Flexibility in the Specificity Site of Serine Proteases†

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ABSTRACT: The statistical availability of tryptophan and tyrosine residues with one ring face fully exposed to solvent was examined for two serine proteases and their derivatives by investigating the formation of charge transfer (CT) complexes between the aromatic donor residues of the protein and the acceptor 1-methylnicotinamide chloride. The availability of the ring face of one of the two exposed tryptophan residues in trypsin has been previously shown to be pH dependent and to parallel the acid side of the pH–activity profile of the enzyme. The present results indicate that, in diisopropylphosphoryltrypsin (DIP-trypsin), this residue [which was identified as Trp-215 in native trypsin (chymotrypsin numbering)] is locked in a relatively rigid, pH-independent conformation with one ring face rotated out toward the solvent. In the zymogen and DIP-zymogen, the ring face is essentially unavailable. Chymotrypsin, like trypsin, has a pH-dependent tryptophan residue available for complexation with the CT acceptor, but unlike trypsin, the pH dependence is apparently associated with dimerization of the enzyme. These and other data suggest this residue is the same as in the homologous trypsin structure, i.e., Trp 215, and that the ring face is mostly buried in the zymogen. Comparison of the crystal structure models of chymotrypsin and chymotypsinogen shows that, as the specificity pocket opens up from its collapsed structure upon zymogen activation, the ring face of Trp-215 moves out and rotates relative to the surface of the enzyme in such a fashion as to become more accessible to solvent. These observations are in accord with the present CT results and provide additional support for the assignment of changes in Trp-215 availability to parallel changes in the conformation of the specificity pocket of these serine proteases. The present investigation also shows that, although a tryptophan ring face is partly exposed in DIP-chymotrypsin, its statistical availability more closely resembles that of the zymogen than the native enzyme. The reverse appears to be true for DIP-trypsin, which suggests the possibility that the specificity pocket in DIP-chymotrypsin may be partially collapsed while the catalytic residues are frozen in the conformation of the acyl-enzyme.

Conformational flexibility in the active site regions of enzymes has frequently been mentioned as a factor in the control of substrate binding and subsequent catalysis. In chymotrypsin (and probably in other serine proteases as well) the charge relay system responsible for the actual catalytic step is intact and apparently functional in both the enzyme and its zymogen, and the much higher activity of the enzyme is primarily the result of conformational changes which allow the formation of a viable specificity pocket (Freer et al., 1970; Morgan et al., 1972; Fersht and Sperling, 1973; Wright, 1973a,b; Porubcan et al., 1975). Less well understood are conformational rearrangements associated with the accommodation of specific substrates and inhibitors by the active enzymes and with the modulation of activity by changes in pH or ionic strength. Small differences in the arrangement of a number of noncatalytic residues in the active site region of DIP-[1] and BA-inhibited trypsin crystals have been observed by Krieger et al. (1974), and significant pH-related changes in conformation have been noted in single crystals of α-chymotrypsin by Tulinsky and co-workers (Tulinsky et al., 1973; Vandlen and Tulinsky, 1973; Mavridis et al., 1974).

We have recently used a charge transfer (CT) method to examine the availability of aromatic donor residues on bovine trypsin in solution, and found a variation in the average exposure of one of the two accessible tryptophan side chains which parallels the pH–activity profile of the enzyme (Deranleau et al., 1975). The pH-dependent residue was identified as Trp-215, whose peptide bond forms a portion of one side of the specificity pocket in both trypsin and in chymotrypsin. Thus, minor variations in the conformation of this residue and/or its surrounding residues may be correlated with the integrity of the substrate binding site in solution. In the current study we pursue our investigations on the availability of aromatic donor residues in serine proteases, with particular emphasis on monitoring changes in the exposure of Trp-215. The interaction of aromatic donor residues with the CT acceptor 1-methylnicotinamide chloride (MNCl) is used to compare the average exposure of the surface tryptophan side chains of trypsin, DIP-trypsin, DIP-trypsinogen, chymotrypsin, DIP-chymotrypsin, and chymotrypsinogen over a wide pH range. Chemical modification reactions utilizing *N*-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNB), as well as crystallographic data, are used to help identify the tryptophan binding sites. Since crystallographic data on trypsinogen are not yet available, it is of particular interest to note here variations in the solution structures of the zymogen and the active enzyme, and their respective DIP derivatives.

Experimental Section

Materials. Bovine trypsin, twice crystallized (No. TRL 2JX), trypsinogen, once crystallized (No. TG 7JH and Tg 6423), α-chymotrypsin (No. CDI 8LK), and chymotrypsinogen A were purchased from Worthington Biochemical Corp. The latter protein had an activity of 47 units/mg toward *N*-

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[1] Abbreviations used: BA, benzamidine; CT, charge transfer; DIP, diisopropylphosphoryl; DFP, diisopropyl phosphorofluoridate; HNB, 2-hydroxy-5-nitrobenzyl bromide; MNCl, 1-methylnicotinamide chloride (1-methyl-3-carbamidopyridinium chloride); NBS, *N*-bromosuccinimide. The chymotrypsin numbering scheme is used throughout the text to identify amino acid residues.

benzoyl-L-tyrosine ethyl ester after activation. *N*-Acetyl-L-tryptophanamide was a Sigma preparation. Diisopropyl fluorophosphate and 2-hydroxy-5-nitrobenzyl bromide were products of Pierce Chemical Co. *N*-Bromosuccinimide was purchased from Eastman Organics and recrystallized before use. Nicotinamide from J. T. Baker Chemical Co. was converted into 1-methylnicotinamide chloride as described by Karrer et al. (1936). All buffers were prepared from reagent grade chemicals.

