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Synthesis and Characterization of a Pancreatic Trypsin Inhibitor Homologue and a Model Inhibitor†

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ABSTRACT: The synthesis and characterization of protein proteinase inhibitor homologues with variations in the amino acid composition in the vicinity of the reactive site should aid the understanding of the mechanism by which inhibition of enzymatic activity occurs. A homologue inhibitor in which the reactive-site residue Ala-16 of basic pancreatic trypsin inhibitor (Kunitz) (BPTI) is replaced by Phe has been synthesized to study the effect of this replacement on the dissociation constants of the enzyme-inhibitor complexes. The replacement of Ala-16 by Phe causes a dramatic increase in the K_I value of the trypsin-BPTI complex while that of the chymotrypsin-BPTI complex remains essentially the same. This cannot be explained simply in terms of increased steric crowding. The Phe replacement probably causes a small change in the local conformation of the reactive site of the inhibitor which leads to a large decrease in the stability of the very tight trypsin-BPTI complex. This conformation change apparently can be tolerated in the less tightly bound chymotrypsin-BPTI complex. On the basis of the known structure of BPTI, a cyclic heptadecapeptide containing one disulfide bond was synthesized as a model inhibitor in order to determine if a smaller peptide can be designed to act as a highly efficient inhibitor for trypsin. This heptadecapeptide which contains all of the amino acid residues of BPTI taking part in the interaction of the proteinase inhibitor with trypsin binds 3×10^7 times more weakly to the enzyme than native BPTI does. It thus appears that even though only a small part of the inhibitor molecule enters directly into interaction with the enzyme, the remaining portions of the molecule which hold the structure of the inhibitor rigid are essential for the strong interaction.

Previous studies in this laboratory (Tan and Kaiser, 1976) have shown that it is possible to use solid-phase peptide synthesis (Merrifield, 1963, 1965; Stewart and Young, 1969; Erickson and Merrifield, 1976) to synthesize a large peptide such as BPTI[1] (Kassell, 1970; Laskowski and Sealock, 1971; Lazdunski et al., 1974) that has properties identical with those of the native inhibitor within experimental error. In our synthesis, we have been able to determine that the dissociation constant of the synthetic BPTI-trypsin complex is 8×10^{-14} M under conditions where a value of 6×10^{-14} M has been reported in the literature for the native BPTI-trypsin complex (Vincent and Lazdunski, 1972). This observation, in addition to the peptide mapping, polyacrylamide gel electrophoresis, circular dichroism spectra, and amino acid analysis data, indicates that we do have a synthetic inhibitor which has properties identical with those of the native inhibitor within experimental error.

Success in the solid-phase synthesis of the peptide possessing the amino acid sequence of native BPTI and meeting high standards of purity opens the way to the preparation of analogues with variations in the amino acid composition in the vicinity of the reactive site of the inhibitor which would be useful in mechanistic studies of the inhibition process. Our studies on an analogue in which the reactive-site residue Ala-16 has been replaced by Phe are reported in this article (Figure 1, top).

Recently, attempts have been made to synthesize small fragments of BPTI that are biologically active (Wiejak, 1974, 1975; Siemion et al., 1973; Weber, 1975). They were hexapeptides or analogues that constituted the reactive site of BPTI (Kunitz). However, these peptides were very weak trypsin inhibitors, of which the best had a K_I of 2×10^{-3} M. Since the strong binding of the proteinase inhibitor is not due solely to the reactive-site sequence, it is not surprising that the peptide fragments made by Wiejak and Weber were weak trypsin inhibitors. The contributions of van der Waals contacts from other parts of the inhibitor molecule are in fact very important also (Blow et al., 1972; Rühlmann et al., 1973). We present here the synthesis and characterization of a cyclic heptadecapeptide containing all of the essential amino acid residues that contribute to the van der Waals contacts and hydrogen bonds of the trypsin-BPTI and chymotrypsin-BPTI complexes. The amino acid sequence of this heptadecapeptide

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[1] Abbreviations used are: BPTI, basic pancreatic trypsin inhibitor (Kunitz); Bz-ArgAnil, *N*-benzoyl-DL-arginine *p*-nitroanilide; OD, optical density; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; STI, soybean trypsin inhibitor.

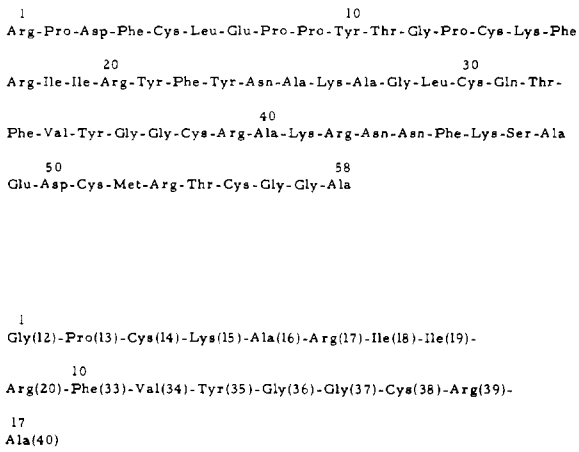

FIGURE 1: (Top) Linear sequence of amino acids in Phe-16 BPTI. Three disulfide linkages are present in the native inhibitor: Cys_5-Cys_{55}; Cys_{14}-Cys_{38}; Cys_{30}-Cys_{51}. (Bottom) Linear sequence of amino acids in the model inhibitor; Cys-3 and Cys-15 form a disulfide linkage. The numbers in parentheses give the positions of the corresponding amino acid residues in the natural BPTI sequence.

which contains a disulfide bridge formed between the two Cys residues is shown in Figure 1.

Experimental Section

Materials. Glass-distilled dichloromethane and chloroform from Burdick and Jackson Laboratory were distilled before use. Trifluoroacetic acid (Aldrich) was distilled and stored in amber bottles with polyethylene-lined screw caps. Analytical grade dimethylformamide from J. T. Baker Chemical Co. was purified and tested for free amine according to the procedure of Stewart and Young (1969) and was then stored under a nitrogen atmosphere at 4 °C in brown bottles over molecular sieves (Linde Type 4A). Triethylamine (Eastman Kodak) was distilled from calcium hydride. Dicyclohexylcarbodiimide from Aldrich Chemical Co. was distilled under reduced pressure.

Chloromethylated styrene–divinylbenzene copolymer (1% cross-linked) was purchased from Schwarz/Mann. The *tert*-butyloxycarbonyl amino acid derivatives used were the following: Ala, Asp (β-benzyl), Asn-*p*-nitrophenyl ester, Glu (γ-benzyl), Gln-*p*-nitrophenyl ester, Gly, Lys (ϵ-2-chlorobenzyloxycarbonyl), Phe, Thr (benzyl), Ser (benzyl), Tyr (2,6-dichlorobenzyl), Cys (*p*-methoxybenzyl), Met, Val, Leu, Ile, and Pro. These were all purchased from Bachem. The purity of each amino acid derivative was checked by thin-layer chromatography on silica gel chromatogram sheets (Eastman) before use (Stewart and Young, 1969).

Sodium acetate, sodium sulfite, sodium carbonate, sodium bicarbonate, acetic anhydride, calcium chloride (Baker Analyzed grade), sodium phosphates (Fisher), β-mercaptoethanol, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine (Eastman Kodak), *N*-acetyltryptophan ethyl ester, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride, Tris (Schwarz/Mann), ammonium persulfate (Mallinckrodt), *N*-benzoyl-DL-arginine *p*-nitroanilide, tosyl arginine methyl ester, *N*-benzoyl-L-tyrosine ethyl ester (Sigma), *N*-methylmorpholinebenzamidine hydrochloride, cyanogen, 5-dimethylamino-1-naphthalenesulfonyl chloride, and ninhydrin (Aldrich) were used without further purification. 5-Nitro-1,2-benzoxathiole 2,2-dioxide from Eastman Kodak was recrystallized from ethanol. Reagent grade urea (Fisher) was recrystallized from 95% ethanol prior to use (Stark et al., 1960). Sephadex G-50 and G-25, CM Sephadex C-25, and Sepharose 4B were obtained from Pharmacia Fine Chemicals. Polyamide layer sheets were obtained from Pierce.

