The Involvement of Tyrosyl and Amino Groups in the Interaction of Trypsin and a Soybean Trypsin Inhibitor*

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SUMMARY

Modification of the tyrosyl and amino groups of trypsin and soybean trypsin inhibitor (Kunitz) with N-acetylimidazole before and after their combination was used to assess the role of these amino acids in the binding process. Trypsin was inactivated by an excess of the inhibitor and the complex isolated by chromatography on Sephadex G-75, at pH 6.8. Prior to complex formation, trypsin and the inhibitor each possessed 4 reactive tyrosyl residues, but after complex formation only 4 of the expected 8 reacted with N-acetylimidazole. Trypsin and the inhibitor had four and three acetylatable amino groups, respectively, but only two of these were reactive in the complex. These results indicated that four tyrosyl and five amino groups had been prevented from reacting with N-acetylimidazole because of their involvement in the formation of the complex. Modified but fully active trypsin and inhibitor could be recovered from the acetylated complex by chromatography on sulfoethyl Sephadex at pH 2.6. Subsequent analysis of each of these isolated components revealed that 2 of the 4 shielded tyrosyl residues of the complex had been derived from trypsin and 2 from the inhibitor. Of the five shielded amino groups in the complex, three were contributed by trypsin and two by the inhibitor.

Despite the intensive amount of study which this system has received (see review in Reference 1), the precise manner in which these two macromolecules combine to form an inactive complex remains to be fully elucidated. It has been shown that trypsin which has been inactivated by chemical modification of its active site is no longer capable of combining with STI2 (5-7). Complementing these observations are the reports that an arginine-isoleucine bond in soybean trypsin inhibitor is split by trypsin under certain well defined conditions (8, 9) and that modification of the arginine residues of STI leads to a loss in inhibitory activity (10, 11). Thus, in many respects, the combination of trypsin with STI appears to resemble the interaction of trypsin with a substrate for which it is specific, although Haynes and Feeney (12), on the basis of studies involving other protease inhibitors, have questioned whether proteolysis is an obligatory feature of the inhibition process. Other than an involvement of the catalytic site of trypsin and an arginine-isoleucine sequence in soybean trypsin inhibitor, little is known about the actual size and chemical features of the contact zone wherein these two macromolecules interact. There is some evidence to indicate, however, that some of the tyrosine and tryptophan residues of trypsin and STI may be located at or near the zone of contact (13, 14).

More extensive characterization of the combining sites of trypsin and STI would undoubtedly lead to a better understanding of the nature of the forces (covalent bonds, ionic attraction, hydrophobic forces, hydrogen bonding, etc.) involved in the formation and stabilization of the trypsin-STI complex. This paper reports an initial attempt to obtain this kind of information by using the following sequence of operations: (a) treatment of the trypsin-STI complex with a group-specific reagent; (b) dissociation of the modified complex under conditions which permit the resolution of each component; (c) determination of the number of amino acid residues in each component which may have escaped modification while in the form of the complex; (d) treatment of these unmodified amino acid residues with the same reagent as used in Step a but now bearing a radioactive or chromophoric label; and (e) isolation and characterization of

The stoichiometric combination of trypsin with naturally occurring inhibitors such as one found in soybeans, soybean trypsin inhibitor,1 has provided the protein chemist with an excellent model system for studying protein-protein interaction.

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1 In view of the fact that several different species of trypsin inhibitors are known to be present in soybeans (1), it is important to point out that the particular species of inhibitor referred to in this paper as STI corresponds to the inhibitor originally isolated by Kunitz (2) and subsequently identified by chromatographic techniques as Fraction STI-A2 (3) or Fz (4).

2 The abbreviations used are: STI, soybean trypsin inhibitor (see Footnote 1); AcIm, N-acetylimidazole; BAEH, benzoyl-L-arginine ethyl ester; TNBS, trinitrobenzenesulfonic acid; DFP, diisopropyl phosphofluoridate.
peptides derived from the labeled regions of trypsin and trypsin inhibitor.

