Chemical–Enzymatic Replacement of Ile^{64} in the Reactive Site of Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: All the reactive amino groups in soybean trypsin inhibitor (Kunitz) were protected by guanidination of 9 out of 10 lysyl residues with *O*-methylisourea and by carbamoylation of the NH_2 terminal Asp with potassium cyanate. This derivative was converted to *modified* inhibitor (Arg^{63}-Ile^{64} reactive site peptide bond hydrolyzed) by incubation with trypsin at pH 3. The NH_2 terminal of Ile^{64} was allowed to react with phenyl isothiocyanate to produce inactive phenylthiocarbamoyl-*modified* inhibitor. Treatment with trifluoroacetic acid formed the anilinothiazolinone of Ile^{64} yielding des-Ile^{64}-*modified* inhibitor. After renaturation and purification, this material coelectrophoresed with *modified* inhibitor but did not form a stable complex with trypsin. Incubation with *tert*-butyloxycarbonyl-(amino acid)-*N*-hydroxysuccinimide esters yielded [*tert*-butyloxycarbonyl-(amino acid^{64})]-*modified* inhibitor. The *tert*-butyloxycarbonyl protective group was removed in trifluoroacetic acid. After renaturation, active [amino acid^{64}]-*modified* inhibitors were obtained for Ile^{64}, Ala^{64}, Leu^{64}, and Gly^{64} replacements. The resynthesis of the reactive-site peptide bond by kinetic control dissociation of the trypsin–inhibitor complex yielded fully active [Ala^{64}]-*virgin* inhibitor. Thus, soybean trypsin inhibitor (Kunitz) has been shown to tolerate the replacement of the P_1' residue with retention of activity. The importance of P_1' residues in the function of protein proteinase inhibitors is discussed.

In order to understand the evolution of proteins it is necessary to know what the constraints preventing the acceptance of mutations of individual amino acid residues in proteins are. Our laboratory is attempting to delineate such constraints on the amino acid residues in the reactive sites of protein proteinase inhibitors. One approach to this problem is the preparation of semisynthetic derivatives of inhibitors in which a single amino acid residue is specifically replaced by another.

The P_1 and P_1' residues (notation of Schechter and Berger, 1967) in the reactive sites of proteinase inhibitors are particularly amenable to removal and replacement since incubation of inhibitors with the proteinases with which they interact leads to the specific hydrolysis of the P_1–P_1' peptide bond. If after replacement of the P_1 or P_1' residue the inhibitor remains active, a facile method for synthesis of the P_1–P_1' peptide bond is at hand since kinetically controlled dissociation of the complex made from the modified (peptide bond hydrolyzed) inhibitor and the enzyme yields predominantly virgin (peptide bond intact) inhibitor and enzyme as products (Hixson and Laskowski, 1970).

In previous work on the replacements of P_1 Arg^{63} in Kunitz soybean trypsin inhibitor (STI)[1] by Lys (Sealock and Laskowski, 1969) and by Trp (Leary and Laskowski, 1973, 1976), carboxypeptidase B was employed for the removal step and the replacement by Lys while carboxypeptidase A was used for the replacement by Trp. That the action of both of these enzymes was strictly limited was highly fortunate.

In this work, the objective was the replacement of the P_1' residue in order to probe its function in the inhibitor. In principle, if an aminopeptidase of an appropriate specificity (i.e., good release of P_1' only) were found, it could have been employed in a manner analogous to the enzymatic P_1 replacements. However, a brief search for such an aminopeptidase failed. Thus, it was decided to remove and replace the P_1' Ile^{64} in modified STI by chemical methods.

The starting material for the removal–replacement reactions was prepared by protection of all reactive amino groups in STI by guanidination followed by carbamoylation (Kowalski and Laskowski, 1972; see also Figure 1). The α-amino group of interest was then liberated by incubation of the amino protected STI with trypsin. A similar maneuver, using guanidinated trypsinogen as the starting material, was used by Robinson et al. (1973) in the study of the amino-terminal Ile in trypsin.

The literature on chemical removal–replacement reactions performed on *proteins* is sparse. Generally, such reactions have been carried out in nonaqueous media on amino terminals of small proteins (e.g., insulin, ferredoxin) with few, if any, reactive functional groups (Borras and Offord, 1970; Krail et al., 1971; Lode, 1973; Lode et al., 1974). It is advantageous to carry out such reactions in aqueous solution since only functional groups accessible in the *native* protein will react. Furthermore, in aqueous solution, the reactivity of different functional groups can be modulated by the control of pH. A modified Edman reaction, performed in water, developed by Africa and Carpenter (1970) was

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[1] Abbreviations used are: STI, soybean trypsin inhibitor (Kunitz) in all of its forms; S, virgin STI (Arg^{63}-Ile^{64} bond intact); S(63,64), modified STI (Arg^{63}-Ile^{64} bond hydrolyzed); I, the product of guanidination of the ϵ-amino groups and carbamoylation of the α-amino group of S; I(63,64), modified I (Arg^{63}-Ile^{64} bond hydrolyzed); des-Ile^{64}-I(63,65), the product of removal of Ile^{64} from I(63,64); [amino acid^{64}]-I(63,64), the product of replacement of Ile^{64} (see Figure 1 for further clarification); PhNHCS, phenylthiocarbamoyl; PhNHCO, phenylcarbamoyl; Boc, *tert*-butyloxycarbonyl; ONSu, *N*-hydroxysuccinimide ester; PTI, bovine pancreatic trypsin inhibitor (Kunitz); ODU, optical density units.

