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Identity of the Tryptic and α-Chymotryptic Reactive Sites on Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: Incubation of soybean trypsin inhibitor (Kunitz) with catalytic quantities of tosyllysine chloromethyl ketone treated bovine α-chymotryspin leads to the hydrolysis of one specific peptide bond in the inhibitor molecule. Upon kinetic control dissociation at pH 2 of a complex made from α-chymotrypsin-modified inhibitor and from trypsin, the hydrolyzed bond is completely resynthesized; therefore, the bond is identified as Arg^{63}–Ile (the tryptic reactive-site peptide bond). In order to show that this peptide bond is the reactive-site peptide bond in the α-chymotrypsin–inhibitor complex, a complex was prepared by incubation of equimolar amounts of trypsin-modified inhibitor (Arg^{63}–Ile hydrolyzed) and of α-chymotrypsin for several hours at pH 7. Kinetic control dissociation of this complex was achieved by incubation with a large excess of the active-site titrant *p*-nitrophenyl *N*-acetyl-*N*-benzylcarbazate. The recovered free inhibitor was predominantly virgin (Arg^{63}–Ile intact). On these bases it is concluded that the principal reactive site for the interaction of soybean trypsin inhibitor (Kunitz) with α-chymotrypsin and trypsin is the same.

Soybean trypsin inhibitor (Kunitz) (STI)[1] is a strong inhibitor of trypsin-like enzymes. However, Kunitz (1947) showed that it also inhibits bovine α-chymotrypsin, albeit considerably more weakly than trypsin. Extensive work in this laboratory (for a review, see Laskowski and Sealock, 1971) has shown that on the surface of the soybean trypsin inhibitor molecule there is a relatively small cluster of amino acid residues, called the reactive site, which comes into intimate contact with the active site of trypsin in the stable trypsin–STI complex. Within this site there is a special peptide bond (Arg^{63}–Ile)[2] which is particularly important to the enzyme–inhibitor interaction and which is referred to as a reactive-site peptide bond. In view of the lack of detailed knowledge of the structure of the stable complex this bond is temporarily only operationally defined, although it is hoped that X-ray crystallographic studies will replace the operational definition by a more fundamental one.

Therefore, it was of interest to inquire whether the interaction of STI with α-chymotrypsin follows a mechanism similar to that of its interaction with trypsin and if it does to identify the α-chymotryptic reactive-site peptide bond. We had expected that this bond would differ from the tryptic reactive-site peptide bond since for several protein proteinase inhibitors, which inhibit both trypsin and α-chymotrypsin, it has been amply demonstrated that the inhibitions occur on separate reactive sites (*e.g.*, turkey ovomucoid (Rhodes *et al.*, 1960; Stevens and Feeney, 1963), soybean inhibitor (Bowman-Birk) (Birk *et al.*, 1967; Odani *et al.*, 1971; Seidl and Liener, 1971, 1972), lima bean inhibitor (Tan and Stevens, 1971a,b; Krahn and Stevens, 1972)).

When preliminary evidence indicated that the tryptic and α-chymotryptic reactive-site peptide bonds were the same, the intent of the work switched from a search for a new reactive site to providing as conclusive a proof as possible that the two sites are identical. The approach used in this paper is therefore closely similar to that employed by Hixson and Laskowski (1970a) in showing that cocoonase and bovine trypsin interact with the same reactive site in STI. However, there are some differences. In the work on cocoonase there was

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[1] Abbreviations used are: STI, soybean trypsin inhibitor (Kunitz) in all of its forms; S, soybean trypsin inhibitor (Kunitz) (Arg^{63}–Ile reactive-site peptide bond intact); S*, modified soybean trypsin inhibitor (Kunitz) (Arg^{63}–Ile peptide bond hydrolyzed); NPABC, *p*-nitrophenyl N_2-acetyl-N_1-benzylcarbazate; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; TPCK, tosylphenylalanine chloromethyl ketone; TLCK, tosyllysine chloromethyl ketone.

[2] This bond was originally identified as Arg^{64}–Ile by Ozawa and Laskowski (1966), and this designation was kept in many of the papers from our and other laboratories. Since the complete amino acid sequence of STI has now been determined (Koide *et al.*, 1972), the numbering system used here is based on that sequence.

a stringent limitation on the quantity of available enzyme—this was not a problem with bovine α-chymotrypsin. On the other hand the weaker interaction of STI with α-chymotrypsin and especially the slow association of modified inhibitor with α-chymotrypsin gave rise to some new experimental problems.

In providing a proof for the identity of the reactive sites it may be worthwhile to summarize the properties which operationally define Arg^{63}–Ile bond as the the tryptic reactive-site peptide bond. They are as follows. (1) Incubation of virgin inhibitor with catalytic quantities of trypsin at any pH value leads to specific hydrolysis of Arg^{63}–Ile bond in the inhibitor (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966) until an equilibrium composition is attained (Niekamp *et al.*, 1969). Both virgin (bond intact) and modified (bond hydrolyzed) inhibitors are active, but virgin inhibitor reacts with trypsin far more rapidly than the modified one (Finkenstadt and Laskowski, 1965; Laskowski *et al.*, 1971; Luthy *et al.*, 1972). (2) Incubation of modified inhibitor with carboxypeptidase B leads to a specific removal of the newly formed COOH-terminal Arg^{63} and produces inactive desarginine^{63}-modified inhibitor (Finkenstadt and Laskowski, 1965). Similarly, chemical modifications of the newly formed NH_2-terminal Ile^{64} lead to complete loss of inhibitor activity (Haynes and Feeney, 1968; Laskowski *et al.*, 1971; Kowalski and Laskowski, 1972). (3) The stable complexes formed by trypsin and by either virgin or modified soybean trypsin inhibitor are indistinguishable by all physical criteria (Laskowski *et al.*, 1971). Most importantly kinetic control dissociation of these complexes leads to formation of trypsin and of predominantly virgin inhibitor (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b). This result clearly requires intimate contact between the active site of the enzyme and reactive site of the inhibitor.

