Interaction of the Kunitz Soybean Trypsin Inhibitor with Bovine Trypsin. Evidence for an Acyl-Enzyme Intermediate during Complexation†

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ABSTRACT: When the complex of trypsin with ^{125}I-labeled Kunitz soybean inhibitor (STI) was exposed to 8 M urea, about 10% of the radioactivity of STI remained bound to trypsin. This nondissociable form of the complex was stabilized by irreversible denaturation with 10% trichloroacetic acid. When this denatured complex was treated with 0.1 N NaOH, the STI component of this complex was converted to a modified molecular species which was identical with the STI obtained by limited proteolysis with trypsin. When the alkaline-treated complex was subjected to reduction and carboxymethylation of disulfide bonds, fragment A of STI (N-terminal region with arginine-63 as C-terminal residue) was released. A similar result was obtained when reductive carboxymethylation preceded alkaline treatment of the complex. Reaction of the denatured complex with NaB^3H_4 followed by acid hydrolysis yielded a radioactive product which was identified as argininol. These results are interpreted as evidence for the existence of a small amount of acyl-enzyme intermediate, in the form of an ester linkage involving arginine-63 of STI and the active site serine residue of trypsin, which is in equilibrium with the predominant tetrahedral adduct form of the complex. This acyl-enzyme intermediate most likely represents an intermediate in the pathway leading to the formation of modified STI (in which the peptide bond between arginine-63 and isoleucine-64 has been split) when virgin STI interacts with trypsin.

The mechanism of action of serine proteinases on their specific substrates is generally formulated according to the following scheme:

$$E + S \rightleftarrows (ES) \rightleftarrows (ES)^* \rightleftarrows (ES)^{**} \rightleftarrows (EP) \rightleftarrows E + P \quad (1)$$

where (ES) and (EP) are Michaelis complexes (enzyme–substrate and enzyme–product, respectively), (ES)* is a transition state intermediate in the form of a tetrahedral adduct, and (ES)** is an acyl-enzyme intermediate. Present evidence (Laskowski and Sealock, 1971; Means et al., 1974; Fritz et al., 1974) suggests that proteinase inhibitors function in a role analogous to that of a substrate so that eq 1 may be rewritten as:

$$E + I \rightleftarrows (EI) \rightleftarrows (EI)^* \rightleftarrows (EI)^{**} \rightleftarrows (EI')^* \rightleftarrows E + I' \quad (2)$$

where (EI)* now represents an inactive complex in the form of a tetrahedral adduct which in the case of STI[1] and trypsin involves arginine-63 of STI and the active site serine residue of trypsin (Blow et al., 1974; Sweet et al., 1974; Huber et al., 1975). This complex can be dissociated, however, by kinetic control (Sealock and Laskowski, 1969) to yield primarily the virgin inhibitor, I. Treatment of STI with catalytic levels of trypsin under acid conditions, however, yields the modified inhibitor, I′, which has now undergone a proteolytic cleavage between arginine-63 and isoleucine-64 (Ozawa and Laskowski, 1966). If eq 2 is valid, the pathway leading to the generation of I′ must necessarily include the formation of the acyl-intermediate, (EI)**.

To date, there has been little or no direct evidence for the existence of this postulated acyl-enzyme intermediate shown in eq 2.[2] In the present paper, evidence is presented which indicates that a small but detectable fraction of the STI–trypsin complex does in fact exist in the form of acyl intermediate (ester bond involving the carboxyl group of arginine-63 and the active serine residue of trypsin) in equilibrium with the predominant tetrahedral species. This demonstration was facilitated through the use of ^{125}I-labeled STI and the subsequent "trapping" of the acyl-enzyme intermediate by irreversible denaturation in presence of 8 M urea and trichloroacetic acid.