In the present study Steps a to c have been successfully accomplished through the use of AcIm which selectively acetylates the phenolic groups of tyrosyl residues and, to a lesser extent, the free amino groups of proteins (15). The data obtained permitted an assessment of the number of tyrosyl residues and amino groups which are involved in the site of interaction between trypsin and STI and the contribution made by each component.

**EXPERIMENTAL PROCEDURE**

**Materials**—Bovine trypsin (twice crystallized) and STI (three times crystallized) were purchased from Worthington. The trypsin was further purified by chromatography on sulfoethyl Sephadex as described previously (16). AcIm was prepared according to the procedure of Staab (17). BAEE, N, O-di-acetyl-L-tyrosine, and N-α-acetyl-L-lysine were purchased from Mann; TNBS was from Nutritional Biochemicals; DFP was from Sigma; and Sephadex G-75 and sulfoethyl Sephadex C-25 were from Pharmacia.

**Isolation of Trypsin-Soybean Trypsin Inhibitor Complex**—To 350 mg of STI, dissolved in 20 ml of 0.05 M NaCl, dissolved in 20 ml of 0.05 M NaCl, were added 200 mg of the chromatographically purified trypsin. The solution was allowed to stand at 0°C for 2 hours with occasional stirring. A small amount of insoluble material which formed during this time was removed by centrifugation and the supernatant solution was applied to a column (4.5 × 108 em) of Sephadex G-75 which had been equilibrated with the pH 6.8 imidazole buffer at 4°C. The column was eluted with the same buffer at a flow rate of 30 ml per hour and the eluate was collected in 10-ml fractions. The absorbance of each fraction was measured at 280 nm, and those fractions containing protein were assayed for trypsin and trypsin inhibitor activity (see below). The contents of tubes corresponding to the trypsin-STI complex (Fig. 1) were pooled, concentrated to approximately 20 mg per ml by ultrafiltration against the imidazole buffer, pH 6.8, and kept frozen until used.

**Acetylation with N-Acetylimidazole**—Minor modifications of the procedure described by Simpson, Riordan, and Vallee (15) and Riordan, Wacker, and Vallee (18) were used to acetylate trypsin, STI, and the trypsin-STI complex with AcIm. A 120-fold molar excess of solid AcIm was added to the protein dissolved in 0.05 M imidazole buffer, pH 6.8, at room temperature. The pH was maintained at 6.8 by running the reaction in a pH-stat (Radiometer, Copenhagen, Denmark). When the acetylation of buried tyrosyl residues was desired, urea was included in the reaction mixture at a level which gave a final concentration of 8 M. Aliquots of the reaction mixture were withdrawn at various periods of time and diluted with an equal volume of 0.2 M sodium phosphate-0.05 M CaCl₂, pH 4.4, which terminates the acetylation reaction and causes a rapid decomposition of excess N-acetyl-imidazole. No subsequent hydrolysis of O- or N-acetyl groups could be detected over a period of several months when these solutions were stored in a frozen state. Zero time controls were obtained by following the same procedure in the absence of AcIm.

**Dissociation of Trypsin-Soybean Trypsin Inhibitor Complex**—Dissociation of the nonacetylated trypsin-STI complex could be achieved by chromatography on sulfoethyl Sephadex C-25 at pH 2.6 with a salt gradient of 0 to 0.5 M NaCl as previously described for the purification of trypsin (16). In the case of the acetylated complex, the STI component of the complex remained bound to the column under these conditions and could only be eluted by introducing 0.3 M sodium acetate buffer, pH 5.6, following the emergence of the trypsin component of the acetylated complex. Fig. 2 shows the chromatographic separation of trypsin and STI from the unmodified and acetylated complex. Based on activity measurements, tubes corresponding to trypsin inhibitor and trypsin inhibitor from the acetylated and nonacetylated complexes were pooled, concentrated by ultrafiltration against 0.001 M HCl-0.05 M CaCl₂, and kept frozen until used.

