Dansyltyrosyl trypsin was loaded onto a 2.5 × 115 cm column of SE-Sephadex C-50 equilibrated with 0.5 M NaCl, 5 mM sodium citrate, pH 3.1, at 5° and protected from light. This same buffer system was used for elution. The method was identical with that used in the initial purification of trypsin and nitrotyrosyl trypsin (Kenner *et al.*, 1968). A chromatogram of dansyltyrosyl trypsin using this system is shown in Figure 1A. The fractions were pooled according to peaks I and II and chromatographed separately on identical columns. Peak I (Figure 1C) appeared essentially free of II. The protein represented under peak II separated into the material in peaks IIA and IIB (Figure 1B). Only proteins in peaks I and IIA were characterized. Each of these two

[2] In a preliminary preparation of dansyl trypsin and trypsinogen, an unexplained high-energy fluorescence emission, in addition to the usual dansyl fluorescence emission, was observed at 405 nm. This emission was not seen when the reactants and products were protected from light. A similar photosensitive change has been observed in this laboratory by Peter W. Schiller during the synthesis of dansyl amino acids and a dansyllysine protein.

fractions was dialyzed against 1 mM HCl, concentrated in a Diaflo to approximately 5 mg/ml, and stored at −15°.

MEASUREMENT OF TRYPTIC ACTIVITY AND ACTIVATION OF TRYPSINOGEN. Native, nitrated, or dansylated trypsinogen was activated by the addition of native bovine trypsin to the zymogens in a molar ratio of 1:10. Activations were carried out at 0° in 0.1 M Tris-HCl (pH 8.1), 0.05 M $CaCl_2$, and at a final trypsinogen concentration of 5–8 mg/ml. The course of activation was followed in a pH-Stat by the increase in hydrolysis of BAEE at pH 7.8 or by spectrophotometric titrations with the active site titrant, NPGB (Chase and Shaw, 1967).

The catalytic efficiency of trypsin is usually reported as a percentage based on a zero-order rate constant, assuming all trypsin protein molecules to be active (Green and Neurath, 1953). However, if trypsin occurs in various molecular forms which differ in specific activity (Ganrot, 1966; Papaioannou and Liener, 1968; Schroeder and Shaw, 1968; Maroux and Desnuelle, 1969) or if partial denaturation has occurred, such calculations need to be based on active site concentration (determined by titration with NPGB). This method has the advantage over titrations with protein inhibitors that the size of the active site titrant (NPGB) is comparable to that of the substrate (BAEE).[3]

SPECTRAL MEASUREMENTS. Absorption and fluorescent measurements were made with a Turner spectrophotometer–spectrofluorometer Spectro 210. Fluorescence data were calculated as described in the preceding paper (Kenner, 1971).

Results

Selectivity of the Dansylation Reaction. As a control for the selectivity of the dansylation reaction, native trypsinogen and trypsin were treated with all the reagents used for reduction of nitrotyrosine and dansylation of aminotyrosine. The resulting enzymes were unaffected by the treatment, *i.e.*, activity of trypsin and activatability of trypsinogen were unchanged. No fluorescence was detected, indicating that neither the α-amino groups nor any other amino acid side chains had reacted with dansyl chloride at pH 5.0. These control experiments also proved the effective removal of excess reagent by the combination of dialysis and treatment with Dowex-1. Presumably dansylation occurred only on the aminotyrosyl residues generated by the initial nitration and subsequent reduction with $Na_2S_2O_4$.

Characterization of Dansyltyrosyl Trypsins and Trypsinogen. ACTIVITIES OF DANSYLTYROSYL TRYPSINS. Dansyltyrosyl trypsins I and II have reduced catalytic efficiencies compared with native trypsin. Dansyltyrosyl trypsin I contained 1.0 dansyltyrosyl residue per molecule and dansyltyrosyl trypsin II 0.7 of a residue. Table I compares the turnover numbers of dansyltyrosyl trypsins I and II, native trypsin, and nitrotyrosyl trypsin toward BAEE. Derivatives I and II were, respectively, 47 and 80% as efficient (per active site) as native trypsin. At low chymotrypsinogen levels (*ca.* 100 mM), both preparations activated bovine chymotrypsinogen at about the same rate per active site but much more slowly than native trypsin. The Michaelis constant, K_m, for native trypsin and dansyltyrosyl trypsin I was calculated (Eadie, 1942) from initial rates of activation of chymotrypsinogen as described by Abita *et al.* (1969). K_m was found to be virtually unchanged by nitration, reduction, and dansylation, *i.e.*, K_m = 0.48 and 0.56 mM for trypsin and dansyltyrosyl trypsin I, respectively. However, V_{max} of native trypsin for the activation of chymotrypsinogen was 5.3 times greater than that of the derivative. Dansyltyrosyl trypsin II failed to follow Michaelis kinetics under these conditions.

