Sequence of Tryptic Cleavages in Porcine Pancreatic Secretory Inhibitor II*

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SUMMARY

The appearance of sequential forms of inhibitor in a mixture of inhibitor and less than equimolar quantities of porcine trypsin at pH 2.75 has been monitored by polyacrylamide gel electrophoresis. Each of the detected forms has been isolated and identified by its Rf in gel electrophoresis, its composition, and its NH2-terminal and COOH-terminal amino acids. The sequence of the bond cleavages by trypsin during temporary inhibition is Lys 14-Ile 15, followed by Arg 40-Gln 41, Lys 48-Ser 49, and Lys 38-Lys 39. The modified inhibitor (with one cleavage at the reactive site) was active while all other forms were inactive. The second cleavage, Arg 40-Gln 41, is the temporary (inactivating) cleavage.

The modifying cleavage of the Lys 14-Ile 15 bond was found to be an obligatory step for the further digestion of the inhibitor. The reversible nature of the reactive site bond cleavage was demonstrated in gel electrophoresis by the examination of separate incubations of virgin and modified inhibitors with trypsin. Starting with either form, a mixture of virgin and modified inhibitor was obtained. The modified inhibitor was susceptible to digestion by a-chymotrypsin.

Laskowski and colleagues (see Ref. 4) have elucidated the general mechanism of inhibition. They have shown that incubation of a trypsin inhibitor with catalytic amounts of trypsin leads to hydrolysis of the reactive site bond of the inhibitor. The reaction attains equilibrium and is reversible. The same trypsin inhibitor complex is formed if either the virgin (reactive site intact) or the modified inhibitor (reactive site cleaved) is the starting material.

We have recently shown (16) that artificial temporary inhibitor prepared (17) from the nontemporary basic bovine pancreatic inhibitor by reducing the 14-38 S—S bond and substituting the newly formed —SH groups with carbamidomethyl groups, is inactivated by the first, modifying cleavage. However, this artificially prepared temporary inhibitor is an exception rather than a representative case. The site of modifying cleavage, Lys 15-Ala 16, is located in close proximity to the 14-38 S—S bond. Presumably the conformational change caused by cleaving these two bonds was sufficient to prevent reformation of the complex.

Preliminary results with a naturally occurring porcine secretory inhibitor (Form I) showed that the modifying cleavage did not inactivate (1). Present studies were undertaken to test the reversibility of this step and to decide whether the loss of inhibitory activity was caused by one cleavage or in steps of gradually decreasing activity.

We chose direct isolation of intermediate forms as a method of study, and selected Form I of porcine secretory inhibitor as a starting material because of its simplicity. We shall describe experiments which show that the modifying cleavage in a naturally occurring trypsin inhibitor does not inactivate, that this cleavage is an obligatory step in temporary inhibition, and that a single (in this case the second) cleavage inactivates. The protein is then digested further. The resulting forms have been isolated and identified.

The abbreviations used are: TPCK-trypsin, 1,1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; PSTI, pancreatic secretory trypsin inhibitor; BAEE, N-α-benzoyl-L-arginine ethyl ester; dansyl, 5-dimethylaminonapthalene-1-sulfonyl; SP-Sephadex, sulfopropyl Sephadex.

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EXPERIMENTAL PROCEDURE

Materials—Porcine trypsin, crystalline, salt-free, was obtained from Novo. TPCK-trypsin was prepared as described by Wang and Carpenter (18) and had a specific activity of 40 units per mg. Carboxypeptidase B (COOHDP) and \( \alpha \)-chymotrypsin, three times crystallized, salt-free (DC103), were obtained from Worthington.

N-\( \alpha \)-Benzoyl-L-arginine ethyl ester, dansyl chloride, L-1-tosylamido-2-phenethyl chloromethyl ketone, and \( \rho \)-nitrophenyl \( \rho \)-guanidino-2-benzolate HCl were purchased from Schwarz-Mann, Pierce Chemical, Calbiochem, and Nutritional Biochemicals, respectively. Acrylamide and \( \alpha \),\( \alpha \)-methylenebisacylamide were purchased from Eastman and recrystallized from chloroform and acetone, respectively.

DEAE-cellulose and CM-cellulose were from Schleicher and Schuell and were prepared according to the manufacturer's instructions. Sephadex G-10, G-50, CM-Sephadex C-25, and SP-Sephadex C-25 were from Pharmacia. Polyamide thin layer sheets were purchased from Gallard Schlesinger, New York.