Trypsin was purchased from Sigma and purified by Sephadex SEC-50 ion exchange chromatography (Schroeder and Shaw, 1968). β-Trypsin was used throughout all experiments. Commercial α-chymotrypsin (Sigma) was purified before use by affinity chromatography on an inhibitor–Sepharose column prepared according to Cuatrecasas (1970). A trypsin–Sepharose column was prepared according to Chauvet and Acher (1972).

General Methods. The optical densities of column effluents were measured with a Gilford spectrophotometer. Circular dichroism spectra were measured on a Cary 60 spectropolarimeter. All kinetic measurements were carried out at 25.0 °C on a Cary 15 spectrophotometer which was equipped with a thermostated cell compartment. Amino acid analysis and automated Edman degradations were performed on a Beckman Spinco Model 121 automatic amino acid analyzer and a Beckman protein peptide sequencer (Model 890-B), respectively. The Beckman Model 990 peptide synthesizer was used for the synthetic work.

All pH measurements were carried out on a Beckman Research pH meter with combined glass-calomel electrodes (Thomas No. 4094L60). The meter was standardized against Fisher Certified standard buffers prior to use. Deionized water was obtained by passing distilled water through a Continental demineralizer.

Determination of Concentrations. Protein Concentrations. Trypsin and α-chymotrypsin concentrations were estimated on the basis of the absorbance of their solutions at 280 nm with a conversion factor to mg/mL of 0.67 and 0.49, respectively.

Conversion factors for the homologue inhibitor and heptadecapeptide model inhibitor were calculated from their absorbances at 280 nm for a certain amount of protein on a weight basis. The following values were used: Phe-16 BPTI, 1.18; heptadecapeptide model inhibitor, 1.26.

Active Enzyme Concentrations. The concentrations of active trypsin molecules in solution were determined by active-site titrations using *p*-nitrophenyl *p'*-guanidinobenzoate according to the method of Chase and Shaw (1967). α-Chymotrypsin was titrated with 5-nitro-1,2-benzoxathiole 2,2-dioxide according to Heidema and Kaiser (1968).

Assays of Trypsin Inhibitors. The inhibitory activity of trypsin inhibitor was measured as a function of the ratio of the concentration of the inhibitor to that of the proteinase (Kassell and Laskowski, 1964). A rate assay method was employed to monitor the titration of trypsin's active site with the inhibitor.

To 3 mL of 0.1 M Tris buffer (pH 7.8, 0.02 M $CaCl_2$), 100 μL of β-trypsin solution (ca. 10^{-5} M) was added. An appropriate amount of inhibitor was added and the resultant solution was incubated for 3 min at 25 °C. Then 100 μL of *N*-benzoyl-DL-arginine *p*-nitroanilide (Bz-ArgAnil) solution (10 mg/mL of dimethylformamide) was added. The ΔOD_{405} per minute was measured with a Cary 15 spectrophotometer. The final concentrations of the enzyme and inhibitor were about 10^{-7} M.

Synthesis of the Protected Linear 58 Amino Acid Residue Polypeptide Chain of the Phe-16 BPTI Homologue. The Boc-Ala-resin ester was prepared essentially as described in the literature (Marglin, 1971) (0.2 mequiv/g of resin) and was placed in the reaction vessel of the automated Beckman Model 990 peptide synthesizer. The instrument was programmed to perform the remainder of the synthesis automatically, as de-

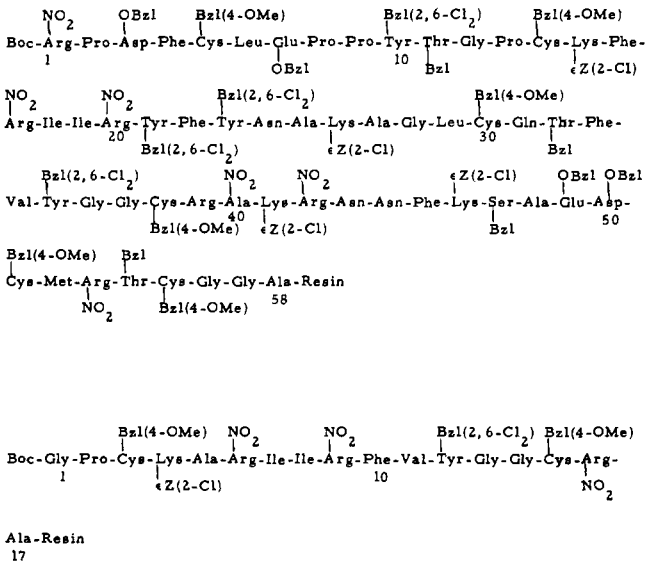

FIGURE 2: (Top) Structure of the fully protected Phe-16 BPTI-resin. (Bottom) Structure of the fully protected heptadecapeptide-resin.

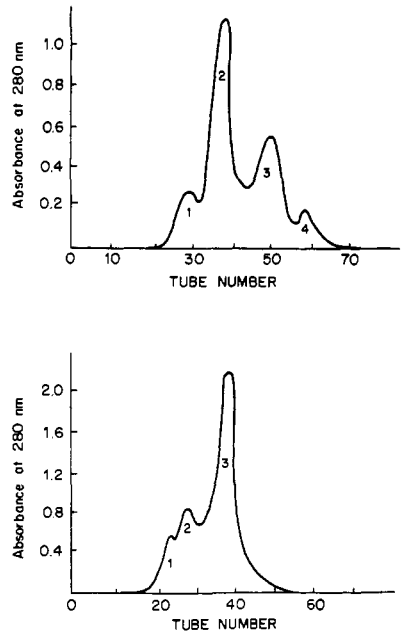

FIGURE 3: (Top) Gel filtration on Sephadex of the crude heptadecapeptide. The column (2 × 80 cm) was eluted with 0.1 M acetic acid; fraction size, 5.5 mL. The crude extract from 300 mg of resin-peptide in 15 mL of 10% acetic acid was applied to the column. Peak 2 (53 mg) had the correct molecular weight and amino acid composition for the heptadecapeptide. (Bottom) Gel filtration on Sephadex G-25 of the reduced-reoxidized heptadecapeptide. The column (2 × 80 cm) was eluted with 0.1 M acetic acid; fraction size 5.5 mL; sample size, 132 mg in 10 mL of 10% acetic acid. Peak 3 (82 mg) corresponded to the cyclic peptide monomer.

scribed in the previous synthesis of BPTI (Tan and Kaiser, 1976). Recoupling was found necessary for the following amino acid residues: Asn-44 (recoupled twice), Asn-43 (recoupled twice), and Asn-24. Following the second recoupling of Asn-43, the ninhydrin test (Kaiser et al., 1970) was still slightly positive. The peptide-resin was therefore acetylated with a mixture of 2 mL of acetic anhydride and 1 mL of *N*-methylmorpholine (Merrifield, 1963) in 25 mL of dimethylformamide (30-min stirring), followed by thorough washing with dimethylformamide and CH_2Cl_2.

Upon completion of the synthesis, the resin was washed with ethanol (3 × 25 mL) and dried in vacuo. The final weight was 1.65 g. Taking into account the amounts removed in the synthesis the crude yield was 75%. The structure of the fully protected BPTI-resin is illustrated in Figure 2 at the top.

Synthesis of the Model Inhibitor. One milliequivalent of Boc-Ala resin ester (5.0 g) was used for the synthesis of this peptide. No recoupling or acetylation was necessary in this synthesis. The crude yield of the protected heptadecapeptide was 87% (7.6 g). The structure of the fully protected heptadecapeptide is shown in Figure 2 at the bottom.

HF Cleavage. The apparatus was similar to that described by Sakakibara et al. (1967). It consisted of three vessels (a reservoir, a reactor, and a trap) molded from Kel-F rods. All valves were made of Teflon. Liquid HF in stainless steel cylinders was obtained from Matheson Co. A typical procedure for cleavage with HF for a 1-g resin-peptide sample has been described in the previous report on the synthesis of BPTI (Tan and Kaiser, 1976).