**Measurement of O-Acetyl Groups**—The number of O-acetyl groups introduced onto the tyrosyl residues of the protein were calculated from the increase in absorbance at 278 nm following the addition of hydroxylamine (15). To 0.5 ml of the acetylated protein solution were added 2.5 ml of 0.1 M phosphate-0.5% EDTA buffer solution, pH 6.8, followed by the addition of sufficient hydroxylamine to provide a final concentration of 0.01 M at 25°C. No further increase in absorbance at 278 nm was observed after 4 hours, and doubling the concentration of hydroxylamine did not effect any further increase in absorbance. The number of O-acetyltyrosyl groups deacetylated (n) was calculated from the expression

\[
n = \frac{\Delta\epsilon_{278}}{\Delta\epsilon_{278}}
\]

where \(\Delta\epsilon_{278}\) is the change in the molar absorptivity of the protein at 278 nm on deacetylation and \(\Delta\epsilon_{278}\) is the change in molar absorptivity at 278 nm for the transition of N, O-di-acetyl- to N-acetyltyrosine. The latter value was found to be 1160 which agrees with the value reported by Simpson et al. (15).
Determination of N-Acetyl Groups—Free amino groups were determined with TNBS according to the procedure of Haynes, Osugi, and Fenney (19). Control experiments with N,N-diacetyltyrosine and N-α-acetylysine indicated that no significant deacetylation of N-acetyl groups occurred under the conditions of this assay (pH 8.5, 25°C). Appreciable autoxidation of trypsin and its partially acetylated derivative does ensue, however, under these conditions, giving rise to anomalously high values for free amino groups. Autoxidation was prevented by adding 0.05 ml of a 10⁻³ M solution of DFP (in isopropyl alcohol) to solutions of the enzyme prior to the assay. The number of N-acetyl groups was calculated as the difference between the number of TNBS-reactive amino groups at any given time and the value observed in the zero time control.

Activity Measurements—Trypsin activity was determined spectrophotometrically with BAEE as the substrate (20). One BAEE activity unit is arbitrarily defined as an increase of 0.001 absorbance unit per min under the conditions of the assay. Trypsin inhibitor activity was determined on mixtures of STI and a 5-fold molar excess of trypsin. Measurement of the residual trypsin activity compared to controls without STI permitted the calculation of trypsin inhibitor activity on the basis of the milligrams of trypsin inhibited per mg of STI.

Protein Concentrations—The concentrations of trypsin, STI, and trypsin-STI complex in milligrams per ml were obtained by multiplying absorbance readings at 280 nm by 0.695 for trypsin (21), 1.06 for STI (22), and 0.765 for the trypsin-STI complex (21). In the case of the acetylated proteins, these factors were applied after deacetylation with hydroxylamine. Protein concentrations determined in this manner were verified by amino acid analysis. Molar concentrations were calculated on the basis of molecular weights of 24,000 for trypsin (23) and 21,700 for STI (24), respectively.

RESULTS

Acetylation of Tyrosyl Residues—The rates at which the tyrosyl residues of trypsin, STI, and the trypsin-STI complex were acetylated in the presence and absence of urea are shown in Fig. 3. In the absence of urea the rate at which the tyrosyl residues of trypsin, STI, and the complex was acetylated was quite similar; 4 tyrosyl residues were acetylated in each case in about 15 min. Thereafter the O-acetyl groups appear to undergo slow hydrolysis, as might be expected at pH 6.8 at which the acetylation was performed (18). In the presence of 8 M urea all of the tyrosine residues known to be present in trypsin and STI, 10 (25) and 4 (22), respectively, were acetylated within 15 min. A total of 14 tyrosyl residues were acetylated in the trypsin-STI complex, a value which represented the sum of the tyrosine residues of each of its components. From these data it may be concluded that (a) 4 out of the 10 tyrosine residues of trypsin are reactive toward AcIm in the native protein, (b) all 4 of the tyrosine residues of STI are similarly reactive, and (c) once the complex is formed, 4 of these 8 reactive tyrosyl residues are no longer capable of reacting with N-acetylindazolamide. The fact that all of the tyrosine residues contained in the complex react with AcIm in 8 M urea may be explained by the observation that urea can effect the dissociation of the complex (26), in which case each component reacts with AcIm in an independent fashion.