DIP-Trypsin was prepared by reacting bovine trypsin (5 mg/ml) with 0.01 M DFP in 0.1 M Tris-HCl buffer containing 0.05 M $CaCl_2$ at pH 8.0. After 1 h, less than 1% of the initial activity (230 units/mg) remained, as measured by TAME hydrolysis, and the preparation was acidified, desalted on Sephadex G-25, and then lyophilized.

DIP-Trypsinogen was prepared following the procedure described by Morgan et al. (1972). After reaction the activatability of the modified trypsinogen was checked with TAME and was less than 35 units/mg, compared with a minimum activatability of 190 units/mg for the unmodified zymogen. The preparation was acidified and extensively dialyzed against 0.001 M HCl.

DIP-Chymotrypsin was prepared according to the procedure of Balls and Jensen as described by Laskowski (1955) and was found to have less than 1% of the initial activity of 45 units/mg (toward *N*-benzoyl-L-tyrosine ethyl ester) before use.

NBS oxidized proteins were prepared by a modification of general procedure described by Spande et al. (1966), adding NBS solutions slowly to 10 mg/ml protein samples such that the final NBS/protein mole ratio was 5:1. For trypsinogen and DIP-trypsin, reaction at pH 5.0, 4 °C, resulted in the oxidation of 2.5 tryptophan residues. For chymotrypsin and chymotrypsinogen, reaction at pH 5.5 resulted in the oxidation of 2.2 tryptophan residues. Charge transfer titrations were then carried out directly on these derivatives in acetate buffer between pH 5.0 and 6.0.

HNB-Trypsinogen was prepared according to the procedure described by Keil-Dlouha and Keil (1972) for specific modification of Trp-215 at pH 3.1. After reaction, the modified protein was desalted on Sephadex G-25 column equilibrated with 0.001 M HCl and concentrated by ultrafiltration. The number of moles of HNB per mole of protein was determined by measuring the concentration of HNB spectrophotometrically at pH 12 (ϵ_{410} 18 000 cm^2/mmol) following the procedure of Horton and Koshland (1965). Alternately, protein concentrations were determined from Rayleigh interference patterns using a synthetic boundary cell in the ultracentrifuge, assuming a refractive index increment of 0.00185 dl g^{-1} at 5464 Å. The mole ratio of HNB to trypsinogen measured was 0.89 to 1. HNB-tryptophan was prepared from *N*-acetyl-L-tryptophanamide following the general procedure of Horton and Koshland (1965) and after centrifugation was used directly in charge transfer studies. The HNB-modified tryptophan residue has a pH-dependent visible absorption band (Koshland et al., 1964), which shifts from ϵ_{max} ~9000 at 320 nm, pH 3.0, to ϵ_{max} 18 900, pH 12.0. Above pH 4.0 this absorption begins to interfere with the observation of the tryptophan-MNCl charge transfer bands. The charge transfer spectra of HNB-trypsinogen plus MNCl and HNB-tryptophan plus MNCl were, therefore, both measured at a constant pH of 3.3 and compared with the spectrum of native trypsinogen plus MNCl under identical conditions. The charge transfer properties of unmodified trypsinogen (vide infra) and tryptophan (Hinman et al., 1974) do not vary significantly from pH 3.0 to 9.0.

Methods. Binding studies were carried out following the constant salt titration method described previously (Deranleau et al., 1975; Hinman et al., 1974). Briefly, the procedure involves monitoring the appearance of charge transfer absorption bands in MNCl–protein mixtures as a function of MNCl concentration from 550 to 300 nm on a Cary 15 spectrophotometer at 25 °C. The individual MNCl–protein solutions were made by mixing, immediately prior to the measurement, cold, dialyzed protein (in 10^{-3} M HCl) with various aliquots of 2 M MNCl and 2 M KCl (in 0.1 M buffers containing 0.05 M $CaCl_2$), such that in the final mixtures the MNCl concentrations varied from 0.02 to 1.0 M but the total MNCl + KCl concentration remained at 1 M. Difference spectra were recorded using MNCl concentrations of 0.5 or 1.0 M in either 0.5 or 1.0 cm path length double tandem cells. Protein concentrations (generally 10 mg/ml) were determined spectroscopically using published extinction values (Walsh and Wilcox, 1970). For studies at pH 8.0, Tris-HCl buffer was used and at lower pH's either a citric acid–phosphate or acetate buffer was chosen.

Results of the titration experiments were analyzed in terms of double-intercept (Scatchard-type) plots of $\bar{\epsilon}/[X]$ vs. $\bar{\epsilon}$, where $\bar{\epsilon}$ is the statistical average extinction coefficient of the mixture (Deranleau, 1975) and [X] is the concentration of free acceptor (very nearly equal to the total acceptor concentration in these experiments). The statistical average extinction coefficient is experimentally equivalent to the observed CT absorbance A divided by the total protein or donor concentration $[P_0]$, and in the case where several classes of sites are being simultaneously titrated (e.g., tryptophan and tyrosine side chains) is given by the expression

$$\bar{\epsilon} = \sum_i \bar{\epsilon}_i = \sum_i n_i \epsilon_i k_i [X]/(1 + k_i[X]) = A/[P_0]$$

Here i is the class of site being observed, n_i is the integral number of titratable residues belonging to that class, and k_i is the association constant for the interaction. The association constants for model donor–MNCl interactions range from 2.1 to 4.7 cm^3/mmol for Trp–MNCl complexes and from 0.55 to 1.2 cm^3/mmol for Tyr–MNCl complexes, depending on the charge on the donor (Hinman et al., 1974). If the association constants obtained with proteins fall below those expected for homomorphic model complexes, we assume this to be the result of hindered collision between the donor side chain and the CT probe, those residues with extremely low k values being essentially unavailable (Coan et al., 1975).