Anaerobic Column Chromatography. In order to minimize the undesirable intermolecular cross-linking of free thiol groups during the column chromatography of the concentrated reduced inhibitor, an anaerobic system (Repaske, 1971) was used. It consisted of a column and an elution buffer vessel, both modified to facilitate anaerobic assembly and operation. Oxygen-free nitrogen gas was used for flushing and bubbling throughout the experiment.

Purification of Synthetic Phe-16 BPTI. The crude peptide from the Phe-16 BPTI obtained after HF cleavage was subjected to the same purification procedures as were employed in the synthesis of BPTI (Tan and Kaiser, 1976). The peptide mixture was first fractionated on a Sephadex G-50 column. The product was reduced and reoxidized, followed by affinity chromatography on a trypsin-Sepharose column. A solution of the active inhibitor was then applied to a CM Sephadex C-25 ion exchange column and eluted with a NaCl gradient (0–0.25 M NaCl).

Purification of the Heptadecapeptide Model Inhibitor. Sephadex G-25 Gel Filtration. Fifteen milliliters of anaerobic 10% acetic acid was used to extract the peptide from a 300-mg resin-peptide mixture. The solution was then fractionated on a Sephadex G-25 column (2 × 80 cm) with 0.1 M acetic acid as the eluent under anaerobic conditions. A typical elution pattern is shown in Figure 3, top. The synthetic product was separated into four peaks. Peak 2 had the correct molecular weight and amino acid composition for the model inhibitor. Peak 1 consisted of interchain disulfide aggregates of the heptadecapeptide and could be reduced to the monomer by conventional methods (Pospisilova et al., 1967). Peak 3 consisted of peptides with lower molecular weights whereas peak 4 was mainly Met and Trp introduced in the HF cleavage step.

Reduction and Reoxidation of the Synthetic Heptadecapeptide. The fractions under peaks 1 and 2 obtained from the Sephadex G-25 fractionation step were combined and reduced as in the case of synthetic BPTI. The reduced peptide was separated from the reagents by using a Sephadex G-25 column (2 × 80 cm) with 0.1 M acetic acid as the eluent under anaerobic conditions. The peptide monomer, which was the only peak in the chromatogram, emerged from the column between tubes 36 and 43. The fractions were combined and then diluted to a concentration of 0.05 mg/mL. The pH was adjusted to 7.5 by addition of solid ammonium bicarbonate. This solution was air oxidized for 4 days at room temperature.

The air-oxidized peptide was lyophilized and fractionated

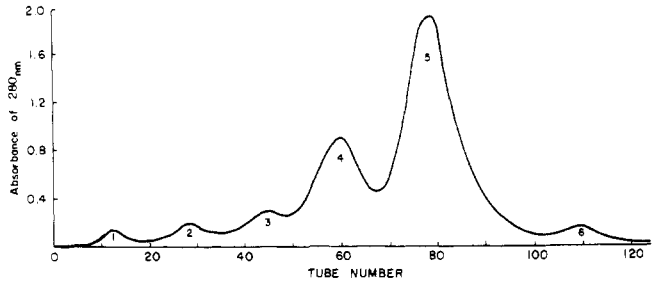

FIGURE 4: Chromatography of the synthetic cyclic heptadecapeptide on CM-Sephadex C-25; column, 2.4 × 20 cm; peptide load, 150 mg; fractions, 2.5 mL each; flow rate, about 40 mL/h. The mixing chamber consisted of a 500-mL flask containing 250 mL of 0.01 M phosphate buffer solution at pH 6.2. The influent to the mixing chamber was the same buffer solution which was also 1 M in NaCl. The peptide fraction under peak 5 (70 mg) showed the correct amino acid composition.

again on a Sephadex G-25 column (2 × 80 cm) by elution with 0.1 M acetic acid. The product was separated into three peaks (Figure 3, bottom). Peak 3 corresponded to the cyclic peptide monomer. Peaks 1 and 2 presumably were interchain disulfide aggregates of the peptide and could be reduced and recycled to form the heptadecapeptide monomer.

CM Sephadex C-25 Chromatography. The partially purified oxidized heptadecapeptide (150 mg) obtained from the second Sephadex G-25 chromatography was applied on a column (2.4 × 20 cm) of CM-Sephadex C-25 preequilibrated with a pH 6.2 sodium phosphate (0.01 M) buffer and eluted with a NaCl linear gradient (0–0.25 M). A typical separation is shown in Figure 4. The peptide under peak 5 (70 mg) showed the correct amino acid composition and was lyophilized after desalting.

Dansylation. About 2–5 nmol of the peptide was used. Dansylation was carried out in Pyrex glass tubes measuring 4 × 75 mm. The procedure for peptides as described by Gray (1972) was followed. Dansyl derivatives were identified by two-dimensional thin-layer chromatography on polyamide sheets using the solvent system H_2O–88% formic acid (100:1.5, v/v) and benzene–acetic acid (9:1, v/v). The chromatograms are shown in Figure 5.

Edman Degradation. Automated Edman degradation (Fietzek and Kuhn, 1972) was performed in a Beckman protein–peptide sequencer (Model 890-B). The phenylthiohydantoins liberated after each cycle were identified and quantitated by gas chromatography on a Beckman GC-45 unit.

Preparation of Trypsin-Modified Heptadecapeptide. Five milliliters of 1.5×10^{-3} M (ca. 15 mg) purified heptadecapeptide was incubated with trypsin (1.5×10^{-6} M) for 5 min in a 0.05 M Tris buffer (pH 8.0, 0.01 M $CaCl_2$). The solution was then fractionated on a column of Sephadex G-25 (2 × 80 cm). The peptide isolated (14 mg) had a molecular weight of about 2000 and moved as a single band slightly faster than the unmodified heptadecapeptide on disc gel electrophoresis (pH 2.3).

Polyacrylamide Gel Electrophoresis. General Method. Standard 7.5% polyacrylamide gels were prepared according to the formulation of Davis (1964). The gels were stacked at pH 4.0 and run at pH 2.3. The unpolymerized gel solution was introduced into 6-mm i.d. glass tubing to a height of approximately 7 cm and allowed to polymerize.

Gel Electrophoresis of Modified Heptadecapeptide. Analytical polyacrylamide disc gel electrophoresis of the heptadecapeptide showed only a single band. At zero time, sufficient trypsin solution was added to the peptide (1.8×10^{-4} M) in

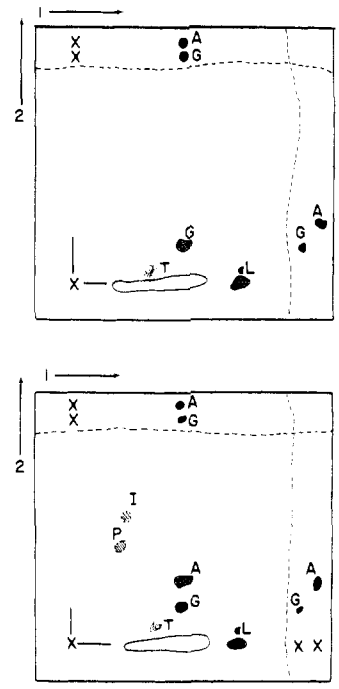

FIGURE 5: Two-dimensional thin-layer chromatography of dansyl derivatives of the purified heptadecapeptide (top) and the modified heptadecapeptide (bottom) on polyamide. Standard dansyl-amino acids (●) used were: alanine (A) and glycine (G); (○) blue, dansyl-OH; (⊕) pink, *O*-dansyltyrosine; all other spots are green (●), Gly (G), Ala (A), Ile (I), Phe (P), Lys (L). Dansylisoleucine and dansylphenylalanine are present in much smaller amounts. Solvents used were: (1) H_2O–88% formic acid (199:1.5, v/v); (2) benzene–acetic acid (9:1, v/v). Samples were applied at the lower left corners; (- - -) solvent front. The spots were examined with the aid of a long-wavelength UV lamp.

a Tris buffer (0.05 M, pH 8.0, 0.1 M $CaCl_2$) to make the reaction 3×10^{-8} M in trypsin. Aliquots (100 μL) were withdrawn periodically and diluted into 250 μL of an 8 M guanidinium hydrochloride solution which quenched the modification. Of this solution 50 μL was applied to the gels. Electrophoresis was carried out for 3.5 h at a constant voltage (70 mV).