Acetylation of Amino Groups—Fig. 4 shows the rate at which the free amino groups of trypsin, STI, and the complex undergo acetylation by AcIm. The amino groups of trypsin and trypsin inhibitor were acetylated at somewhat different rates. The amino groups of trypsin were slowly acetylated, and only after 50 or 60 min did it appear that the reaction had subsided to the point at which approximately eight amino groups had been modified. The amino groups of STI, on the other hand, were more rapidly acetylated, and the reaction was essentially complete in 40 min, at which time about four amino groups had been acetylated. The trypsin-STI complex underwent acetylation in a fashion which seems to reflect the acetylation of the amino groups of STI in the early part of the reaction and the amino groups of trypsin in the latter stage.

Prior to acetylation it was found that only 12 of the 15 amino groups of trypsin and only 5 of the 12 amino groups of STI were reactive toward TNBS. The nonacetylated complex was found to possess 12 TNBS-reactive amino groups, which means that
TABLE I

Tyrosyl residues of trypsin and soybean trypsin inhibitor before and after complex formation as measured by their reactivity towards N-acetylimidazole

| Tyrosyl residues                       | Before complex formation | Trypsin-ST1 complex | After dissociation from acetylated complex |
|----------------------------------------|--------------------------|---------------------|-------------------------------------------|
| Trypsin                                | 10.0                     | 14.0                | 10.0                                      |
| ST1                                    | 3.8                      | 4.0                 | 2.0                                       |
| Theoretical total                      | 14.0                     | 18.0                | 12.0                                      |
| O-Acetyl derivative                    | 4.1                      | 4.0                 | 2.0                                       |
| Unreactive towards AcIm                | 3.0                      | 1.8                 | 2.3                                       |
| Shielded in complex                    | 4                        | 2                   | 2                                         |

* Values after 15 min of acetylation (see Fig. 3).
* This value has been corrected for the partial hydrolysis of O-acetyl groups that accompanies the isolation of STI from the acetylated complex (Fig. 2).
* Sum of O-acety tyrrosyl residues in trypsin and STI minus O-acetyltyrosyl residues in complex.
* O-acety tyrrosyl residues before complex formation minus O-acetyltyrosyl residues after dissociation from acetylated complex.
* Number of tyrosyl residues, to the nearest integer, which are unreactive toward AcIm.

TABLE II

Amino groups of trypsin and STI before and after complex formation as measured by their reactivity toward AcIm

| Amino groups      | Before complex formation | Trypsin-ST1 complex | After dissociation from acetylated complex |
|-------------------|--------------------------|---------------------|-------------------------------------------|
| Trypsin           | 15.0                     | 12.0                | 15                                         |
| STI               | 12.0                     | 5.0                 | 12                                         |
| TNBS-reactive, prior to acetylation  | 7.0                      | 1.8                 | 11                                         |
| TNBS-reactive, after acetylation      | 4.1                      | 3.2                 | 2.3                                         |
| N-Acetyl derivative | 5.3                      | 3.2                 | 2.3                                        |
| Unreactive towards AcIm                | 5                        | 3                   | 2                                         |

* Values after 15 min of acetylation.
* Difference in TNBS-reactive amino groups before and after acetylation (see Fig. 4).
* Difference in TNBS-reactive amino groups before and after dissociation from the acetylated complex.
* Sum of N-acetylamino groups in trypsin and STI minus N-acetylamino groups in complex.
* N-Acetylamino groups before complex formation minus N-acetylamino groups after dissociation from acetylated complex.
* Number of amino groups, to the nearest integer, which are unreactive toward AcIm.

Fig. 4. Rate of reaction of AcIm with amino groups of trypsin, STI, and trypsin-ST1 complex. See text for details. Each point is the average of at least two determinations.