Experimental Section

Materials and Methods. Virgin soybean trypsin inhibitor (Kunitz) (lot B7303) was obtained from Gallard-Schlesinger Chemical Corp. Bovine α-chymotrypsin (three-times crystallized) (lots 6033 and CDIOLC), bovine trypsin (lots TRL 71C and TRL 1DA), and porcine carboxypeptidase B (lot COBC 81A) were obtained from Worthington Biochemical Corp. Electrophoretically purified trypsin modified STI (S*) was prepared by Dr. Carl Niekamp in our laboratory (Niekamp *et al.*, 1969). NPABC was the product of Nutritional Biochemical Co. NPGB, TLCK, and TPCK were purchased from Cyclo Chemical Co. Sodium cacodylate·$6H_2O$ was obtained from K & K Laboratories, Inc. Sephadexes G-75 and G-200 were purchased from Pharmacia Fine Chemicals. Glycine, acrylamide, *N,N*-bisacryamide, and Napthol Blue-Black were products of Eastman Organic Chemicals. Ultra Pure Tris was purchased from Mann Research Laboratories. All other chemicals were at least reagent grade.

pH measurements were made on an Orion digital pH meter with a Fischer microprobe combination electrode or on a Radiometer pH meter with a Radiometer combination electrode, type GK2302C. Water used in experiments was distilled, deionized by a mixed-bed Barnstead column deionizer, and sometimes redistilled.

The molarity of active trypsin was determined using the NPGB active-site titration procedure of Chase and Shaw (1967). The molarity of active α-chymotrypsin was determined by the NPABC procedure of Elmore and Smyth (1968). Measurements were made on a Cary 14 spectrophotometer using the 0.10 slide-wire. First, titrant was introduced into sample and reference cuvets, and a base line was set at 400 nm. An aliquot of enzyme was added to the sample cuvet using a Hamilton syringe; an identical aliquot of buffer was added to the reference cuvet. Recording was begun 15 sec after addition of the samples.

Analytical Gels. Small pore acrylamide gels were prepared according to the formulation of Davis (1964). pH 9.2 gels described by Niekamp *et al.* (1969) were used to monitor α-chymotrypsin–STI complex formation (Figure 4). pH 9.4 gels with a pH 9.3 Tris–glycine buffer in the Sephadex G-200 stacking phase were used to separate virgin, modified, and des-63-arginine-modified forms of STI (R. Sealock, unpublished experiments). Gels were run at a constant 2 mA/tube for 4–5 hr. Gels were stained in Naphthol Blue-Black, electrophoretically destained, scanned with a Gilford Model 2400 spectrophotometer and linear transport at 625 nm, and recorded on a Sargent Model SR recorder. Peaks were resolved and integrated using a DuPont 310 curve resolver.

Preparation of Proteins. To block any trypsin impurity in the α-chmotrypsin, the protein was treated with TLCK according to the procedure of Shaw *et al.* (1965). TPCK treatment of α-chymotrypsin to test for a trypsin impurity followed the procedure of Schoellman and Shaw (1963). Tris–maleate buffer (0.01 M, pH 6) and $CaCl_2$ (0.005 M) were used to stabilize any trypsin. An equilibrium preparation of α-chymotrypsin-modified STI was made by incubating with 5 mole % TLCK–α-chymotrypsin at pH 5.0 for several weeks. STI was separated from α-chymotrypsin by chromatography on a G-75 column equilibrated with 6 M guanidine hydrochloride–0.01 M Tris (pH 7.5)–0.10 M NaCl, similar to Sealock and Laskowski (1969).

Monitoring of Virgin ⇄ Modified Inhibitor Interconversion. The reactive-site hydrolyses and resyntheses were studied using 10^{-4} M STI in 0.50 M KCl–0.05 M $CaCl_2$. The pH was adjusted with 0.1 N HCl or 0.1 N NaOH. Aliquots of reaction mixtures were withdrawn and directly treated in the manner described for analytical gels. Incubations to study virgin STI–α-chymotrypsin and trypsin-modified STI–α-chymotrypsin complex formation were conducted in 0.05 M sodium cacodylate to simplify the use of the NPABC assay.

Kinetic Control Dissociation of Complex. Three methods were used to effect a rapid, irreversible dissociation of complex: (1) The α-chymotrypsin active-site titrant, NPABC (Elmore and Smyth, 1968), afforded a convenient means of dissociating α-chymotrypsin–STI complexes at pH 7.0. It is used as an extremely fast, irreversible inhibitor of α-chymotrypsin since it acylates the enzyme very rapidly but deacylates very slowly. NPABC, dissolved in acetonitrile, was added in 20–40 mole excess over the total α-chymotrypsin available in the reaction mixture. The final acetonitrile concentration was at most 10%, and typically less than this. After sufficient time had been allowed for dissociation of complex (~10 min for α-chymotrypsin–STI) an appropriate aliquot was analyzed on the pH 9.4 gel system. (2) A less convenient method was to drop the pH of a solution of α-chymotrypsin inhibitor complex rapidly to 2 and quickly add guanidine hydrochloride to a final 6 M concentration. Separation of native STI from denatured protein was accomplished as described in Sealock and Laskowski (1969). (3) Finally, kinetic control dissociation of the trypsin–STI complex was accomplished by the pH drop–ammonium sulfate procedure described by Finkenstadt and Laskowski (1967).

