The Tyrosine Residues of the Basic Trypsin Inhibitor of Bovine Pancreas. Spectrophotometric Titration and Iodination*

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ABSTRACT: The basic trypsin inhibitor of bovine pancreas has 4 tyrosine residues in the known sequence of 58 amino acids. Spectrophotometric titration shows that three of these residues are rapidly and reversibly ionized, with the midpoint of the curve ranging from pH 9.7 to 10.9, depending upon the conditions of titration. Titration of the fourth tyrosine residue occurs only above pH 12, is time dependent, and is irreversible. Alkaline conditions up to pH 12.65 do not inactivate the inhibitor and do not cause appreciable hydrolysis of disulfide linkages. Iodination of the inhibitor at pH 8.5 with 2 equiv of iodine/tyrosine residue formed monoiodotyrosine from the tyrosine residues in positions 10 and 21 and formed diiodotyrosine from the tyrosine residue in position 35. Tyrosine 23 was not iodinated. The lack of effect of iodination on the inhibition of trypsin makes it likely that the tyrosine residues are not involved in the protein–protein interaction. A method is presented for the determination of mono- and diiodotyrosine, using the amino acid analyzer after alkaline hydrolysis of peptides containing these amino acids.

The reaction of the basic trypsin inhibitor with trypsin is an interesting example of a protein–protein interaction. When two proteins combine, it is likely that a surface of one comes into contact with a surface of the other. Both hydrophobic and hydrogen bonds may participate in this contact. It was therefore of interest to determine by spectrophotometric titration how many of the four tyrosine residues of the inhibitor are exposed, and also to study the effect of iodination of these residues on the inhibition. Both approaches, described in the present paper, indicate variations in the tyrosine residues from completely exposed to completely buried positions.

Materials and Methods

The trypsin inhibitor, trypsin for activity determinations, RCM[1] derivatives (Crestfield *et al.*, 1963), and acetylated dialysis bags were all prepared as described previously (Kassell *et al.*, 1963). Urea was deionized (Benesch *et al.*, 1955). MIT and DIT were obtained from Mann Research Laboratories, New York. Amino acid analyses were carried out as described by Moore and Stein (1963) with single 24-hr HCl hydrolysates of 1-mg samples. A Beckman–Spinco, Model 120B, amino acid analyzer was used. The iodinated inhibitor and the peptides were also subjected to alkaline hydrolysis with 5 N NaOH in Teflon vials (Neumann *et al.*, 1962). The amount of native inhibitor in solution was determined from the absorbance at 280 nm (1.20 mg/A_{280} unit). After iodination, the exact amount of protein in the samples used for activity and for spectrophotometric titration was determined by amino acid analysis of an aliquot. Trypsin inhibition was measured by the method of Erlanger *et al.* (1961).

For the spectrophotometric titrations of the tyrosine and iodinated tyrosine residues, the procedures of Wolff and Covelli (1966) based on the method of Edelhoch (1962) were used. The titrations were made using a Beckman DB spectrophotometer and a Sargent Model DR pH meter with a microelectrode. Scans were made with a Honeywell Electronik 19 recorder. The inhibitor (or iodinated inhibitor) was dissolved in 0.1 M potassium phosphate buffer of pH 3.90, containing 0.1 M KCl (except where stated otherwise), and titrated in the cuvet with 2 N NaOH. The solution was mixed with a magnetic stirrer. Further details are given in the legends and table footnotes. The concentration of inhibitor (about 0.09 mM) was chosen to keep the observed absorbance values below 1.0, in order to minimize stray light effects. In the range of absorbance and at the wavelengths used, Beer's law was observed (*cf.* Wetlaufer, 1962). To construct uniform curves, the absorbance values were corrected to 0.1 mM concentration of inhibitor.[2]

The iodination was carried out at 23 ± 1° in Tris-HCl buffer of pH 8.5 or in 8 M urea in the same buffer, as described by Covelli and Wolff (1966), with 1 μmole (6.5 mg) of inhibitor and 8 equiv of iodine (the amount

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: RCM, reduced carboxymethyl; MIT, monoiodotyrosine; DIT, diiodotyrosine.

[2] Except for the iodinated inhibitor.

calculated to convert four tyrosine residues into diiodotyrosine). At the end of the reaction, excess iodine was destroyed with thiosulfate. The products were dialyzed in acetylated bags and were lyophilized.