Method—TSI Form II was prepared essentially as described by Cerwinsky et al. (6) from the 0.6-saturated ammonium sulfate precipitate of an extract of whole porcine pancreas purchased from Eli Lilly. This material corresponded to Step 2 of the original method (19). Instead of desalting with Sephadex G-25, a Pharmacia column (21.5 x 100 cm) packed with Sephadex G-50 was used. A considerable purification was achieved in this step. Table I summarizes the purification procedure. The separation into two forms was accomplished in the last step, as in the original method. All pH measurements and chromatographic steps were carried out at 25°. Trypsin was assayed spectrophotometrically according to Schwert and Takenaka (20). Inhibitor was assayed by the inhibition of trypsin esterase activity. Concentrations of trypsin, \( \alpha \)-chymotrypsin, and all forms of the inhibitor were determined spectrophotometrically using optical factors of 0.74, 0.49, and 2.0 at 280 nm, respectively.

Amino acid analyses were performed on 22 hour hydrolysates in 6 N HCl at 110°. A Beckman Spinco analyzer, model 120B, was used for all analyses.

Digestion of modified inhibitor by \( \alpha \)-chymotrypsin was done by mixing 0.56 mg of inhibitor with 0.024 mg of enzyme in 1 ml of 0.05 M Tris-HCl 0.1 M NaCl, pH 8. At various time intervals 50-\( \mu \)l aliquots were removed and added directly to 1 ml of 0.1 M sodium Veronal-0.02 M CaCl\(_2\), pH 8.3, containing 0.05 mg of trypsin. After a 5-min incubation 10 \( \mu \)l of 0.1 M \( \rho \)-nitrophenyl guanidino-2-benzolate HCl in dimethyl formamide were added. The increase in absorbance at 410 nm was measured and the fraction of inactive inhibitor calculated as described by Kowalski and Laskowski, Jr. (21).

Polyacrylamide gel electrophoresis was performed according to the method of Davis using the anionic buffer 2-amino-2-methyl-1,3-propanediol described by Tamura and Ui (22).

End Group Determination—COOH-terminal lysine and arginine were determined from analysis of reaction mixtures of inhibitor and carboxypeptidase B. A 0.6-mg to 1-mg sample of inhibitor was incubated with 0.3 mg of carboxypeptidase B in 1 ml of 0.2 M sodium borate-0.1 M NaCl, pH 8.0, at 37°. Half of the enzyme was added at 0 time and the remainder after 8 hours of incubation. After 22 hours at 37°, 1 ml of 0.2 M sodium citrate, pH 2.2, was added and the entire mixture was charged on the short column of the analyzer.

NH\(_2\)-terminal residues were determined by reaction with dansyl chloride (23). The dansyl amino acids were identified by thin layer chromatography on polyamide sheets in solvent systems described by Hartley (25).

RESULTS

Fig. 1 represents the primary structure of Form II of the porcine secretory inhibitor, and is based on data of Greene et al. (11, 12) and Tschesche and Wachter (15). It is reproduced here for the purpose of facilitation of presentation. The 6 basic amino acids are shown in capital letters to indicate the six potentially susceptible bonds. The three S-S bonds are shown as triple lines.

Fig. 2 shows the results of experiments in which the effect of pH on the rate of reappearance of tryptic activity was studied. It is evident that the latent period increases with increasing pH. It is also obvious that once the digestion of inhibitor has started it is much faster at pH 8.0 than at acid pH values. To illustrate

### Table I

| Step                  | Am. units | Inhibitor units | Specific activity of inhibitor (units/Am.) | Per cent recovery |
|----------------------|-----------|----------------|------------------------------------------|------------------|
| Ammonium sulfate precipitate |           |                |                                          |                  |
| Sephadex G-50        | 6.9 x 10^4 | 10.4 x 10^4    | 150                                      | 92               |
| CM-Cellulose         | 1.6 x 10^4 | 6.2 x 10^4     | 200                                      | 60               |
| DEAE-Cellulose       | 1.0 x 10^4 | 4.1 x 10^4     | 200                                      | 10               |
| SP-Sephadex          | 1.3 x 10^4 | 1.9 x 10^4     | 430                                      | 46               |
| Form I               | 1.1 x 10^4 | 5.3 x 10^4     | 485                                      | 13               |