Results

Preliminary Identification of the Hydrolyzed Bond. In screening experiments soybean trypsin inhibitor (Kunitz) was incubated with catalytic quantities of TLCK-treated α-chymotrypsin at a variety of pH values. Aliquots of the incubated solution were withdrawn at various times and subjected to disc gel electrophoresis. As the incubation proceeded the original virgin STI band diminished in intensity and a new, faster moving band appeared. The optimal pH for this conversion appeared to be pH 5.2. The mobility of the new band was the same as that of trypsin-modified soybean trypsin inhibitor (Arg^{63}–Ile bond hydrolyzed). However, this was initially taken to mean only that some peptide bond had been hydrolyzed in the new species and that this species had one more negative charge than the virgin STI in the pH 9.4 gel system (COO^-H_2N replacing C(O)NH) (Niekamp *et al.*, 1969).

To test whether some of the observed hydrolysis arose as a result of a small tryptic contamination we subjected partially α-chymotrypsin-modified samples to hydrolysis by carboxypeptidase B. Carboxypeptidase B quantitatively removes the COOH-terminal Arg from trypsin-modified STI (Finkenstadt and Laskowski, 1965) and, therefore, converts it to an even faster moving component on disc gel electrophoresis. To our great surprise all of the α-chymotrypsin-modified band was converted to a faster moving component indistinguishable from $desArg^{63}$-modified STI. This result formally allowed us only to conclude that the TLCK-treated α-chymotrypsin preparation hydrolyzes a Lys–X or an Arg–X bond in STI. We have assumed, however, that the bond was Arg^{63}–Ile and proceeded with an identification on this basis.

Definite Assignment of the Hydrolyzed Bond. In order to obtain more definitive data virgin STI was incubated at pH 5.0 with 5 mole % of the following enzymes: trypsin, TLCK–α-chymotrypsin, TPCK–α-chymotrypsin, and TLCK– and TPCK–α-chymotrypsin. The data (Figure 1) show that both trypsin and TLCK–α-chymotrypsin affected the conversion and that with either enzyme the same final composition 82 ± 2% modified 18 ± 2% virgin was obtained. This composition corresponds to $K_{hyd} = 4.5$. Closely similar values are now repeatedly found in our laboratory at pH 5.0 for the trypsin catalyzed conversion of STI to the modified form with Arg^{63}–Ile bond hydrolyzed (J. Mattis, unpublished results). It should also be noted in Figure 1 that the trypsin-catalyzed conversion is accurately zero order (the curvature near the equilibrium position arises only from the effect of the reverse reaction (J. Mattis, unpublished results)), while the α-chymotrypsin-catalyzed conversion is roughly first order. The difference in the order of the reaction clearly emphasizes that the α-chymotrypsin catalyzed conversion is not caused by a trypsin impurity. This is even more dramatically emphasized by the result that TPCK-treated α-chymotrypsin does not catalyze the conversion.

In order to show that α-chymotrypsin acts on the same bond as trypsin does a sample of pure, trypsin-modified STI (Arg^{63}–Ile bond hydrolyzed) was incubated at pH 5.0 with 5 mole % of α-chymotrypsin. As shown at the top of Figure 1, α-chymotrypsin converts it to an equilibrium mixture of virgin and modified inhibitor by resynthesizing the hydrolyzed bond in 18% of the molecules. Note that if α-chymotrypsin had acted on another bond one would expect additional hydrolysis rather than resynthesis.

The best proof that α-chymotrypsin hydrolyzes in STI the Arg^{63}–Ile tryptic reactive-site peptide bond is provided

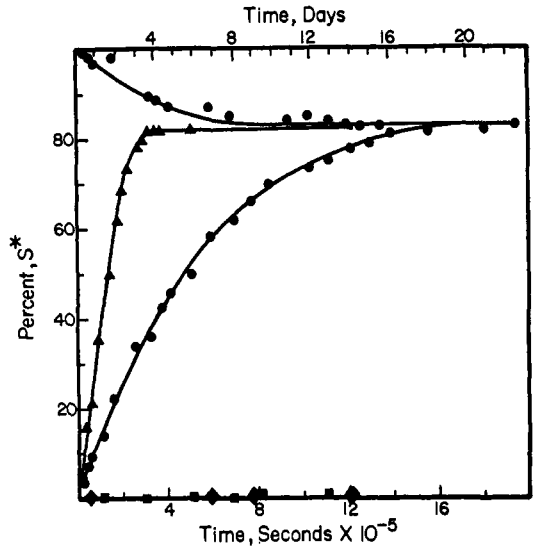

FIGURE 1: Conversion of virgin and pure modified soybean trypsin inhibitor into the pH 5.0 equilibrium mixture at 20°. Concentrations were 1.0×10^{-4} M for STI and 1.4×10^{-4} M for modified STI. The solvent was 0.5 M KCl–0.05 M $CaCl_2$. Enzyme concentrations were 5 mole % in: (●) TLCK-treated α-chymotrypsin, (▲) trypsin, (■) TPCK-treated α-chymotrypsin, and (◆) TLCK,TPCK-treated α-chymotrypsin. For convenience the time scales are shown both in days and in seconds.