TABLE I: Esterase Activity of the Dansyltyrosyl Trypsins.

Species	BAEE Activity (μmoles/min per μmole)[a]
Dansyltyrosyl trypsin I	800
Dansyltyrosyl trypsin II	1350
Nitrotrypsin[b]	1700
Native trypsin	1700

[a] See text for definition of BAEE activity. [b] An average of one nitrotyrosyl residue per trypsin molecule.

Activation of Dansyltyrosyl Trypsinogen. The modified trypsinogen, containing 1.0 dansyltyrosyl residue per molecule, could be activated with native trypsin to about 40% of the maximum number of potential active sites of unmodified trypsinogen. The product of activation had a BAEE turnover number of 720 min^{-1} or 40% of that of native trypsin. Figure 2 compares the activation of three species of trypsinogen. Native and nitrotyrosyl trypsinogen were activated to the same level. The autoactivation of nitrotyrosyl trypsinogen went to completion, but each active site was only 81% as efficient as an esterase (k = 1380 min^{-1}) as native trypsin. Reduction of the nitrotyrosyl residue to an aminotyrosyl residue changed neither the time course of activation nor the esterase efficiency. The rate of activation of dansyltyrosyl trypsinogen was so slow that two to three times the usual amount of native trypsin was needed to reach the maximum activity in 4–8 hr (Figure 2).

Fluorescence of Dansyltyrosyl Trypsin and Trypsinogen. The fluorescent properties of these dansylated proteins were generally those of the dansyltyrosine model compounds (Kenner, 1971). The quantum efficiencies, Q, and wavelengths of maximum fluorescence emission, $\lambda_{em\ max}$, at pH 4.8 are tabulated in Table II. Fluorometric pH titrations of dansyltyrosyl trypsin I and dansyltyrosyl trypsinogen revealed half-titration values for the dimethylamino groups at pH 3.0 (Figure 3).

Changes in Fluorescence of Dansyltyrosyl Trypsinogen during Activation. During the activation of the dansylated trypsinogen, a large change in fluorescence occurred. The corrected excitation spectra recorded during activation (Figure 4) showed the same shift in excitation maxima as a series of corrected excitation spectra of the model compound ADTA in ethanol–water mixtures (Kenner, 1971). A significant shift in wavelength of maximum excitation was noted

[3] Tryptic activity was calculated relative to the concentration of active sites rather than to protein since, according to experience, the active site concentration relative to protein can vary between about 70 and 90% (depending on source and subsequent purification of trypsin preparations). Similar considerations apply to the degree to which trypsinogen can be activated. The trypsinogen preparation used in this investigation was 92% activatable (as determined by the active sites generated, using NPGB as titrant) and contained no detectable tryptic activity. Since nitration is accomplished under optimum conditions of autocatalytic activation of trypsinogen, absence of initial tryptic activity is essential. If more than 0.01% trypsin is initially present, an unacceptable amount of nitrotyrosyl trypsin is synthesized.

