Changes in the Exposure of the Tyrosyl and Tryptophyl Residues in Trypsin Due to Diisopropylphosphoryl and Benzamidine Inhibition*

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ABSTRACT: The exposure of the tyrosyl and tryptophyl residues in diisopropylphosphoryl (DIP)-trypsin and in benzamidine-inhibited trypsin was investigated by means of the solvent perturbation technique of difference spectroscopy. About 0.2–0.3 additional tryptophyl residue and half to a full additional tyrosyls or their equivalents are found to be buried in the α and β forms of DIP-trypsin, relative to the same two forms of the parent enzyme. About the same degree of tryptophyl and tyrosyl burial is observed, relative to α- and β-trypsin, with benzamidine as an inhibitor. In α- and β-trypsin 4–6 of the 10 tyrosyl and 2–3 of the 4 tryptophyl residues have been found to be exposed, with the remaining groups apparently buried to the approach of solvent. The increased burial of tryptophyl and tyrosyl groups due to DIP and benzamidine inhibition is attributed to induced conformational changes at or near the active center of the enzyme or to a combination of conformational changes and direct shielding of some of the chromophoric groups by the bound inhibitors.

Phosphorylation of the active center of trypsin by diisopropyl (DIP) fluorophosphate is known to produce changes in the aromatic region of its ultraviolet absorption spectrum (Oppenheimer and Hess, 1963). Spectral changes (measured by difference spectral methods) have also been detected as a result of benzamidine inhibition of trypsin (East and Trowbridge, 1968). The additional observations, that the covalently linked DIP group introduced into the active center is found to protect some of the tryptophyl and tyrosyl residues against oxidation by *N*-bromosuccinimide (Spande *et al.*, 1966) and reaction with cyanuric fluoride (Hachimori *et al.*, 1966), indicate strongly that the active center of the enzyme is altered as a result of inhibition. During the course of activation of trypsinogen to trypsin no measurable changes have been observed in the tryptophan fluorescence (Kenner and Neurath, 1971) nor in the exposure of the tryptophyl groups (Villanueva and Herskovits, 1971). There was, however, a change in the burial of tyrosyl residues (Villanueva and Herskovits, 1971). In addition, Kenner and Neurath (1971) have observed a change in the fluorescence of a dansyltyrosyl residue during the activation of a dansyl derivative of trypsinogen and have concluded that the environment of this residue changes in the course of activation. In contrast to these observations our preliminary investigations on commercial trypsin and DIP-trypsin have suggested that inhibition of trypsin is accompanied by changes in both tryptophyl and tyrosyl exposure (Herskovits and Villanueva, 1969). The more detailed investigation presented in this paper examines the effects of DIP and benzamidine inhibition on the exposure of the tryptophyl and tyrosyl residues in the purified α and β forms of trypsin (Schroeder and Shaw, 1968). We also report the results of some related circular dichroism measurements on trypsin, on DIP-trypsin, and on trypsin in the presence of benzamidine.

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Experimental Section

Materials. The commercial trypsin and DIP derivative of trypsin (DIP-trypsin) used in this study were Worthington twice-crystallized products. Both proteins were chromatographically purified using essentially the procedures of Schroeder and Shaw (1968). In the case of the DIP-trypsins the use of pH 2.9 potassium formate in the final step of fractionation was omitted. Since the DIP moiety is unstable in acidic solutions, the use of 0.001 M HCl was also omitted. Only for the studies involving the effects of acid on the regeneration of enzymic activity of DIP-trypsin were any of the DIP-trypsin solutions exposed to pH's other than 7.1 (0.1 M Tris-HCl buffer containing 0.01 M $CaCl_2$ was used in most of our experiments). All the reagents and solvents used for the activity and other measurements were the same as those in our previous studies on trypsin (Herskovits and Villanueva, 1969; Villanueva and Herskovits, 1971).