Experimental Procedure

Materials. STI and crystalline bovine trypsin, purchased from Worthington Biochemical Corp., were further purified as described by Frattali and Steiner (1968) and Papaioannou and Liener (1968), respectively. $Na^{125}I$ (2 mCi/mL) and NaB^3H_4 (320 mCi/mmol) were purchased from New England Nuclear and Amersham/Searle Co., respectively. Argininol was synthesized from arginine methyl ester by modification of the method of Karrer et al. (1948) as follows: A solution containing 2 mmol of L-arginine methyl ester and 20 mmol of $NaBH_4$ in 20 mL of DMF was stirred overnight at room temperature. The solution was acidified with 5.7 N HCl and the DMF removed by repeated extractions with chloroform. The aqueous phase was dried under vacuum and redissolved in absolute methanol. Argininol was identified on the basis of

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[1] Abbreviations used: STI, Kunitz soybean trypsin inhibitor; [^{125}I]STI, STI iodinated with ^{125}I; STI*, trypsin-modified STI; [^{125}I]STI*, trypsin-modified [^{125}I]STI; STC, STI–trypsin complex; [^{125}I]STC, [^{125}I]-STI–trypsin complex; STCd, STC which had been denatured by treatment with trichloroacetic acid and urea; [^{125}I]STCd, [^{125}I]STC which had been denatured in a similar fashion; DMF, *N,N′*-dimethylformamide; RCM, reduced and carboxymethylated.

[2] A possible exception is the report by Moroi and Yamasaki (1974) that α_1-antitrypsin forms an inactive complex with trypsin via the formation of an acyl bond between a carbonyl carbon of the inhibitor and active site serine of trypsin.

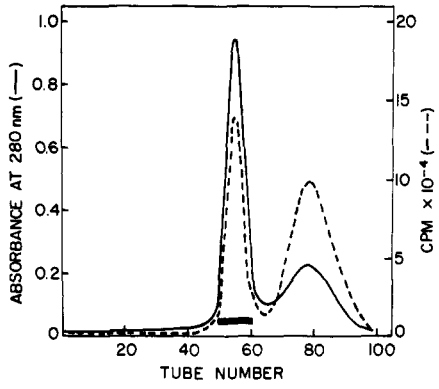

FIGURE 1: Isolation of complex of trypsin and ^{125}I-labeled STI. A mixture of trypsin (15 mg) and [^{125}I]STI (20 mg) in 10 mL of 0.1 N $NaHCO_3$ was applied to a column (2.2 × 110 cm) of Sephadex G-75 and eluted wth the same buffer. Flow rate, 30 mL per h; volume of each fraction, 3 mL. Tubes corresponding to the complex, [^{125}I]STC, were pooled as denoted by horizontal bar, dialyzed, and lyophilized.

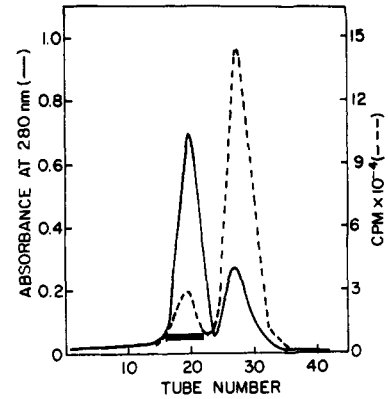

FIGURE 2: Chromatography of [^{125}I]STC on Sephadex G-75 in the presence of 8 M urea. A solution of 20 mg of [^{125}I]STC in 5 mL of 0.1 M acetate buffer, pH 5.2, containing 8 M urea, was applied to a column (2.2 × 74.5 cm) of Sephadex G-75 equilibrated against the same buffer. Flow rate, 36 mL per h; volume of each fraction, 6 mL. Tubes corresponding to first peak denoted by horizontal bar were pooled and subsequently treated with trichloroacetic acid as described in the text to give the preparation designated as [^{125}I]STCd.

its ability to form formaldehyde after periodate oxidation (Rees, 1958) and by its positive reaction with ninhydrin and Sakaguchi's reagent. All other reagents were of analytical grade and used as purchased.