When the association constants are small, the maximum obtainable saturation fraction $s_i = k_i[X]/(1 + k_i[X])$ is low and consequently very little information (in bits) is available from the binding study (Deranleau, 1969). In such cases, an approximate method utilizing a *linear* fit to the (curved) double-intercept plot was used to obtain estimates of the required parameters (Deranleau, 1975). Although linear fitting is equivalent to the assumption of a single set of identical sites, the spectral distribution of the CT absorbance is such that Trp–MNCl interactions can be estimated independently, and Tyr–MNCl interactions can either be obtained by difference techniques or by chemical destruction of the exposed Trp side chains (Hinman et al., 1974).

The following notation is used to distinguish the various parameters of the double-intercept plots:
High saturation intercept

$$\lim_{[X]\to\infty} \bar{\epsilon} = \sum_i n_i \epsilon_i$$

Table I: Estimated Number of Exposed Tryptophyl Residues from Linearly Fitted Double Intercept Plots of CT Data at 400 nm.[a]

Protein	Low Satn Intercept[b] $\sum_i n_i\epsilon_i k_i$	Apparent Slope k_{app}	Apparent High Satn Intercept $(n\epsilon)_{app}$	Estimated No. Trp Residues[c]	pH Variation pH 3.0–9.0
Trypsin, pH 8.0	2040	3.0	680 ± 80	2.0	Yes
pH 4.0	1500	2.5	600 ± 100	1.8	
DIP-Trypsin	1820	2.4	760 ± 50	2.2	No
Trypsinogen	720	3.0	240 ± 50	0.7	No
DIP-Trypsinogen	580	3.7	250 ± 60	0.7	No
α-Chymotrypsin, pH 8.0	1080	3.0	360 ± 40	1.1	
pH 4.0	150	~0.5	300 ± 200	(0.9)	Yes
DIP-Chymotrypsin	165	~0.5	330 ± 200	(1.0)	No
Chymotrypsinogen A	195	~0.6	280 ± 200	(1.0)	No

[a] At pH 8.0 unless otherwise indicated. [b] Values in this column have been obtained very near to the real intercept, and are thus not dependent on the method of curve fitting. [c] Calculated from the values in the preceding column, assuming $\epsilon_{Trp} = 340$ cm^2/mmol at 400 nm (see Deranleau et al., 1975). Values in parentheses are subject to potentially large errors because of the small total CT absorbance; compare, for example, the low saturation intercepts in the second column.

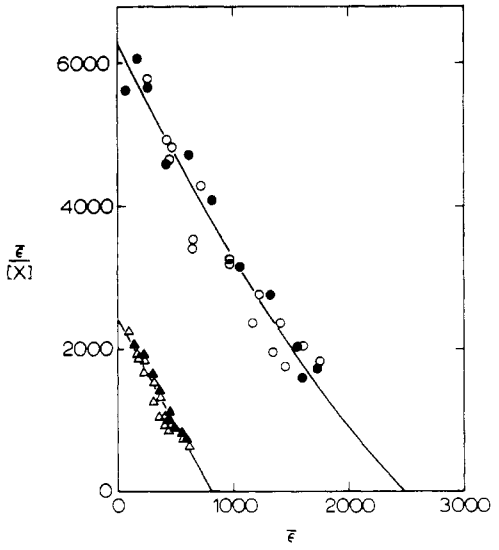

FIGURE 1: Double intercept plots of 350-nm CT data for trypsin (○), DIP-trypsin (●), trypsinogen (△), and DIP-trypsinogen (▲) at pH 8.0. Solid lines are calculated from the parameters given in Table III and the extinction coefficients determined from model complexes by Hinman et al. (1974).

Low saturation intercept

$$\lim_{[X]\to 0} \bar{\epsilon}/[X] = \sum_i n_i\epsilon_i k_i$$

Slope of the plot

$$\frac{d(\bar{\epsilon}/[X])}{d\bar{\epsilon}} = \frac{\sum_i n_i\epsilon_i k_i^2(1 + k_i[X])^{-2}}{\sum_i n_i\epsilon_i k_i(1 + k_i[X])^{-2}}$$

For a single class of identical and independent sites (straight line fit, whether as an actual fit of the data or as an approximation), the sums drop out, and the intercepts and slope are respectively $nk\epsilon$, $n\epsilon$, and k.

Results

Initial Estimates of the Numbers of Exposed Tryptophan Residues. All of the proteins studied here showed a CT absorbance band in the long wavelength region of the CT spectrum where tyrosine contributions are negligible. Double-intercept plots of the CT titration data were initially analyzed by a linear fitting procedure, and the numbers of exposed tryptophan residues were determined as described by Deranleau et al. (1975) for trypsin. The results at 400 nm are shown in Table I. It is apparent that, while DIP-trypsin (like trypsin itself) has two tryptophan residues which are sufficiently exposed to interact with MNCl, all of the other proteins in the table have only a single available tryptophan residue. Also, the small apparent slopes (association constants) and low saturation intercepts of the last three entries in the table strongly suggest that in these cases the exposed tryptophan residue is statistically much less available than it is in the other proteins listed.

Trypsin and DIP-Trypsin. The charge transfer properties of bovine trypsin have been previously investigated in detail from pH 3.0 to 9.0 (Deranleau et al., 1975). Two Trp residues and about five Tyr residues are available on the native enzyme at all pH values studied, and an analysis of the *curved* double-intercept plots according to a system of three independent classes of sites gave the following intrinsic association constants for the donor–MNCl interactions: Trp-215 (pH dependent), $k = 0.7$ cm^3/mmol at pH 4.0, 2.3 at pH 8.0; Trp-237 (tentative identification), $k = 3.8$ at both pH 4.0 and 8.0; Tyr (five unidentified residues considered as a single class), $k = 0.87$ at pH 4.0 and 8.0.