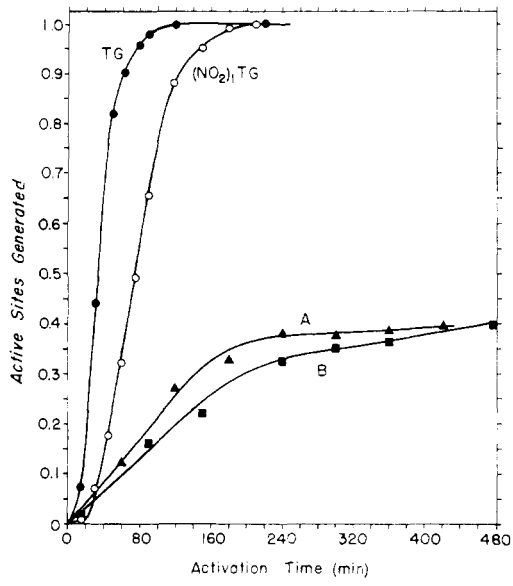

FIGURE 2: Tryptic activation of native, nitrotyrosyl, and dansyltyrosyl trypsinogen. Nitrotyrosyl trypsinogen was modified with 1.0 nitrotyrosyl residue per molecule and dansyltyrosyl trypsinogen with 1.0 dansyltyrosyl residue per molecule. Native (TG) and nitrotyrosyl [(NO_2)TG] trypsinogen were activated with trypsin in a molar ratio of 1:15. Dansyltyrosyl trypsinogen was activated with trypsin in a molar ratio of 1:5 (curve A) or 1:10 (curve B). Activation was accomplished at pH 8.1, 0°, 0.1 M Tris-HCl, 0.05 M $CaCl_2$. The zymogen concentration was approximately 5 mg/ml.

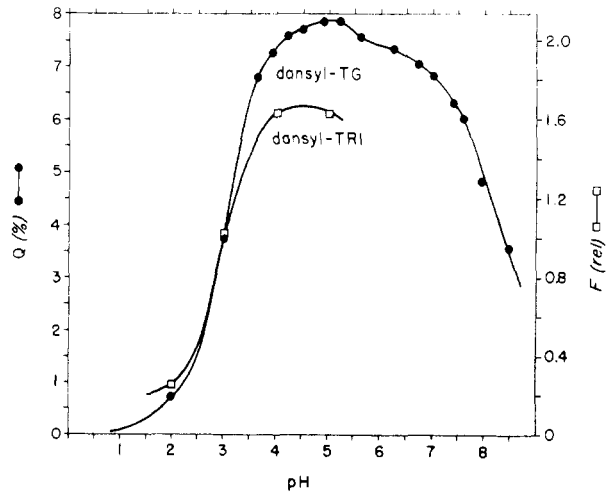

FIGURE 3: Fluorometric pH titration of dansyltyrosyl trypsin I and dansyltyrosyl trypsinogen. The absolute quantum efficiency, Q (%), for dansyltyrosyl trypsinogen or the area under the corrected fluorescence emission spectrum, F(rel), for dansyltyrosyl trypsin I is plotted as a function of pH.

in the 330-nm region. The wavelength of maximum excitation for dansyltyrosyl trypsinogen (zero time) corresponds to that of ADTA in 80% (v/v) ethanol. The value at 200 min activation corresponds to ADTA in about 30% (v/v) ethanol.

Tryptophanyl and dansyltyrosyl fluorescence can be independently quantitated on the same sample. In both native and dansyltyrosyl trypsinogen, tryptophanyl residues fluoresce at a wavelength maximum of 335 nm and dansyltyrosyl residues between 535 and 546 nm, depending on the extent of tryptic activation of dansyltyrosyl trypsinogen (Table II). Both types of residues are excited by light of 280 nm to yield two distinct fluorescence emission spectra, one with a maximum at 335 nm (tryptophanyl fluorescence) and a second one with a maximum between 535 and 546 nm (dansyltyrosyl fluorescence). However, if the exciting light is in the wavelength range of 320–340 nm, only a single fluorescence emission spectrum corresponding to dansyltyrosyl fluorescence is observed, *i.e.*, the absorptivity of tryptophan is zero in the region of 320–340 nm. The overlap of the tryptophanyl fluorescence emission band ($\lambda_{em\ max}$ 335 nm) with the absorption band (Kenner, 1971) of dansyltyrosyl residues (ϵ 4600 M^{-1} cm^{-1} for EADT and ADTA) allows for long-range radiationless transfer of singlet excitation energy (Förster, 1966; Eisinger *et al.*, 1969) from tryptophanyl to dansyltyrosyl residues.

In dansyltyrosyl trypsinogen, the chromophores substantially quench tryptophanyl fluorescence by exchange of singlet excitation energy. The quantum efficiency of tryptophanyl fluorescence in native trypsinogen was 13.4% in the zymogen (Teale, 1960) and 14.7% in the enzyme. The quan-

TABLE II: Emission Maxima and Quantum Efficiencies of Dansyltyrosyl Trypsins and Dansyltyrosyl Trypsinogen.