Preparation of Radioactive Derivatives of STI. STI labeled with ^{125}I ([^{125}I]STI) was prepared by dissolving 88 mg of STI in 10 mL of 1 M glycine buffer, pH 8.8, containing 0.6 mL of 0.02 M ICl (prepared according to Hugli, 1965) and 0.4 mL of Na^{125}I. After 20 min at room temperature, the solution was dialyzed against distilled water at 4 °C and lyophilized. The trypsin inhibitor activity of this preparation was equivalent to that of the original STI when measured on benzoyl-DL-arginine-*p*-nitroanilide as the substrate (Kakade et al., 1969). Trypsin-modified STI (STI*) and [^{125}I]STI ([^{125}I]STI*) were prepared by treating STI or [^{125}I]STI with trypsin at a ratio of 60:1 (w/w) in 0.05 M $CaCl_2$, pH 3.75, as described by Ozawa and Laskowski (1966). After 48 h at room temperature, the pH was raised to 7 by the addition of 0.1 M NH_4HCO_3.

Preparation of Complexes of Trypsin-STI Complexes. Complexes of trypsin with STI (STC) or [^{125}I]STI ([^{125}I]-STC) were prepared by mixing a 1.15 M excess of inhibitor with trypsin followed by separation of the complex from excess inhibitor by chromatography on Sephadex G-75 (Papaioannou and Liener, 1970).

Trapping of Acyl-Enzyme Intermediate. Lyophilized samples of STC or [^{125}I]STC were dissolved in 0.1 M acetate buffer, pH 5.2, containing 8 M urea and passed through a column of Sephadex G-75 equilibrated with the same buffer. The first peak to emerge from this column was mixed with an equal volume of 20% trichloroacetic acid in the same urea-containing acetate buffer. After 40 min at room temperature, the reaction mixture was dialyzed against distilled water, which had been acidified to pH 6 with dilute acetic acid, and finally lyophilized. The denatured complexes obtained in this fashion with STC and [^{125}I]STC will be referred to as STCd and [^{125}I]STCd, respectively.

Identification of Argininol in STCd. STCd was dissolved in 1.5 mL of 0.05 N HCl, to which was then added 0.1 mL of pepsin solution (2.3 mg of crystalline pepsin, Worthington, per mL of water). After 24 h at room temperature, the digest was lyophilized. To 14 mg of the peptic digest was added 1.6 mg of NaB^3H_4 dissolved in DMF, and the mixture was stirred for 24 h at room temperature, at the end of which time it was acidified by adding 2 mL of 6 N HCl. DMF was removed by extraction with chloroform and the remaining chloroform eliminated by immersion in a boiling water bath for 2 to 3 min. The resulting solution was reduced to dryness at 110 °C for 24 h and subsequently extracted with anhydrous ethanol, and the ethanol extract, after being reduced to dryness, was examined for the presence of argininol by paper electrophoresis. The latter was carried out on sheets (15.2 × 57 cm) of Whatman 3MM paper at 2000 V for 1 h in a solvent system composed of pyridine–glacial acetic acid–water (200:34:1766, v/v), pH 6. The strips of paper were air-dried and cut into segments (2.5 cm) which were immersed in scintillation counting fluid for measurement of radioactivity.

Results

Isolation of Acyl-Enzyme Intermediate. When a mixture of trypsin and a slight molar excess of ^{125}I-labeled STI was chromatographed on Sephadex G-75, the complex so formed, [^{125}I]STC, emerged as a single peak, well separated from the residual uncomplexed inhibitor (Figure 1). This peak was devoid of antitryptic activity, and electrophoresis on cellulose acetate revealed the presence of only a single component.