Fig. 5. The number of amino groups in the trypsin-ST1 complex rendered inaccessible to acetylation as a function of the time of exposure to AcIm. Values on the ordinate (SHIELDED AMINO GROUPS) were calculated from the data in Fig. 4 by the following expression:

\[
\text{No. of shielded amino groups} = (N\text{-acetyl groups of trypsin} + N\text{-acetyl groups of STI}) \text{ minus (N-acetyl groups of complex)}
\]

out of the total of 17 theoretical trinitrobenzene sulfonic acid-reactive amino groups in the complex, five of these were no longer capable of reacting with TNBS after complex formation. If, from the acetylation data on Fig. 4, one plots the number of amino groups which escaped acetylation as a consequence of complex formation as a function of time, the curve shown in Fig. 5 may be constructed. This plot reveals that the maximum number of amino groups which were shielded from reacting with AcIm is five, a value which was attained rather abruptly after 15 min of acetylation. Longer periods of acetylation gave lower values for the number of protected amino groups, indicating that some of the masked amino groups may react more slowly with AcIm. It may be reasonably concluded that the five amino groups which are masked during complex formation (as measured by their failure to react with TNBS) can likewise be shown to be refractory to acetylation provided that the time of acetylation is carefully controlled.

Analysis of Components Derived from Acetylated Complex—Although the foregoing data provide information on the total number of tyrosyl and amino groups which are rendered unreactive when trypsin and STI combine, no decision could be made...
TYROSINE RESIDUES

\[
\begin{array}{c}
\text{Tr} \quad \overset{\text{AcIm}}{\rightarrow} \quad \text{Tr} \\
+ \quad \overset{\text{AcIm}}{\rightarrow} \\
\text{pH 6.8} \\
\hline
\text{Tr} \quad \overset{\text{AcIm}}{\rightarrow} \quad \text{Tr} \\
\rightarrow \quad \overset{\text{AcIm}}{\rightarrow} \\
\text{pH 2.6} \\
\hline
\text{Tr} + \text{STI} \\
\end{array}
\]

AMINO GROUPS

\[
\begin{array}{c}
\text{Tr} \quad \overset{\text{AcIm}}{\rightarrow} \quad \text{Tr} \\
+ \quad \overset{\text{AcIm}}{\rightarrow} \\
\text{pH 6.8} \\
\hline
\text{Tr} \quad \overset{\text{AcIm}}{\rightarrow} \quad \text{Tr} \\
\rightarrow \quad \overset{\text{AcIm}}{\rightarrow} \\
\text{pH 2.6} \\
\hline
\text{Tr} + \text{STI} \\
\end{array}
\]

Fig. 6. Scheme showing the involvement of tyrosine residues in the interaction of trypsin (Tr) and STI. - - - ( unmodified tyrosyl residues; \( \times \) - - (, O-acetyltyrosyl residues; \( \bigcirc \) - - (, shielded tyrosyl residues which are unreactive toward AcIm.

Fig. 7. Scheme showing the involvement of amino groups in the interaction of trypsin (Tr) and STI. - - - ( unmodified amino groups (TNBS-reactive); \( \times \) - - (, N-acetylamino groups; \( \bigcirc \) - - (, shielded amino groups which are unreactive toward AcIm.

TABLE III

Activity of trypsin and STI before and after acetylation and after dissociation from acetylated complex

| State of modification | Tr | STI |
|-----------------------|----|-----|
|                       | Activity* | O-Acetyl groups | N-Acetyl groups | Activity* | O-Acetyl groups | N-Acetyl groups |
| Unmodified            | 23 | 0   | 0   | 1.15 | 0   | 0   |
| Acetylated**          | 19 | 4*  | 4e  | 1.15 | 4*  | 3e  |
| After dissociation    | 20 | 2   | 1   | 1.15 | 2   | 1   |

Activity was measured on BAEE. Units are defined in text.

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* Milligrams of trypsin inhibited by 1 mg of STI.

* Acetylated trypsin was inhibited by STI and acetylated STI to the same extent as unmodified trypsin.

* Values after 15 min of acetylation (see Table I).

* Values after 15 min of acetylation (see Table II).

Trypsin and STI derived from the acetylated complex were found to possess 2.1 and 2.0 O-acetyl groups, respectively, compared to 4 tyrosyl residues in each protein which could be acetylated prior to complex formation. Therefore, the 4 tyrosine residues shielded from reaction with AcIm in the complex, 2 are derived from trypsin and 2 from STI. These conclusions are presented in a diagrammatic fashion in Fig. 6.