Methods. Difference spectral measurements were made in a Cary 14 recording spectrophotometer equipped with a tenfold scale-expander accessory. The difference spectral techniques used have been fully described in previous publications (Herskovits and Laskowski, 1962; Herskovits, 1967). Protein solutions containing benzamidine were made up from acidic trypsin stock solutions of known optical density and concentration. Initially 4:1 or 9:1 volumetric dilutions were made using protein, concentrated benzamidine, $CaCl_2$, and Tris buffer to give 0.1 M pH 7.1 Tris solutions, containing from 0 to 1.0 mM benzamidine. After about 30-min incubation twofold volumetric dilutions were made using these protein solutions, 0.1 M pH 7.1 Tris buffer, and 40% ethylene glycol and glycerol containing 0.1 M Tris. DIP-trypsin solutions were similarly diluted using the various perturbants listed in Table I. Protein concentrations were based on optical density measurements on the final protein solutions containing no perturbant or benzamidine. For both trypsin and DIP-trypsin the extinction coefficients of 15.2 and 15.0 at 280–282 nm for the α and β components (Villanueva and Herskovits, 1971), respectively, were used. Circular dichroism measurements were made in a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment. Enzymatic ac-

TABLE I: A Summary of the Difference Spectral Parameters of α- and β-DIP-trypsin.[a]

Perturbant 20%[b]	Molar Absorbance Differences of DIP-trypsin		Apparent No. of Exposed Residues in DIP-trypsin		Changes in Molar Absorbance Difference Due to DIP Inhibition[c]		Groups Buried Due to DIP Inhibition of Trypsin[d]	
	$\Delta\epsilon_{290-292}$	$\Delta\epsilon_{284-286}$	Tryptophyls	Tyrosyls	$\Delta\Delta\epsilon_{290-292}$	$\Delta\Delta\epsilon_{284-286}$	Tryptophyls	Tyrosyls
				α-Trypsin				
Dimethyl sulfoxide	1135	1095	2.1	4.0	−105	−160	0.2	0.6
Ethylene glycol	715	690	2.5	3.9	−90	−115	0.2	0.3
Glycerol	690	820	2.4	5.7	−160	−150	0.3	0.6
Propylene glycol	770	700	2.6	3.9	−30	−10	0.1	0.2
Tetramethylurea	1260	1220	2.1	4.0	−50	−125	0.1	0.5
Hexaethylene glycol[e]	950	1020	1.4	5.4	−165	−175	0.2	0.6
Sucrose	345	485	1.9	(7.9)[f]	−205	−200	(0.9)[f]	(0.9)[f]
		Av values	2.1 ± 1	4.5 ± 1			0.2	0.5
				β-Trypsin				
Dimethyl sulfoxide	1200	1160	2.2	4.2	−120	−175	0.2	0.6
Ethylene glycol	780	745	2.6	4.1	−80	−150	0.1	1.1
Glycerol	700	820	2.4	5.7	−210	−185	0.5	0.7
Propylene glycol	775	760	2.6	4.0	−145	−120	0.5	0.4
Tetramethylurea	1350	1270	2.2	4.1	85	−250	−0.1	1.1
Hexaethylene glycol[e]	880	935	1.3	4.7	−290	−415	0.4	2.1
Sucrose	435	585	2.1	(8.1)[f]	−215	−140	(1.1)[f]	(1.4)[f]
		Av values	2.2 ± 1	4.5 ± 1			0.3	1.1

[a] Solvent, 0.1 M, pH 7.1 Tris buffer, 0.01 M $CaCl_2$. [b] With the exception of sucrose 20 volumes of liquid perturbant was used per 100 volumes of solutions. Sucrose solutions contained 21.6 g of solid/100 ml of solution (20%, w/w). [c] Differences between trypsin and DIP-trypsin $\Delta\epsilon_M$ values of columns 1 and 2. The trypsin data were taken from Table III of Villanueva and Herskovits (1971). [d] Differences in exposure between trypsin and DIP-trypsin values give in columns 3 and 4. The α-trypsin tryptophyl and tyrosyl values used for the perturbants listed are, respectively, 2.3 and 4.6, 2.7 and 4.2, 2.7 and 6.3, 2.7 and 4.1, 2.2 and 4.5, 1.6 and 6.0, and 2.8 and 8.8, while for β-trypsin the corresponding values are 2.4 and 4.8, 2.7 and 5.2, 2.9 and 6.4, 3.1 and 4.4, 2.1 and 5.2, 1.7 and 6.8, and 3.2 and 9.5 (Villanueva and Herskovits, 1971). [e] Carbowax 300; average number of polymerization = 6. [f] Not included in the estimates of average exposure. See text and the discussion of Villanueva and Herskovits (1971) concerning this item.