The CT data for DIP-trypsin are indistinguishable from the trypsin data at all wavelengths at pH 8.0, as shown, for example, in the double-intercept plot of the 350-nm data in Figure 1, suggesting that the tryptophan and tyrosine availability is virtually identical in the two proteins. In fact, the DIP-trypsin data can be fitted using the same parameters as for trypsin. A comparison of the observed CT spectrum of DIP-trypsin with a spectrum calculated on the basis of the numbers of residues, association constants, and extinction coefficients deduced for native trypsin (Deranleau et al., 1975) is shown in Figure 2. An independent check on the similarity of tyrosine exposure between the two proteins was made by titrating NBS-oxidized DIP-trypsin with MNCl. The CT spectrum of NBS-oxidized DIP-trypsin was found to be in close correspondence with that of NBS-oxidized trypsin.

Unlike the charge transfer properties of native trypsin, the CT properties of the DIP-trypsin–MNCl complex are constant from pH 3.0 to 9.0 and the spectrum shown in Figure 2 remained unchanged throughout this pH range.

Trypsinogen and Derivatives. From the preliminary com-

Table II: Linear Fitting Parameters at 350 nm (pH 8.0) for Proteins with a Single MNCl-Available Tryptophan Residue, Assuming the Tyrosine Contribution to be Negligible.

Protein	Slope, k (cm^2/mmol)	Intercept ϵ (cm^3/mmol)
Trypsinogen	3.0 ± 0.3	815 ± 70
DIP-Trypsinogen	3.2 ± 0.3	780 ± 70
DIP-Chymotrypsin	0.62 ± 0.13	840 ± 180
Chymotrypsinogen A	0.70 ± 0.10	790 ± 180

parison of charge transfer data presented above (see Table I), it is clear that the number of tryptophan residues on trypsinogen is about half that for trypsin and DIP-trypsin, and the apparent extinction coefficient indicates that, at most, one residue is available on the zymogen for complexation with MNCl. To provide an independent measure of the tyrosine–MNCl interaction on trypsinogen, the charge transfer properties of NBS-oxidized trypsinogen were investigated. The increase in absorbance observed upon addition of MNCl to solutions of NBS-oxidized trypsinogen was barely distinguishable from baseline error and scatter, such that within the limitations of the present method it appears that the tyrosine contribution to the trypsinogen charge transfer spectrum is zero.

The long wavelength titration data, indicating at most one available tryptophan, and the data on NBS-oxidized trypsinogen, indicating no available tyrosine residues, suggest that a simple one site binding model is appropriate. The double-intercept plot can thus be suitably analyzed by linear methods. The double-intercept plot of the trypsinogen data at 350 nm is compared with those of trypsin and DIP-trypsin in Figure 1 and the least-squares slope and high saturation intercept are presented in Table II. The full spectrum is given in Figure 2. At wavelengths higher than 350 nm, the extinction values for trypsinogen reproducibly fall slightly below that for an average tryptophan–MNCl complex. Similar variations in the spectral distribution of the CT bands have been observed among model MNCl complex spectra as a function of solvent environment in both aqueous media and in guanidine hydrochloride (Hinman et al., 1974; Coan et al., 1975) and the trypsinogen–MNCl complex could involve just such a slightly shifted tryptophan band.

The effects of pH on the charge transfer properties of the trypsinogen–MNCl complex were investigated from pH 3.0 to 9.0. The titration data at pH 3.0 and 9.0 were identical with those shown at pH 8.0 in Figures 1 and 2, and no significant variations in spectra or binding parameters were observed over the entire pH range.

The charge transfer properties of the DIP-trypsinogen–MNCl complex were similarly investigated. Comparison of the data for trypsinogen and DIP-trypsinogen complexes shows that the total exposure of aromatic residues on trypsinogen is not significantly affected by the presence of the inhibitor in the active site. On both trypsinogen and DIP-trypsinogen, we find only one tryptophan–MNCl binding site.

To assist in identifying this single pH-independent tryptophan residue on trypsinogen and DIP-trypsinogen, the charge transfer properties of HNB-trypsinogen were examined. Keil-Dlouha and Keil (1972) have shown that Trp-215 of trypsinogen is uniquely reactive with 2-hydroxy-5-nitrobenzyl

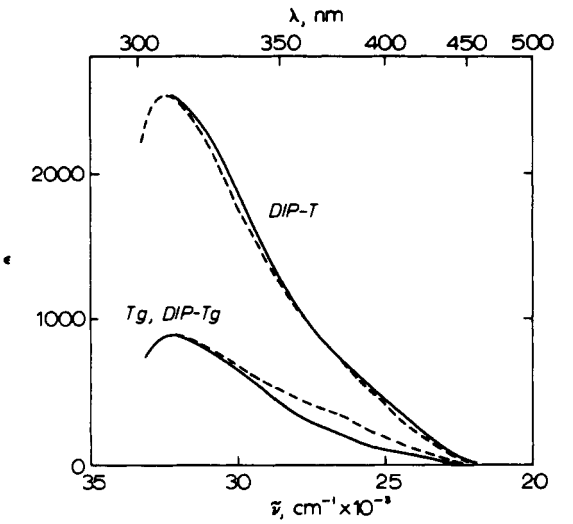

FIGURE 2: Charge transfer spectra of MNCl complexes with DIP-trypsin (upper curves) and trypsinogen and DIP-trypsinogen (lower curves). Solid lines are the observed difference spectra of the complex, and dashed lines are theoretical curves calculated from model complexes using the parameters in Table III and the extinction coefficients of model complexes given by Hinman et al. (1974). The experimental difference spectra for trypsinogen and DIP-trypsinogen are indistinguishable and have been shown as a single line.