To locate the iodinated tyrosine residues, a 50-mg sample of the inhibitor was iodinated in Tris buffer. The modified inhibitor was reduced and carboxymethylated. The derivative (10 mg) was digested for 5 hr at a constant pH of 8.5 in 0.01 M $CaCl_2$ solution with 1 mg of trypsin. The trypsin had been pretreated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (Worthington Biochemical Corporation, Freehold, N. J.) to prevent chymotryptic cleavages (Schoellmann and Shaw, 1963; Kostka and Carpenter, 1964). After digestion, the solution was acidified with 1 N acetic acid and was heated for 3 min in boiling water. The precipitate, containing the trypsin, was separated by centrifugation and was discarded; the supernatant solution was lyophilized. The dry material was dissolved in 0.2 ml of 0.1 M ammonium hydroxide and was streaked in a line (20 cm long) onto a sheet of Whatman No. 3MM paper. Electrophoresis was carried out in a Model D Electrophorator (Gilson Medical Electronics, Middleton, Wis.) at 3000 V and 140–150 A for 90 min in a pyridine acetate buffer of pH 3.7 (Katz *et al.*, 1959). The known tryptic peptides of the noniodinated RCM inhibitor (Kassell *et al.*, 1965) were put on the same paper for comparison. The peptides containing phenolic groups were located by a modified Pauly reaction (O'Sullivan, 1966) carried out on a strip cut from one side of the electropherogram. The ninhydrin reaction was used to locate the peptides on a strip from the opposite side of the paper. Peptides from the iodinated inhibitor were found in positions that were slightly retarded relative to the three normal tyrosyl peptides in their migration toward the cathode. The main portion of the zones containing phenolic residues was cut out and eluted.

The peptides were hydrolyzed for 16 hr at 110° with 5 N NaOH in Teflon vials. Tyrosine is recovered to the extent of 95% after alkaline hydrolysis (Brand and Kassell, 1939). The short column of the amino acid analyzer, with a resin height of 9 cm, was used to determine MIT and DIT. Figure 6a shows a tracing of an elution diagram. Determinations with standard solutions gave relative absorbance values of 0.77 for MIT and 0.85 for DIT, taking the value of the equivalent amount of lysine as 1.0, and correcting for a small amount of tyrosine in the MIT. The tyrosine in the MIT was determined on the amino acid analyzer and was also detected on a thin-layer chromatogram (Figure 8). Alkaline hydrolysis of the standard solutions as described above gave a recovery of 78% for MIT and 88% for DIT. No recovery experiments were run in the presence of the protein because of an interfering substance (*cf.* Figure 6b); therefore no corrections have been applied to the results below.

Amino-terminal MIT in one peptide was identified as the PTH derivative, using a manual modification (D. Eaker, personal communication) of the "sequenator" method of Edman and Begg (1967). Thin-layer chromatography on silica gel (Silicar TLC-7GF, Mallinckrodt Chemical Works, New York) in the solvent chloroform–98% formic acid (95:10, v/v) separated the PTH derivatives of tyrosine, MIT and DIT; their R_F values were 0.48, 0.78, and 0.92, respectively.

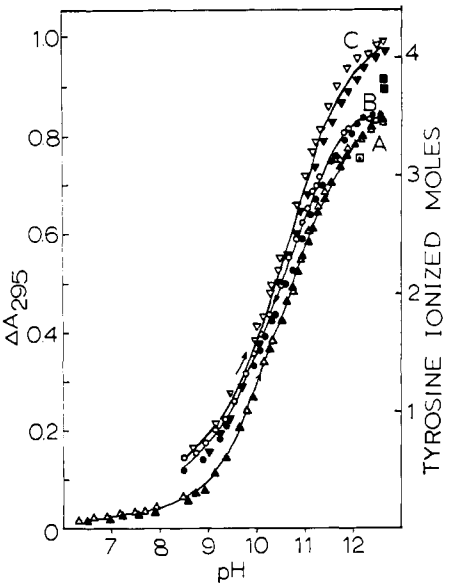

FIGURE 1: Spectrophotometric titration of the native inhibitor in 0.05 M potassium phosphate buffer, containing 0.1 M KCl, at 295 nm and 23.5°, at 0.1 mM concentration, based on the value of ϵ 2400. (⊡) Rapid adjustment to pH 12.13. (A) (▲△—▲△) Duplicate forward titrations from acid to alkaline regions. (■) Triplicate solution held for 1.5 and 3 hr at pH 12.65. (B) (●○—●○) Back-titration within 20 min. (C) (▼▽—▼▽) Second forward titration immediately after back-titration.

Results

Titration of the Native Inhibitor. Figure 1 shows three consecutive spectrophotometric titrations (forward, back, and forward again, in duplicate). The first forward titrations (curve A) show that 3.3 residues are ionized. However, these titrations took 45 min. When the solution was adjusted within 5 min to pH 12.13, two experiments gave 3.1 residues ionized[3] (the point outlined by a square). When equivalent solutions, after titrating to pH 12.65, were allowed to stand, the absorbance gradually increased, as indicated by the solid squares obtained at 1.5 and 3 hr. Thus, three tyrosine residues are rapidly titrated; the midpoint of the ionization curve for three residues is 10.2. Ionization of the fourth tyrosine residue is time dependent, with a pK_a value above 12.