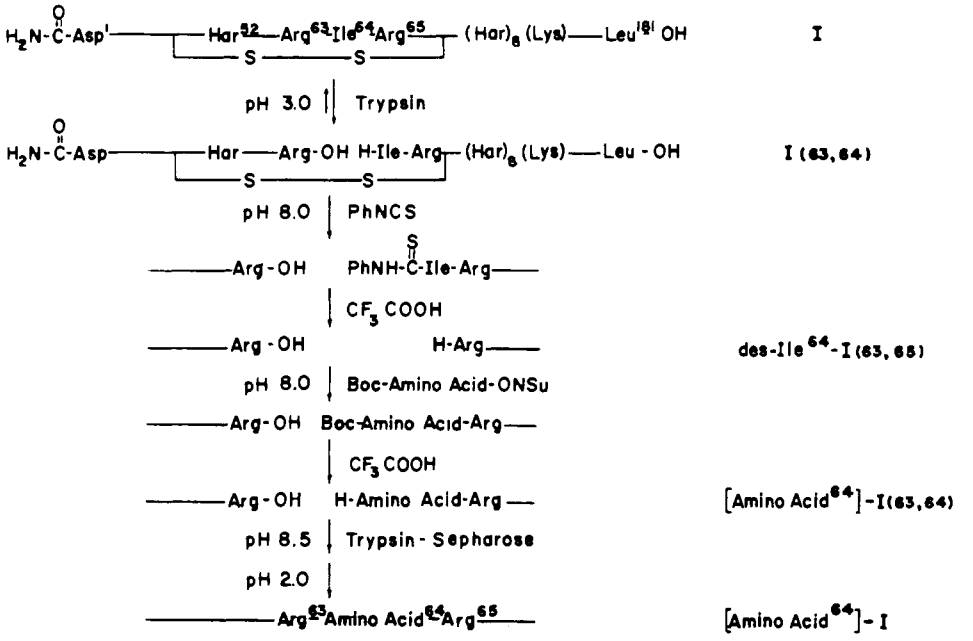

FIGURE 1: The sequence of reactions employed in the removal of Ile^{64} from I(63,64)[1] and the replacement of Ile^{64} by other amino acid residues. Guanidination of 9 of the 10 ϵ-amino groups of STI and carbamoylation of the amino-terminal Asp were previously described (Kowalski and Laskowski, 1972). The single ϵ-amino group which is not guanidinated is unreactive in the native protein. The positions of various residues are based on the sequence of STI determined by Koide et al. (1972).

used for the removal of the P_1' Ile in trypsin-modified STI. For the replacement of the P_1' Ile, *tert*-butyloxycarbonyl-(amino acid)-*N*-hydroxysuccinimide esters were used in aqueous solution following the suggestion of Anderson et al. (1964).

During the course of this work, problems in the nomenclature of the semisynthetic derivatives were encountered. While the nomenclature for synthetic modifications of the *normal* amino terminal of proteins and peptides is lucid (IUPAC–IUB Commission on Biochemical Nomenclature, 1967), no provisions were made for an amino-terminal residue resulting from a specific chemical or enzymatic cleavage of a protein where the two chains are held together either by noncovalent interactions (e.g., ribonuclease-S, staphylococcal nuclease-T) or noncovalent and covalent interactions (proteinase inhibitors with their reactive site peptide bond hydrolyzed, α-trypsin). The nomenclature used in this work indicates the hydrolyzed peptide bond by the residue numbers of the new carboxyl and amino terminals. For example, trypsin-modified STI (previously called S*), which is hydrolyzed at the Arg^{63}-Ile^{64} peptide bond, is S(63,64). The product resulting from the carboxypeptidase B catalyzed removal of Arg^{63} from S(63,64) is des-Arg^{63}-S(62,64), Tyr^{62} being the new carboxyl terminal. The abbreviations for semisynthetic derivatives of STI prepared in this work are shown below[1] and in Figure 1.

Experimental Procedures

Materials. Kunitz soybean trypsin inhibitor (SI-1AA) and bovine trypsin (TRL-1DA) were obtained from Worthington Biochemical Corp. Bovine α-trypsin, prepared as described by Luthy et al. (1973), and bovine trypsin-Sepharose, prepared as described by Kassell and Marciniszyn (1971), were kindly provided by Dr. Sarah Herbert. Phenyl isothiocyanate, *N*-ethylmorpholine, and trifluoroacetic acid, all Sequanal grade, were products of Pierce Chemical Co. and were stored under N_2 at 4 °C. Boc-(amino acid)-*N*-hydroxysuccinimide esters were obtained from either Fox Chemical or Bachem Inc. 4-Methylumbelliferyl *p*-guanidinobenzoate was a gift of Dr. Harry Hixson. Porcine anhydrotrypsin was kindly provided by Hoy Widener. All other chemicals were reagent grade or were the best available.

General Methods. The molarity of trypsin active sites was determined by the method of Chase and Shaw (1967). Relative trypsin activity was determined using the fluorescent burst titrant, 4-methylumbelliferyl *p*-guanidinobenzoate (Roberts et al., 1971), as described by Sealock and Laskowski (1973). Analytical disc gel electrophoresis was performed as previously described by Kowalski and Laskowski (1972). For molecular weight determinations on sodium dodecyl sulfate gels, the procedure of Fairbanks et al. (1971) with the modifications described by Leary and Laskowski (1976) was employed. Amino-terminal residues were analyzed using the dansyl technique (Gray, 1972; Weiner et al., 1972). Solutions were prepared with distilled, deionized water. When determined spectrophotometrically (280 nm), STI concentrations were obtained using the optical factor 1.1 mg ml^{-1} (ODU)$^{-1}$. Molecular weights used were 24 000 for trypsin and 20 000 for STI. All experiments were performed at 21 ± 1 °C unless otherwise indicated.