Determination of the Dissociation Constant of the Phe-16 BPTI–Trypsin Complex. This was determined by carrying out an investigation in detail near the equivalence point of an inhibition curve obtained by adding increasing amounts of inhibitor to a fixed amount of trypsin (4.06×10^{-8} M, in 0.1 M Tris buffer containing 0.02 M $CaCl_2$ at pH 8.0). After 25 min of incubation, residual trypsin activity was monitored by rate assay employing tosyl-L-arginine methyl ester (Hummel, 1959). K_I was calculated by the method of Green and Work (1953).

Determination of the Dissociation Constant of the α-Chymotrypsin–Phe-16 BPTI Complex. The inhibition curve for the determination of K_I was obtained by adding increasing amounts of inhibitor to a fixed amount of α-chymotrypsin (2.38×10^{-8} M (pH 8.0), 0.02 M $CaCl_2$, 0.1 M Tris). The enzyme was incubated with the inhibitor for 25 min. Residual enzyme activity was then monitored by rate assay employing 0.5 mM *N*-benzoyl-L-tyrosine ethyl ester (Hummel, 1959).

Inhibition of Trypsin by the Heptadecapeptide Model Inhibitor. The inhibition of the tryptic hydrolysis of Tos-Arg-OMe and Bz-ArgAnil by the heptadecapeptide was monitored by the use of standard rate assay methods for the enzyme (Hummel, 1959; Walsh and Wilcox, 1970; Erlanger et al., 1961). Conditions used were as follows: for the Tos-Arg-OMe

TABLE I: Summary of Yields and Inhibitory Activities Obtained for Synthetic Phe-16 BPTI.[a,b]

Stage of Synthesis or Purification	Synthetic Phe-16 BPTI Yield (%)	Synthetic Phe-16 BPTI Inhibitory Act. against Trypsin (%)
(1) Protected inhibitor–resin	75	
(2) Crude, cleaved peptide	70	
(3) Inhibitor monomer isolated by gel filtration through Sephadex G-50	60	Very low
(4) Inhibitor isolated by affinity chromatography	1	80–90
(5) Inhibitor isolated after ion-exchange chromatography	80	95

[a] The overall yield at the end of the fifth stage was 0.25% for synthetic Phe-16 BPTI. [b] When sulfonated native BPTI which had been treated with HF was purified by the procedures outlined in the table a final yield of 50% of material which was 100% active as a β-trypsin inhibitor was obtained (Tan and Kaiser, 1976).

TABLE II: Amino Acid Composition of Purified Phe-16 BPTI.[a]

Amino Acid	Expected	No. of Residues Found
Arg	6	5.69
Lys	4	4.32
Asx	5	5.02
Thr	3	2.80
Ser	1	0.82
Glx	3	2.97
Pro	4	3.89
Gly	6	6.10
Ala	5	5.23
Val	1	0.83
Met	1	0.88
Ile	2	0.96[b]
Leu	2	2.00
Tyr	4	3.65
Phe	5	4.95
Cys	6	Not determined

[a] Number of residues found is the average value of duplicate analysis. [b] See Dlouha et al., 1965.

assay, the enzyme concentration was 4.7×10^{-8} M in a buffer containing 0.05 M Tris at pH 8.0, with 0.01 M $CaCl_2$ at 25 °C. The increase in absorbancy at 247 nm was followed. For the Bz-ArgAnil rate assay the enzyme concentration was 2.0×10^{-7} M, and the same buffer system was employed. The increase in absorbancy at 405 nm was followed.

Results

Synthetic Phe-16 BPTI

Solid Phase Synthesis. The yield of crude, uncleaved peptide obtained in this work was 75% (Table I) based on the starting Boc-Ala content. This suggests that the average yields of the individual steps in the synthesis were very high, and that little peptide must have been lost from the resin during the course of the synthesis. The conventional protecting groups for the hydroxyl function of tyrosine (Bzl) and for the ϵ-amino function of lysine (Z) are not very satisfactory for the stepwise synthesis of long peptide chain and their loss during synthesis can easily lead to extensive chain branching (Erickson and Merrifield, 1973; Yamashiro and Li, 1973). To reduce this problem, the Bzl (2,6-Cl_2) and ϵ-Z (2-Cl) protecting groups were used for tyrosine and lysine, respectively.

In our synthetic work the individual coupling steps and deprotecting steps were monitored by the ninhydrin test (Kaiser et al., 1970). Repeated coupling was used to ensure complete reaction when the ninhydrin test was positive after the initial coupling had occurred. This was found to be necessary only in the cases of three asparagines in the synthesis of Phe-16 BPTI. Thus, it appears that the growing peptide chain was able to react rapidly and in high yield with added amino acids.

Purification of Synthetic Phe-16 BPTI. Procedures for purification of the homologue inhibitor Phe-16 BPTI were the same as those for the synthetic BPTI (Tan and Kaiser, 1976). Gel filtration on Sephadex G-50 of an acetic acid extract of the resin–peptide mixture obtained after the HF cleavage gave five peaks, one of which contained a polypeptide of approximately the correct molecular weight. After treatment with β-mercaptoethanol and passage again through a Sephadex G-50 column, the solution was diluted to a concentration of 0.01 mg/mL polypeptide. The latter solution was air oxidized for 4 days (at pH 4.7) and applied to a trypsin–Sepharose column. The yield of fully active inhibitor obtained from affinity chromatography was only 1%. As indicated in Table I, the overall yield was thus only 0.25%, in contrast to the 2.9% overall yield in the case of the synthetic BPTI. This might be due to the fact that with Phe-16 in place of Ala-16, the reduced, denatured polypeptide was unable to renature as efficiently as the unsubstituted polypeptide since Phe, a much better β sheet forming amino than Ala (Chou and Fasman, 1974), is very close to Cys-14 which forms a disulfide bridge with Cys-38. Thus, to increase the yield in this case a possible approach might be the application of the methods of directed bond formation (Hiskey et al., 1975).

Characterization of the Synthetic Phe-16 BPTI. Amino Acid Analysis of the Synthetic Phe-16 BPTI. The highly purified Phe-16 BPTI had the expected overall amino composition (Table II) with a Phe in place of an Ala.

Inhibitory Activity of Synthetic Phe-16 BPTI. Bz-ArgAnil was used as the substrate to monitor residual tryptic activities. The trypsin concentration used was 4.0×10^{-7} M. The stoichiometry of inhibition was followed by adding increasing quantities of synthetic Phe-16 BPTI (stock solution = 6.54×10^{-6} M) and by measuring the residual activity after 25 min of incubation (Figure 6, solid line). Complete inhibition was obtained at an inhibitor to trypsin ratio of 1.05:1, which indicated that the highly purified Phe-16 BPTI was at least 95% active. An error of several percent might be involved in the determination of the concentration of the inhibitor.

Circular Dichroism Spectrum of the Phe-16 BPTI. The CD spectrum of synthetic Phe-16 BPTI is almost identical with that of the natural inhibitor. Apparently the Phe-16 substitution did not measurably perturb the conformation of the inhibitor.

Polyacrylamide Gel Electrophoresis. The synthetic Phe-16 BPTI moved as a single band in 7.5% disc gel (stacked at pH 4.0 and run at pH 2.3). This indicated a high degree of homogeneity.