An attempt was made to dissociate the noncovalently[3] bound components of this complex by gel filtration in the presence of 8 M urea. This is similar to the technique employed by Sealock and Laskowski (1969) who used 6 M guanidine hydrochloride to recover STI from its complex with trypsin. As shown in Figure 2, two peaks were obtained under these conditions, and the bulk of the radioactivity was associated with the second peak. That this peak was indeed [^{125}I]STI was confirmed by electrophoresis on cellulose acetate and the fact that it displayed antitryptic activity after removal of the urea by dialysis. Although, on the basis of the molecular weights for trypsin and STI (25 000 and 22 000, respectively), one might not expect a clear-cut separation of these two components by gel filtration, trypsin apparently becomes sufficiently unfolded in 8 M urea so that it behaves as a molecule having a higher molecular weight than STI. This conclusion was confirmed by companion experiments in which trypsin and STI were run separately in 8 M urea on the same column of Sephadex G-75. The elution volumes of trypsin and STI corresponded to the first and second peaks, respectively, in Figure

[3] The term "noncovalent" in this instance is used in a strictly operational sense to denote bonds which are disrupted by 8 M urea.

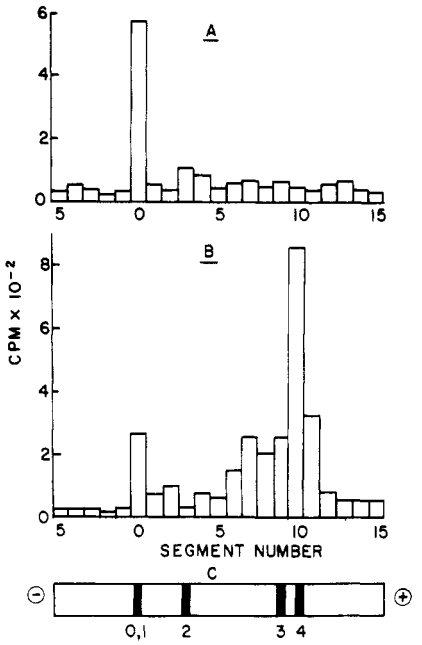

FIGURE 3: Electrophoretic behavior of [^{125}I]STCd before and after alkaline hydrolysis. [^{125}I]STCd (1 mg) was dissolved in 0.2 mL of 0.1 N NaOH, allowed to set at 37 °C for 20 min, and then dialyzed and lyophilized. Electrophoresis was conducted on strips (2.5 × 7.6 cm) of cellulose acetate at pH 8.8 (0.2 M Tris–barbiturate buffer) for 30 min, 2000 V. The strips were stained for protein with Ponceau S dye and then cut into 0.15-cm segments which were immersed into 10 mL of scintillation fluid and the radioactivity was measured in a scintillation counter. (A) Distribution of radioactivity before alkaline treatment; (B) distribution of radioactivity after alkaline treatment; (C) strip stained for protein showing the origin (0) and the position of denatured trypsin (1), [^{125}I]STC (2), [^{125}I]STI (3), and [^{125}I]STI* (4).

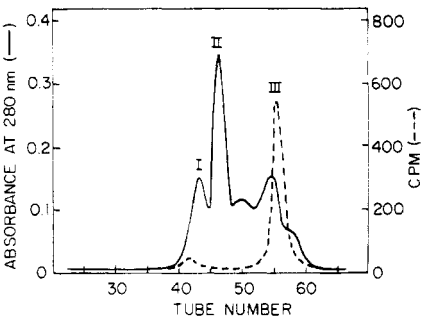

FIGURE 4: Chromatography of [^{125}I]STCd which had been subjected to alkaline hydrolysis followed by reduction and carboxymethylation. [^{125}I]STCd (2 mg) was dissolved in 0.1 mL of NaOH and allowed to set at 37 °C for 20 min. STI* (15 mg) was added and the mixture dissolved in 3.3 mL of 0.2 M Tris buffer, pH 8.6, containing 8M urea, and 0.1 mL of β-mercaptoethanol. After 4 h at room temperature, 300 mg of iodoacetic acid in 1.4 mL of 2 N NaOH was aded. After 30 min the reaction mixture was applied to a column (2.2 × 110 cm) of Sephadex G-75 equilibrated and subsequently eluted with 50% acetic acid. Flow rate, 7 mL per h; volume of each fraction, 3.5 mL. See text for identification of peaks. The slight lag in the elution of the radioactive peak III in relation to the protein peak is due to the interaction of the iodinated label with Sephadex.