Preparation of I(63,64). Guanidination of the reactive amino groups of STI followed by carbamoylation of the amino-terminal Asp to yield an amino protected inhibitor (simply called I herein) was previously described (Kowalski and Laskowski, 1972). After incubation of I with 2 mol % porcine trypsin at pH 3.0 for 2 days, a mixture containing 85% I(63,64) and 15% I was obtained. The trypsin–inhibitor complex as well as any inhibitor dimer were removed by passage through a Sephadex G-75 column (4.7 × 100 cm) equilibrated with 0.01 M Tris-HCl–0.1 M NaCl (pH 8.4).[2]

Preparation of PhNHCS-I(63,64). Coupling of the pro-

[2] The molarities of the buffers refer to the total concentration of the buffering species at the indicated pH.

tein with phenyl isothiocyanate was performed by a method similar to the modified Edman degradation of Africa and Carpenter (1970). Throughout, care was taken to eliminate O_2 in order to avoid oxidative desulfurization of the PhNHCS derivative (Edman, 1970). The I-I(63,64) mixture was dissolved to the extent of 4 mg/ml in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) which was first degassed (via a water aspirator) and thoroughly flushed with N_2. Phenyl isothiocyanate (2 μl/ml) was added, the reaction vessel sealed with parafilm, and the biphasic reaction allowed to proceed at 21 °C with magnetic stirring. In the kinetic studies, samples (30 μg of protein) of the reaction mixture were run immediately on pH 9.4 disc gels previously charged with a fourfold excess of porcine trypsin in the G-200 stacking medium. While the coupling reaction is much faster at 40 °C, this temperature was unsuitable since under these conditions new components, which increase with time, were detected by disc gel electrophoresis.

After approximately 24 h, the reaction mixture was extracted five times with 1 to 2 vol of benzene (Edman, 1970). The extraction was performed in centrifuge tubes, the samples mixed on a Vortex mixer, and the emulsion broken up by centrifugation (bench top centrifuge). After removal of the last benzene extract, the turbid aqueous phase was separated from any remaining phenyl isothiocyanate globules, then kept under a stream of dry N_2 to evaporate any remaining benzene, and finally diluted 10 times into water (first thoroughly flushed with N_2) and lyophilized.

Preparation of Des-Ile^{64}-I(63,65). Trifluoroacetic acid (Konigsberg and Hill, 1962) containing 0.2% (v/v) mercaptoethanol was added to the lyophilized PhNHCS-I(63,64) to achieve a protein concentration of 10–20 mg/ml. After 5 min, 5 vol of anhydrous ethyl acetate was added to precipitate the protein. A small volume (10–100 μl) of concentrated HCl was added when necessary to aid in the precipitation. After centrifugation and removal of the supernatant, the precipitate was washed with a smaller volume of ethyl acetate, centrifuged, and dried under N_2 (after decanting the supernatant). Enough 0.2 M *N*-ethylmorpholine acetate (pH 8.0) was added to the amorphous white solid until the final protein concentration was 10 mg/ml. The mixture was magnetically stirred for 3 days to dissolve and renature the protein. Des-Ile^{64}-I(63,65) was purified on Sephadex G-75 and trypsin-Sepharose as described in the Results section.

Reaction of Purified Des-Ile^{64}-I(63,65) with Boc-(amino acid)-ONSu Esters. The purified des-Ile^{64}-I(63,65) (see Results) at 2 mg/ml in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) was treated with 1/9 the volume of 0.05 M Boc-(amino acid)-ONSu in dioxane (50-fold molar excess, 10% (v/v) dioxane) for 12 h in the case of the amino acids Gly, Ala, and Leu. Boc-Ile-ONSu was 0.1 M in dioxane and the protein solution was given two treatments (12 h each) with 1/19 the volume of the reagent. The solutions were then dialyzed vs. H_2O and 10^{-3} M ammonium acetate (pH 9), and lyophilized.

In kinetic studies of the reaction, samples (30 μg of protein) of the reaction mixture were first treated with citraconic anhydride (see below) and then subjected to pH 9.4 disc gel electrophoresis.

Deblocking of [Boc-(amino acid^{64})]-I(63,64). Removal of the Boc group was effected by treatment of [Boc-(amino acid^{64})]-I(63,64) for 15 min with trifluoroacetic acid containing 0.2% (v/v) mercaptoethanol (protein, 20 mg/ml). The protein was precipitated from the trifluoroacetic acid with anhydrous ethyl acetate as described above. After drying under N_2, the protein was taken up in 6 M guanidine-HCl and heated to 70 °C for 30 min to ensure complete denaturation. After cooling to room temperature, the solution was dialyzed vs. two changes of 0.01 M Tris-HCl–0.1 M NaCl (pH 8.4) over a 24-h period.

Test for Reactive Amino Groups by Citraconylation. Reaction of various STI derivatives with citraconic anhydride followed by pH 9.4 disc gel electrophoresis was previously shown to give a reliable picture of the state (free or blocked) of the α-amino groups of Asp^1 and Ile^{64} (Kowalski and Laskowski, 1972). Citraconic anhydride (Dixon and Perham, 1968) was used as a 1.0 M solution (9% v/v) in dioxane and the solution was stored at 4 °C. The reaction was carried out in 0.2 M *N*-ethylmorpholine acetate (pH 8.0–8.5). The protein (1–4 mg/ml) was treated two times (10 min each) with a 20-fold molar excess of reagent per free amino group. The number of reactive amino groups (generally zero or one in amino protected STI derivatives) were then assessed by the separation distance on disc gels between the protein and the citraconylated protein, the distance between S(63,64) and des-Arg^{63}-S(62,64) being defined as one charge unit.

This method is equivalent in principle to color reactions of amino groups with ninhydrin or trinitrobenzenesulfonic acid but it yields much more information. Since the citraconylated protein is viewed on disc gels, the number of reactive amino groups per component, rather than an average number over all components, is obtained.

Results

The reactions described in this work were limited to the α-amino group of Ile^{64} in the trypsin-modified STI by prior guanidination of 9 of the 10 ϵ-amino groups of STI and carbamoylation of the amino-terminal Asp (Kowalski and Laskowski, 1972). This amino protected STI, simply called I herein,[1] was then incubated with trypsin in order to liberate the α-amino group of Ile^{64}, the product designated as I(63,64) (see Figure 1). The sequence of reactions employed in the removal of Ile^{64} from I(63,64) and the replacement of Ile^{64} by other amino acids is shown in Figure 1. I(63,64) has only one reactive amino group, that of Ile^{64}. Under conditions where the protein is in the native form, the single Lys, which is not guanidinated, is unreactive.