Trypsin dissociated from the acetylated complex contained approximately one N-acetyl group compared to the four which were obtained in the absence of STI. Therefore, three amino groups of trypsin are apparently prevented from reacting with AcIm in the presence of STI. STI dissociated from the acetylated complex likewise had only one N-acetyl group which may be compared to the approximately three amino groups capable of being acetylated in the absence of trypsin. Hence, it may be
concluded that two of the amino groups of ST1 escape acetylation when the latter is combined with trypsin. These conclusions are embodied in the diagram shown in Fig. 7.

Activity Measurements—Data pertaining to the activities of trypsin and ST1 before and after acetylation as well as after dissociation from the acetylated complex are presented in Table III. It is evident that the acetylation of the tyrosyl and amino groups of trypsin or ST1 as performed in these studies had no significant effect on their activities, neither did these proteins suffer any appreciable impairment in activity following their dissociation from the acetylated complex.

DISCUSSION

The extent to which the tyrosyl residues of trypsin may be modified and the changes in activity attendant upon such modification are highly dependent on the reagent and conditions which are used. Of the 10 tyrosine residues present in the trypsin molecule, spectrophotometric evidence indicates that 5 to 6 are exposed (24-29). At least 4 of these exposed tyrosyl residues can be nitrated (30-32) or can react with cyanuric fluoride (33) without appreciable loss in activity toward synthetic substrates. On the other hand, reports on the effect of acetylated tyrosyl residues with AcIm on the activity of trypsin have been highly variable. As little as 1.7 to as high as 6.7 tyrosyl residues have been reported to be modified by N-acetylimidazole, with activities ranging from completely inactive to superactive, depending on the experimental conditions (34-39). The conditions that we used were such that 4 tyrosyl residues of trypsin could be acetylated with AcIm with no significant effect on activity. In this respect, our results agree with reports that 4 tyrosine residues of trypsin could be acetylated with AcIm (36) or nitrated with tetranitromethane (32) with very little effect on activity. The fact that four of the amino groups of trypsin could also be acetylated by AcIm without affecting its activity was not unexpected since all of the ε-amino groups of trypsin can be acetylated with acetic anhydride without loss in activity (41).

Although the 4 tyrosine residues of ST1 titrate normally (23), they appear to react somewhat sluggishly with certain chemical modifying reagents. Thus only 1 to 2 tyrosines react with cyanuric fluoride (42), and 2 tyrosine residues can be iodinated without loss in activity (43). Only 1.5 (44) to 3 (42) tyrosine residues of ST1 have been reported to be reactive toward AcIm, values which are lower than the value of 4 reported here. It is important to note that these lower values were obtained in experiments conducted according to the original procedure of Riordan et al. (18) which involves an acetylation time of 1 hour. Our own time course studies of the acetylation of ST1 have shown that a maximum number of 4 tyrosine residues are acetylated at the end of 15 min, followed by a slow decline (deacetylation) to a value of 3 at the end of 1 hour. This would suggest that one of the O-acetyl groups of acetylated ST1 is rather unstable, and its presence would be overlooked if the time of acetylation were prolonged. The acetylation of the 4 tyrosine residues of ST1 and three of its amino groups did not interfere with its ability to inhibit trypsin. Other workers (10, 11, 20, 43) have also shown that the activity of ST1 toward trypsin is unaffected by extensive modification of its amino groups.

Most studies designed to elucidate the amino acid residues involved in the interaction of trypsin and ST1 have used chemical modification of certain amino acids prior to the formation of the complex (5, 7, 11, 43, 45, 46). Any conclusion regarding the nature of the combining sites based on this approach must take into account the possibility that chemically induced conformational changes might indirectly prevent the interaction of these two proteins. Since there is no assurance that chemical modification will be limited to those residues which are directly involved in the combining sites, conformational changes, leading to an inability of the two proteins to combine with each other, could occur in regions of the molecule quite removed from the actual site of contact. Conversely, the failure of chemical modification to affect the ability of trypsin and ST1 to interact does not necessarily preclude the possibility that groups which have been chemically modified might still be located at or near the zone of contact. Witness the fact that although acetylation of the tyrosine and amino groups of ST1 and trypsin does not affect their ability to interact, nevertheless, as reported here, some of these same residues are evidently located at or near the site of interaction. How does one explain this apparent paradox? It would appear that chemical modification of an amino acid residue located at or near the zone of contact need not interfere with the formation of whatever bonds are involved in the binding process. Once these bonds have been allowed to form, however, these same residues may be located in an environment which renders them sterically inaccessible to the modifying agent.