tivities were determined according to the procedure described by Hummel (1959) using *p*-toluenesulfonyl-L-arginine methyl ester as substrate.

Results

Difference Spectral Studies on DIP-trypsin. Figure 1 presents a comparison of the solvent perturbation difference spectra of α- and β-trypsin with the same two forms of the diisopropylphosphorylated enzyme isolated by essentially the same chromatographic procedures (Schroeder and Shaw, 1968). The lower amplitudes of the difference spectral maxima at the 290- to 292-nm tryptophyl and the 284- to 286-nm tyrosyl[1] peaks (data represented by the solid lines) are characteristic of the two DIP-trypsins relative to the parent enzymes. Table I presents a summary of the perturbation data of the two forms of DIP-trypsin obtained with seven perturbants. The estimates of the apparent number of exposed tryptophyl and tyrosyl residues, listed in columns 3 and 4, were obtained using the procedures of Herskovits and Sorensen (1968a,b). The same method of analysis was employed for the related α- and β-trypsin data (Villanueva and Herskovits, 1971). The model curves based on the tabulated model compound data of the *N*-acetyl ethyl esters of tryptophan and tyrosine (Herskovits and Sorensen, 1968a; Villanueva and Herskovits, 1971) were plotted and compared to the protein curves (Figures 2 and 3) according to the relationship

$$\Delta\epsilon(\text{protein}) = a\Delta\epsilon_\lambda(\text{Trp}) + b\Delta\epsilon_\lambda(\text{Tyr}) \quad (1)$$

where a represents the number of exposed tryptophyl residues, b the number of exposed tyrosyls, and $\Delta\epsilon_\lambda$(Trp) and $\Delta\epsilon_\lambda$(Tyr), respectively, the molar absorbance difference values of free tryptophan and tyrosine as a function of wavelength, designated by the subscript, λ. The problems involved in the fitting and interpretation of protein and model compound data have been fully described in previous publications from our laboratory (Herskovits and Sorensen, 1968a,b). The loss and obliteration of difference spectral detail of the tryptophyl and tyrosyl maxima and minima around 290–292, 284–286, and 288–289 nm often observed with protein curves such as in Figures 2 and 3, relative to the much steeper maxima and minima of model curves (data represented by dotted lines), have been attributed to the presence of partly exposed chromophoric groups and to the heterogeneity of the amino acid

[1] Actually in a protein both tyrosyl and tryptophyl groups contribute to the difference spectra at this wavelength. The maximum, however, is due to the tyrosyl difference spectrum which peaks at this wavelength region.

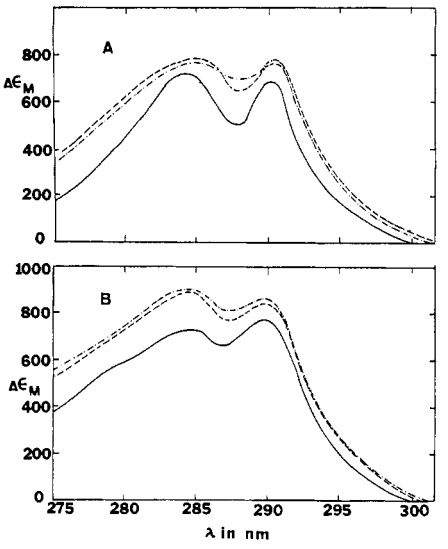

FIGURE 1: A comparison of the solvent perturbation difference spectra of α- and β-trypsin with the DIP derivatives, α- and β-DIP-trypsin, obtained with 20% ethylene glycol as perturbant. (A) ———, α-DIP-trysin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; -·-·-·-, α-trypsin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; – – – – –, α-DIP-trysin after 30-min exposure to pH 3.1 followed by neutralization, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$. (B) ———, β-DIP-trypsin, pH 7.1, 0.01 M $CaCl_2$; -·-·-·-·-, β-trypsin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; ------, β-DIP-trypsin after 30-min exposure to pH 3.1 followed by neutralization at pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$. Protein concentration 2.3×10^{-5} to 4.6×10^{-5} M.