Coupling of I(63,64) with Phenyl Isothiocyanate. Reaction of a mixture containing 85% I(63,64) and 15% I with phenyl isothiocyanate was shown by pH 9.4 disc gel electrophoresis (with prior addition of excess porcine trypsin) to lead to selective inactivation of I(63,64).[3] On the basis of previous chemical modification studies (Kowalski and Laskowski, 1972), it was assumed that the inactivation of I(63,64) was due solely to the substitution on the α-amino group of Ile^{64} giving PhNHCS-I(63,64).

The rate of the reaction at 21 °C (pH 8.0) was followed by the loss of inhibitory activity as monitored by pH 9.4 disc gel electrophoresis (7-cm gels) of samples mixed with excess porcine trypsin. The reaction is pseudo first order, $t_{1/2}$ = 5.5 h. Allowing for the difference in temperature and solubility of the reagent, the $t_{1/2}$ is similar to those determined for the coupling of phenyl isothiocyanate with the amino-terminals Gly^{A1} ($t_{1/2}$ = 1 h) and Phe^{B1} ($t_{1/2}$ = 30

[3] The statement that the inhibitor is inactive is only operational, defined in terms of the methods used to detect inhibitory activity. This is not meant to imply that there is no interaction between the enzyme and the "inactive" inhibitor (see Kowalski and Laskowski, 1976).

min) of insulin by a similar method at 40 °C (Africa and Carpenter, 1970).

In large scale preparations, the reaction was typically terminated at 24 h by extraction of the reagent and lyophilization (see Experimental Procedures).

Removal of Ile^{64} from PhNHCS-I(63,64). Treatment of PhNHCS-I(63,64) with trifluoroacetic acid very rapidly liberates a new amino group as shown by reaction with citraconic anhydride and subsequent disc gel electrophoresis (pH 9.4). PhNHCS-I(63,64) shows no reaction with citraconic anhydride, while after a 5-min trifluoroacetic acid treatment, the bulk of the material reacts with citraconic anhydride giving a monocitraconyl derivative (for the reaction of *purified* des-Ile^{64}-I(63,65) with citraconic anhydride see Figure 3, zero time gels). Longer incubations (up to 1 h) in trifluoroacetic acid produce no change in the disc gel pattern of the protein after citraconylation.

Mass spectral analysis (Fairwell and Lovins, 1971) of the 1,2-dichloroethane extracts of the residue after evaporation of the trifluoroacetic acid showed a fragmentation pattern identical with that of the isoleucyl phenylthiohydantoin standard. The molecular ion at *m/e* 248 (100%) corresponds to either isoleucyl phenylthiohydantoin or leucyl phenylthiohydantoin. A peak at *m/e* 205 corresponding to the loss of the isopropyl group of the Leu side chain was notably absent. Further proof that Ile^{64} was removed was demonstrated using purified des-Ile^{64}-I(63,65) described below.

Renaturation and Purification of Des-Ile^{64}-I(63,65). After precipitation from trifluoroacetic acid, the protein slowly dissolved in 0.2 M *N*-ethylmorpholine acetate (pH 8.0). Renaturation was allowed to proceed over a period of 3–4 days at 21 °C. In control experiments with a trifluoroacetic acid treated I(63,64)–I mixture, regain of inhibitor activity was first order, $t_{1/2} \approx 6$ h. By this renaturation procedure, 55% of the total activity was regained and chromatography on Sephadex G-75 showed that approximately the same amount of the total protein was native.

Analysis of the renatured des-Ile^{64}-(63,65) on pH 9.4 disc gels gave a pattern much more complicated than the original PhNHCS-I(63,64). Addition of excess porcine trypsin prior to electrophoresis eliminated all of the new bands, presumably by digestion, restoring the original pattern, albeit of much lower intensity.

The renatured material was purified by chromatography on Sephadex G-75 as shown in Figure 2. The first peak, which ran with the column void volume, appeared on pH 9.4 disc gels (7 cm) as a series of faint bands spanning the region from the position of the native inhibitor to the top of the gel (i.e., components larger in size and/or more positive than native STI). Addition of excess porcine trypsin to the sample prior to electrophoresis resulted in the complete obliteration of the bands. Analysis of this first peak on sodium dodecyl sulfate gels showed that the material consisted mainly of the two fragments of des-Ile^{64}-I(63,65) (residues 1–63 and 65–181). Attempts to renature the material in the first peak were futile. Thus, the protein was assumed to be unfolded but apparently chemically damaged.

The material in the second peak (Figure 2) behaved on sodium dodecyl sulfate gels the same as that in the first peak but was much more homogeneous on pH 9.4 disc gels, running as a broad band, just above the native inhibitor. This band too was largely eliminated by prior addition of trypsin to the sample.

The third peak (Figure 2) eluted in the position of native STI; in fact, in the chromatogram shown, the third peak

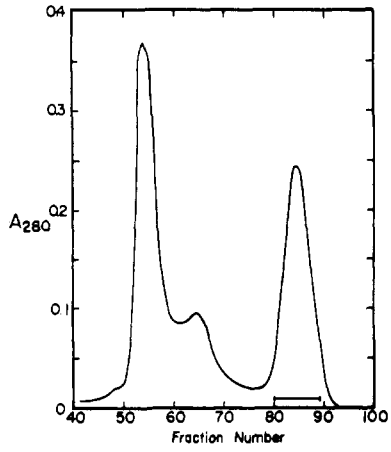

FIGURE 2: Purification of des-Ile^{64}-I(63,65) on a column (2.5 × 91 cm) of Sephadex G-75 equilibrated with 0.01 M Tris-HCl (pH 8.4)–0.1 M NaCl. The fraction volume is 3 ml. The first peak is at the column void column. The third peak corresponds to the elution volume of native STI and is a mixture of des-Ile^{64}-I(63,65), I, and I(63,64). The third peak was pooled (as indicated by the bar) and the inactive des-Ile^{64}-I(63,65) separated from the active I and I(63,64) by passage through a column of trypsin-Sepharose (see text for details).

was a mixture of des-Ile^{64}-I(63,65), unreacted I(63,64), and I, since the starting material was a mixture of 85% I(63,64) (ca. 90% labeled with phenyl isothiocyanate) and 15% I. Disc gels (pH 9.4) of this material showed two bands corresponding to I and I(63,64); prior addition of excess porcine trypsin left a band which corresponded to I(63,64) (presumably des-Ile^{64}-I(63,65)) and a band which ran in the position of the enzyme–inhibitor complex.