Some of the uncertainties described in the preceding paragraph can be largely obviated by comparing the reactivity of certain amino acid residues before and after formation of the trypsin-ST1 complex. Such a study was made many years ago by Kunitz (47) who measured the amino groups of trypsin and ST1 by formal titration before and after complexation. From his data it may be calculated that 5.8 amino groups were no longer detected after the two proteins had combined; this value is not too far removed from the value of 5 reported here. Physical measurements, including spectral changes, fluorescence, and optical rotation, have implicated tryptophan and tyrosine as being located within the confines of the combining site (13, 48). When trypsin was combined with ST1, 2 residues of tryptophan and 3 residues of tyrosine were shielded from reaction with N-bromosuccinimide and iodine, respectively (14). We conclude from our data, based on the acetylation of tyrosine residues by AcIm, that 4 tyrosine residues are involved in the site of contact. This discrepancy of 1 residue may be due to the difference in the reactivity of tyrosine residues toward iodine and AcIm.

Since tyrosine residues and amino groups are common to both trypsin and ST1, the foregoing studies do not serve to distinguish these shielded residues which originate from trypsin from those which are derived from ST1. The present study is believed to represent the first successful attempt to answer this
question by dissociating and isolating the trypsin and STI components of the chemically modified complex. A comparison of these recovered proteins with free trypsin and STI revealed that, of the 4 tyrosine residues presumably located at or near the zone of contact, 2 were derived from trypsin and 2 from STI. Of the 5 amino groups so involved, 3 were contributed by trypsin and 2 by STI.

It is significant to note that the partially acetylated derivatives of trypsin and STI which could be isolated from the acetylated complex were fully as active as the original proteins. This is a strong indication that no gross conformational change had occurred in either protein as a result of complex formation or of contact. Steiner (14), however, is of the opinion that the conformational change that is observed spectrally when trypsin amino groups even if these groups were not located in the zone of contact could lead to a masking of tyrosyl and nitrated inhibitor isolated from both complexes. The same 2 tyrosine residues were identical with the tyrosine residues nitrated in the free inhibitor.

It is conceivable that a conformational change could lead to a masking of tyrosine residues 48 and 137 of trypsin and STI, which could be isolated from the acetylated complex were fully as active as the original proteins. This is a question by dissociating and isolating the trypsin and STI components of the chemically modified complex. A comparison of these recovered proteins with free trypsin and STI revealed that, of the 4 tyrosine residues presumably located at or near the zone of contact, 2 were derived from trypsin and 2 from STI. Of the 5 amino groups so involved, 3 were contributed by trypsin and 2 by STI.

Since the ultimate objective of this study is to delineate the regions of the molecules which are involved in the interaction of trypsin and STI, it would not be amiss perhaps to speculate on this point on the basis of the evidence thus far available, at least with respect to trypsin. If one makes the reasonable assumption that the 4 tyrosine residues of trypsin which are acetylated by AcIn are the same as the 4 which are most readily nitrated by tetranitromethane, then these tyrosine residues can be tentatively identified as residues 11, 28, 48, and 137 (30, 31).

In view of the evidence that the modification of histidine-46 or trypsin by 1-chloro-3-tosylamido-7-aminot septanone prevents its interaction with STI (7), it is not unlikely that the zone of contact would include tyrosine-48 as well. This may very well be the same tyrosine residue which becomes buried when trypsin interacts with low molecular weight synthetic substrates (51) or inhibitors (52, 53). Dluhos, Kell, and Sörm (54) postulate that the site of contact of trypsin with the pancreatic inhibitor embraces a region of the trypsin molecule which includes aspartic acid-139. If this site is the same as the one involved in the combination of trypsin with STI, then tyrosine-137, by virtue of its proximity to aspartic acid-139, could qualify as the other tyrosine residue located at or near the zone of contact. It is tentatively proposed therefore that tyrosine residues 48 and 137 of trypsin are part of the binding site with STI.

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