Curve B shows that the titration is only partly reversible, even though the back-titration was begun within 20 min. The amount of irreversibility corresponds approximately to the fraction of the fourth residue titrated. Other experiments, with immediate back titration from pH 12.1 only, showed the reversal curve to be much closer to the forward titration. The second forward titration (curve C) resulted in the rapid

[3] Similar phenomena have been noted by others, *e.g.*, Inada (1961) with insulin, lysozyme, and catalase.

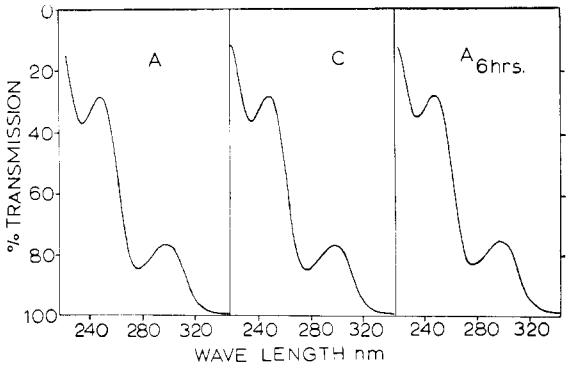

FIGURE 2: Scans made at pH 12.65 on solutions of Figure 1, after dilution with buffer of the same pH. (A) At the end of the first forward titration (curve A of Figure 1). (C) At the end of the second forward titration (curve C of Figure 1). ($A_{6\,hr}$) Diluted solution A after 6 hr at room temperature.

ionization of all four tyrosine residues; this curve was shifted about 0.3 pH unit toward a lower midpoint value.

In order to attribute the increase in absorbance unequivocally to ionization of the fourth tyrosine residue and not to hydrolysis of disulfide bonds by alkali (Donovan, 1967; Garratt and Walson, 1967), the three absorption spectra of Figure 2 were compared. Scan A was made by diluting part of the solution at the end of curve A of Figure 1 with a buffer of pH 12.65 and scanning immediately. Scan C was made at the end of curve C of Figure 1 with a dilution that gave an equivalent value for A_{295}. These two scans are superimposable. In proteins that showed disulfide-bond hydrolysis (references above), there was a larger increase in absorbance in the region of lower wavelength than at 295 nm, as

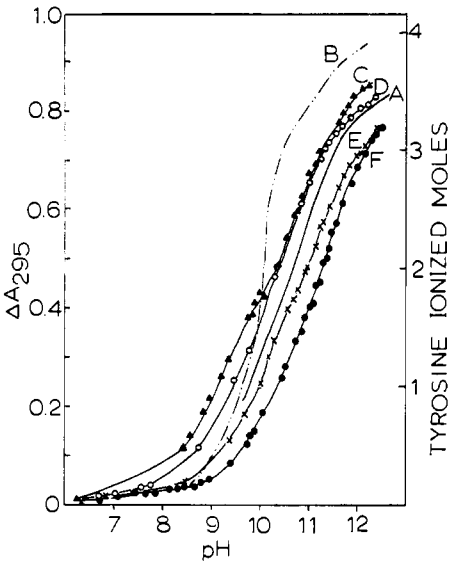

FIGURE 3: Spectrophotometric titration of the native inhibitor at 295 nm under varying conditions. (A) (———) Same forward titrations as Figure 1A. (B) (—··—) Titration of 0.4 mM tyrosine at 15–17° (from Inada, 1961). (C) (▲—▲) Titration in water solution at 25°. (D) (○—○) Titration in 0.1 M acetic acid at 25°. (E) (×—×) Titration in buffer–KCl as for A, but at 14–15°. (F) (●—●) Titration in 8 M urea at 25°.

TABLE I: Stability of the Inhibitor under Various Conditions.

Conditions[a]	No. of Detn	Activity[b]
Control solution, pH 3.9	5	2.78 ± 0.10
Rapid adjustment to pH 12.3	2	2.78
24 hr at pH 12.3	2	2.73
Titrated to pH 12.3, and back-titrated to pH 8.5	1	2.75
Second forward titration to pH 12.65 (as in Figure 1)	1	2.97
24 hr at pH 12.65	1	3.12
7 hr at pH 12.8	1	2.50
24 hr at pH 12.8	2	1.84

[a] After the treatment indicated, all solutions were diluted with 0.05 M Tris-HCl buffer of pH 8.2 and mixed with trypsin 5 min before addition to the substrate. [b] Micrograms of trypsin inhibited per microgram of inhibitor.

well as a marked change in the shape of the spectrum. When the solution of scan A was measured again after 6 hr, there was a slight change in the shape of the spectrum. The difference between the maximum at 247 nm and the minimum at 232 nm decreased by 13%. This small change indicates little destruction of the S–S linkages even on prolonged standing at pH 12.65.