Determination of the Dissociation Constant of the Trypsin–Synthetic Phe-16 BPTI Complex. This was carried out essentially according to Green and Work (1953). An inhibition

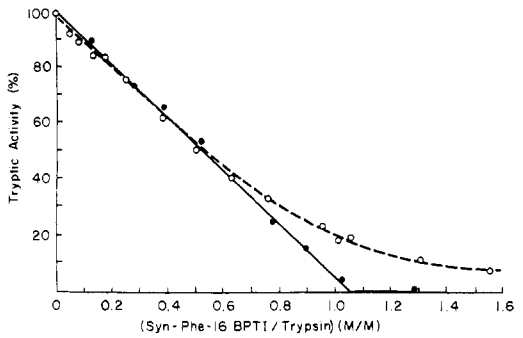

FIGURE 6: (●–●) Assay of highly purified synthetic Phe-16 BPTI. The titration of synthetic BPTI was carried out employing increasing inhibitor to β-trypsin ratios. The titration was monitored by assaying the remaining β-trypsin activity using a rate assay method. Synthetic Phe-16 BPTI was incubated with β-trypsin for 25 min, followed by addition of the substrate Bz-ArgAnil. Conditions used were: trypsin concentration, 4.0×10^{-7} M; inhibitor concentration, $0.52\text{–}5.12 \times 10^{-7}$ M; 0.1 M Tris buffer (pH 7.8) containing 0.02 M $CaCl_2$. (O- - -O) Curve for the inhibition of trypsin by highly purified synthetic Phe-16 BPTI. An increasing amount of the inhibitor was added to a fixed amount of β-trypsin at a concentration of 4.0×10^{-8} M (0.1 M Tris (pH 8.0)–0.02 M $CaCl_2$). Tos-Arg-OMe was used as the substrate to monitor remaining tryptic activity.

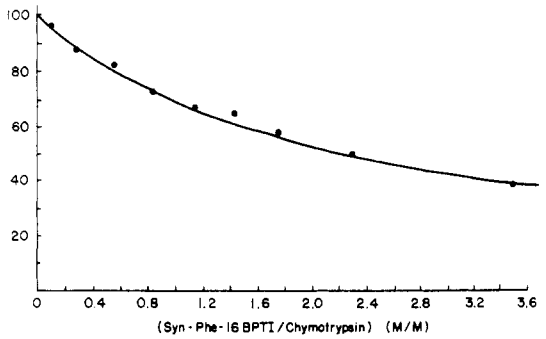

FIGURE 7: Curve for the inhibition of α-chymotrypsin by highly purified synthetic Phe-16 BPTI. An increasing amount of the inhibitor was added to a 0.1 M Tris buffer (pH 8.0) with a chymotrypsin concentration of $1.9\text{–}2.38 \times 10^{-8}$ M. *N*-Benzoyl-L-tryosine ethyl ester was used as the substrate to monitor remaining chymotryptic activity.

curve (Figure 6, dashed line) was obtained by adding an increasing amount of the inhibitor to a fixed amount of trypsin at a concentration of 4.0×10^{-8} M (0.1 M Tris (pH 8.0)–0.02 M $CaCl_2$). Tos-Arg-OMe was used as a substrate for the assay of remaining trypsin activity. The residual tryptic activity in the presence of different amounts of the inhibitor was then used to calculate the dissociation constant of the trypsin–Phe-16 BPTI complex.

From eq 1, since $[EI] = [\text{trypsin}]_{tot} - [\text{trypsin}]_{free}$ and $[I]_{free} = [I]_{tot} - [EI]$, eq 2 can be obtained:

$$K_I = \frac{[E]_{free}[I]_{free}}{[EI]} \quad (1)$$

$$\frac{[I]_{tot}}{[EI]} = \frac{K_I}{[E]_{free}} + 1 \quad (2)$$

The K_I value obtained from a plot of $[I]_{tot}/[EI]$ vs. $1/[E]_{free}$ was $(1.70 \pm 0.25) \times 10^{-9}$ M.

Determination of the Dissociation Constant of the Chymotrypsin–Synthetic Phe-16 BPTI Complex. Essentially the same method as described above was used to evaluate the K_I value for the α-chymotrypsin–Phe-16 BPTI complex (Figure 7). The experimental conditions used were: α-chymotrypsin concentration, $1.9\text{–}2.38 \times 10^{-8}$ M; pH 8.0; 0.1 M Tris at 25 °C. The K_I was found to be $(3.31 \pm 0.48) \times 10^{-8}$ M, equivalent to an association constant (K_A) of 30 μM^{-1}. This value was close to the association constant of the α-chymotrypsin–natural BPTI complex obtained by Vincent and Lazdunski (1973) (K_A = 110 μM^{-1}). This indicates that the Phe-16 substitution does not significantly affect the binding of the inhibitor with α-chymotrypsin. In contrast, the binding of the natural inhibitor with trypsin is 3×10^5 times stronger than that of the Phe-16 BPTI.

TABLE III: Summary of Yields Obtained for Synthetic Heptadecapeptide.

State of Synthesis or Purification	Yield (%)
1. Protected heptadecapeptide	87
2. Crude, cleaved peptide	65
3. After gel filtration	62
4. After ion-exchange chromatography	46
Overall yield	16

Heptadecapeptide Model Inhibitor

Solid Phase Synthesis. The high yield (87%) (Table III) of the protected heptadecapeptide and the fact that no repeated coupling was required indicated that the growing peptide chain was also able to react in high yield with the added Boc amino acids.

Purification of the Synthetic Heptadecapeptide. Since the heptadecapeptide lacks the rigid structure of the natural inhibitor, it was expected that it would probably be digested by trypsin slowly. Thus, the powerful affinity chromatography method cannot be used for purification in this case.

Gel filtration on Sephadex G-25 of an acetic acid extract of the cleaved resin–peptide mixture gave a peptide with approximately the correct molecular weight. After reduction by β-mercaptoethanol and passage through the same Sephadex column, the solution was diluted to a concentration of 0.05 mg/mL at a pH of 7.0. The solution was then air oxidized for 4 days and lyophilized. The white powder (132 mg) obtained was dissolved in a small amount of 0.1 M acetic acid and fractionated again using the same Sephadex column. As shown in Figure 3 (bottom), there were three peaks which presumably corresponded to polymer, dimer, and the desired cyclic heptadecapeptide (82 mg) (62%), respectively. The first two peaks could be reduced and recycled to form the desired cyclic heptadecapeptide. Peak 3 was lyophilized and fractionated on a column of CM-Sephadex C-25. Six peaks appeared in the elution pattern (Figure 4), of which peak 5 was found to be the fraction of polypeptide with the correct amino acid composition.

Since this heptadecapeptide contains only two cysteine residues, there is only one possible mode of disulfide pairing. The yield of the cyclic monomer during the air oxidation was, however, highly dependent upon the pH of the peptide solution. For example, it was found that at lower pH (4.5) the yield of cyclic monomer after 4-days air oxidation was only 10–20%.

Characterization of the Synthetic Heptadecapeptide. Amino Acid Analysis of the Synthetic Heptadecapeptide. The purified synthetic heptadecapeptide has the expected amino acid composition (Table IV) except for Ile, which gave only 1 (theory 2) mol equiv after 24-h hydrolysis. This also happened in the hydrolysis of natural BPTI. This presumably is due to the fact that the two isoleucine residues are next to each other in the sequence and that it is difficult to hydrolyze this

TABLE IV: Amino Acid Composition of Purified Synthetic Heptadecapeptide.

Amino Acid	No. of Residues Found[a] Expected	Heptadeca-peptide	Modified Peptide
Lys	1	0.95	0.98
Arg	3	2.98	2.65
Pro	1	0.98	0.90
Gly	3	3.00	3.00
Ala	2	2.13	1.75
Val	1	0.83	0.78
Ile	2	0.99	0.75
Tyr	1	0.78	0.73
Phe	1	0.97	0.86
Cys	2	Not determined	Not determined

[a] Average of duplicate analysis.