Passage of the pooled third peak through a column of trypsin-Sepharose (25 ml packed bed in a 25-ml syringe) equilibrated with 0.05 M sodium borate (pH 8.5), 0.5 M KCl, and 0.01 M $CaCl_2$ removed the active inhibitor, the des-Ile^{64}-(63,65) passing through as a skewed peak. The overall yield of refolded des-Ile^{64}-I(63,65) was ca. 20%, based on the PhNHCS-I(63,64). The skewing of the des-Ile^{64}-I(63,65) peak was taken as an indication of weak interaction between the protein and trypsin. PhNHCS-I(63,64) shows no such skewing under the same conditions.

Properties of Purified Des-Ile^{64}-I(63,65). Purified des-Ile^{64}-I(63,65) co-chromatographs (Sephadex G-75) and co-electrophoreses (pH 9.4 gels) with native I(63,64). The fragments produced upon reduction of the disulfide bonds are of the same molecular weight as those of native I(63,64) as shown by sodium dodecyl sulfate gel electrophoresis (shown later in Figure 5). Des-Ile^{64}-I(63,65) is not digested by trypsin, attesting to its conformational stability.

Dansyl end group analysis of the purified protein shows dansyl-Arg as well as ϵ-dansyl-Lys and a large amount of *O*-dansyl-Tyr (STI has four Tyr; Koide et al., 1972). Arg is the expected amino terminal since it is the residue penultimate to Ile^{64}. The fact that Arg was the only amino terminal shows that no peptide bond cleavage occurred in passing the inactive inhibitor through trypsin-Sepharose (described under the section on purification above). The presence of ϵ-dansyl-Lys shows that the single Lys which was not covered to Har (hence presumably buried) is available for dansylation in 33% acetone and therefore did not react with phenyl isothiocyanate under the conditions employed. Likewise, the bulk of the Tyr residues appear to be free for dansylation. This is in agreement with the results of Africa and Carpenter (1970) on insulin.

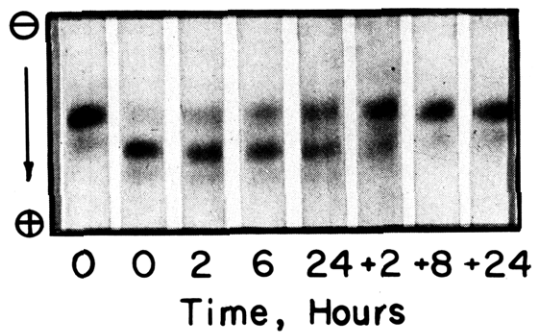

FIGURE 3: Disc gels (pH 9.4, 15 cm) showing the time course of the reaction of Boc-Ile-ONSu with des-Ile^{64}-I(63,65) as monitored by citraconylation of the free α-amino group. The zero time gels are des-Ile^{64}-(63,65) before and after citraconylation, in that order. After 24 h, a second addition of Boc-Ile-ONSu was made. The time intervals after this addition are indicated by +. Reaction conditions are: des-Ile^{64}-I(63,65), 2 mg/ml in 0.2 M *N*-ethylmorpholine acetate (pH 8.0); two treatments, 24 h each, with a 100-fold molar excess of 0.5 M Boc-Ile-ONSu in acetonitrile.

Citraconylation of purified des-Ile^{64}-I(63,65) produces a new band on pH 9.4 disc gels that is one charge more negative than the starting material (see Figure 3; zero time gels), showing the presence of only one reactive amino group. From the end group analysis, this reactive group is the α-amino of Arg^{65}. Approximately 20% of the total protein did not react with citraconic anhydride and was presumed to have a blocked amino group as a result of oxidative desulfurization (Edman, 1970), i.e., PhNHCO-I(63,64). When care was not taken to exclude O_2 in the coupling and cleavage steps, the amount of this noncitraconylatable material was much higher. Contamination of des-Ile^{64}-(63,65) by this material was of no consequence to this work, however, since this material is inactive as an inhibitor and not reactivatable.

Taken together, the above results show that except for the absence of Ile^{64} and inhibitory activity, des-Ile^{64}-I(63,65) shares many of the properties of I(63,64). It was assumed, then, that the conformations of I(63,64) and the des-Ile derivative were very similar, if not identical. This is supported by the weak interaction of des-Ile^{64}-I(63,65) and trypsin observed on the insoluble enzyme column. Strong evidence for a very similar conformation in the area of the reactive site comes from preliminary experiments (D. Kowalski, unpublished) showing the formation of a stable complex of des-Ile^{64}-I(63,65) with porcine anhydrotrypsin (see Discussion). With a reactive site conformation similar to I(63,64), closure of the Arg^{63} to Arg^{65} peptide bond in des-Ile^{64}-I(63,65) catalyzed by trypsin might be possible. Attempts toward such an end were futile suggesting that Arg^{65} is simply too remote from Arg^{63} for synthesis to take place. The separation of those residues could be stabilized by secondary interactions of the P′ residues with the remainder of the molecule. The failure to synthesize the Arg^{63} to Arg^{65} peptide bond stands in contrast to the ability to *insert* an amino acid in the reactive site by synthesis of the Arg^{63} to (amino acid)^{63A} peptide bond catalyzed by trypsin (see Kowalski and Laskowski, 1976).

The ultimate test of the chemical integrity of des-Ile^{64}-I(63,65) is the restoration of Ile^{64} with regain of inhibitory activity. Results of experiments toward this end are discussed below.