Species	$\lambda_{em\ max}$ (nm)	Q (%)
Dansyltyrosyl trypsin I	540	9.4
Dansyltyrosyl trypsin II	544	7.4
Dansyltyrosyl trypsinogen	535	8.3
Dansyltyrosyl trypsin[a]	546	5.4

[a] The dansyltyrosyl trypsin formed from the activation of dansyltyrosyl trypsinogen (40% activated).

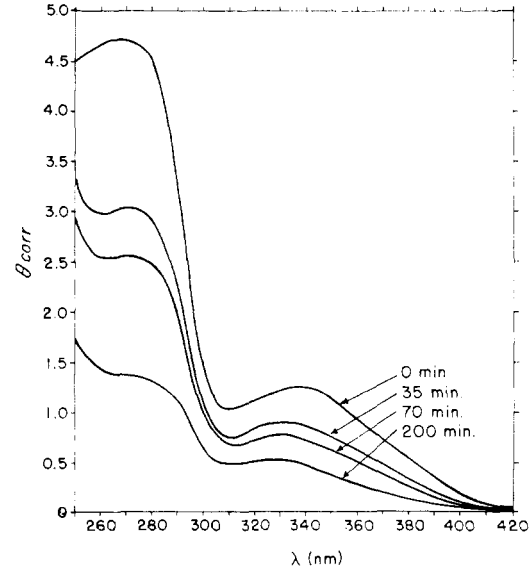

FIGURE 4: Fluorescence excitation spectra during activation of dansyltyrosyl trypsinogen. Activation of dansyltyrosyl trypsinogen was at pH 8.1, 0°, by the addition of trypsin in a molar ratio of 1:10. Excitation spectra were measured at pH 4.8 in 0.05 M K acetate buffer, 25°, at the times indicated on the spectra. The fluorescence, Q_{corr}, was measured with the emission monochromator set at 535 nm with a band width of 10 nm, and the excitation light, λ (nm), from the excitation monochromator with a band width of 2.5 nm.

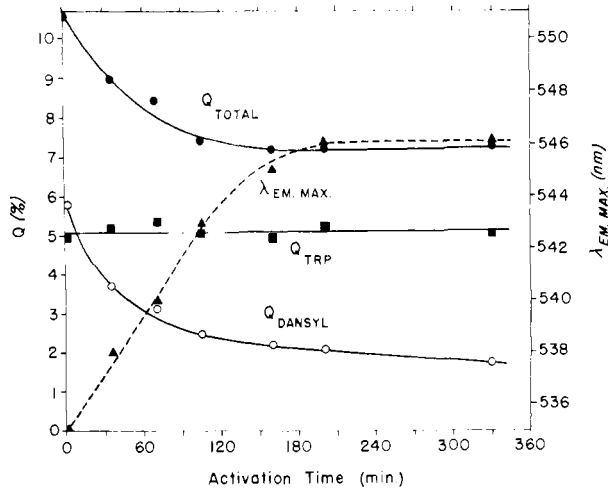

FIGURE 5: Changes in the fluorescence properties of dansyltyrosyl trypsinogen during activation. Activation of dansyltyrosyl trypsinogen was at pH 8.1, 0°, by the addition of trypsin in a molar ratio of 1:10. The quantum efficiencies, Q (%), were measured at pH 4.8 in 0.05 M K acetate buffer at 25°. For definitions of symbols, consult the text. $\lambda_{em\ max}$ is the wavelength of maximum fluorescence emission of dansyltyrosyl fluorescence.