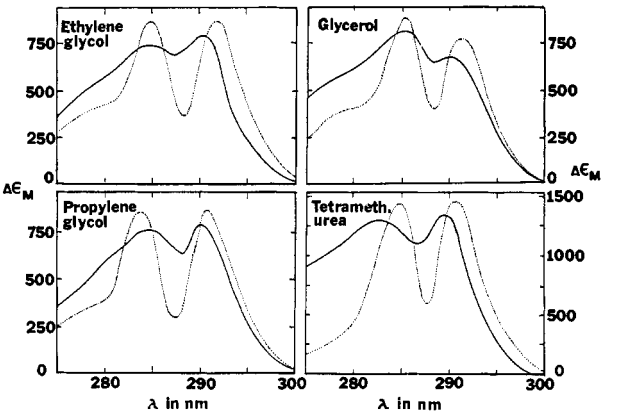

FIGURE 2: A comparison of the solvent perturbation difference spectra of β-DIP-trypsin with the calculated model curves based on 20% ethylene glycol, glycerol, propylene glycol, and tetramethylurea data. The solid lines represent the protein data while the dotted lines represent the calculated curves based on eq 1 of the text and the best-fit values of the *a* and *b* parameters. These values are listed in columns 3 and 4 of Table I as apparent number of exposed tryptophyl and tyrosyl residues. The protein concentrations used ranged from 2.3×10^{-5} to 4.2×10^{-5} M.

environment of exposed groups (Herskovits, 1967; Herskovits and Laskowsi, 1962; Herskovits and Sorensen, 1968b).

The $\Delta\epsilon_\lambda$ values of free tryptophan at the 291- to 293-nm tryptophyl maximum obtained with the various perturbants of this study range from 192 to 569 (Herskovits and Sorensen, 1968b; Villanueva and Herskovits, 1971). Thus the observed decrease in $\Delta\epsilon_\lambda$ for the two DIP-trypsins relative to trypsin, at 290–292 nm, ranging from −30 to −290 represent the burial of about 0.2–0.5 of a tryptophyl residue or its equivalent, assuming that the contribution from the exposed tyrosyls can be neglected at this wavelength. With both the tryptophyl and tyrosyl contributions taken into account, about the same estimates of tryptophyl burial are obtained. The average estimates of tryptophan burial obtained with six perturbants, leaving out sucrose, are 0.2 and 0.3 tryptophyl and 0.5 and 1.1 tyrosyls, respectively, for the α and β forms of the enzyme. These estimates, given in the last two columns of Table I, represent the differences between the apparent number of tryptophyl and tyrosyl groups exposed in the two trypsins and corresponding DIP-trypsins. The trypsin values used for these estimates were taken from our previously published results (Villanueva and Herskovits, 1971). These values are listed in footnote *d* of this table while the DIP values are given in columns 3 and 4. The sucrose values given in parentheses in the last two rows of this table have not been included in the final averages because of their relatively high values. The possible reasons for the high absorbance difference values have been discussed in the previous paper dealing with the solvent perturbation of trypsin.

Both the enzymatic activity and the perturbation parameters characteristic of the two purified forms of trypsin can be regenerated by exposure of the DIP-trypsins to acidic pH's (pH 3.0–3.1) for relatively brief periods of time. This is clearly shown by the data of Figure 1 represented by the dashed lines and the difference spectral and activity data of the DIP-trypsins relative to the parent enzymes, following 30-min exposure to pH's 3.0–3.1 and neutralization to pH 7.1. The latter results are compared in Table II.