Restoration of Ile in Des-Ile^{64}-I(63,65). (a) Reaction of Des-Ile^{64}-I(63,65) with Boc-Ile-ONSu. Control experiments with an I–I(63,64) mixture showed that I(63,64) was selectively inactivated by Boc-Ile-ONSu (Kowalski and Laskowski, 1976). Thus, the substitution of functional groups other than the α-amino group of Ile^{64}, if indeed it occurred at all, was of no consequence as far as inhibitory activity was concerned.

The reaction of des-Ile^{64}-I(63,65) with Boc-Ile-ONSu was monitored by the loss of the free α-amino group of Arg^{65} as shown in Figure 3. The starting material, des-Ile^{64}-I(63,65), reacted with citraconic anhydride to give a species one charge unit more negative (Figure 3, second zero time gel). The product, [Boc-Ile^{64}]-I(63,64), having no free amino group, did not react with citraconic anhydride (Figure 3, + 24 h gel). The reaction of Boc-Ile-ONSu with des-Ile^{64}-I(63,65) was much slower than the same reaction with I(63,64) (Kowalski and Laskowski, 1976). For des-Ile^{64}-I(63,65), two additions of the reagent were necessary due to the competing hydrolysis of the reagent. The slower rate of reaction with des-Ile^{64}-I(63,65) is interpreted to mean that the α-amino group of Arg^{65} is less accessible to the reagent than is the α-amino group of Ile^{64} in I(63,64). That the backbone of Arg^{65} itself is inaccessible is attested to by the fact that trypsin fails to hydrolyze the Arg^{65}-Phe^{66} peptide bond in P_1' α-amino blocked-I(63,64) or des-Ile^{64}-I(63,65). The x-ray crystallographic structure of the STI–porcine trypsin complex clearly shows that both the amido hydrogen and carbonyl oxygen of Arg^{65} are directed into the STI molecule, although it is not clear whether these groups are involved in intramolecular hydrogen bonds (Janin et al., 1974).

(b) Removal of Boc from [Boc-Ile^{64}]-I(63,64) and Regain of Activity. Experiments on the product of the reaction of Boc-Ile-ONSu with I(63,64), i.e. *endo*-Boc-Ile^{63A}-I(63,63A), showed that the removal of the Boc group in trifluoroacetic acid was essentially complete in less than 30 min with ca. 8% of the material not deblocking (Kowalski and Laskowski, 1976). [Boc-Ile^{64}]-I(63,64) was given a 15 min treatment with trifluoroacetic acid, and, after precipitation of the protein as described under Experimental Procedures, was dissolved in 6 M guanidine-HCl and subjected to a denaturation–renaturation cycle. Native STI denatures very slowly in 6 M guanidine-HCl at 21 °C, taking 14 days to reach completion; at 70 °C, however, the denaturation is complete in 15 min (Fish and Leach, 1973). Renaturation of the denatured inhibitor proceeds with the regain of activity. Denaturation–renaturation experiments with S(63,64) and I(63,64) showed that the above was also true for the modified inhibitor; however, activity regain proceeded more slowly. Since [Ile^{64}]-I(63,64) should be identical with I(63,64), the 6 M guanidine-HCl denaturation–renaturation was utilized (see Experimental Procedures) instead of the slower renaturation in 0.2 M *N*-ethylmorpholine acetate described for des-Ile^{64}-I(63,65).

Renatured [Ile^{64}]-I(63,64) showed a disc gel pattern similar to that of I(63,64) which was treated in the same manner. Addition of excess porcine trypsin to the renatured [Ile^{64}]-I(63,64) prior to electrophoresis showed significant amounts of active inhibitor present as the enzyme–inhibitor complex (25–35% of the total starting material). Of the remaining inactive material, ca. 45–55% was digested by the trypsin (hence, presumably unfolded protein) and ca. 20–30% migrated in the position of I(63,64). This inactive but undigestible protein was assumed to be blocked at Ile^{64} by PhNCO and/or Boc.

The regain of inhibitory activity upon restoration of Ile^{64}

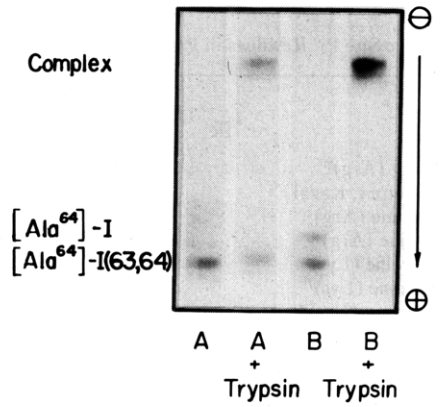

FIGURE 4: Disc gels, pH 9.4 (7 cm): (A) crude [Ala^{64}]-I(63,64) without and with a fourfold molar excess of porcine trypsin; (B) [Ala^{64}]-I(63,64) after purification on trypsin-Sepharose without and with a fourfold molar excess of porcine trypsin. Approximately 30% of the material is [Ala^{64}]-I (i.e., 63,64 peptide bond intact).

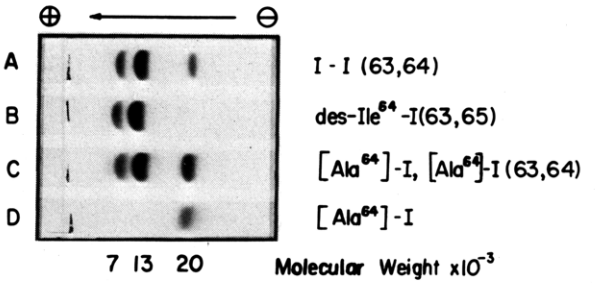

FIGURE 5: Sodium dodecyl sulfate gels comparing the starting material with the purified products of removal and replacement of Ile^{64}: (A) I–I(63,64) mixture (15%:85%); (B) purified des-Ile^{64}-I(63,65); (C) [Ala^{64}]-I(63,64) after purification on trypsin-Sepharose; (D) material from gel C after binding to trypsin-Sepharose and subjecting it to a kinetic control dissociation as described in the text.

serves as a final proof of the authenticity of the material called des-Ile^{64}-I(63,65). Since the enzyme–inhibitor complex is observed directly on the gels, rather than as the average inhibitory activity of the entire sample, 25–35% of the molecules in the Ile^{64} restored material are 100% active. Finally, the restoration of Ile^{64} by reaction with Boc-Ile-ONSu and subsequent deblocking serves as an excellent control for the replacement of Ile^{64} by other amino acids using the same method.