A previous titration of this inhibitor by Scholtan and Rosenkranz (1966) produced two S-shaped curves. Two tyrosine residues titrated with a pK_a of 10.8; the other two had a pK_a of 12. The latter part of the titration was irreversible. Our conditions of titration above differed from theirs in the salt concentration of the solution (0.1 M KCl *vs.* no added salt, respectively), probably in temperature (room temperature), and slightly in concentration (0.09 *vs.* 0.04 mM).

To determine if the difference in conditions accounted for the difference between our results and those of these other investigators, additional titrations are compared in Figure 3 with the titration of Figure 1 (curve A in both figures) and with the titration of tyrosine itself (B, pK_a = 10.0). The titration curves in water (C) and at lower temperatures (E) both show indications of two curves, with a break at two residues. Titration in water shifts the curve to lower pK_a values; titration at lower temperatures or in urea (F) shifts the ionization to higher pK_a values. The midpoints of the curves range from 9.7 for water to 10.9 for urea. The slopes of all the curves are similar, and much lower than the slope of the titration curve of tyrosine itself (B). It is clear that the shape and position of the titration curves do, indeed, differ with the conditions of titration.

To test the possibility that dimer present in the inhibitor preparation at the start of the titration has an effect, a solution of the inhibitor was left overnight in

TABLE II: Iodination of the Inhibitor with 8 equiv of Iodine.

Expt	Solvent	Spectrophotometric Titration[a] DIT	Spectrophotometric Titration[a] Slowly Ionizing Tyrosine	Activity[b] Iodinated	Activity[b] Control
1	Tris[c]	[0.32][d]	0.70	2.25	2.18
2	Tris	0.93	1.13	1.94	2.18
3	Tris	1.05	0.94	2.06	2.67
Av	Tris	0.99	0.92	2.08	2.34
4	Urea[e]	0.89	1.11	3.10	*f*
5	Urea	1.38	0.97	2.56	*f*
Av	Urea	1.14	1.04	2.83	

[a] The values are residues per mole of protein (6513 molecular weight). Calculations are based on the following data. Slowly ionizing tyrosine: ΔA_{290}, pH 12.0–12.9, ϵ 2100. The absorbance at pH 12.9 was measured after 3 hr; DIT: ΔA_{325}, pH 4.8–7.3, ϵ 4100. [b] Activity is expressed as micrograms of trypsin inhibited per micrograms of inhibitor. [c] 0.1 M Tris-HCl buffer (pH 8.5). [d] Omitted from average. [e] 8 M urea in the same buffer as *c*. [f] Lost.

0.1 M acetic acid to dissociate any dimer (Anderer and Hörnle, 1966), then was titrated. The curve (D) of this solution falls between those of water (C) and buffer–KCl solution (A). Therefore, the dimer–monomer change is probably not involved.

Back-titration and retitration of the solutions of Figure 3 (not shown) gave results similar to Figure 1, both in the shift of the curves to the left, and in the rapid retitration of the fourth tyrosine residue.

Thus, ionization of three tyrosine residues is in the normal range and reversible, while ionization of the fourth one is time dependent and irreversible.

Effect of Alkaline Conditions on Inhibiting Activity. The activity determinations in Table I show that the inhibitor remains active during the entire course of the titrations of Figure 1, and even after standing for 24 hr at pH 12.65. This is the upper limit of stability; at pH 12.8, there is a slow loss of activity on standing.

The Effect of Iodination. Typical titration curves of the inhibitor iodinated in buffer and in 8 M urea are shown in Figures 4 and 5, respectively. No significant difference is apparent in the two preparations, either in the shape of the curves or in the calculated results. There was considerable overlap in the titration of DIT and MIT, and of MIT and tyrosine. For the calculation of DIT, we used the change in absorbance at 325 nm between pH 4.8 and 7.3. The latter was the point at which the 305-nm curve began to rise more steeply than the 325-nm curve, *i.e.*, the point at which the MIT titration began to predominate. The results (Table II) indicate that approximately one residue of DIT was present. The overlapping of the MIT and tyrosine titration curves made quantitative evaluation almost impossible, except for the titration of the fourth, slowly ionizing tyrosine residue. This single tyrosine was calculated from the difference in the absorbance at 290 nm, read after holding the solutions at pH 12.9 for at least 3 hr, and that determined at pH 12, where the fourth tyrosine began to ionize. The results in Table II show that all of this

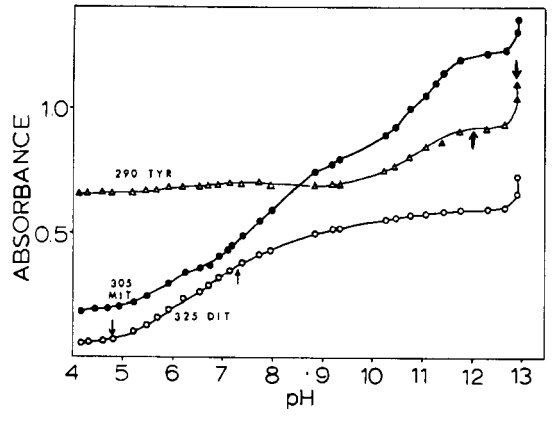

FIGURE 4: Spectrophotometric titration of 0.0754 mM inhibitor iodinated in buffer (sample 2 of Table II) at 290, 305, and 325 nm, 23.5°. The arrows indicate the portions of the curves used for calculating the data of Table II.