**Fig. 1.** Primary structure of pancreatic secretory inhibitor. Form II, according to Greene et al. (11, 12) and Tschesche and Wachter (15).
This point an experiment was performed (Fig. 3) in which complex was formed at pH 3.4 and was allowed to remain at that pH until about 30% of trypsin activity had reappeared. At that time one-half of the sample was withdrawn and readjusted to pH 8.0. As seen from Fig. 3 the rate of return of trypsin activity is much faster at pH 8 than at pH 3.4. A practical conclusion from these experiments (Figs. 2 and 3) is that the best chance of preserving the intermediate forms is at acid pH values (e.g. pH 2.75).

An attempt was then made to correlate the observed reappearance of trypsin activity with the appearance of intermediate forms of the partially digested inhibitor. To this end the complex was formed at pH 2.75 and allowed to stand at room temperature. At intervals aliquots were withdrawn, trypsin was removed by precipitation with trichloroacetic acid, and the solution of inhibitor was adjusted to pH 9.5 and subjected to gel electrophoresis (Fig. 4). The gels were stained and scanned in a Gilford spectrophotometer, model 2400. Unfortunately, uniform staining could not be ascertained and therefore the areas under the peaks are only roughly proportional to the quantity of material and are not comparable from one aliquot to another. At 4 hours the modified (first intermediate) form plus the virgin form account for approximately 85% of the total material and the second intermediate form for about 15%. At that time about 16% of trypsin activity is observed (Fig. 2). The result is suggestive that the modified form is fully active, the next form inactive. These implications were confirmed by direct isolation of intermediate forms.

A large scale experiment was performed starting with 122 mg of the virgin inhibitor, Form II. The inhibitor was incubated with less than an equivalent amount of trypsin (388 mg) at pH 2.75 for 75 min. The pH was then adjusted to 1.8 to dissociate the complex and the mixture was passed through a Sephadex G-50 column (Fig. 5). The active peak was collected and the protein chromatographed on CM-Sephadex as described in the legend to Fig. 6. The peaks marked I and II contained activity. Each was collected. Peak I was identified as virgin inhibitor by disc electrophoresis and by determination of termini. Peak II was further purified as described in the legend to Fig. 7. The first peak of Fig. 7 contained only traces of activity and represented fragments of the trypsin molecule. The second peak contained inhibitory activity. Specific activity was essentially constant across the peak, suggesting a high degree of homogeneity. The peak material was collected and lyophilized. The material was identified as modified inhibitor (Lys 14-Ile 15 bond cleaved, see Fig. 1), by first oxidizing the material with performic acid.
acid, separating the two resulting peptides (Fig. 8) and performing amino acid analyses on each peptide. Table II shows the results which unequivocally establish that the active material represents the modified form.

In order to isolate subsequent forms of temporary degradation, experiments similar to that of Fig. 5 were performed. The starting material for isolation of two-hit (C) was virgin inhibitor. It was incubated with less than equimolar amount trypsin at pH 2.75 for 10 hours. The incubation mixture was chromatographed on Sephadex G-50 (Fig. 9). The first large peak contained active trypsin. The material marked Peak I was desalted, lyophilized, and chromatographed on SP-Sephadex (Fig. 10). The fraction that appeared after the eluting buffer was changed to 0.1 M acetate, pH 5.4, was identified as two-hit (C) by the amino acid composition and by NH₂ and COOH termini (Table III). The material was inactive.

**FIG. 5.** Gel filtration on Sephadex G-50 of reaction mixture of virgin PSTI (122 mg) and TPCK-trypsin (388 mg) in 60 ml of 0.05 M citrate (sodium), pH 2.75. After a 75-min incubation the mixture was adjusted to pH 1.8 and charged on the first of two columns (2.5 x 90 cm) connected in tandem. Elution in 4-ml fractions was with 0.01 M HCl at a flow rate of 60 ml per hour. The fractions indicated were pooled and purified further. ---, absorption at 280 nm; ○, specific activity of inhibitor.

**FIG. 6.** Chromatography on CM-Sephadex of pooled fractions, from Fig. 5. The desalted pool was dissolved in 30 ml of 0.01 M citrate, adjusted to pH 2.4, and charged on a column (2.5 x 90 cm). The column was equilibrated with 0.02 M citrate, pH 4, and was eluted with the same buffer for 120 ml. A linear gradient was started which was from 0 to 2 M NaCl in 0.02 M citrate, pH 4 (1800 ml per bottle). Elution was at 25 ml per hour in 3.7-ml fractions. Concentration of NaCl was monitored by conductivity ---, absorption at 280 nm; ○, concentration of NaCl; ○, specific activity of inhibitor.