by kinetic control dissociation of a complex made by combining equimolar quantities of TLCK–α-chymotrypsin hydrolyzed inhibitor (55% modified, 45% virgin) (gel A, Figure 2) with trypsin at pH 7. This complex was subjected to rapid dissociation at pH 2, and the free inhibitor was isolated by precipitation with ammonium sulfate at pH 2. The recovered inhibitor was 100% virgin (gel B, Figure 2). Thus, association with trypsin followed by rapid dissociation led to complete resynthesis of the bond hydrolyzed by α-chymotrypsin. As discussed by Finkenstadt and Laskowski (1967) and by Hixson and Laskowski (1970) the kinetic control resynthesis of a hydrolyzed peptide bond occurs only at the reactive-site peptide bond of the inhibitor for the enzyme with which it is combined in complex.

The experiments described above clearly show that α-chymotrypsin specifically hydrolyzes the Arg^{63}–Ile peptide bond in soybean trypsin inhibitor (Kunitz). They do not as yet show that this bond is the reactive site peptide bond for α-chymotrypsin. Furthermore, a remote possibility exists that the hydrolysis was catalyzed by an enzyme impurity present in the α-chymotrypsin preparation. However, such an enzyme impurity is not trypsin-like since the impurity (if it exists) is inactivated by TPCK treatment but not by TLCK treatment.

Demonstration that Arg^{63}–Ile Bond is the Reactive-Site Peptide Bond for α-Chymotrypsin. Having now shown that α-chymotrypsin specifically hydrolyzes the Arg^{63}–Ile bond, we still need to show that this bond is the reactive-site peptide bond for inhibition of α-chymotrypsin. Therefore, we must demonstrate that a stable 1:1 complex made from either virgin or modified STI and α-chymotrypsin is the same chemical substance.

Formation of α-chymotrypsin–STI complex was monitored by two methods—reaction with the molecular titrant NPABC introduced by Elmore and Smyth (1968) and disc gel electrophoresis, where bands due to free inhibitor and to α-chymotrypsin–STI complex could be observed (since α-chymo-

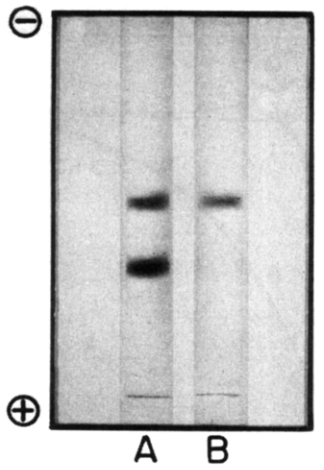

FIGURE 2: Kinetic control dissociation of the stable complex of trypsin with α-chymotrypsin-modified STI. α-Chymotrypsin-modified STI was prepared by incubating 5 mole % TLCK-treated α-chymotrypsin with STI at pH 5.0 in 0.05 M sodium acetate with 0.5 M KCl–0.05M $CaCl_2$. The α-chymotrypsin was removed by chromatography on a 6 M guanidine hydrochloride column according to Sealock and Laskowski (1969). α-Chymotrypsin- (5×10^{-5} M) modified STI incubated with an equal amount of trypsin was subjected to kinetic control dissociation by the pH drop–ammonium sulfate precipitation method (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b). Gel A: initial α-chymotrypsin-modified STI mixture; gel B: STI obtained from kinetic control dissociation of the complex of trypsin with material shown in gel A.

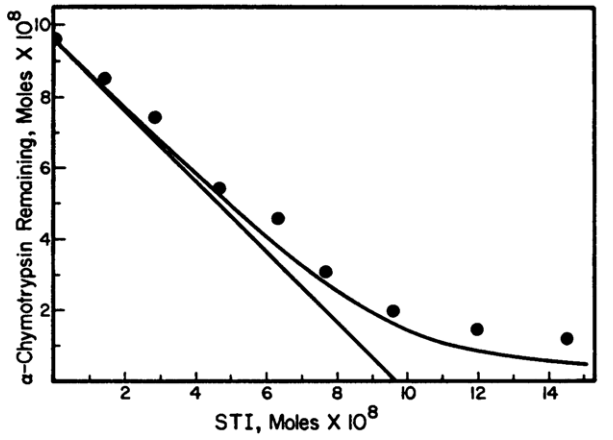

FIGURE 4: NPABC titration of mixtures of α-chymotrypsin with virgin STI. α-Chymotrypsin was held at 1×10^{-4} M. Sodium cacodylate (0.05 M, pH 7.04) was used as solvent. The solid curve is a theoretical curve generated using $K_{assoc} = 3 \times 10^5$ M^{-1}. The solid line represents the case of an infinitely large K_{assoc}. The data were extrapolated to zero time to obtain the concentration of free α-chymotrypsin.

trypsin is cationic it does not enter the gels at this stacking pH). The NPABC assay of α-chymotrypsin–STI mixtures exhibits a behavior strictly analogous to the NPGB titration of trypsin–ovoinhibitor mixtures described by Zahnley and Davis (1970). A rapid *p*-nitrophenoxide burst (Figure 3) is followed by a slower first-order release (half-time ~45 sec) of *p*-nitrophenoxide. The total release corresponds to the total amount of α-chymotrypsin present, but the amount of burst portion decreases and the amount of the slow release portion correspondingly increases as more inhibitor is added. Clearly, the rapid burst is a measure of free α-chymotrypsin in solution, while the slow release arises from the reaction of NPABC with α-chymotrypsin which is produced by dissociation of complex. Since the slow phase of the release is still relatively rapid and since NPABC has a sizeable spontaneous hydrolysis rate, the quantitative separation of the burst and slow release portions is rather poor. However, it could be used to measure the fractions of free and complexed α-chymotrypsin with an accuracy of 5–10%. This appearance of the slow release phase in the α-chymotrypsin–STI system is in contrast to results with the trypsin–STI complex (monitored by NPGB), where at neutral pH the complex dissociation is too slow to be usefully monitored in this way. On the time scale of the experiments only a burst due to free trypsin is observed. This difference is another demonstration of the well known fact that trypsin–STI complexes are strong and dissociate slowly while α-chymotrypsin–STI complexes are weak and dissociate rapidly.