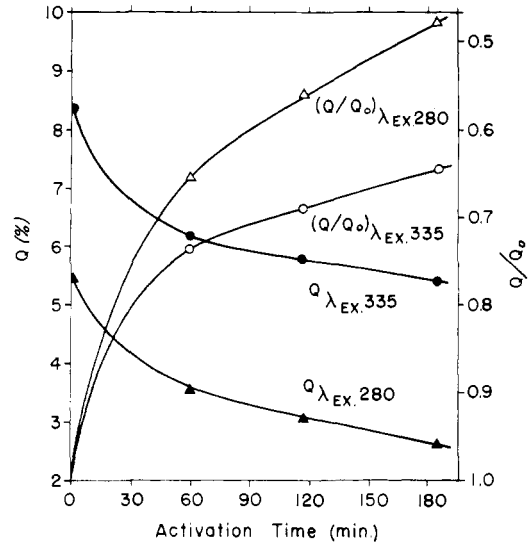

FIGURE 6: Tryptophanyl fluorescence during the activation of dansyltyrosyl trypsinogen. The conditions for activation and fluorescence measurements are the same as those in Figure 5. Measurements of the quantum efficiencies of dansyltyrosyl fluorescence were made by either exciting dansyltyrosyl residues only at 335 nm, $Q\lambda_{ex335}$, or by exciting both dansyltyrosyl and tryptophanyl residues at 280 nm, $Q\lambda_{ex280}$. Q_0 is the fluorescence quantum efficiency at the start of activation and Q at times during activation.

tum efficiency for tryptophanyl fluorescence in dansyltyrosyl trypsinogen was only 5.2%.

An experiment designed to follow dansyl and tryptophanyl fluorescence during the activation of dansyltyrosyl trypsinogen is illustrated in Figure 5. The quantum efficiency of tryptophanyl fluorescence, Q_{Trp}, was essentially constant for the entire activation. The dansyltyrosyl fluorescence quantum efficiency, Q_{dansyl}, when excited at 280 nm (same as tryptophanyl excitation) decreased to about 30% of zero time (Q_{dansyl} = 5.8–1.75%). The change in the total quantum efficiency, Q_{total} (dansyltyrosyl plus tryptophanyl fluorescence), approximately paralleled the decrease in quantum efficiency of dansyltyrosyl fluorescence (Figure 5). By exciting dansyltyrosyl trypsinogen either at 280 nm (dansyltyrosyl plus tryptophanyl) or at 325 nm (dansyltyrosyl exclusively), the ratio of the quantum efficiency at zero time, Q/Q_0, for each of the exciting wavelengths was calculated and plotted as a function of activation time (Figure 6). The quantum efficiency of dansyltyrosyl fluorescence resulting from excitation at 280 nm, $(Q/Q_0)_{\lambda ex280}$, decreased slightly more during the activation than the quantum efficiency of dansyltyrosyl residues exclusively excited at 325 nm, $(Q/Q_0)_{\lambda ex325}$.

Measurements of esterase activity generated during tryptic activation of dansyltyrosyl trypsinogen were made concomitant with the measurement of quantum yield of fluorescence. A plot of percentage activity generated as a function of the percentage change in quantum efficiency gave nearly a straight line with the expected slope of 45° (Figure 7).

Discussion

Vincent *et al.* (1970) have recently drawn attention to the side reactions which may accompany the nitration of trypsinogen, trypsin, and other proteins with TNM and which might make it difficult to relate the effects of chemical modification to biological function. Evidence for such side reactions are polymerization, presumably by cross-linking, and heterogeneity of monomeric fractions. Moreover, there is often a disparity between the values of nitrotyrosine determined by amino acid analysis and by spectrophotometry, respectively. Finally, nitration of trypsin has been reported by these workers to lead to a gradual loss of esterase activity, approaching complete inhibition after prolonged treatment with TNM. Under proper precautions, homogeneous nitrotyrosyl—as well as aminotyrosyl—trypsin could be obtained which retained full activity toward small substrates but was less effective than the native enzyme toward proteins.