**FIG. 7.** Chromatography on SP-Sephadex of Pool II, from Fig. 6. Desalted Pool II was dissolved in 15 ml of 0.025 M ammonium acetate, pH 5.4, and applied to a column (2.5 x 94 cm) which was equilibrated with 0.05 M ammonium acetate, pH 5.4. Elution was with equilibrating buffer. Fractions of 4 ml were collected at a flow rate of 20 ml per hour. Fractions indicated were pooled. ---, absorption at 280 nm; ○, specific activity of inhibitor.

**FIG. 8.** Gel filtration on Sephadex G-50 of 2 mg of modified PSTI (Fig. 5) after performic acid oxidation. Two columns (1.5 x 90 cm) were connected in tandem and equilibrated with 0.01 M HCl. Elution was at 20 ml per hour in 3.5-ml fractions. The fractions indicated by the bars were combined and subjected to amino acid analysis.

| Amino Acid | Peptides |
|------------|----------|
| Residues 1-14 | Expected | Observed |
| Residues 15-52 | Expected | Observed |

- Lysine 1 1.04 3 2.03
- Arginine 1 0.84 1 1.06
- Aspartic acid 2 1.34 4 3.50
- Cysteic acid 2 0.80 4 3.33
- Serine 2 1.78 3 1.34
- Methionine 2 3.10 3 1.30
- Glycine 1 1.25 3 2.05
- Proline 1 0.76 0 0.22
- Alanine 1 0.19 0 0.22
- Valine 0 0 0 0
- Isoleucine 2 0.24 3 2.42
- Leucine 3 0.16 2 1.48
- Tyrosine 0 0.15 2 1.46
- Phenylalanine 0 0 0 0

**TABLE II**

Amino acid composition of peptides from performic acid oxidized modified inhibitor

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- Analysis of 20-hr hydrolysates.
- Calculated on the basis of Glutamic acid = 2 residues.
- Calculated on the basis of Glutamic acid = 4 residues.
The material of the peak marked I was collected, desalted, lyophilized, and chromatographed on SP-Sephadex (Fig. 11). A complex pattern was obtained. Peaks marked D and E were identified as three-hit (D), and four-hit (E), respectively (Table 11). Both were inactive.

Gel electrophoresis was used throughout this study to ascertain identity as well as purity of the isolated fractions. Fig. 12 shows electrophoretic mobilities of the isolated forms. The $R_F$ values were used for the identification of individual forms. In tube A a small amount of virgin inhibitor (A) was added to a large amount of modified inhibitor (B) to indicate resolution. Forms C, D, and E are, respectively, two-, three-, and four-hit material.

Of three inactive forms only the two-hit (C) contained all of the original amino acids. The third cleavage removes the 40-48 octapeptide Gln-Thr-Pro-Val-Leu-Ile-Gln-Lys. For simplicity the next form is called 4-hit (E), in which the Lys 38-Lys 39 bond is cleaved and the dipeptide Lys 39-Lys 40 is removed. However, the present data cannot distinguish between a single cleavage of a dipeptide or two cleavages of single residues in rapid succession. The four-hit (E) differs from that of Taeschke (24) since it still has Arg 1.

Of all forms isolated, only the modified form, one-hit (B) was active, confirming our preliminary results (1) obtained with the porcine secretory inhibitor Form I. Thus, the original observation of Rigbi and Greene (25) that the modified form of the bovine pancreatic secretory inhibitor is active is now extended to both forms of porcine secretory inhibitor.

If the general mechanism of inhibition established by Laskowski, Jr. and his colleagues (4) is applicable to naturally occurring temporary inhibitors of trypsin, one would predict the reversibility of this step of the reaction. An experiment was therefore performed using the gel electrophoresis technique first described by Niekamp et al. (26). Fig. 13 shows the results. In tubes 1 to 4 virgin inhibitor was used and in tubes 1' to 4' modified inhibitor was used. Each pair of tubes (1 and 1', 2 and 2' etc.) was exposed to 1 mole % of TPCK-trypsin at pH 2.75, for 1, 24, 52, 73 hours. After each time interval the complex was dissociated, trypsin was removed, and inhibitors were subjected to gel electrophoresis. At 73 hours (tubes 4 and 4') an essentially identical mixture was observed whether the starting material was a virgin or a modified form. This experiment establishes the reversibility of the first step and extends the application of the general mechanism of inhibition (4) to the naturally occurring temporary inhibitors of trypsin. Sealock and Laskowski (27) using the bovine inhibitor and different methods came to the same conclusion.