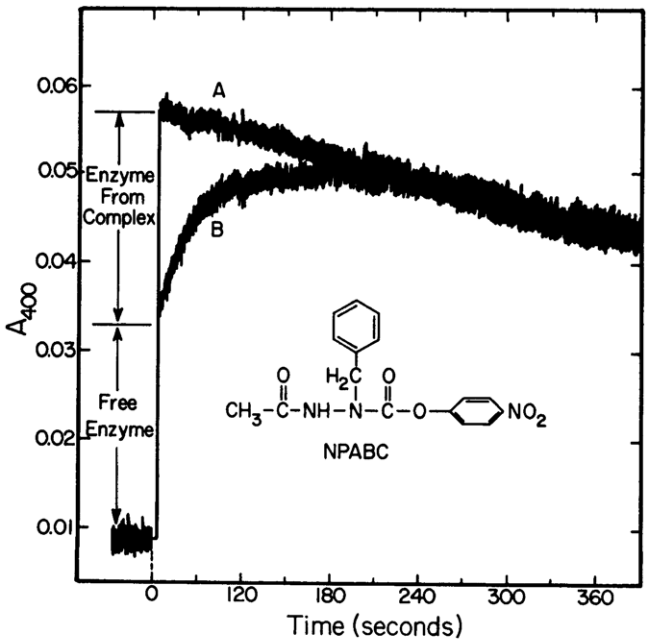

FIGURE 3: Time course of NPABC assay of α-chymotrypsin and α-chymotrypsin–STI mixtures at pH 7.04. Curve A: α-chymotrypsin; curve B: α-chymotrypsin–STI mixture (1:1.55). The vertical dashed line represents the time at which enzyme was added to the sample cuvet and buffer was added to the reference cuvet.

On our time scale the association of virgin STI with α-chymotrypsin was essentially instantaneous. The data (Figure 4) are consistent with a 1:1 complex formation and K_{assoc} between 2×10^5 and 5×10^5 M^{-1}. This compares very favorably with K_{assoc} of 5×10^5 M^{-1} calculated by Laskowski and Laskowski (1954) from the data of Kunitz (1947) but differs a good deal from the value of 1×10^7 M^{-1} obtained by Kasche (1970) using gel equilibrium dialysis. While a 1:1 complex is strongly indicated, the data are too scattered to allow exclusion of the possibility that another molecule of α-chymotrypsin may be inhibited as well, although more weakly (Wu and Laskowski, 1955).

In contrast to the interaction with virgin inhibitor, the rate of association of α-chymotrypsin with modified STI is extremely slow and shows rather complex kinetic behavior. With 10^{-4} M α-chymotrypsin and 10^{-4} M modified STI the association had a half-life of roughly 30 min and progressed very slowly thereafter. We have rather arbitrarily chosen 20 hr as the time at which the reaction was at equilibrium. The

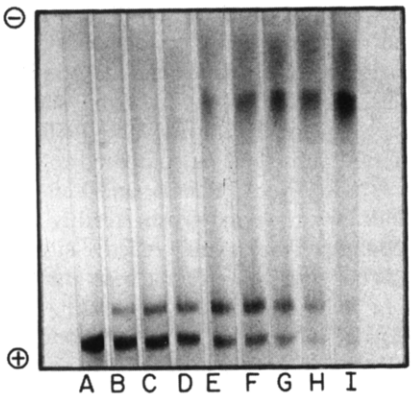

FIGURE 5: Dependence of complex formation on the mole ratio of α-chymotrypsin to trypsin-modified STI. Mixtures were dissolved in 0.05 M sodium cacodylate (pH 7.04) and incubated for 20 hr.

Gel	A	B	C	D	E	F	G	H	I
(α-Chymotrypsin) / (total STI)	0.0	0.25	0.37	0.50	0.75	0.87	1.00	1.25	1.75

NPABC titration data are again consistent with a 1:1 association and K_{assoc} of 2×10^5 M^{-1}, closely similar to that obtained with virgin inhibitor.

The disc gel monitoring of complex formation consisted simply of withdrawing aliquots from α-chymotrypsin–inhibitor mixtures and running them on disc gel electrophoresis. A typical result is shown in Figure 5. The α-chymotrypsin–STI complexes stack very poorly in all electrophoretic systems tried, in contrast with trypsin–STI complexes which stack relatively well (S. Herbert, unpublished experiments). This may be another reflection of the weakness and rapid dissociation of α-chymotrypsin–STI complexes.