2. A similar observation was reported by Sealock and Laskowski (1969) using guanidinium hydrochloride as the denaturant.

Although the first peak, after removal of the urea, had tryptic activity, it is important to note that this peak contained a low but significant level of radioactivity (about 10% of the total radioactivity recovered in the column effluent). Since this radioactivity could only have come from [^{125}I]STI, it was assumed at this point that this peak contained not only the free trypsin which was originally noncovalently bound to the inhibitor but also a small amount of [^{125}I]STI covalently bound to trypsin. In order to stabilize this covalent complex, the pooled contents of the tubes corresponding to the first peak were mixed with an equal volume of 20% trichloroacetic acid. After 40 min this mixture was exhaustively dialyzed against distilled water and lyophilized. This preparation, hereafter referred to as [^{125}I]STCd, will constitute the preparation for which evidence will be presented for the probable presence of an acyl-enzyme intermediate in the form of an ester linkage.

Alkaline Lability of [^{125}*I*]*STCd.* Since it is well known that an ester linkage is readily hydrolyzed under alkaline conditions (Ruch and Vagelos, 1973), evidence for the presence of such a linkage in [^{125}I]STCd was sought by examining this preparation by electrophoresis before and after alkaline hydrolysis. As shown in Figure 3A, prior to alkaline treatment most of the radioactivity remained at the origin. The protein associated with this activity, however, includes not only the covalent complex of trypsin and [^{125}I]STI but also the free trypsin which had been irreversibly denatured by prior treatment of [^{125}I]STCd with urea and trichloroacetic acid. Control experiments had shown that trypsin itself when subjected to denaturation under these same conditions remains at the origin upon electrophoresis. However, after exposure to 0.1 N NaOH under the conditions described in Figure 3, most of the radioactivity was shifted toward the anode in a position which coincided with location of trypsin-modified [^{125}I]STI ([^{125}I]STI*) seen in the reference strip in Figure 3C. These results indicate therefore that there must be present in [^{125}I]STCd a linkage, presumably an ester bond, which, when hydrolyzed by alkaline treatment, leads to the formation of what is equivalent to trypsin-modified STI, that is, that form of the STI molecule in which a bond has been split between arginine-63 and isoleucine-64 (Ozawa and Laskowski, 1966). Control experiments indicated that the conditions employed here for alkaline hydrolysis caused no cleavage of peptide bonds in [^{125}I]STI or [^{125}I]STC.

If the arginine-63 residue of STI is in fact esterified to the active site serine residue of trypsin, then alkaline treatment of [^{125}I]STCd followed by reduction and carboxymethylation of disulfide bonds should yield a peptide which represents the first 63 residues of STI and a larger peptide of 118 residues. These two fragments would correspond to fragments A and BCD respectively which Koide and Ikenaka (1973a) obtained when they subjected trypsin-modified STI to reductive carboxymethylation. Accordingly, [^{125}I]STCd which had been treated with 0.1 N NaOH and then subjected to reductive carboxymethylation was passed through a column of Sephadex G-75. Unlabeled trypsin-modified STI (STI*) was added as a carrier prior to reductive carboxymethylation in order to provide increased yields of fragments A and BCD.

On the basis of their amino acid composition (data not presented) peaks I, II, and III in Figure 4 were identified as the RCM derivatives of intact STI, fragment BCD, and fragment A, respectively. Most of the radioactivity was associated with fragment A since this portion of the STI molecule contains 3 of the 4 tyrosine residues in STI (Koide and Ikenaka, 1973b). The minor nonradioactive peak observed between peaks II and III is most likely derived from the denatured trypsin present in [^{125}I]STCd.