TABLE V: Results on Edman Degradation of the Purified Heptadecapeptide.[a]

Degradation Cycle	Amino Acid Residue Expected[b]	Amino Residue Obsd	Amount (nmol)
1	Gly	Gly	425
2	Pro	Pro	390
		Gly	45
3	Cys[c]	Cys	75
		Pro	30
4	Lys	Lys	255
5	Ala	Ala	375
6	Arg[d]	Arg	135
		Ala	35
7	Ile	Ile	250
8	Ile	Ile	285
9	Arg[d]	Arg	105
10	Phe	Phe	125
		Ile	5
11	Val	Val	120
		Phe	5
12	Tyr	Tyr	25[e]
		Val	15
13	Gly	Gly	108
14	Gly	Gly	120
15	Cys[c]	Cys	5
		Gly	20
16	Arg[d]	Arg	75
17	Ala	Ala	25

[a] The phenylthiohydantoins were identified as such and quantitated by gas chromatography. [b] Refer to the amino acid sequence in Figure 1. [c] Identified and quantitated as cysteic acid. [d] Identified and quantitated by back conversion. [e] The low yield of Tyr might be due to formation of halogen derivatives during performic acid oxidation. The possibility that the 2,6-dichlorobenzyl protecting group was not completely removed from Tyr during the HF cleavage step has been eliminated since a chlorine analysis of the purified intact peptide revealed the presence of less than 0.1% Cl.

part of the peptide chain completely in 24 h (Dlouha et al., 1965).

Polyacrylamide Gel Electrophoresis. The synthetic heptadecapeptide moved as a single band on disc polyacrylamide gel electrophoresis (7.5% gel, stacked at pH 4.0 and run at pH 2.3). This indicated that the synthetic peptide had a high degree of homogeneity.

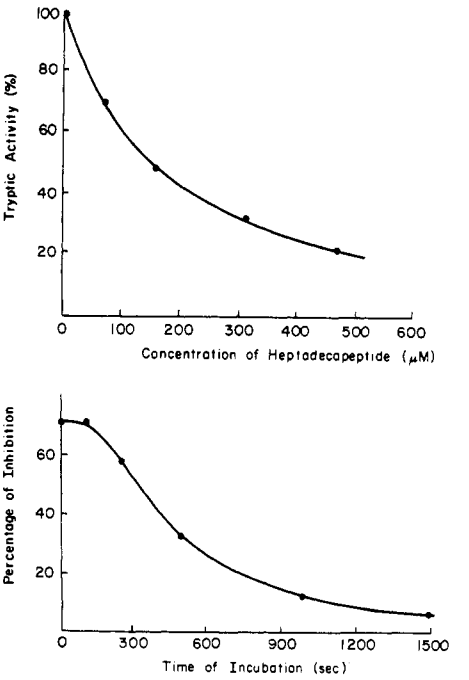

FIGURE 8: (Top) Inhibition of tryptic hydrolysis of Tos-Arg-OMe by the heptadecapeptide. Conditions used were: Tos-Arg-OMe, 1 mM; trypsin concentration, 4.7×10^{-8} M in a pH 8.0 buffer containing 0.05 M Tris and 0.01 M $CaCl_2$ at 25 °C. (Bottom) Time dependency of the inhibition of the tryptic hydrolysis of Tos-Arg-OMe by the heptadecapeptide. Conditions used were: Tos-Arg-OMe, 1 mM; 0.05 M Tris buffer, pH 8.0, containing 0.01 M $CaCl_2$. Trypsin (6.5×10^{-8} M) was incubated with heptadecapeptide (300 μM) for various periods of time before being assayed.

Dansylation. About 2–5 nmol of the purified peptide was dansylated and hydrolyzed according to Gray (1972). The dansyl derivatives were identified by two-dimensional thin-layer chromatography and examination under a long-wavelength ultraviolet (UV) lamp. The chromatogram is shown in Figure 5 at the top. The only N-terminal residue detected for the heptadecapeptide was Gly-1.

Edman Degradation. The purified heptadecapeptide (900 nmol) was oxidized by performic acid (Hirs, 1956) and subjected to Edman degradation. Results as shown in Table V indicate that the purified heptadecapeptide has the correct amino acid sequence.

Inhibition of Trypsin by the Heptadecapeptide. Inhibitory Behavior. The curve for the inhibition of trypsin resulting from varying the concentration of the heptadecapeptide is shown in Figure 8 at the top. Tryptic hydrolysis of Tos-Arg-OMe was inhibited appreciably by the synthetic heptadecapeptide. At pH 8.0, the peptide concentration required to inhibit 50% of the tryptic activity was 150 μM.

This inhibition was, however, time dependent (Figure 8, bottom). The heptadecapeptide (300 μM) was incubated with trypsin (6.5×10^{-8} M) in a pH 8.0 Tris buffer (0.05 M) containing 0.01 M $CaCl_2$ at 25 °C for different periods of time before the rate assay was carried out. It was found that if the incubation time was less than 80–100 s, the extent of inhibition remained the same. Thereafter, as the incubation time increased, the heptadecapeptide became less effective as an inhibitor and, if the peptide was incubated with trypsin for 1500 s before the assay, it became almost totally ineffective as an inhibitor of the tryptic hydrolysis of Tos-Arg-OMe.

Similar phenomena occurred also in the inhibition of tryptic hydrolysis of Bz-ArgAnil by the heptadecapeptide. Since

Bz-ArgAnil has a higher K_m (ca. 1 mM; Erlanger et al., 1961) toward trypsin than Tos-Arg-OMe (1.25×10^{-5} M; Trowbridge et al., 1963) does, the heptadecapeptide is more efficient in inhibiting the tryptic hydrolysis of this substrate. Thus, when trypsin was added to a pH 8.0 Tris buffer containing heptadecapeptide (180 μM) and Bz-ArgAnil (4.02×10^{-4} M), practically no hydrolysis of Bz-ArgAnil took place for the first few hundred seconds. Trypsin started to hydrolyze the substrate slowly 300 s after it had been added. This "lag" period in tryptic hydrolysis of Bz-ArgAnil disappeared if the heptadecapeptide was preincubated with trypsin (at a concentration of 3×10^{-8} M) for 300–400 s before carrying out the rate assay.

The Modified Heptadecapeptide. The above results indicated that the heptadecapeptide binds to trypsin fairly strongly. The inhibition was, however, a temporary one. The peptide appeared to be slowly "modified" by trypsin with a corresponding decrease in its binding ability with trypsin. Amino acid analysis (Table IV) of this modified peptide gave an amino acid composition that was essentially the same as that of the natural heptadecapeptide. Thus, the "modified" peptide was probably a partially digested heptadecapeptide. The peptide bond most likely to be cleaved was Lys_4-Ala_5, the reactive-site peptide bond in the natural BPTI. Dansylation of the modified peptide indicated the presence of two major N-terminal residues: Ala and Gly (Figure 5, bottom). There were, however, small amounts of dansyl-Phe and dansyl-Ile. This showed that the nonspecific hydrolysis of Agr_6-Ile_7 and Arg_9-Phe_{10} did occur to some extent. The severed pieces of the peptide chain produced by this partial hydrolysis were probably held together by the disulfide bridge.

Disc gel electrophoresis (7.5%, pH 2.3) of the heptadecapeptide incubated with trypsin for various periods of time provided additional evidence for the hypothesis. The "modified" heptadecapeptide, if partially cleaved, should migrate faster than the natural peptide because of an additional positive charge from the α-amino group liberated upon the peptide bond cleavage. Indeed, an additional band which migrated slightly faster appeared after the heptadecapeptide had been incubated with trypsin. This new band gradually increased in intensity while the intensity of the more slowly moving band which corresponded to the natural peptide decreased correspondingly with increasing incubation time.

Determination of the Inhibition Constant of the Heptadecapeptide toward Trypsin. Under the standard conditions used for studies on the inhibitory activity of the heptadecapeptide using Tos-Arg-OMe as the substrate (trypsin concentration, 6×10^{-8} M; heptadecapeptide concentration, 300 μM, at pH 8.0) the degree of inhibition remained constant for nearly 100 s (Figure 8, bottom). Since a Tos-Arg-OMe rate assay could be done within 20–30 s, it was thus possible to obtain a satisfactory value for the inhibition constant of the heptadecapeptide toward trypsin despite the fact that the peptide was slowly digested by the trypsin.

Since the K_I value (estimated to be 10^{-6} M) is much smaller than the peptide concentration (10^{-4} M) used in the kinetic studies, the substrate concentration (1 mM), or the K_m value (1.25×10^{-5} M), the standard Dixon plot (Dixon and Webb, 1964) could not be used to give an accurate inhibition constant.