Kinetic control dissociation (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970) experiments on a complex made from α-chymotrypsin- and trypsin-modified STI (Arg^{63}–Ile bond hydrolyzed) were undertaken. A series of solutions with an initial trypsin-modified[3] inhibitor concentration of 5×10^{-5} M and variable TLCK–α-chymotrypsin concentration was prepared. After incubation at pH 7.04 for 20 hr at 20° to achieve maximal complex formation, monitoring by disc gel electrophoresis gave results similar to those of Figure 5. To achieve kinetic control dissociation NPABC in acetonitrile was added to each sample in at least 20 molar excess with respect to total α-chymotrypsin.[4] After sufficient time to allow dissociation to proceed to completion, the samples were subjected to disc gel electrophoresis. All of the α-chymotrypsin–STI complex bands disappeared.

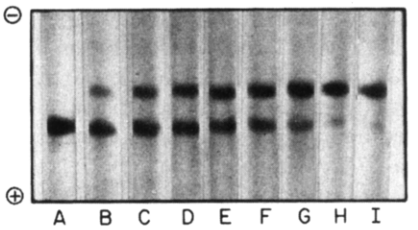

FIGURE 6: Kinetic control dissociation of α-chymotrypsin–trypsin-modified STI mixtures with NPABC. Inhibitor (0.5×10^{-4} M) was preincubated in 0.05 M sodium cacodylate (pH 7.04) for 20 hr with TLCK-treated α-chymotrypsin before addition of NPABC.

Gel	A	B	C	D	E	F	G	H	I
(α-Chymotrypsin) / (total STI)	0.00	0.19	0.28	0.36	0.49	0.63	0.85	1.14	1.42

The distribution between virgin and modified STI with increasing α-chymotrypsin is shown in Figure 6. The fraction of virgin inhibitor in each of these samples was measured and the results are plotted in Figure 7. The identification of the virgin inhibitor band as authentic virgin STI was confirmed by tryptic conversion of this material to modified inhibitor, and this modified inhibitor was further converted to des-63-Arg-modified STI by carboxypeptidase B. Qualitatively, the results of Figure 7 clearly indicate that upon kinetic control dissociation, the complex of α-chymotrypsin with trypsin-modified STI yields predominantly virgin inhibitor. In a separate experiment kinetic control dissociation of the complex of α-chymotrypsin with virgin STI also predominantly yields virgin inhibitor. Therefore, the two complexes are the same chemical substance, completing our proof that Arg^{63}–Ile is the reactive-site peptide bond for interaction with α-chymotrypsin.

Quantitatively, these results are much harder to interpret than those on kinetic control dissociation of trypsin-modified inhibitor complex (Figure 5 of Hixson and Laskowski, 1970b). The reasons for this are: (a) K_{assoc} is relatively low (10^5–10^6 M^{-1}); therefore, the concentration of complex is not equal to the concentration of α-chymotrypsin added; the result

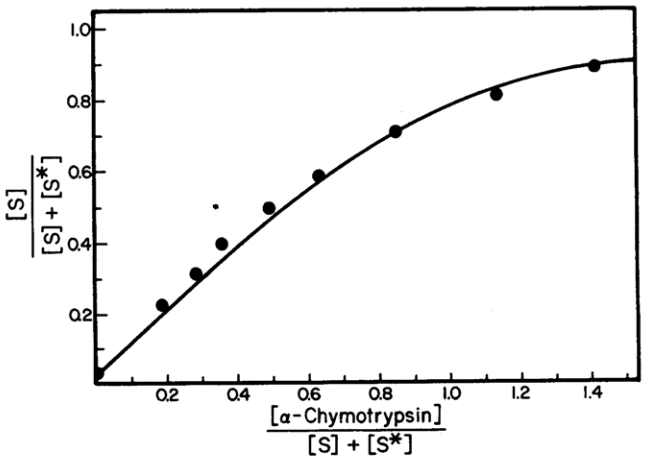

FIGURE 7: Fraction of virgin inhibitor obtained after kinetic control dissociation of samples described in Figure 6. The solid line represents a theoretical curve generated using $K_{assoc} = 3 \times 10^5$ M^{-1}.

[3] Since it has now been shown that incubation of virgin inhibitor with catalytic quantities of either trypsin or of α-chymotrypsin leads to the hydrolysis of the same Arg^{63}–Ile bond, the distinction between trypsin-modified and α-chymotrypsin-modified inhibitor is now redundant. It is nonetheless kept throughout the Results section solely as a convenient shorthand for the experimental procedure employed. Similarly, it has already been shown that the complexes of trypsin with virgin and modified inhibitors are the same substance (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b). It is the major conclusion of this paper that the complexes of α-chymotrypsin with virgin and with modified inhibitor are the same substance. However, the distinction between these substances is again maintained in the Results section for the sake of clarity.

[4] The use of molecular titrants to cause kinetic control dissociation is a better procedure than either dissociation in acid (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b) or in 6 M guanidine hydrochloride (Sealock and Laskowski, 1969). These later methods require that the enzyme and inhibitor be separated, and the separation procedures may possibly introduce some bias into the virgin:modified inhibitor ratio. No such bias is introduced here since there is no need to remove the inactive α-chymotrypsin, and the whole sample can be subjected to analysis.

is a smooth curve rather than two line segments with a sharp bend found for trypsin; (b) a long incubation time was needed for complex formation, and, therefore, α-chymotrypsin was able to partially reequilibrate the original 3% virgin–97% modified mixture toward the equilibrium distribution at pH 7 (33% virgin–67% modified). The occurrence of this effect can be seen by inspection of the gels in Figure 4 where the ratio of virgin to modified inhibitor for the free STI species in the presence of complex is affected in favor of virgin inhibitor by large amounts of α-chymotrypsin.