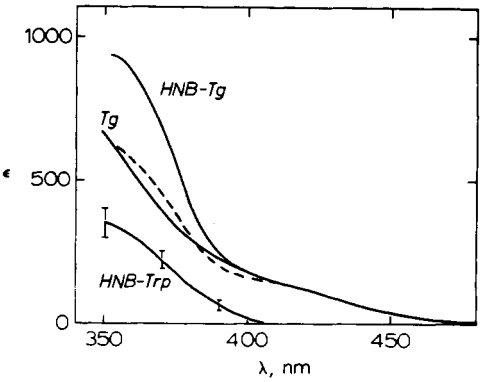

FIGURE 3: Charge transfer spectra of HNB-trypsinogen (upper curve), trypsinogen (middle curve), and HNB-tryptophan (approximate values, lower curve). The dashed portion of the middle curve is the CT difference spectrum between HNB-trypsinogen and HNB-tryptophan. All curves obtained with identical protein concentrations and 1 M MNCl.

bromide. The spectra of HNB-trypsinogen in 1 M MNCl and trypsinogen in 1 M MNCl are compared in Figure 3. Below 400 nm a complex between HNB modified tryptophan residues and MNCl begins to absorb and the spectrum of this interaction, as approximated from a spectrum of a mixture of HNB-modified tryptophan and MNCl under identical experimental conditions, is included in the figure. Clearly the HNB-trypsinogen–MNCl spectrum equals in magnitude the CT spectrum of a full tryptophan and an HNB-modified tryptophan. Thus, a second tryptophan residue (other than the HNB-modified Trp-215) is available on HNB-trypsinogen which appears to have a charge transfer absorbance within experimental error of that found for the tryptophan–MNCl transition on native trypsinogen or DIP-trypsinogen.

α-Chymotrypsin. The data in Table I indicate that only one tryptophan residue is available to MNCl on the surface of α-chymotrypsin at pH 8.0. A linear analysis is therefore appropriate at wavelengths where tyrosine complexes do not absorb, and we estimate the association constant as 3.0 ± 0.2 cm^3/mmol from double-intercept plots at each 10 nm from 380

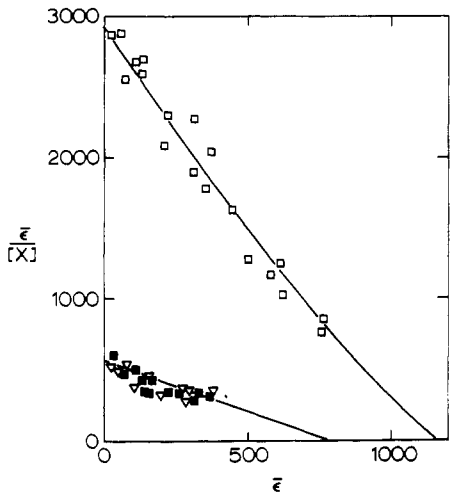

FIGURE 4: Double intercept plot of 350-nm CT data for α-chymotrypsin (□), DIP-chymotrypsin (■), and chymotrypsinogen (▽) at pH 8.0. Solid lines are theoretical curves calculated as in the legend to Figure 1.

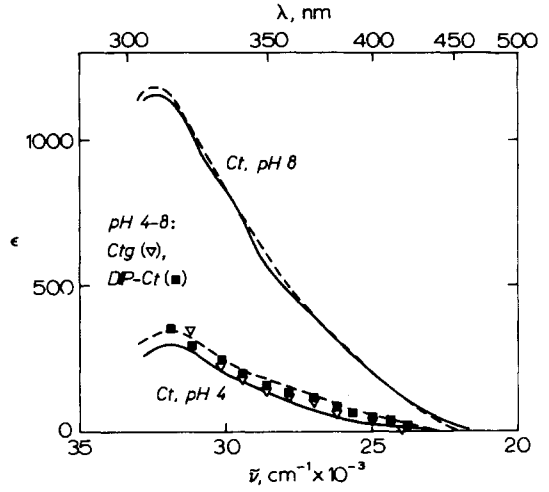

FIGURE 5: Charge transfer spectra of MNCl complexes with α-chymotrypsin at pH 8.0 (upper curve) and at pH 4.0 (lower curve). As in the legend to Figure 2, the solid lines are the observed difference spectra of the complexes and the dashed lines the calculated spectra. The observed difference spectra for MNCl complexes of DIP-chymotrypsin (■) and chymotrypsinogen (▽) are shown as points in the lower portion of the figure, and these spectra are independent of pH in the range pH 4.0 to pH 8.0.

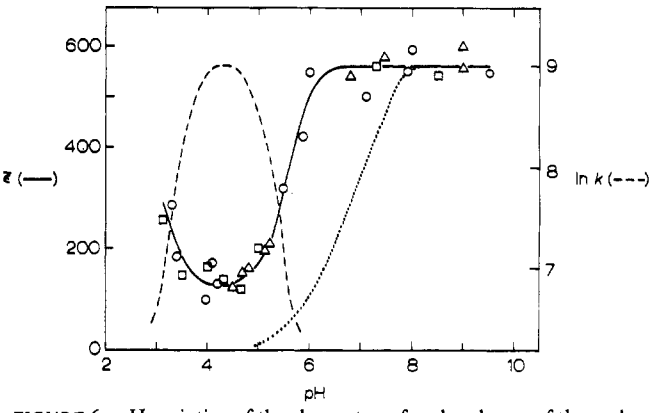

FIGURE 6: pH variation of the charge transfer absorbance of the α-chymotrypsin–MNCl complex at 350 nm. The solid line is drawn to fit the results of solutions either made up independently at the pH in question (○), or by changing the pH of a solution made up at pH 8.0 or at pH 4.0 (△, □). Protein concentrations were 10 mg/ml in 0.5 M MNCl plus 0.5 M KCl. The dashed curve shows the logarithm of the dimerization association constant as a function of pH (Aune and Timasheff, 1971), and the dotted curve shows the approximate pH–activity profile of α-chymotrypsin, normalized to the maximum of the statistical average extinction maximum (adapted from Hess, 1971).