In the present work, the chemical derivatives of trypsinogen

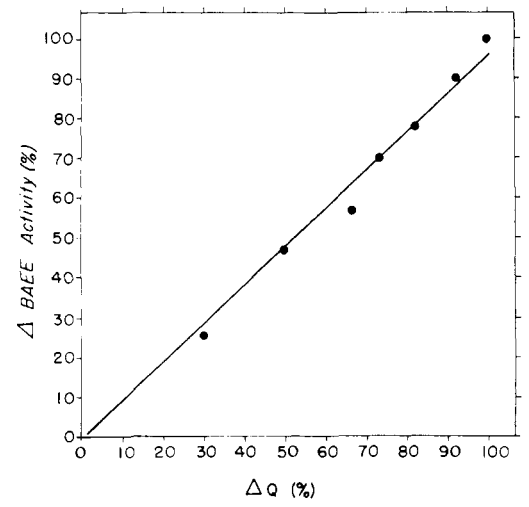

FIGURE 7: Correlation of increase in esterase activity with decrease in dansyltyrosyl fluorescence quantum efficiency during dansyltyrosyl trypsinogen activation. The percentage increase in BAEE activity, ΔBAEE activity (%), is plotted against the percentage decrease in fluorescence quantum efficiency, ΔQ (%). Activation conditions and quantum efficiency measurements are the same as those in Figure 5. The fluorescence quantum efficiencies of dansyltyrosyl fluorescence were measured by using excitation light at 335 nm.

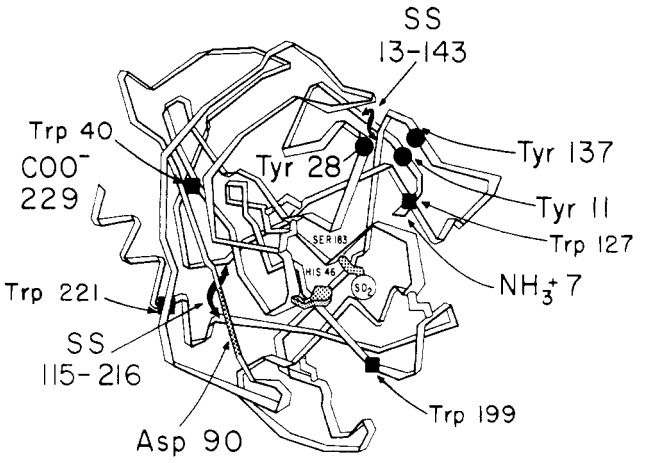

FIGURE 8: Three-dimensional model of trypsin. The model is based entirely on the assumption of "conformational homology" between trypsin and chymotrypsin and the three-dimensional structure of tosyl-α-chymotrypsin as elucidated by Sigler *et al.* (1968). The numbering used is that of trypsinogen (Walsh and Neurath, 1964).

and trypsin were prepared under conditions which would minimize polymerization, heterogeneity, and loss of enzymatic function. The preparation of nitrotyrosyl proteins was conducted in low protein concentrations and the resulting products were fractionated by gel filtration (Kenner *et al.*, 1968). The derivatives contained, on the average, only one nitrotyrosyl residue per molecule and retained full enzymatic activities or activatability. After reduction and dansylation, ion-exchange chromatography was employed first for removing excess reagent (Dowex 1-X4) and subsequently for resolution of the heterogeneous product into distinct fractions (SE-Sephadex). However, even under these conditions, the dansylated aminotyrosyl proteins had reduced biological activity since the trypsinogen derivative could be activated to only 40% of the potential number of active sites of unmodified trypsinogen, and dansyltyrosyl trypsin was maximally only 80% as effective as an esterase, compared with trypsin, and much less effective in its catalytic activation of chymotrypsinogen. In analogy to the behavior of nitrotyrosyl trypsin (Vincent *et al.*, 1970), the rate retarding factor in the activation of chymotrypsinogen was found to be V_{max} rather than K_m.

The spectral characteristics of both dansyltyrosyl trypsin I and dansyltyrosyl trypsinogen resemble closely those of the dansyltyrosine model compounds, EADT and ADTA (Kenner, 1971). The half-titration value at approximately pH 3 observed for both sets of compounds (Figure 3) is about one pH unit lower than that of dansyl amide and dansyllysine and about one-half pH unit higher than that of the dimethylamino group in dansyl lysozyme (Lagunoff and Ottolenghi, 1966; Young, 1963). Phenolic ionization with a half-titration between pH 8.5 and 9.0 is responsible for fluorescence quenching at the higher pH values. Dansyltyrosyl trypsinogen shows a weak shoulder on the titration curve with a half-titration at approximately pH 5.5. The corresponding value for ADTA is pH 6.25. The only effect of an interaction of the fluorophore with the protein is to decrease the half-titration value by about one pH unit. This titration shoulder then must originate from the particular conformation of the fluorophore and the interaction of its titratable groups.