Effects of Benzamidine Inhibition. The effects of benzamidine on the solvent perturbation difference spectra of trypsin are qualitatively similar to the effects of DIP inhibition. Above the

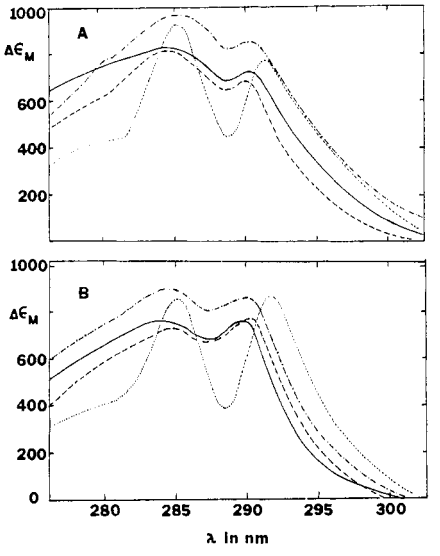

FIGURE 3: A comparison of the effects of benzamidine and DIP on the solvent perturbation difference spectra of α- and β-trypsin. (A) ———, α-trypsin, 0.2 mM benzamidine; -·-·-·-, α-trypsin alone; – – – – –, α-DIP-trypsin; . . . . . , model curve calculated for 2.4 exposed tryptophyls and 5.7 tyrosyls. Perturbant, 20% glycerol. (B) ————, β-trypsin, 0.2 mM benzamidine; -·-·-·-·-, β-trypsin alone; – – – – –, β-DIP-trypsin; . . . . . , model curve calculated for 2.6 exposed tryptophyls and 4.1 tyrosyls. Perturbant, 20% ethylene glycol. The protein curves obtained in the presence of benzamidine were corrected for benzamidine contribution to the difference spectra (see text and Figure 4). All the solutions contained 0.1 M pH 7.1 Tris buffer and 0.01 M $CaCl_2$. The protein concentrations ranged from 2.0×10^{-5} to 4.8×10^{-5} M.

TABLE II: Effects of Exposure to Acidic pH of α- and β-DIP-trypsin.[a]

pH Conditions and Treatment of Solutions	Molar Absorptivity Differences Due to 20% Ethylene Glycol $\Delta\epsilon_{290-292}$	$\Delta\epsilon_{285-286}$	Enzyme Act.[b]
DIP-α-trypsin, pH 7.1	715	690	0
DIP-α-trypsin at pH 7.1 after 30-min exposure to pH 3.0	775	775	272
α-Trypsin, pH 7.1	805	770	295
α-Trypsin, pH 3.1	810	770	292[c]
DIP-β-trypsin, pH 7.1	730	720	0
DIP-β-trypsin at pH 7.1 after 30-min exposure to pH 3.0	845	885	438
β-Trypsin, pH 7.1	860	895	440
β-Trypsin, pH 3.1	840	900	438[c]

[a] Solvent, 0.1 M, pH 7.1 Tris buffer, 0.01 M $CaCl_2$. [b] Activities expressed in micromoles of *p*-toluenesulfonyl-L-arginine methyl ester hydrolyzed per minute per milligram of enzyme. [c] Assayed at pH 7.1 after exposure to pH 3.0–3.1 followed by neutralization.

saturation levels of 0.1–0.2 mM benzamidine the molar absorbance difference values at the 290- to 292-nm tryptophyl and the 284- to 286-nm tyrosyl peaks, corrected for benzamidine contribution, are reduced by about 13–50% (Figure 3). Since benzamidine has a slight to moderate difference spectrum in the 284- to 292-nm region of interest (Figure 4B), all the difference spectra obtained in the presence of benzamidine were corrected by subtracting the benzamidine difference spectrum from each of the protein curves. Figure 4 presents some of the difference spectra of trypsin plus benzamidine (curves in Figure 4A), benzamidine alone (Figure 4B), and the corrected protein difference spectra obtained at several benzamidine concentrations (Figure 4C). The perturbant used for these experiments was 20% ethylene glycol. Figure 5 shows the effects of increasing benzamidine concentration on the corrected $\Delta\epsilon_M$ values for β-trypsin at 285 and 290 nm, obtained with 20% ethylene glycol. A summary and analysis of the difference spectral data obtained on the α and β forms of trypsin in the presence of benzamidine similar to our DIP-trypsin results are given in Table III. These results are based on data obtained with both 20% ethylene glycol and glycerol as perturbants (Figures 3 and 4).