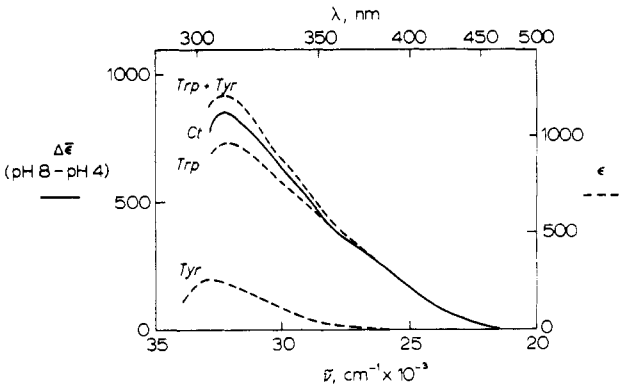

FIGURE 7: Difference spectrum between the charge transfer of the α-chymotrypsin–MNCl complex at pH 8.0 and 4.0 (solid line). Protein concentration 10 mg/ml in 0.5 M MNCl plus 0.5 M KCl. The dashed curves are the spectra of model complexes as indicated in the figure. The ordinate on the right-hand side has been adjusted to compensate for the fact that the comparisons are between the *statistical average extinction* $\bar{\epsilon} = A/[P_0]$ (which depends on the degree of saturation of the complex) and the extinction *coefficient* ϵ, a fundamental property of the complex.

to 420 nm. NBS-Oxidized α-chymotrypsin was found to have two tyrosine residues available for complexation with MNCl. A two-class model ($n_{Trp} = 1$; $n_{Tyr} = 2$) with $k_{Trp} = 3.0$, $k_{Tyr} = 0.87$ was used to fit the experimental data on the double-intercept plots at a series of wavelengths, and an example of the fitted curve is shown for the 350-nm data in Figure 4. Although curvature due to the tyrosine contribution is not large at this wavelength, it becomes very apparent at shorter wavelengths. The observed CT spectrum of the chymotrypsin–MNCl complex at pH 8.0 is compared with the spectrum calculated from the association constants deduced above and the extinction coefficients of homomorphic model complexes in Figure 5.

A significant finding was that the intensity of the charge transfer band of α-chymotrypsin changes with pH, and in a manner similar to that of trypsin. The variation in CT absorbance with pH is shown in Figure 6 at 350 nm from pH 3.0 to 9.0. Rather than following the pH–activity profile of the enzyme as is the case with trypsin, the curve is shifted to the acid side of the activity profile. At least part of the drop in MNCl binding efficiency is due to dimerization, and the dotted curve in Figure 6 represents the natural logarithm of the dimerization equilibrium constant as a function of pH (sedimentation equilibrium data; Aune and Timasheff, 1971). The difference between pH 8.0 and pH 4.0 spectra is compared in shape with homomorphic model tryptophan and tyrosine complexes in Figure 7, which clearly shows that at least one tryptophan, and probably a tyrosine as well, is involved in the pH variation of the charge transfer data.

DIP-Chymotrypsin and chymotrypsinogen A were similarly investigated by charge transfer techniques from pH 3.0 to 9.0. The CT spectra of both were found to be independent of pH over this range and to closely resemble that of α-chymotrypsin at pH 4.0 (i.e., when the active protein is almost completely dimerized in solution; see Figure 5). In Figure 4, we compare double-intercept plots of binding data at 350 nm for both zymogen and inhibited enzyme with that of α-chymotrypsin at pH 8.0, and in Table II the slopes and intercepts of the linearly fitted plots are summarized. Binding of the probe to DIP-

Table III: Binding Parameters for Charge Transfer Complexes of MNCl with Two Serine Proteases and Their Zymogens and Derivatives, and Tentative Identification of the Tryptophan Binding Sites.[a]

Protein	No. of Available Trp	Estimated Assoc Constant and Tentative Identification[b]	No. of available Tyr	Approx Assoc Constants
Trypsin, pH 8.0	2	2.3 (215), 3.8 (237)	5	0.87
pH 4.0	2	0.7 (215), 3.8 (237)	5	0.87
DIP-Trypsin	2	2.3 (215), 3.8 (237)	~5	0.87
Trypsinogen	1	3.0 (237)	1?	<0.2
DIP-Trypsinogen	1	~3.0 (237)	1?	<0.2
α-Chymotrypsin,				
pH 8.0	1	3.0 (215)	2	0.87
pH 4.0	1	0.5 (215)	1?	<0.2
DIP-Chymotrypsin	1	0.6 (215)	1?	<0.2
Chymotrypsinogen A	1	0.7 (215)	1?	<0.2

[a] At pH 8.0 unless otherwise indicated. [b] The association constant is given first in $cm^3/mmol$, followed by the residue number in parentheses. Chymotrypsin numbering is used in all cases. [c] Tyrosine residues are considered as a single class of experimentally indistinguishable residues all having the same apparent association constant.

chymotrypsin or chymotrypsinogen A is obviously very weak and accordingly the signal to noise ratio is very much worse than it is for the other proteins studied here. Comparing the least-squares parameters at 350 nm (Table II) with those of model complexes, we estimate that one tryptophan residue on DIP-chymotrypsin or chymotrypsinogen A can make a very weak complex with MNCl but that its availability as compared with α-chymotrypsin at pH 8.0 is less than 20%. Studies of NBS-oxidized-chymotrypsinogen indicate an extremely weak tyrosine contribution but the magnitude is so small as to preclude assignment of either numbers of residues or association constants from data on this derivative.