Assuming that the trypsin was inhibited competitively, under the condition that $[S] \gg K_m$ (Bieth, 1974), eq 3 could be used to calculate K_I, where v_I and v_0 are the rates of reaction with and without inhibitor I and [S] is the substrate concentration. Substrate activation by Tos-Arg-OMe of tryptic hydrolysis was neglected here because the range of substrate concentration used was too small to have a significant effect.

TABLE VI: Determination of the Inhibition Constant of the Heptadecapeptide.[a,b]

[Tos-Arg-OMe] ($\times 10^3$) (M)	[Peptide] ($\times 10^6$) (M)	v_I/v_0[c]	Calcd K_I (M)
1.0	150	0.49	1.80×10^{-6}
1.0	200	0.40	1.67×10^{-6}
1.0	300	0.34	1.93×10^{-6}
0.5	150	0.34	1.93×10^{-6}
0.5	180	0.28	1.75×10^{-6}
0.5	300	0.18	1.64×10^{-6}
0.25	120	0.24	1.89×10^{-6}
0.25	200	0.15	1.76×10^{-6}
0.25	300	0.11	1.86×10^{-6}
		Av K_I:	1.80×10^{-6}

[a] Conditions used were: β-trypsin, 4.7×10^{-8} M at pH 8.0 in a 0.05 M Tris buffer containing 0.01 M $CaCl_2$. [b] K_m of Tos-Arg-OMe toward trypsin is 1.25×10^{-5} M (Trowbridge et al., 1963). [c] $v_I/v_0 \times 100\%$ is the percentage of activity remaining.

$$K_I = \frac{(v_I/v_0)K_m[I]}{[S][1 - (v_I/v_0)]} \quad (3)$$

Values of K_I calculated from various substrate and peptide concentrations are shown in Table VI. The average value was 1.8×10^{-6} M.

Determination of the Inhibition Constant of the Modified Heptadecapeptide toward Trypsin. Under standard conditions of tryptic hydrolysis of Tos-Arg-OMe, the modified peptide, at a concentration of 10^{-4} M, showed hardly any inhibition of enzyme action. This indicated that the modified peptide had a much higher inhibition constant (K_I) than the natural heptadecapeptide. Thus, to evaluate K_I, a substrate with a larger K_m such that the extent of inhibition by the modified peptide is measurable had to be used. Bz-ArgAnil ($K_m \simeq 10^{-3}$ M; Erlanger et al., 1961) was found to be a suitable substrate for this purpose.

Inhibition of tryptic hydrolysis at three different Bz-AgAnil concentrations (1.76×10^{-4}, 4.02×10^{-4}, and 7.66×10^{-4} M) was used to obtain a standard Dixon plot. The value of K_I obtained from the intersection point of the three lines (Dixon and Webb, 1964) was 5.5×10^{-4} M.

Discussion

Chemical and Physical Evidence for the Purity of the Synthetic Peptides. Synthetic Phe-16 BPTI. Due to the low overall yield of the synthesis, the limited amounts of active Phe-16 BPTI isolated did not allow as extensive characterization of the synthetic material as in the case of the native inhibitor (Tan and Kaiser, 1976). It had the expected overall amino acid composition and was homogeneous as judged by CM Sephadex C-25 ion exchange chromatography and polyacrylamide gel electrophoresis. The K_I value found for Phe-16 BPTI was 1.6×10^{-9} M (with trypsin) but there is no K_I value measured for this inhibitor species produced by another route with which a comparison can be made. The affinity chromatographically purified inhibitor was 95% active against trypsin using Bz-ArgAnil as substrate. However, it should be noted that an error of several percent might be involved in the determination of the concentration of the inhibitor. In view of the purity of the synthetic BPTI obtained and the fact that the

TABLE VII: Binding of Proteinase Inhibitors and Derivatives to Proteinase.[a]

Inhibitors	Proteinase	Binding	K_I[b] (M)	Reference
BPTI (Kunitz)	Trypsin	+	6×10^{-14}	*k*
BPTI	Chymotrypsin	+	9×10^{-9}	*l*
BPTI	Anhydrotrypsin	+	1.1×10^{-13}	*m*
BPTI	Pseudotrypsin[c]	+	9×10^{-9}	*k*
Phe-16 BPTI	Trypsin	+	1.7×10^{-9}	This work
Phe-16 BPTI	Chymotrypsin	+	3.3×10^{-8}	This work
Arg-15 BPTI	Trypsin	+[d]	n.d.[j]	*n*
Arg-15 BPTI	Chymotrypsin	+[e]	n.d.	*n*
Trp-15 BPTI	Trypsin	Weak	n.d.	*n*
Trp-15 BPTI	Chymotrypsin	+	3.6×10^{-10}	*n*
Phe-15 BPTI	Trypsin	Weak	n.d.	*n*
Phe-15 BPTI	Chymotrypsin	+	3.6×10^{-10}	*n*
STI (Kunitz)[f]	Trypsin	+	1.1×10^{-10}[g]	*o*
STI	Chymotrypsin	+	3.3×10^{-6}	*o*
Lys-63 STI	Trypsin	+[h]	n.d.	*p*
Trp-63 STI	Trypsin	−	−	*q*
Trp-63 STI	Chymotrypsin	+	10^{-9}	*q*
Phe-63 STI	Trypsin	+	2×10^{-9}	*o*
Phe-63 STI	Chymotrypsin	+	1.5×10^{-5}	*o*
Leu-64 STI	Trypsin	+[i]	n.d.	*r*
Ala-64 STI	Trypsin	+[i]	n.d.	*r*
Gly-64 STI	Trypsin	+[i]	n.d.	*r*

[a] All reactive-site analogues except the Phe-16 BPTI were prepared by enzymatic replacement (Sealock and Laskowski, 1969) or enzymatic chemical replacement (Kowalski and Laskowski, 1976) methods. The Phe-16 BPTI was a synthetic peptide prepared by the solid-phase method. [b] All values at pH 8.0, 25 °C unless otherwise specified. [c] Trypsin with Lys-176–Asp-177 bond cleaved. [d] Probably as strong an inhibitor as BPTI. [e] Arg-15 BPTI is a weaker inhibitor of bovine chymotrypsin than native BPTI. [f] Reactive site of STI is Arg-63–Ile-64. [g] Measured at pH 7.0, 20 °C. [h] K_I slightly larger than for native STI. [i] Probably as strong an inhibitor as native STI. [j] n.d., not determined. [k] Vincent and Lazdunski, 1972. [l] Vincent and Lazdunski, 1973. [m] Vincent et al., 1974. [n] Jering and Tschesche, 1974, 1976. [o] Kowalski et al., 1974. [p] Sealock and Laskowski, 1969. [q] Kowalski et al., 1974. [r] Kowalski and Laskowski, 1976.

synthesis of the Phe-16 BPTI was carried out essentially the same way as that of the BPTI, we believe that it is unlikely that impurities are present in Phe-16 BPTI which do not have the designed reactive-site sequence.

Synthetic Heptadecapeptide. The purified peptide had the expected amino acid composition and moved as a single band on polyacrylamide gel electrophoresis. Dansylation of the peptide showed that the only N-terminal residue for this peptide was glycine. Edman degradation of the peptide gave the expected results (cf. Table IV; the amino acid sequence of this peptide is given in Figure 1 at the bottom). However, it is still possible that the synthetic peptide is contaminated by small amounts of impurities.

Effect of the Phe-16 Replacement on the Interaction of BPTI with Enzymes. Jering and Tschesche (1974, 1976) have shown that replacing the reactive-site residue P_1 of BPTI by aromatic amino acids such as tryptophan and phenylalanine changed the trypsin-chymotrypsin inhibitor into a chymotrypsin inhibitor. Replacing the P_1 residue (Arg-63) of soybean trypsin inhibitor (STI) by tryptophan also essentially abolished the anti-tryptic activity of the inhibitor (Kowalski et al., 1974). However, Phe-63 STI is a better trypsin than chymotrypsin inhibitor, in spite of its chymotrypsin specific residue P_1 (Table VII). Apparently, the strong interaction between proteinase inhibitor and proteinase is due to the hand-in-glove perfect fit of the two rigid molecules. The P_1 residue usually does not play a decisive role unless it is too bulky (such as tryptophan at P_1 in the STI analogue). It is interesting to note that while the Phe-63 residue can be tolerated in the trypsin-STI complex (K_I only 10 times larger than for the native inhibitor) replacement by Phe-15 causes a drastic decrease in the stability of the trypsin-BPTI complex. Thus, whether substitution by a given bulky amino acid affects proteinase-proteinase inhibitor binding depends upon the structure of the reactive site of the particular inhibitor.