From the time event study (Fig. 4) it is evident that the modifying cleavage is an obligatory stage in temporary in-
hination. Until the reactive site bond has been cleaved virgin inhibitor is resistant to further tryptic digestion. Interestingly, the same is true with respect to chymotryptic digestion. Fig. 14 shows that the modified inhibitor is rapidly digested with α-chymotrypsin, while the virgin inhibitor is not.

**Discussion**

The first step of temporary inhibition conforms to the general scheme established by Laskowski and co-workers (see the review (4)). This step is reversible. We have shown reversibility by establishing the same pseudoequilibrium regardless of whether

**Table III**

Amino acid composition and termini of consecutive forms isolated in course of temporary inhibition

| Amino Acidsa | Virgin | Modifiedb | 2 hit [C]c | 3 hit [D]c | 4 hit [E]c |
|--------------|--------|-----------|------------|------------|------------|
| Lysine       | 4      | 4.06      | 3.10       | 3.05       | 1.92       |
| Arginine     | 2      | 2.01      | 1.91       | 1.91       | 0.85       |
| Aspartic acid| 4      | 3.04      | 4.00       | 4.00       | 4.00       |
| Threonine    | 5      | 4.95      | 4.70       | 4.70       | 4.70       |
| Serine       | 5      | 4.80      | 4.84       | 4.84       | 4.84       |
| Glutamic acid| 6      | 6.00      | 5.05       | 5.05       | 5.05       |
| Proline      | 4      | 3.73      | 0.60       | 0.60       | 0.60       |
| Glycine      | 4      | 4.21      | 4.12       | 4.12       | 4.12       |
| Alanine      | 1      | 1.21      | 1.25       | 1.25       | 1.25       |
| Cystine/2d   | 6      | 4.85      | 5.28       | 5.28       | 5.28       |
| Valine       | 4      | 3.77      | 2.80       | 2.80       | 2.80       |
| Methionine   | 0      | 0.00      | 0.00       | 0.00       | 0.00       |
| Isoleucine   | 3      | 2.84      | 1.82       | 1.82       | 1.82       |
| Leucine      | 2      | 1.63      | 0.94       | 0.94       | 0.94       |
| Tyrosine     | 2      | 1.74      | 1.49       | 1.49       | 1.49       |
| Phenylalanine| 0      | 0.00      | 0.00       | 0.00       | 0.00       |

COOH-Termini

Lysine          | Arg    | 0.67    | 1.60      | 1.60       | 1.60       |
| Arginine       | Arg    | 1.60    | 1.10      | 1.10       | 1.10       |
| NH2 -Termini   | Arg    | 0.60    | 1.10      | 1.10       | 1.10       |

Bonds cleaved

Arg 14-Ile 15   | Arg 14-Ile 15 | Arg 14-Ile 15 | Arg 14-Ile 15 | Arg 14-Ile 15 |
| Arg 38-Lys 39  | Arg 38-Lys 39 | Arg 38-Lys 39 | Arg 38-Lys 39 | Arg 38-Lys 39 |

| RF            |
|---------------|
|Virgin         | 0.09    | 0.18    | 0.36      | 0.41       | 0.60       |
|Modified       | 0.09    | 0.18    | 0.36      | 0.41       | 0.60       |

...Analysis of 20-hr hydrolysates. Figures in parenthesis differ from the expected.

...Residues calculated on the basis of Glutamic=6.

...Residues calculated on the basis of Aspartic=4.

dValues for Cystine/2 for all virgin inhibitor analyses were consistently lower than expected.

**Fig. 12** (left). Disc electrophoresis of intermediates isolated from reaction mixtures of PSTI and TPCK-trypsin. Electrophoresis and staining were performed as described in Fig. 4. In the Gel A small amount of virgin inhibitor was added as a reference to the modified inhibitor, tube B contains only the modified inhibitor (Fig 7). Form C is a pool from Fig. 10 Forms D and E are pools from Fig. 11.