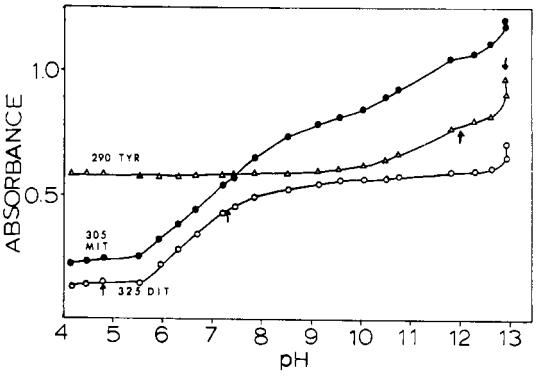

FIGURE 5: Spectrophotometric titration of 0.0773 mM inhibitor iodinated in 8 M urea (sample 4 of Table II) at 290, 305, and 325 nm, 23.5°. The arrows indicate the portions of the curves used for calculating the data of Table II.

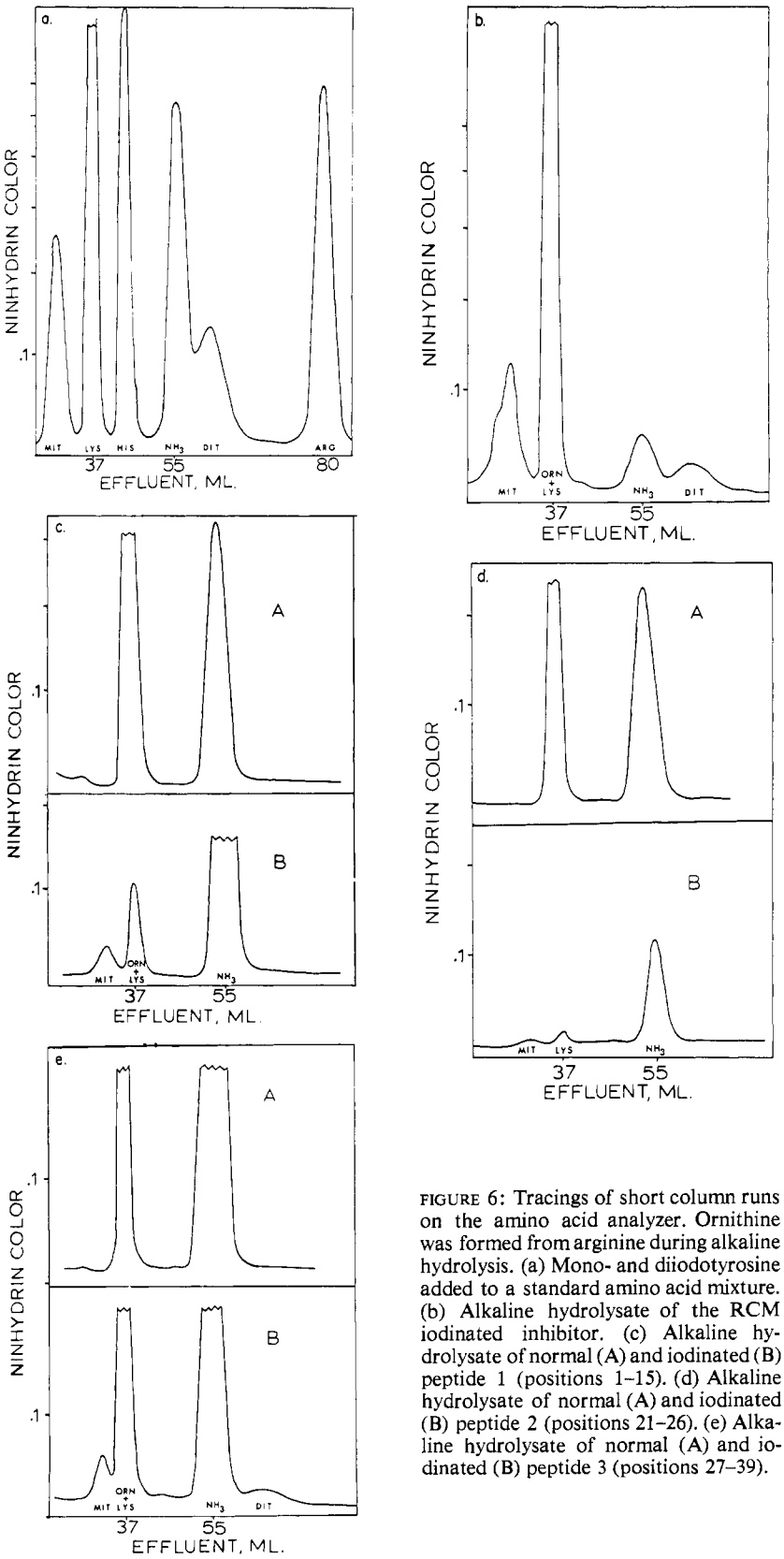

FIGURE 6: Tracings of short column runs on the amino acid analyzer. Ornithine was formed from arginine during alkaline hydrolysis. (a) Mono- and diiodotyrosine added to a standard amino acid mixture. (b) Alkaline hydrolysate of the RCM iodinated inhibitor. (c) Alkaline hydrolysate of normal (A) and iodinated (B) peptide 1 (positions 1–15). (d) Alkaline hydrolysate of normal (A) and iodinated (B) peptide 2 (positions 21–26). (e) Alkaline hydrolysate of normal (A) and iodinated (B) peptide 3 (positions 27–39).

tyrosine residue was unchanged in the iodinated inhibitor. The portion of the 305-nm curve between pH 7.3 and 12, corresponding to the other two residues, was not resolved; it included MIT and any rapidly ionizing tyrosine still present. The MIT was determined from the peptide analyses below.

Confirmation of the presence of MIT and DIT was obtained by amino acid analysis of the RCM derivative of the iodinated inhibitor after alkaline hydrolysis. Figure 6b shows a tracing of the short-column run. The two iodotyrosine peaks were in the same position as the MIT and DIT in an analysis with standard solutions of the amino acids (Figure 6a). A quantitative calculation for Figure 6b was not made because of an interfering substance that overlapped MIT; this material was also detected on alkaline hydrolysis of the noniodinated inhibitor. It is likely from its position on the chromatogram that the peak just before MIT is lysinoalanine (N^{ϵ}-(2-amino-2-carboxyethyl)lysine) identified by Bohak (1964) in alkali-treated proteins. The inhibitor contains no tryptophan; the latter is eluted just before and separates from MIT upon chromatography of a standard mixture of amino acids.