Nonetheless, we were able to fit the data reasonably well by considering only the effect of (a) ignoring (b). This is shown by the solid curve in Figure 7. To make the calculation we assumed that only a 1:1 complex forms, that K_{assoc} is 3×10^5 M^{-1}, and that kinetic control dissociation of pure complex (in absence of any free inhibitor) yields essentially 100% virgin inhibitor. Unfortunately, the correctness of the last assumption cannot be accurately checked from our data. We can only state that the inhibitor obtained from the dissociation is at least 90% virgin.

Aside from providing the direct proof that the Arg^{63}–Ile bond is the reactive-site peptide bond and not simply a bond that happens to be hydrolyzed by α-chymotrypsin, the kinetic control resynthesis described above eliminates the unlikely possibility that the hydrolysis of the Arg^{63}–Ile was caused by an unknown enzyme impurity. Kinetic control resynthesis requires 1 mole of enzyme/mole of resynthesized material. For this resynthesis to be caused by an impurity there would have to be more moles of this impurity enzyme than of α-chymotrypsin in the α-chymotrypsin preparations.

The results presented here do not exclude the possibility that there may be another site on STI for interaction with α-chymotrypsin. On incubation of des-63-Arg-modified STI with equimolar amounts of α-chymotrypsin under conditions used to study the interaction of virgin $\rightleftharpoons$ modified STI with α-chymotrypsin no complex is demonstrable on disc gels, and little interaction is apparent by NPABC assay. Some precipitation occurred, however when several molar excess α-chymotrypsin was added to des-63-Arg-modified STI at 10^{-4} M, indicating an interaction. The additional interaction may be either nonspecific or involve another very weak reactive site. Addition of massive amounts of α-chymotrypsin (1 mM) to a solution of des-63-Arg-modified inhibitor (0.5 mM) at very low ionic strength produces very poorly stacked bands in the region corresponding to α-chymotrypsin–STI complex. Therefore, the conclusions of this paper apply only to the major site of α-chymotrypsin–STI interaction.

Discussion

The work reported here shows not only that Arg^{63}–Ile is a reactive-site peptide bond for the α-chymotrypsin–soybean trypsin inhibitor interaction but also that there are several similarities between the interaction of this inhibitor with trypsin and with α-chymotrypsin. These are: (1) a more rapid association of the virgin than of modified STI with α-chymotrypsin to form a complex, and (2) the production of primarily virgin STI upon kinetic control dissociation of enzyme–inhibitor complex. These regularities are not specifically demanded by the reactive-site mechanism but appear to be common to interactions of trypsin and α-chymotrypsin with various inhibitors thus far examined (Laskowski and Sealock, 1971). The surprising finding that soybean trypsin inhibitor (Kunitz) utilizes the same reactive-site peptide bond (Arg^{63}–Ile) for interaction with trypsin and with α-chymotrypsin clearly points out the primacy of the proper reactive-site conformation over the type of residue contributing the carbonyl group to the reactive-site peptide bond. However, α-chymotrypsin is capable of hydrolyzing some Lys–X and Arg–X bonds (Inagami and Sturtevant, 1960), but these are not the preferred substrates as compared to the more commonly split Trp–X, Tyr–X, Phe–X, and Leu–X bonds (Hill, 1965). In addition, α-chymotrypsin readily hydrolyzes the Lys^{28}–Ser^{29} bond at the tryptic reactive-site peptide bond of reduced carboxymethylated lima bean inhibitor (Tan and Stevens, 1971a,b; Krahn and Stevens, 1972). It preferentially hydrolyzes Lys^{15}–Ala^{16} and Arg^{39}–Ala^{40} bonds in pancreatic trypsin inhibitor whose Cys^{14}–Cys^{38} disulfide bridge was selectively reduced (Rigbi, 1971).

The observation of specific Arg^{63}–Ile hydrolysis by α-chymotrypsin is even more surprising when we recall that the preceding residue is Tyr^{62} (*i.e.*, Tyr-Arg-Ile, Koide *et al.*, 1972) and that this tyrosyl residue is exposed to solvent (Niekamp, 1971). However, examination of the three dimensional structure of the Lys^{15}–Ala^{16} reactive site of pancreatic trypsin inhibitor (Kunitz) (Huber *et al.*, 1970, 1971) shows that the NH of the Ala^{16} residue is hydrogen bonded to the backbone of the protein, while the amido group of Lys^{15} is freely exposed to the solution. It seems reasonable that such a peptide N–H hydrogen-bonding pattern is a necessary (but not sufficient) requirement for a reactive site of all protein proteinase inhibitors. We have argued that in most or all protein proteinase inhibitors the NH_2-terminal partner amino acid is strongly attached to the protein backbone (Laskowski, 1970; Laskowski and Sealock, 1971); the hydrogen bonding of its amino group may well aid in this function. On the other hand the exposed amido hydrogen of Lys^{15} (and the corresponding residues of other inhibitors) may be required for recognition by the enzyme (the acylamido site, Cohen and Schultz, 1968). If these presumptions are correct, then it follows that, in a sequence P_2–P_1–P_1'–P_2' (using the notation of Schechter and Berger, 1967) where P_1–P_1' is the reactive-site peptide bond for one serine esterase, neither P_2–P_1 (Tyr^{62}–Arg^{63} in STI) nor P_1'–P_2' can serve as a reactive-site peptide bond for any other serine esterase.