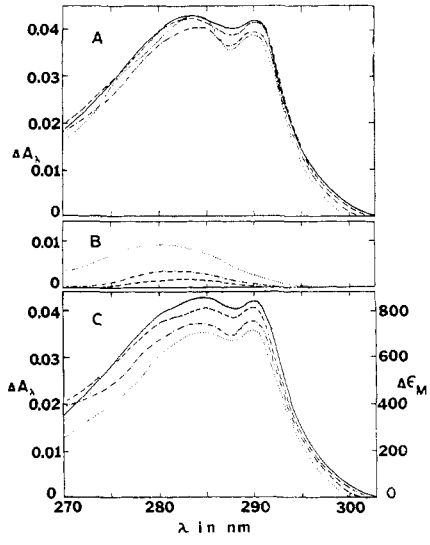

FIGURE 4: The effects of benzamidine on the solvent perturbation difference spectra of β-trypsin obtained with 20% ethylene glycol as perturbant. (A) Uncorrected difference spectra obtained with 0–0.3 mM benzamidine. (B) Difference spectra of 0.05–0.3 mM benzamidine. (C) Corrected protein difference spectra (curves of panel A minus curves of B of the same benzamidine concentration). Benzamidine concentration: ———, 0 M; – – – – –, 0.05 mM; – · – · – · –; 0.1 mM; . . . . . , 0.3 mM. All the solutions were buffered by 0.1 M pH 7.1 Tris buffer and contained 0.01 M $CaCl_2$. Protein concentration used was 4.8×10^{-5} M.

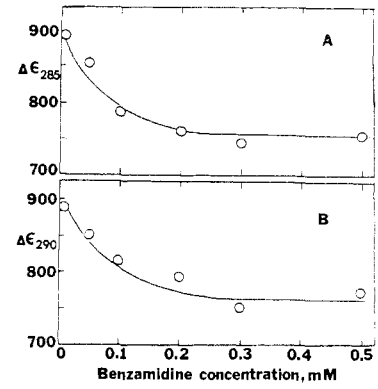

FIGURE 5: The effects of benzamidine concentration of the corrected absorbance difference values of β-trypsin, $\Delta\epsilon_{285}$ and $\Delta\epsilon_{290}$, obtained with 20% ethylene glycol as perturbant. The protein concentration used was 4.8×10^{-5} M.

Circular Dichroism Studies. The CD spectra of β-trypsin obtained in the far- and near-ultraviolet absorption region are shown in Figure 6A,B. The mean residue ellipticity of β-trypsin at the 209- to 210-mμ trough is -4320 (deg cm^2)/dmole. Diisopropylphosphorylation or benzamidine inhibition of the active center of the enzyme seems to have little or no effects on the observed CD spectra in the peptide-absorbing far-ultraviolet region (Figure 6A). The mean residue ellipticity of α-trypsin at 209–210 nm was found to be very nearly the same as that of the β form of the enzyme, -4215 (deg cm^2)/dmole. Again DIP or benzamidine inhibition was found to have little or no effect on the CD spectra (Villanueva, 1972).