Replacement of Ile^{64} in I(63,64) by Other Amino Acids. The amino acids Leu, Ala, and Gly were allowed to react as their Boc-(amino acid)-ONSu esters with des-Ile^{64}-I(63,65) and monitored by the loss of the free α-amino group of Arg^{65} as described above. The rate of reaction in all cases was much faster than that for Boc-Ile-ONSu and therefore only one addition of reagent was necessary to achieve complete replacement. Since under these conditions (10% dioxane), all Boc-(amino acid)-ONSu esters were soluble, the slow rate for Ile is probably due to steric hindrance by the side chain.

After trifluoroacetic acid treatment to remove the Boc group followed by denaturation in 6 M guanidine-HCl and renaturation, the [amino acid^{64}]-I(63,64) derivatives were analyzed on pH 9.4 disc gels with and without prior addition of excess porcine trypsin (for example, see Figure 4, gels A, for [Ala^{64}]-I(63,64)). In all cases ([Leu^{64}]-, [Ala^{64}]- and [Gly^{64}]-I(63,64)), large amounts (25–35% of the total protein) of inhibitory activity were detected as the enzyme–inhibitor complex. Thus, for STI, it is apparently the α-amino group in the P_1' position and not the nature of the side chain which is critical for inhibitory activity.

Replacement of Ile^{64} by several other amino acid residues was also attempted but no active derivatives were obtained. This should not be regarded as evidence that other amino acids at the P_1' position inactivate the inhibitor but rather that the proper conditions for introduction of the amino acid, deblocking of the Boc derivative, or renaturation of the final product were not found.

Purification and Properties of [Ala^{64}]-I(63,64). [Ala^{64}]-I(63,64), prepared as described in the preceding section, was purified by binding the active material to a trypsin-Sepharose column (7.5 ml packed bed in a 10-ml syringe). A large fraction of the material (65% of the total A_{280}) passed through the column unretarded. Analysis of this material on pH 9.4 disc gels showed several weak bands. The bands were very much weaker than expected from the A_{280} of the pooled peak indicating that the material consisted largely of peptides resulting from the digestion of unfolded protein. The active semisynthetic inhibitor was released from the column by washing with 0.05 M glycine (pH 2.0). Disc gel electrophoresis (pH 9.4) showed that the active inhibitor released was 70% [Ala^{64}]-I(63,64) and 30% [Ala^{64}]-I as shown in Figure 4 (gel B). The increase in purity of the active inhibitor is evidenced by the increased staining of the enzyme–inhibitor complex, since gels A and B (Figure 4) carry about the same amount of total protein. The presence of [Ala^{64})-I was verified by electrophoresis on sodium dodecyl sulfate gels after reduction of the disulfide bonds. As shown in Figure 5, gel C, indeed, a large fraction of the material corresponds to a molecular weight of 20 000.

If carefully executed, dissociation of the complex of inhibitor with trypsin-Sepharose at low pH results in a kinetically controlled distribution of products in which the virgin (peptide bond intact) form of the inhibitor predominates. A portion of the active peak from above was bound to a small trypsin-Sepharose column (1.5 ml packed bed of a 2.5-ml syringe) at pH 8.5 and then dissociated with 0.1 M HCl at a high flow rate as described by Sealock and Laskowski (1973). Analysis of this material on pH 9.4 disc gels showed ca. 90% [Ala^{64}]-I and only 10% [Ala^{64}]-I(63,64). Electrophoresis on sodium dodecyl sulfate gels after reduction of the disulfide bonds confirmed that the semisynthetic inhibitor was almost completely in the peptide bond intact form (Figure 5, gel D). A parallel experiment with a mixture of 85% I(63,64) and 15% I gave 100% virgin inhibitor (I) upon dissociation.

Finally, [Ala^{64}]-I(63,64) was compared with I(63,64) in titrations against bovine α-trypsin and the results are shown in Figure 6. As evidenced by the linearity of the titration, [Ala^{64}]-I(63,64) forms a very strong complex ($K_{assoc} > 10^9$ M^{-1}) with trypsin. No difference in the titrations, within experimental error, is seen between [Ala^{64}]-I(63,64) and I(63,64). Finally, during the assay, no dissociation of the semisynthetic inhibitor–trypsin complex is observed. Therefore, the *upper limit* of the rate constant for dissociation is 10^{-3} s^{-1}.

Discussion

The results presented here show that reaction of the α-amino group of P_1' Ile^{64} with phenyl isothiocyanate inactivates I(63,64) (see Figure 1) and that the protein remains inactive as an inhibitor after cyclization of phenylthiocarbamoyl-Ile to yield des-Ile^{64}-I(63,65).[3] Reaction of des-Ile^{64}-I(63,65) with a Boc-(amino acid)-ONSu ester to yield

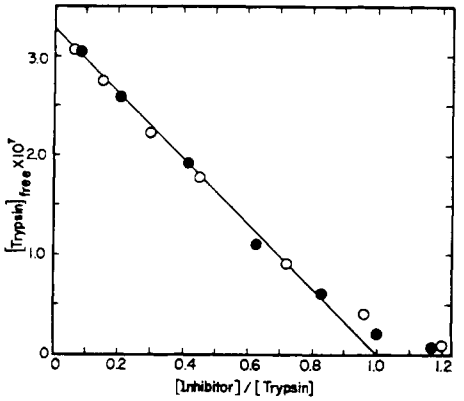

FIGURE 6: Titration of bovine α-trypsin with an [Ala^{64}]-I-[Ala^{64}]-I(63,64) mixture (30%:70%, ○) and an I-I(63,64) mixture (15%:85%, ●). Data points are averages of duplicate determinations. A 1:1 stoichiometry of inhibitor to enzyme was assumed. The relative concentration of free trypsin was determined using the fluorescent burst titrant 4-methylumbelliferyl *p*-guanidinobenzoate as described by Sealock and Laskowski (1973). The absolute concentration of trypsin active sites in the trypsin stock solution was determined using *p*-nitrophenyl *p'*-guanidinobenzoate by the method of Chase and Shaw (1967).