**Fig. 13** (right). Disc electrophoresis of separate reaction mixtures of virgin PSTI (2 mg per ml) and modified PSTI (2 mg per ml) and 1 mole % TPCK-trypsin at pH 2.75. Incubation conditions, sampling, and electrophoresis were carried out as described for Fig. 4. Tubes 1 to 4 are reaction mixtures containing virgin PSTI after 1 hour, 24 hours, 52 hours, and 73 hours of incubation. Tubes 1' to 4' are analogous except that they contain the modified inhibitor. The apparent identity of 4 and 4' indicates that a pseudoequilibrium mixture of virgin and modified forms has been established.
the starting material was virgin or modified inhibitor. Seabock and Laskowski (27) came to the identical conclusion on the basis of kinetic and thermodynamic data.

Two lines of evidence indicate that the modifying cleavage is a prerequisite for subsequent inactivation. The time course of inactivation (Fig. 4) shows that the third sequential form appears when the concentration of the modified form approaches a maximum. This indicates a pathway: virgin → modified → two-hit (C). No evidence of a form with one bond cleaved other than Lys 14-Ile 15 has been found. Secondly, under conditions which do not favor the transition of modified → two-hit (C), a pseudoequilibrium between virgin and modified form is established (Fig. 13).

In the present work we used Form II of the porcine secretory inhibitor. In the preliminary report (1) we used Form I. Rigbi and Greene (25) and Seabock and Laskowski (27) used bovine secretory inhibitor. In all three cases the reactive site was hydrolyzed to produce modified inhibitor which was active. Third is not a sufficient number to make a claim that all naturally occurring temporary inhibitors have an active modified form. However, if one takes into consideration that many nontemporary inhibitors have active modified forms the above generalization appears highly probable. The only exception known so far is an artificially prepared temporary inhibitor (16) where the modifying cleavage inactivates.

The isolated modified form (Fig. 7) consistently showed specific activity about 12% lower than the virgin form. We normally tested for activity at pH 6 using large excess of trypsin and adding substrate 5 min after mixing inhibitor with trypsin. When Seabock and Laskowski found that the modified form react with trypsin 25-fold slower than the virgin, we retested specific activity allowing 1 hour for the inhibitor-trypsin reaction. The same difference in specific activities of about 12% was observed. It is conceivable that the time allowed for complex formation was still too short. Another alternative, however, must be considered.

By analogy with the soybean inhibitor let us assume that the modified inhibitor is fully active. The molecule of the modified soybean inhibitor can react with trypsin only in one way by

![Graph showing time course of inactivation of virgin and modified PST1 by 1 mol \( \alpha \)-chymotrypsin at pH 8. The concentration of virgin and modified inhibitor was \( 1 \times 10^{-3} \) mol in 0.05 M Tris buffer pH. The fraction of inactive inhibitor was determined by \( \alpha \)-nitrophenyl \( p \)-guanidinoacetate titration of trypsin in trypsin digest mixtures as described in the text.](http://www.jbc.org/)

formimg a complex in which the reactive site of the inhibitor is brought into close proximity with the active site of trypsin. No other approach by the inhibitor molecule is possible because it is still resistant to trypsic digestion. With the pancreatic secretory inhibitor this is not the case. The modified inhibitor may approach the active site of trypsin either by its reactive site (Lys 14-Ile 15) or by the temporary site (Arg 40-Gln 41). The first leads to reformation of the stable complex, the second to digestion of the inhibitor and formation of a substrate. The new form two-hit (C) is totally inactive.

The relative preference for the approach by the reactive site over the temporary site depends on pH and trypsin concentration. Under the conditions of assaying for specific activity (pH 8, and excess of trypsin) the route leading to the complex is favored, even though the second is not totally excluded. If now we postulate that 12% (14) represents one approach in eight by the temporary site the experimental findings fit. Should measurement of the specific activity be performed under conditions which prohibit the approach by the temporary site we think that 100% activity would be found for the modified form (one-hit).

After this work was completed, through the courtesy of Dr. Tschese we had an opportunity to read the abstract of Tschese, H. et al. to be presented during the Second Conference on Protease Inhibitors to be held in Cologne in October 1974. The authors use Form I of porcine secretory inhibitor and find in agreement with us that the loss of activity coincides in time with the cleavage of the Arg 44-Gln 45 bond.

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