Discussion

The results obtained with the two forms of DIP-trypsin, summarized in Table I, indicate that on an average about 0.2–0.3 tryptophyl and about a half to a full tyrosyl residue or its equivalent become buried as the result of the blocking of the active site of the enzyme by the added DIP group. In the two parent enzyme fractions about 4 to 6 of the 10 tyrosyl residues and 2 to 3 of the 4 tryptophyls have been found to be exposed to the various perturbants of this study (Villanueva and Herskovits, 1971). The studies of Spande *et al.* (1966) with *N*-bromosuccinamide as a tryptophan modifying reagent have shown about the same degree of protection of the tryptophyl groups in DIP-trypsin relative to the parent enzyme as our results. DIP-trypsin is also found to be less reactive toward the tyrosine specific reagent, cyanuric fluoride (Hachimori

TABLE III: Effects of Benzamidine (0.2 mM) on the Difference Spectral Parameters of α- and β-Trypsin.[a]

	Molar Absorbance Differences of Benzamidine-Inhibited Trypsin[b]		App No. of Exposed Residues in Benzamidine-Inhibited Trypsin		Changes in Molar Absorbance Difference Due to Benzamidine Inhibition[c]		Groups Buried Due to Benzamidine Inhibition[d]	
Perturbant 20%	$\Delta\epsilon_{290-292}$	$\Delta\epsilon_{284-286}$	Tryptophyls	Tyrosyls	$\Delta\Delta\epsilon_{290-292}$	$\Delta\Delta\epsilon_{284-286}$	Tryptophyls	Tyrosyls
				α-Trypsin				
Ethylene glycol	690	655	2.3	3.4	−115	−115	0.4	0.8
Glycerol	730	830	2.4	5.7	−120	−140	0.3	1.2
				β-Trypsin				
Ethylene glycol	760	770	2.5	4.2	−100	−125	0.2	1.1
Glycerol	705	850	2.3	5.3	−205	−155	0.6	1.1

[a] Solvent, 0.1 M, pH 7.1 Tris, 0.01 M $CaCl_2$ plus 0.2 mM benzamidine except in the case of β-trypsin–20% ethylene glycol data where the saturation values based on the 0.2–0.5 mM benzamidine results were used (see Figure 5). [b] Corrected for benzamidine contribution (see text and Figure 4 for detail). [c] Difference between trypsin and trypsin–benzamidine values of columns 2 and 3. Trypsin data taken from Table III of Villanueva and Herskovits (1971). [d] Differences between trypsin and benzamidine-inhibited trypsin values of columns 3 and 4. The α-trypsin values are 2.7 and 4.2 and 2.7 and 6.3 tryptophyls and tyrosyls for 20% ethylene glycol and glycerol, respectively. The values for β-trypsin are 2.7 and 5.2 and 2.9 and 6.4 for the same two perturbants (Villanueva and Herskovits, 1971).

et al., 1966). A maximum of 6 tyrosyls is modified in trypsin by this reagent, while at most only about 4 groups are found to be modified in DIP-trypsin.[2] While no related chemical modification studies have been reported with benzamidine as inhibitor, it is significant that nearly the same number or fraction of tryptophyl and tyrosyl residues are found to be blocked to solvent access by both the covalently linked DIP groups and the noncovalently bound inhibitor, benzamidine (see Figure 3 and Table III).

Changes in the burial of tryptophyl and tyrosyl residues may be attributed to one of two factors: the direct blocking or shielding of these groups from solvent access by the inhibitor, or by induced conformational changes in the enzyme associated with the flexibility of the active site (Koshland, 1958). It is also possible that a combination of both of these effects are operative during the course of the initial binding step of the enzyme–inhibitor or enzyme–substrate complex formation. While we are not in a position to present an unequivocal answer favoring one of these three possibilities, our findings that nearly a full tyrosyl and a part of a tryptophyl group is buried due to the introduction of the DIP group and the binding of benzamidine would argue in favor of the latter two possibilities. In this connection it is important to note that DIP and benzamidine groups are comparable in size to a tyrosyl side chain. Thus it is unlikely that the burial of a full tyrosyl group and a fraction of a tryptophyl could be affected in β-trypsin by the binding of the inhibitor alone. This contention is also supported by the fact that actually two tyrosyl residues seem to be protected by the introduced DIP group in trypsin against cyanuric fluoride (Hachimori *et al.*, 1966).