On the basis of the results given here it is tempting to speculate that if Arg^{63} in STI were replaced by Trp, Tyr, Phe, or Leu the resultant protein would be a strong inhibitor of α-chymotrypsin. Such a surmise gains additional support from the results of Tan and Stevens (1971a,b) showing that in the double-headed lima bean inhibitor the specific reactive site for α-chymotrypsin and the specific reactive site for trypsin (Krahn and Stevens, 1972) have almost identical sequences and differ primarily by a Lys (tryptic site) to Leu (chymotryptic site) replacement. If STI derivatives with Trp^{63}, Tyr^{63}, Phe^{63}, or Leu^{63} made by an enzymatic replacement similar to that of Sealock and Laskowski (1969) could be obtained and if as expected they would inhibit α-chymotrypsin strongly, it would be of great interest to find out whether they would also be weak inhibitors of trypsin. Preliminary results (T. R. Leary, unpublished data) suggest that these expectations are indeed realized. On the other hand, all trypsin inhibitors thus far studied could be easily assigned as either Arg or Lys type, and so far there is no hint of a naturally occurring weak inhibitor with some other residue at its reactive site.

The inhibition of α-chymotrypsin at the strong trypsin reactive site is probably not unique for protease inhibitors. The studies of Rigbi (1971) on bovine pancreatic trypsin inhibitor (Kunitz) lend strong support—but no proof—that the Lys^{15}–Ala^{16} tryptic reactive-site peptide bond of that

inhibitor is also its α-chymotryptic reactive-site peptide bond. Unfortunately, his studies cannot be as definitive as ours since the modified (Lys^{15}–Ala bond hydrolyzed) form of pancreatic inhibitor was thus far not obtained in any laboratory without previous chemical alteration of the covalent structure of the inhibitor. In a recent detailed study of the interaction of α-chymotrypsin with pancreatic trypsin inhibitor (Kunitz) based on the three-dimensional models of both proteins, Blow *et al.* (1972) assumed that Lys^{15}–Ala was the reactive-site peptide bond for the interaction with α-chymotrypsin. The fit was excellent.

However, rather extensive literature exists pointing out that neither chicken ovomucoid nor bovine or porcine pancreatic secretory inhibitors (Kazal) inhibit α-chymotrypsin even though they have clearly defined reactive sites for inhibition of trypsin (Wu and Laskowski, 1955; Feeney *et al.*, 1963; Burck *et al.*, 1967; Fritz *et al.*, 1967). Two explanations for the results that some trypsin inhibitors inhibit α-chymotrypsin weakly at their reactive sites and others do not are possible. The simpler of these is that the weak α-chymotrypsin inhibition phenomenon will simply be observed only for very strong trypsin inhibitors. Since the association equilibrium constants for interaction of either soybean trypsin inhibitor (Kunitz) or of pancreatic trypsin inhibitor (Kunitz) with α-chymotrypsin are several orders of magnitude weaker than those for interaction of these inhibitors with trypsin, one may simply argue that in case of relatively weak trypsin inhibitors the interaction with α-chymotrypsin will be too weak to be observed. The presently available K_{assoc} data for various inhibitors are not sufficiently accurate or sufficiently comparable to make this conclusion quantitative. Alternately, the ability of some trypsin inhibitors to inhibit and of others not to inhibit α-chymotrypsin at their reactive sites is another reflection of secondary specificity of trypsin inhibitors. Such secondary specificity is already clearly manifested in the ability of some P_1 Lys and P_1 Arg inhibitors of bovine trypsin to inhibit plasmin or human trypsin, which are not inhibited by other P_1 Lys and P_1 Arg inhibitors of bovine trypsin (Feeney *et al.*, 1969).

Finally, we wish to point out that the results of this paper invalidate the conclusion reached in the discussion of the paper by Hixson and Laskowski (1970a) that the interaction of both cocoonase and trypsin with soybean trypsin inhibitor at the same reactive-site Arg^{63}–Ile provides additional evidence for classification of cocoonase as a trypsin-like enzyme. Clearly, finding that an unknown enzyme interacts with Arg^{63}–Ile site of STI is not a satisfactory criterion for classifying it as trypsin or α-chymotrypsin like. Needless to say our results do not affect the earlier conclusions of Kafatos *et al.* (1967a,b) that cocoonase is a trypsin-like enzyme.

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CORRECTIONS

"The State of the Tyrosines of Bovine Pancreatic Ribonuclease in Ethylene Glycol and Glycerol," by Jake Bello, Volume 8, Number 11, November 1969, page 4535.

On page 4537, column 2, third line from the bottom, the sentence: "The T_m values of Figure 1 were taken from the descending limbs of melting profiles such as that shown in Figure 4" should read: "The T_m values of Figure 1 were taken from the ascending limbs of rotatory melting profiles such as C in Figure 4."

"Conversion of 6-Methylsalicylic Acid into Patulin by *Penicillium urticae*," by P. I. Forrester and G. M. Gaucher, Volume 11, Number 6, March 14, 1972, page 1102.

In Table II the initial concentration in fermentor for [G-^{3}H]*m*-cresol should be 0.044 μmole/ml.

"Synthesis and Biological Activity of Several 6-Substituted 9-β-D-Ribofuranosylpurine 3′,5′-Cyclic Phosphates," by Rich B. Meyer, Dennis A. Shuman, Roland K. Robins, Randy J. Bauer, M. K. Dimmitt, and Lionel N. Simon, Volume 11, Number 14, July 4, 1972, page 2704.

In Table II, compound VII, the value under the 10^{-6} concentration should be 0.90 instead of 0.09.