The calculated and observed spectra of chymotrypsin at pH 4.0, DIP-chymotrypsin, and chymotrypsinogen A are shown in Figure 5. The curves can be approximately fitted by assuming a single tryptophan residue which can bind MNCl with a $k \simeq 0.5$ (see Tables I and II). Although low k values make accurate curve fitting impossible, the shapes of the charge transfer spectra of chymotrypsinogen and DIP-chymotrypsin indicate that an extremely weak tyrosine–MNCl interaction also is present on these derivatives. In Figures 4 and 5, we therefore have calculated the spectra by assuming a single weak tryptophan ($k \simeq 0.5$) and a very weak tyrosine ($k \simeq 0.2$) interaction per molecule of protein.

In Table III, the results of the present work are summarized in terms of the number of MNCl-available tryptophan and tyrosine residues and the association constants of their respective MNCl complexes. In addition, a tentative identification of the tryptophan residues is included, based on chemical and crystallographic studies to be discussed below.

Discussion

The exposure of one whole ring face of Trp-215 in native trypsin to the charge transfer probe 1-methylnicotinamide chloride parallels the pH–activity profile of the enzyme, and it seems likely that changes in the exposure of this residue reflect concomitant changes in the conformation of the specificity pocket (Deranleau et al., 1975; see also Arrio et al., 1973). In the crystalline state at neutral pH, DIP-trypsin appears to be very similar to trypsin in the conformation of groups lining the specificity pocket and in the orientation of Trp-215 (Krieger et al., 1974), and the present CT results suggest that this is also the case in solution at neutral pH. Specifically, the results indicate that the exposed Trp residue in DIP-trypsin which corresponds to the active site Trp residue in trypsin (presumably Trp 215) is locked into a conformation similar to that of the native enzyme at pH 8.0, with its ring face rotated out in such a fashion so as to be accessible to the CT probe.

It is known that the binding of DFP to Ser-195 essentially freezes the *catalytic* residues in the active site into the conformation of an acylated enzyme. Although entrance to the adjacent specificity pocket is partially blocked by an isopropyl group in DIP-trypsin, the pocket itself appears to be potentially functional (Stroud et al., 1974; Krieger et al., 1974). If the exposure of the ring face of Trp-215 in DIP-trypsin reflects the conformational state of the specificity pocket as seems to be the case in trypsin, then the present results suggest that the *specificity site* residues also remain frozen in the active or open conformation in solution. [An interesting speculation is whether formation of the acyl-enzyme also prevents the sandwiching effect noted by Krieger et al. (1974) when benzamidine is present in the specificity pocket. Stabilization of the specificity pocket in a more open or expanded conformation would tend to decrease the magnitude of the dispersion interactions between the substrate side chain and the groups comprising the lining of the pocket, thereby possibly facilitating the release of the acyl-enzyme hydrolysis product.]

Solvent perturbation and chemical modification studies show that the general surface availability of tryptophan residues (without regard to the degree of exposure of individual residues) is similar in trypsin and trypsinogen at neutral pH (Villanueva and Herskovits, 1971; Spande et al., 1966). Like trypsin, trypsinogen reacts with DFP (Morgan et al., 1972) and binds pancreatic trypsin inhibitor (Dlouha and Keil, 1969; Stewart and Doherty, 1971), emphasizing certain overall similarities in the active site regions of the enzyme and its zymogen. However, pancreatic trypsin inhibitor does not bind as tightly to trypsinogen as to trypsin, and DFP is incorporated more slowly into trypsinogen than into trypsin. These observations and homological comparisons with chymotrypsin and its zymogen have suggested that the specificity pocket is not functional in trypsinogen. Nevertheless, crystallographic data establishing the conformation of the specificity pocket residues in trypsinogen have not yet been reported, and the charge transfer data are of some interest.

The CT data show that trypsinogen and HNB-trypsinogen both have a single fully exposed tryptophan side chain. Since HNB-trypsinogen is specifically modified only at position 215,

the unmodified residue which is available for interaction with MNCl is not Trp-215. Because of this and the uniqueness of the reaction with HNB (Keil-Dlouha and Keil, 1972), we conclude that Trp-215 is statistically unavailable for interaction with MNCl in native trypsinogen. The reactivity of Trp-215 with HNB but not with MNCl can be explained if we assume that the region of the protein in the vicinity of Trp-215 is flexible to the extent that at any given moment a small fraction of the total population of trypsinogen molecules have a full ring face of Trp-215 exposed to solvent. Chemical reaction with HNB will irreversibly perturb the equilibrium distribution between the exposed and buried conformations of Trp-215 and lead to eventual modification of this residue in the entire population. On the other hand, the interaction with MNCl will not appreciably perturb the equilibrium distribution because the free energy of interaction (~1 kcal/mol; Deranleau and Schwyzer, 1969) is less than that available from thermal energy. In view of the homology with chymotrypsin and its zymogen (Freer et al., 1970; Wright, 1973a,b), we may also speculate (from the lack of pH dependence of the charge transfer properties) that the single fully exposed tryptophan residue in trypsinogen is the same as the pH-independent residue in trypsin, which we have tentatively identified as Trp-237.

The charge transfer properties of DIP-trypsinogen are the same as those of trypsinogen within experimental error. It is significant that modification of the active site serine residue in trypsinogen does not increase the availability of aromatic residues, in particular Trp-215. The inability of this residue to bind MNCl in both the zymogen and DIP-zymogen indicates that the tertiary structure of the enzyme and the zymogen differ specifically in the conformation of the substrate binding pocket in solution. Presuming that the increasing availability of Trp-215 is related to the opening of the specificity pocket in trypsin, then the pocket appears to be collapsed in the zymogen, in agreement with current homology arguments. Furthermore, freezing the charge relay residues of the zymogen into the conformation of an acyl-enzyme complex apparently does not result in concomitant formation of the specificity pocket.