Alternatively, it should also be possible to release fragment A from [^{125}I]STCd by carrying out the alkaline cleavage after reduction and carboxymethylation of disulfide bonds. Re-

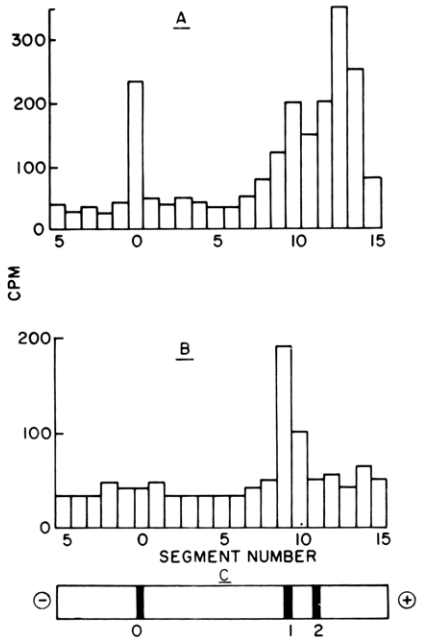

FIGURE 5: Electrophoresis of [^{125}I]STCd subjected to reduction and carboxymethylation followed by alkaline hydrolysis. Conditions for reductive carboxymethylation are the same as those described in legend to Figure 4 followed by exposure to 0.1 N NaOH for 20 min at 37 °C. Electrophoresis on cellulose acetate was carried out as described in legend to Figure 3. (A) Distribution of radioactivity after reductive carboxymethylation *without* subsequent alkaline treatment; (B) distribution of radioactivity after reductive carboxymethylation followed by alkaline treatment; (C) strip stained for protein showing the origin (0), fragment A (1), and RCM-[^{125}I]STI (2).

duction and carboxymethylation of [^{125}I]STCd without subsequent alkaline hydrolysis produced a rather heterogeneous distribution of radioactivity when subjected to electrophoresis on cellulose acetate (Figure 5A). Some of this heterogeneity may arise from the fact that STI may be attached to different species of trypsin which have undergone limited proteolysis (Schroeder and Shaw, 1968). Nevertheless it is clear that, if reductive carboxymethylation is followed by alkaline hydrolysis, most of the radioactivity then appeared in a position which coincided with fragment A (Figure 5B) rather than intact RCM [^{125}I]STI.

Reduction of STCd with NaB^{3}H$_4$. Thus far the evidence for an acyl intermediate in the form of an alkaline-labile ester bond has been largely indirect. More direct evidence for the presence of such a bond was sought by taking advantage of the fact that esterified carboxyl groups of proteins can be reduced to their corresponding amino alcohols with $LiBH_4$ (Chibnall and Rees, 1958). If there is in fact present in STCd an ester linkage between the carboxyl group of arginine-63 of STI and the hydroxyl group of serine-195 of trypsin, reduction with NaB^3H_4, should yield [1-^{3}H]argininol. Because it was only slightly soluble in water, STCd was predigested with pepsin prior to treatment with NaB^3H_4. As shown in Figure 6, when the acid hydrolysate of this preparation was examined by paper electrophoresis, a radioactive band corresponding to argininol was readily identified. About 70% of the total radioactivity incorporated into STCd was recovered as argininol; the remainder of the radioactivity is presumably due to nonspecific tritiation of other amino acids. As a control trypsin-modified STI (STI*), in which the carboxyl group of arginine-63 is intact, did not yield any detectable argininol when treated under precisely the same conditions as STCd.

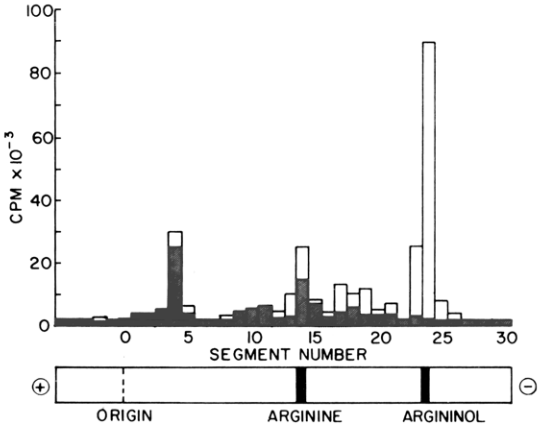

FIGURE 6: Paper electrophoresis of acid hydrolysate of STCd which had been reduced with NaB^3H_4. Peptic digest of STCd was reduced with NaB^3H_4, hydrolyzed with acid, and subjected to paper electrophoresis at pH 6 as described in text. The distribution of radioactivity in each segment (2.5 cm) for STCd is shown by the open bars; the closed bars show the distribution of activity obtained with trypsin-modified STI which had been subjected to identical treatment. Shown at the bottom of figure is the location of reference samples of arginine and argininol, as revealed by staining with ninhydrin and Sakaguchi's reagent.