[2] Five to six of the ten tyrosyl residues in trypsin are also reactive toward tetranitromethane (Kenner *et al.*, 1968) and are found to ionize rapidly and reversibly (Smillie and Kay, 1961; Inada *et al.*, 1964; Lazdunski and Delaage, 1965). A more detailed discussion and comparison of the chemical reactivity, spectrophotometric titration, and solvent perturbation data of trypsin and trypsinogen is presented in our previous paper (Villanueva and Herskovits, 1971).

Several recent investigations have suggested that the active center of trypsin contains a hydrophobic binding site (Mares-Guia and Shaw, 1965; Heidberg *et al.*, 1967; Seydoux *et al.*, 1969; Mares-Guia and Figueiredo, 1970). The burial of tyrosyl and tryptophyl side chains by these inhibitors while consistent with this interpretation does not preclude the possibility of changes in the conformation of the enzyme prior to, or as a result of, hydrophobic interactions between these side chains and the introduced DIP group or benzamidine. Other evidence concerning the possibility of inhibitor-induced conformational changes is suggested by the DIP- and benzamidine-induced difference spectra of trypsin in the aromatic absorption region (Oppenheimer and Hess, 1963; East and Trowbridge, 1968). However, no significant changes in the ORD and the CD spectra of trypsin have been found in the peptide-absorbing, far-ultraviolet region (Figure 6A; Villanueva, 1972) due to DIP or benzamidine binding. Thus the structural changes induced by these inhibitors must be relatively limited, probably in-

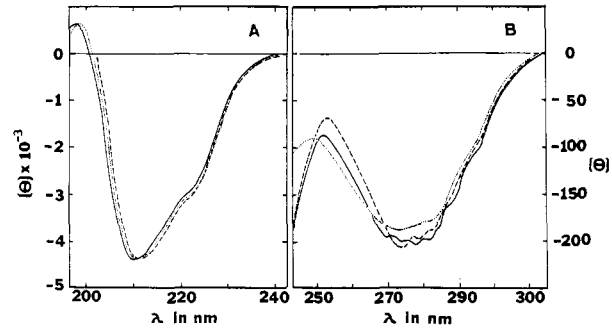

FIGURE 6: Circular dichroism spectra of β-trysin in the far-ultraviolet region (A) and near-ultraviolet, aromatic region (B). ———, β-trypsin; – – – –, β-trypsin in the presence of 0.2 mM benzamidine; . . . . . , β-DIP-trypsin. All the solutions were buffered by 0.1 M pH 7.1 Tris buffer and contained 0.01 M $CaCl_2$. The protein concentrations used ranged from 0.04 to 0.1%.

volving none of the helical or β-structured regions of the enzyme.[3]

Perhaps the most convincing evidence concerning the flexibility and adaptability of the active site is suggested by the fact that the conformations of the α and β forms of the enzyme are found to be slightly different (Villanueva and Herskovits, 1971), while the conformations of the DIP- and the benzamidine-inhibited enzymes appear to be almost identical (Table I and Figure 3). In α- and β-trypsin the average exposure of tyrosyls obtained with 6 of the perturbants used in this study is 5.0 and 5.5 groups, respectively (Villanueva and Herskovits, 1971), while in the two forms of the DIP-enzyme the average exposure of tyrosyls is found to be the same. In both the α and β forms of the DIP-inhibited enzyme an average of 4.5 tyrosyls or their equivalents is exposed to solvent (Table I). However, it is important to note that both enzyme forms have essentially identical ORD and CD spectra, with DIP and benzamidine having little or no effect on the 230-nm ORD and the 209- to 210-nm CD troughs (Villanueva and Herskovits, 1971; Villanueva, 1972; and Figure 6), suggesting also identical or nearly identical folding and structural organization of the polypeptide chains. The latter results underline the fact that the conformational differences between the two forms of the enzyme must be relatively small and subtle.

[3] The possibility of compensating structural changes due to the inhibition of trypsin should of course be entertained. For example, the conversion of a helical or β segment of the enzyme to the unordered or random conformation may perhaps be compensated for by the simultaneous conversion of an unordered segment to helical or β structure in another part of the enzyme. Such structural changes should leave the ORD and CD spectra of the enzyme unaltered.

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