The present study of α-chymotrypsin shows that only one of the eight tryptophan residues is sufficiently exposed for interaction with MNCl, although a total of two to three residues are solvent available at neutral pH (Bello, 1970; Williams et al., 1965; Williams and Laskowski, 1965). The residue which binds nicotinamide is more or less fully exposed in the native enzyme at pH 8.0, but the availability of the ring face drops markedly as the pH is decreased. α-Chymotrypsin is known to dimerize at low pH, and under the conditions of the present experiments (protein concentration ca. 10 mg/ml) the enzyme is about 75% dimerized at pH 4.4. Crystallographic studies of the native enzyme clearly show that the low pH dimer contact region involves the active site regions of both monomeric units, and that Trp-215 is buried in the interface (Birktoft and Blow, 1972; Tulinsky et al., 1973). Thus it is highly likely that Trp-215 is the residue which binds MNCl in native α-chymotrypsin, as evidenced by the reciprocal behavior of the dimerization equilibrium constant and the statistical availability of the exposed tryptophan residue (Figure 6). Also, although α-chymotrypsin has not been studied with respect to reaction with HNB, Katrukha et al. (1973) found that Trp-215 is uniquely reactive with HNB in chymotrypsinogen.

The fact that the availability of this residue does not appear to follow the pH–activity profile of the enzyme as it does in the case of trypsin deserves comment. In trypsin, we postulate that the decrease in availability of Trp-215 with pH corresponds to a collapse of the specificity pocket, and chymotrypsin should show a similar behavior if the structure is functionally homologous to that of trypsin. However, the structure of the dimer is such that strong interactions elsewhere in the molecule force the region around Ser-217–Ser-218, very close to Trp-215, to adopt strained configurations in order to preserve the intermolecular contacts (Tulinsky et al., 1973). The active site region may be further stabilized by contacts between Tyr-146 of one monomer unit and His-57 of the other, and dimer formation may thus prevent collapse of the specificity pocket with decreasing pH. On the other hand, it is disturbing that a decrease in the availability of the exposed Trp residue does not set in closer to pH 7, where very little dimer is present (Figure 6).

Crystallographic data on DIP-chymotrypsin are not sufficiently refined to determine side chain orientations (Sigler et al., 1966), but several solution studies indicate a slightly smaller net exposure or reactivity of tryptophan residues in comparison with the native enzyme (Williams et al., 1965; Oppenheimer et al., 1963; Oppenheimer and Hess, 1963). The CT data suggest that here the ring face of Trp-215 is almost completely unavailable, and the CT properties more closely resemble the zymogen than the native enzyme. This result is in contrast to that of trypsin, where the DIP-enzyme is like the native enzyme at pH 8.0. This may indicate that, although the catalytic residues are frozen in position in both DIP-trypsin and in DIP-chymotrypsin, the specificity pocket conformations are different in the two structures. If Trp-215 availability is strictly correlated with formation or collapse of the specificity pocket, these results could be interpreted to mean that the pocket is at least partly collapsed in DIP-chymotrypsin. Whatever the conformation of the specificity pocket may be, however, the pH dependence of tryptophan availability appears to reinforce the concept that once the active site is frozen by reaction of DFP with Ser-195, *flexibility* in the substrate binding region is lost in both trypsin and chymotrypsin.

A comparison of the crystal structure models of chymotrypsin and chymotrypsinogen shows not only that the specificity pocket is collapsed in the zymogen, but that opening of the pocket is associated with a net movement of Trp-215 outward with respect to the enzyme surface, and a concomitant rotation of the ring plane which makes the ring face more accessible (Freer et al., 1970; Wright, 1973a,b). The present charge transfer results are in complete accord with these observations, and lend further support to current views concerning the viability of the specificity pocket in serine proteases and their zymogens.

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The Interaction of Actin Monomers with Myosin Heads and Other Muscle Proteins†

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ABSTRACT: The simplest interacting unit of actomyosin, viz., single myosin heads (subfragment 1) with actin monomers, has been studied at physiological ionic strength, by isolating the actin molecules from each other on a solid support. The interaction is characterized by a binding constant of 10^5 to 10^6 M^{-1} in the temperature range 4–30 °C. It is endothermic with a standard enthalpy of 24 ± 10 kcal mol^{-1}, and a standard entropy of 110 ± 40 eu. It is thus, like many protein–protein association processes, entropy-driven. Despite the high affinity of the association, which is comparable in its binding constant to that of subfragment 1 with F-actin, there is only very small activation of myosin ATPase. The ionic-strength dependence of the interaction shows unusual features. Binding of the proteins of the relaxing system to the monomeric actin was also examined: troponin binds both in the presence and absence of calcium ions, but neither tropomyosin nor the tropomyosin–troponin complex was found to bind significantly. Monomeric actin has also been examined as a function of ionic strength by spectroscopic methods; it appears that conformational differences between the G and the F state are the consequence of polymerization, and not of the change in ionic strength required to bring the conversion about.

In approaching a complete description of the mechanism by which the interaction of myosin heads with the components of the actin helices, constituting the thin filaments of skeletal muscle, leads to relative motion, it is necessary to define each binding process individually in terms of its intrinsic thermodynamic and kinetic parameters. Compared with the function of a single multi-subunit enzyme, which can be described in terms of a small number of equilibrium constants, the system is obviously extremely complex. It is still a matter of debate whether the two heads on a single myosin molecule are functionally identical, and practically nothing is known about the interaction of each head with its binding site on a single actin molecule. This interaction must be studied in isolation if the nature of the interaction of the two-headed myosin molecule with the constellation of accessible monomers in the actin filament, both in the presence and absence of ATP, is to be understood, as well as the relation of the process to the reported

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