Discussion

The postulated acyl-enzyme intermediate in which the acyl moiety of the substrate is covalently linked to the active site of the enzyme has actually been isolated in the case of the interaction of chymotrypsin with *p*-nitrophenyl acetate (Balls and Aldrich, 1955; Oosterbaan and van Adrichem, 1958; Wählby, 1970) and more recently with the β chain of insulin (Dahlquist and Wählby, 1975). The isolation of an acyl-enzyme intermediate involving macromolecular substrates, however, has proved much more difficult because of their low concentration and marked instability. In an analogous fashion STI may be viewed as playing the role of a substrate with which trypsin interacts in a highly specific fashion to form a stable complex which appears to exist primarily as a tetrahedral intermediate (Blow et al., 1974; Sweet et al., 1974). Proteolytic cleavage ensues, however, when the inhibitor is treated with catalytic levels of trypsin under acid conditions leading to the formation of a modified form of the inhibitor in which a specific peptide bond, arginine-63/isoleucine-64, has been cleaved (Ozawaka and Laskowski, 1966). Implicit in this analogy to the mechanism of action of a serine proteinase on its substrate is the existence of an acyl-enzyme intermediate in the step preceding the formation of the modified inhibitor.

In the studies reported here, the use of ^{125}I-labeled STI permitted the recognition of any STI that remained covalently bound to trypsin when the complex was subjected to conditions which favor the dissociation of the complex, that is, in the presence of 8 M urea. That portion of the complex, however, which remained resistant to dissociation by virtue of a covalent bond was irreversibly trapped by further denaturation with 10% trichloroacetic acid. It could be estimated that about 10% of the total radioactivity present in the [^{125}I]STI-trypsin complex could be recovered in this manner as a nondissociable, covalent complex. The judgment that this complex does in fact represent an acyl-enzyme intermediate involving an ester linkage is based on the following lines of evidence: (1) treatment of the denatured complex with 0.1 N NaOH caused the release of a new radioactive component which exhibited the same electrophoretic behavior as trypsin-modified STI (Figure 3). (2) Alkaline treatment of the denatured complex followed by reductive carboxymethylation of disulfide bonds resulted

in the release of fragment A of STI which represents the N-terminal portion of STI with arginine-63 as the C-terminal amino acid (Figure 4). (3) Fragment A was also produced when reductive carboxymethylation preceded alkaline treatment of the denatured complex (Figure 5). (4) Finally, argininol was identified in the acid hydrolysate of the denatured complex which had been reduced with NaB^3H_4 (Figure 6).

We interpret these results as strong supporting evidence for the existence of a true acyl intermediate in which the carboxyl group of arginine-63 of STI has formed an ester linkage with the active site serine of trypsin. This small amount of acyl-enzyme intermediate is probably in equilibrium with the tetrahedral complex which constitutes the predominant molecular species when STI and trypsin interact. The precise amount of acyl intermediate that is actually in equilibrium with the tetrahedral intermediate under nondenaturing conditions is difficult to assess since the true equilibrium in the complex would undoubtedly be disturbed during the course of the rather slow denaturing process employed here. Furthermore, the tetrahedral intermediate may have collapsed, at least in part, to the acyl-enzyme as the latter becomes trapped by denaturation in trichloroacetic acid. The amount of acyl intermediate actually present in the complex is probably so small that it would be impossible to detect by present crystallographic techniques. Despite these reservations the results presented in this paper would appear to support the conclusion that there is in fact an acyl intermediate in the reaction pathway leading to the formation of a modified inhibitor from a stable tetrahedral complex between trypsin and STI.

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