Dansyltyrosyl trypsinogen has a quantum efficiency of fluorescence of 8.3% with a maximum emission at 535 nm (Figure 6). During tryptic activation, the quantum efficiency drops to 5.4% and the wavelength of maximum emission increases to 546 nm. As judged from the fluorescence of EADT and ADTA, these spectral changes accompanying activation suggest a movement of the dansyltyrosyl side chain from a less to a more polar environment. Since only 40% of the dansyltyrosyl trypsinogen was activated under the conditions employed (Figure 2), the maximum changes in quantum efficiency and emission maximum should be even larger.

Fluorescence excitation spectra measured during tryptic activation of dansyltyrosyl trypsinogen show a characteristic shift to shorter wavelengths (Figure 4). Excitation spectra of ADTA in going from a low polarity solvent to water demonstrated an expected shift in the same direction (Kenner, 1971). Environmental changes around the dansyltyrosyl side chain obviously occur as a result of the conformational change during the transition from the zymogen to the enzyme. A large environmental change, however, does not necessarily imply a corresponding conformational change. It is more likely a measure of side-chain orientation or side-chain interaction which can occur without gross rearrangement of the peptide chains.

The pertinent information on dansyltyrosyl trypsin and trypsinogen is summarized in Figure 8, which has been redrawn from the electron-density map of tosyl-α-chymotrypsin developed by Sigler *et al.* (1968), assuming homology in both primary sequence (Walsh and Neurath, 1964) and three-dimensional structure (Hartley *et al.*, 1965; Sigler *et al.*, 1968; Steitz *et al.*, 1969; Keil *et al.*, 1968) between trypsinogen and chymotrypsinogen. With these assumptions, and based on the peptide analysis of nitrotyrosyl trypsin and trypsinogen (Kenner *et al.*, 1968), the positions of tyrosyl residues that could be dansylated in dansyltyrosyl trypsins I and II and dansyltyrosyl trypsinogen have been designated in the model. The positions of the four tryptophanyl residues of trypsin that occupy homologous positions in chymotrypsinogen are also indicated.

Concomitant with activation of dansyltyrosyl trypsinogen, a rearrangement of the peptide chain takes place which influences the environment around the cluster of tyrosyl residues 137, 11, and 28 (Figure 8). It seems plausible to assume that immediately following release of the highly charged hexapeptide (Davie and Neurath, 1955; Desnuelle and Fabre, 1955), the newly formed α-amino group and the series of hydrophobic residues following in the primary sequence refold to a more stable configuration. Tyrosyl residue 137 is in a region of the peptide chain immediately adjacent to the amino terminal region of trypsin. Movement of the newly generated amino terminal region would influence the environment and thus the fluorescence emission of dansyltyrosyl residue 137.

About two-thirds of the tryptophanyl fluorescence in dansyltyrosyl trypsinogen is quenched by exchange of singlet excitation energy with dansyltyrosyl residues. The model of trypsin presented in Figure 8 reveals that, of the four tryptophanyl residues, only Trp 127 is near the site of dansylation. This close proximity of Trp 127 to dansyltyrosyl residue 137 could account for much of the exchange of singlet excitation energy between tryptophanyl and dansyltyrosyl residues.[4]

[4] It is in the region of Trp 127 that Schroeder and Shaw (1968) identified an interchain split between Lys 131 and Ser 132 in one species of

The method which we have described for the introduction of a fluorescence reporter of conformation is highly sensitive for detecting changes in the environment of tyrosyl residues in proteins and enzymes. The dansylation of tyrosyl residues in the proteins investigated is highly specific, limited only by the selectivity of the initial nitration reaction. Dansyl groups attached to "reactive" or "exposed" tyrosyl residues which play no direct role in catalysis represent a chemical modification of enzymes that may be of considerable use in probing the motility of enzyme conformation.

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trypsin. If this split is generated but to a limited extent during the tryptic activation of dansyltyrosyl trypsinogen, a minor reorientation of the side chain of Trp 127 would be expected. A change in either distance or orientation from dansyltyrosyl residue 137 would produce a change in the efficiency of quenching of tryptophanyl fluorescence by the dansyltyrosyl residue. A small change in quenching efficiency was observed (Figure 6).

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