Amino acid analyses after acid hydrolysis gave the expected reconversion of the iodotyrosines into tyrosine, with no change from the original composition in other amino acids, except for loss of slightly over two half-cystine residues of the original six. However, when the iodinated inhibitor was first converted into the RCM derivative, and then analyzed, 5.5 moles of carboxymethylcysteine were found. Thus the cystine residues were not oxidized in the protein during iodination, but were oxidized by the iodine liberated from the iodotyrosines during acid hydrolysis. Such an oxidation also occurred with ribonuclease (Cha and Scheraga, 1963). The analysis, therefore, indicates that iodination caused no change except in tyrosine residues. There is no histidine in this protein.

The activity (Table II) of the different samples of the iodinated inhibitor, while somewhat variable, did not differ significantly from the native inhibitor. The higher activities of the samples iodinated in urea are probably related to a higher proportion of the active monomeric form of the inhibitor after urea treatment (*cf.* Kassell and Chow, 1966).

Location of the Iodinated Tyrosine Residues. The four tyrosine residues of the inhibitor occur in three high-yield peptides when the RCM derivative is digested with trypsin (Kassell *et al.*, 1965). In the known sequence[4] shown in Figure 7, these peptides are:

positions 1–15: Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-
10
Tyr-Thr-Gly-Pro-Cys-Lys

21 23
positions 21–26: Tyr-Phe-Tyr-Asn-Ala-Lys

35
positions 27–39: Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-
Gly-Gly-Cys-Arg

[4] The structure shown in Figure 7 (Kassell and Laskowski, 1965; Anderer and Hörnle, 1966) is now agreed upon by other workers who originally published slightly different structures (Acher and Chauvet, 1967; Dlouhá *et al.*, 1968).

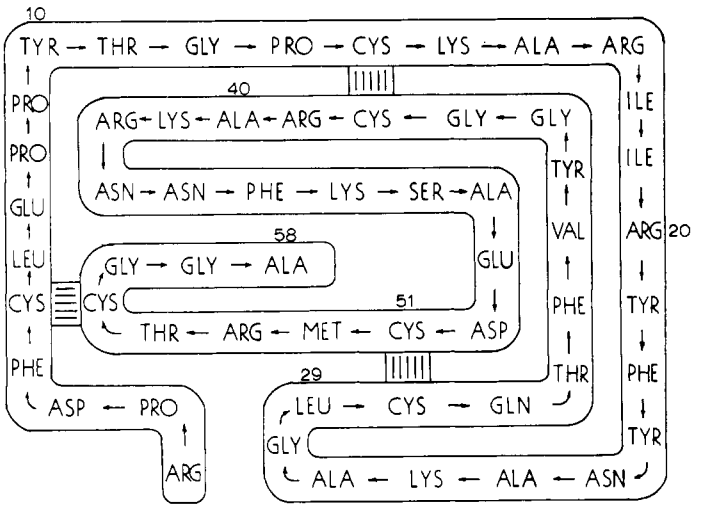

FIGURE 7: A schematic representation of the structure of the trypsin inhibitor. Tyrosine residues occur at positions 10, 21, 23, and 35.