One would expect the same principle to apply to the effects of replacement of the P_1' residue of the reactive site. Indeed, for STI, since P_1' itself is a bulky isoleucine, its replacement by less bulky amino acid residues such as glycine, leucine, and alanine did not significantly affect the binding (Kowalski and Laskowski, 1976). Inspection of models of the three-dimensional structure of the trypsin-BPTI and the chymotrypsin-BPTI complexes revealed that while phenylalanine is more bulky than alanine, Phe-16 BPTI might be expected to behave similarly to native BPTI in its binding ability because the phenylalanine side chain in the Phe-16 BPTI could be accommodated in the enzyme-inhibitor without much steric hindrance. It is thus interesting to note that whereas the K_I value for chymotrypsin-Phe-16 BPTI complex (3.3×10^{-8} M) is comparable to that of the chymotrypsin-native BPTI complex (9×10^{-9} M), K_I for the trypsin-Phe-16 BPTI complex (1.7×10^{-9} M) is much higher than that for the trypsin-native BPTI complex (6×10^{-14} M; Vincent and Lazdunski, 1972) (Table VII). This drastic decrease in stability (6.5 kcal/mol) of the trypsin-inhibitor complex does not appear to be explained by the simple steric effect. Rather, from inspection of a three-dimensional model of the trypsin-BPTI complex it appears more likely that the replacement of Ala by Phe at the reactive site caused a change in the local conformation of the reactive site of BPTI. This weakened the ionic interaction of the ϵ-amino group of Lys-15 of BPTI with the β-carboxylate of Asp-177 of trypsin which contributes to the difference in the stability of the trypsin-BPTI and chymotrypsin-BPTI complexes, thereby decreasing the discrimination of the inhibitor in its interaction with trypsin and chymotrypsin. It is interesting to note that cleaving the Lys_{176}-Asp_{177} bond of trypsin

(forming pseudotrypsin) also has a similar effect on the dissociation constant of the trypsin–BPTI complex (Table VII).

The Cyclic Heptadecapeptide as a Model of BPTI. As discussed earlier in this paper, the short peptide fragments made by Wiejak (1974, 1975) and Weber (1975) were weak trypsin inhibitors. In contrast to such peptide fragments, the cyclic heptadecapeptide we synthesized contains all of the essential amino acid residues that contribute to the van der Waals contacts and hydrogen bonds of the trypsin–BPTI and chymotrypsin–BPTI complexes. Our heptadecapeptide, which had a K_I value of 1.8×10^{-6} M against trypsin, was bound much better to the enzyme than the previously synthesized model hexapeptides. The K_I value we observed was still 3×10^7 times larger though than the K_I value for the trypsin–BPTI complex. Also, the heptadecapeptide was slowly partially digested by trypsin. The partially digested peptide (the modified heptadecapeptide) could also bind to trypsin, but the K_I measured (5.5×10^{-4} M) was 500 times larger than that of the native heptadecapeptide. This difference is presumably due to the fact that after partial digestion, the modified heptadecapeptide, while still having essentially the same amino acid composition as the native heptadecapeptide, possessed a looser structure. This increase in "flexibility" of the peptide chains may result in an unfavorable increase in the entropy of binding. Thus, for the partially digested model inhibitor to bind strongly to the enzyme, the molecule would have to be more rigid, so that cleavage of a peptide bond would not greatly affect the conformation of the reactive site.

In natural BPTI, while the majority of the amino acid residues do not enter into direct contact with the enzyme, they contribute to the rigidity of the inhibitor which is essential for the strong binding observed. Binding of a rigid, correctly folded protein molecule to the enzyme may be achieved without major changes in the internal degrees of freedom of the inhibitor (Sweet et al., 1974). This seems to explain why proteinase inhibitors as a class are characterized by their rigidity. Furthermore, the smallest serine proteinase inhibitor, the pancreatic secretory trypsin inhibitor II (Kazal), contains 52 amino acid residues (Tschesche et al., 1969). The anti-tryptic fragment obtained from the cleavage of the double-headed soybean Bowman-Birk inhibitor contains 38 amino acid residues (Odani and Ikenaka, 1973). Thus, except for inhibitors containing unusual residues, the minimum number of amino acids required for a naturally occurring polypeptide to have a sufficiently rigid structure to enable it to bind to enzyme highly effectively appears to be on the order of 30–50 residues.

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Interaction of Substrates and Inhibitors with the Homoserine Dehydrogenase of Kinase-Inactivated Aspartokinase I†

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ABSTRACT: The aspartokinase activity of the aspartokinase-homoserine dehydrogenase complex of *Escherichia coli* was affinity labeled with substrates ATP, aspartate, and feedback inhibitor threonine. Exchange-inert ternary adducts of Co(III)-aspartokinase and either ATP, aspartate, or threonine were formed by oxidation of corresponding Co(II) ternary complexes with H_2O_2. The ternary enzyme-Co(III)-threonine adduct (I) had 3.8 threonine binding sites per tetramer, one-half that of the native enzyme. The binding of threonine to I was still cooperative as determined by equilibrium dialysis (n_H = 2.2) or by studying inhibition of residual dehydrogenase activity (n_H = 2.7). Threonine still protected the SH groups of I against 5,5′-dithiobis(2-nitrobenzoate) (DTNB) reaction but the number of SH groups reacting with thiol reagents (DTNB) was reduced by 1-2 per subunit in the absence of threonine. This suggests either that Co(III) is bound to the enzyme via sulfhydryl groups or that 1-2 SH groups are buried or rendered inaccessible in I. The binding of threonine to sites not blocked by the affinity labeling produced changes in the circular dichroism of the complex comparable to changes produced by threonine binding to native enzyme and also protected against proteolytic digestion. The major conformational changes produced by threonine are thus ascribable to binding at this one class of regulatory sites. The interactions of kinase substrates with various aspartokinase-Co(III) complexes containing ATP, aspartate, or threonine and a threonine-insensitive homoserine dehydrogenase produced by mild proteolysis were studied. The inhibition of homoserine dehydrogenase by kinase substrates is not due to binding of these inhibitors at the kinase active site but was shown to be due to binding to sites within the dehydrogenase domain of the enzyme. L-α-Aminobutyrate, a presumed threonine analogue, also inhibits the dehydrogenase by binding at the same or similar sites in the dehydrogenase domain and not at a threonine regulatory site.

The feedback inhibition of the aspartokinase I-homoserine dehydrogenase I complex by threonine is one regulatory mechanism controlling L-threonine biosynthesis in *E. coli*. The tetrameric enzyme carries two activities on each of its identical polypeptide chains (Falcoz-Kelly et al., 1972). The two reactions catalyzed are:

aspartate + Mg-ATP → aspartyl phosphate + Mg-ADP

and

aspartate-β-semialdehyde + NADPH → homoserine + $NADP^+$

In addition to four pairs of active centers, aspartokinase-homoserine dehydrogenase possesses eight threonine binding sites (Falcoz-Kelly et al., 1972; Véron et al., 1973). Nuclear relaxation studies with 1-[^{13}C]threonine revealed that at least one threonine site is adjacent to the kinase metal-ion cofactor site. The threonine C_1 to Mn(II) distance is 4.4 ± 0.3 Å (Tilak et al., 1976). This distance estimate was corroborated by the discovery that L-threonine could be specifically incorporated into the inner sphere of an exchange-inert Co(III)-aspartokinase complex (Wright et al., 1976a). Both substrates of the kinase reaction are stably incorporated into similar complexes

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