Figure 6c–e (part B) shows tracings of the amino acid analyses of the alkaline hydrolysates of these peptides from the tryptic digest of the RCM-iodinated inhibitor (see Methods). The upper part A of each figure shows the analysis of the alkaline hydrolysate of the corresponding peptide of the noniodinated RCM inhibitor. Only the short-column runs are shown to demonstrate the iodinated tyrosines. The long-column analysis determined uniodinated tyrosine; it also gave sufficient information on composition to identify each of the three peptides and to permit an estimation of their purity.

Figure 6c shows that tyrosine 10 of peptide 1 was present as MIT. This peak corresponded to 0.71 mole of MIT, and 0.24 mole of tyrosine was present. The other amino acids found were those known to be present; peptide 1 was pure.

From Figure 6d, MIT was identified in peptide 2, although this peak was too small to calculate the amount. The tyrosine peak was slightly larger than the MIT peak, indicating that one of the tyrosines of this peptide was unchanged. Titration data above also indicated the presence of an unchanged tyrosine residue. Since tyrosine 21 was the amino terminus, it was possible to determine which of the two tyrosine residues of this peptide had been iodinated by the PTH method. PTH-MIT was identified by thin-layer chromatography; no PTH-tyrosine or PTH-DIT was detected (Figure 8). Thus, tyrosine 21 was the one converted into MIT.

Interpretation of Figure 6e is more complex. Both MIT and DIT were present in the hydrolysate of peptide 3. However, the total analysis showed the presence of proline, indicating that peptide 3 was not pure, and was contaminated with peptide 1. The DIT can be unequivocally assigned to peptide 3, since there was none in peptide 1 (Figure 6c). Further evidence is the ratio of DIT to valine in the hydrolysate (1:1.01); peptide 3 contains the *single valine* of this protein. Similarly, because peptide 1 contains *all four* proline residues of the protein, it can be calculated that the MIT was *all* de-

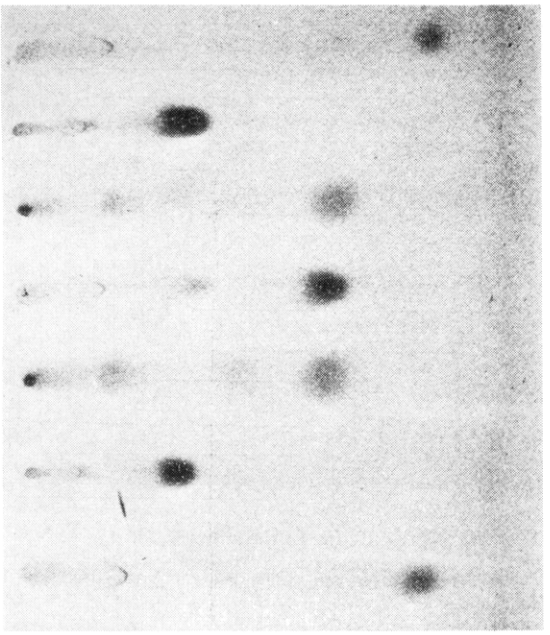

FIGURE 8: Thin-layer identification of the PTH derivative of MIT derived from tyrosine 21. For method, see text. The spots marked 1λ and 2λ peptide are samples taken from the extracted PTH derivatives after Edman degradation of peptide 2. The others are standards. The photograph was taken under ultraviolet light Ascending migration is pictured from left to right.

rived from peptide 1. In the analysis of pure peptide 1 (Figure 6c), the ratio of proline to MIT was 4:1.06; in the analysis of the mixture of peptides 1 and 3 of Figure 6e, this ratio was 4:0.86. These two analyses are within experimental error, and show that there was no MIT in excess of that present in peptide 1. Therefore, tyrosine 35 of peptide 3 was converted into DIT. The amount of tyrosine found in the hydrolysate was very small.

This analysis of the three peptides shows that two tyrosine residues, 10 and 21, were converted into MIT, but that this conversion was not quite quantitative, since a small amount of tyrosine was found in the hydrolysates. The DIT by titration was 1.05 moles, and by amino acid analysis was 1.0 mole from tyrosine 35. The slowly ionizing tyrosine, the one residue not iodinated, was 23.

Discussion

Several conclusions can be drawn regarding the structure of the inhibitor molecule. From the spectrophotometric titration of the native inhibitor, three tyrosine residues appear to be exposed. The iodination experiments, however, indicate that they are exposed to varying degrees. Although there was enough iodine present to convert all four tyrosine residues into DIT, only tyrosine 35, was actually converted into DIT. This therefore appears to be the most exposed residue. Tyrosines 10 and 21 formed only MIT; and while it is likely from titration that the phenolic groups are on the surface, the aromatic rings are probably shielded in part by other residues. For iodination to occur at a significant rate, both the aromatic ring and the phenolic group must be exposed to the solvent (Edelhoch, 1965).

Considering these differences, the spectrophotometric titration curve of these three groups of the native inhibitor may represent the overlapping titration of three groups with pK_a values within a range of 1.5–2 pH units, having 10.2 as an average midpoint value. The lower slope of this curve (Figure 3, curve A), when compared with the titration of tyrosine itself (curve B), supports this conclusion, which is the same as that drawn by Hermans (1962) from a similar titration curve for myoglobin. Previous titrations of the inhibitor, carried out by other workers, also suggest that there is progressive ionization of the tyrosine residues. A fluorimetric titration curve (Cowgill, 1967) corresponded to the ionization of a single tyrosine residue at pH 9.8, and could not be continued by this method to more alkaline regions. Scholtan and Rosenkranz (1966) found two S-shaped curves.[5]

The fourth tyrosine residue, which ionized only slowly and at high pH in the native inhibitor, behaved in the same manner in the iodinated inhibitor, and therefore was not one of the residues iodinated. This is tyrosine 23 of the Tyr-Phe-Tyr sequence, and is presumably in a hydrophobic region. The greater lack of reversibility the higher the final pH and the normal second forward titration (Figure 1) of this tyrosine residue indicates that a configurational change occurred that did not reverse when the pH of the solution was lowered. Since activity was retained (Table I), it will be of interest to make some further studies on this altered inhibitor.

The ionization curve of the inhibitor shifted to a region of higher pH when the temperature was lowered and also when the ionic strength was raised. The sensitivity of the ionization of tyrosine residues of proteins to these conditions is well known. For example, serum albumin (Tanford and Roberts, 1952) and trypsinogen (Smillie and Kay, 1961) showed this type of shift with temperature. In addition, as the temperature was lowered, trypsinogen resembled the inhibitor in the appearance of breaks in the curves. Changes in ionic strength have produced changes in both directions. For example, with ribonuclease (Tanford *et al.*, 1956) the pK_a decreased with increasing ionic strength while with methemoglobin (Rupley, 1964) the pK_a increased with increasing ionic strength.

A comparison of the behavior of the inhibitor in buffer and in 8 M urea indicates that it was not denatured in urea. First of all, the fourth tyrosine residue was not readily titrated in either solvent (Figure 3). Secondly, the change in the midpoint of the titration curve of the first three tyrosine residues from pH 10.2 in buffer–KCl to pH 10.9 in the same solvent containing 8 M urea (Figure 3, curves A and F) was not a denaturing effect; the latter would be expected to make the tyrosine residues more available, as it did with lysozyme, which showed the expected shift in the opposite direction (Donovan, 1964). Thirdly, the degree of iodination in buffer solutions and in urea was similar (Table

[5] Their conditions were different (*cf.* Results).

II). In this respect also, the inhibitor differed from lysozyme (Covelli and Wolff, 1966); with the latter, considerably more DIT was formed in urea. Fourthly, the inhibitor remained active after exposure to urea during iodination (Table II).

The iodination and titration experiments may be tentatively interpreted as indicating that none of the four tyrosine residues is required for the combination of the inhibitor with trypsin. In the iodinated inhibitor, activity was retained (Table II), although three residues were altered; the bulky iodine atoms as well as the change in pK of the phenolic groups would be expected to interfere with the reaction if these groups were essential. The fourth tyrosine was completely ionized in 3 hr at pH 12.65; even so, at this pH the inhibitor retained its activity overnight. Therefore, it is likely that a configurational change can occur in the region of this residue without inactivation.

The part of the molecule near tyrosine residue 35 is of special interest. The proximity of this single residue to disulfide linkage 14–38, which is the linkage susceptible to specific reduction and alteration (Kress and Laskowski, 1967, 1968) without loss of activity, and also its susceptibility to conversion into DIT, make it likely that this region is on the surface of the molecule. Yet the inhibitor was not susceptible to tyrosinase oxidation (Avineri-Goldman *et al.*, 1967) nor was this tyrosine residue nitrated by tetranitromethane (Meloun *et al.*, 1968). In contrast, residues 10 and 21 were nitrated, but were converted only into MIT upon iodination. This area of the molecule requires further study.

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