[Boc-(amino acid^{64})]-I(63,64) still yields no activity, since the α-amino group of P_1' is acylated. Removal of the Boc group, however, regenerates inhibitory activity in the Ile control as well as in replacements by Leu, Ala, and Gly. Characterization of [Ala^{64}]-I(63,64) shows that the gross properties of authentic I(63,64) are preserved. These results will first be discussed in terms of the known P_1' residues in other proteinase inhibitors and then correlated with the three-dimensional information on the PTI–bovine trypsin complex (Ruhlmann et al., 1973) and information on the structure of the STI–porcine trypsin complex (Janin et al., 1974; Sweet et al., 1974).

Table I lists the P_1' residues in some of the better known reactive sites of protein proteinase inhibitors. It can be seen that many different amino acid residues occupy the P_1' position. On the basis of this variability, it is quite easy to conclude that the P_1' residue should not be strongly conserved and that the activity of [Gly^{64}]-, [Ala^{64}]-, and [Leu^{64}]-STI is not surprising. However, while all small protein proteinase inhibitors interact in an analogous manner with the enzyme they inhibit (Laskowski, 1970), they are not all homologous, and, therefore, the above conclusion is not justified.

Based on amino acid sequence data, some of the inhibitors in Table I can be grouped into three homologous families: (1) pancreatic secretory trypsin inhibitors (Kazal) (five species of mammals), chicken ovomucoid, Japanese quail ovomucoid (domains I, II, and III) and the inhibitors from porcine seminal plasma and dog submandibulary gland (domains I and II); (2) PTI, snail K, and inhibitors from bovine colostrum, turtle egg white, and Russell's viper; (3) double-headed inhibitors from leguminous plants (four species and one variant). While the amino acid sequences of other inhibitors are known, these inhibitors either have as yet no recognizable homologue (notably STI) or show a weak sequence similarity to other inhibitors (e.g., *Streptomyces albogriseolus* and family 1; Ikenaka et al., 1974). In the latter case, homology (divergent evolution) or analogy (convergent evolution) cannot be decided. Within the three homologous families, the P_1' residue is highly variable (family 1), conservative (family 2), or invariant (family 3).

Table I: Reactive Site P_1' Residues in Proteinase Inhibitors.[a]

Ile

Soybean, Kunitz (Arg)[b]
Pancreatic secretory, Kazal
- Bovine (Arg)[c]
- Ovine (Arg)[d]
- Porcine (Lys)[e]
- Canine (Lys)[f]
- Human (Lys)[g]

Guinea pig seminal vesicles (Arg)[h]
Chicken ovoinhibitor (Phe)[i]

Ala

Bovine pancreatic, Kunitz (Lys)[j]
Bovine colostrum (Lys)[k]

Snail K (Lys)[l]
Chicken ovomucoid (Arg)[b]
Wheat (Arg)[m]
Rye (Arg)[m]

Leu

Dog submandibulary, domain I (Arg)[n]
Corn (Arg)[r]

Val

Streptomyces albogriseolus (Met)[aa]

Ser

Double-headed inhibitors from legumes
- Soybean (Lys, Leu)[o]
- Lima bean (Lys, Leu)[p]
- Lima bean (Lys, Phe)[q]
- Garden bean (Ala, Arg)[r]
- *Phaseolus vulgaris* var. *nanus* (Lys, Leu)[s]

Peanut (Arg)[t]
Potato IIA and IIB (Lys)[u]

Gly

Turtle egg white (Lys)[v]
Russell's viper (Arg)[w]

Asp

Potato I (Leu)[x,y]
Japanese quail ovomucoid, domains II and III (Lys)[z]
Dog submandibulary, domain II (Met)[n]

Gln

Porcine seminal plasma (Arg)[bb]

[a] P_1' residues known thus far in active inhibitors are shown in boldface, and below them the sources of the various proteinase inhibitors are listed. P_1 residues are shown in parentheses. For the double-headed inhibitors from legumes, the order of the P_1 residues is that which appears in the molecule. [b] Ozawa and Laskowski, 1966. [c] Rigbi and Greene, 1968. [d] Hochstrasser et al., 1968. [e] Tschesche and Wachter, 1970. [f] Tschesche et al., 1971. [g] Greene et al., 1974. [h] Fink et al., 1971. [i] Feinstein and Gertler, 1972. [j] Chauvet and Acher, 1967; Kress and Laskowski, 1968. [k] Cechova et al., 1969. [l] Dietl and Tschesche, 1974. [m] Hochstrasser and Werle, 1969. [n] Hochstrasser and Fritz, 1975. [o] Birk and Gertler, 1971; Odani and Ikenaka, 1972; Seidl and Liener, 1971, 1972. [p] Krahn and Stevens, 1972. [q] Stevens et al., 1974. [r] Wilson and Laskowski, 1974. [s] Belitz and Fuchs, 1973. [t] Hochstrasser et al., 1970. [u] Iwasaki et al., 1973a,b. [v] I. Kato, unpublished experiments; Laskowski et al., 1974. [w] Takahashi et al., 1974. [x] Kiyohara et al., 1973. [y] Richardson and Cossins, 1974. [z] Kato et al., 1976. [aa] Ikenaka et al., 1974. [bb] Fritz and Tschesche, 1975.

Thus, in some homologous families of inhibitors the P_1' residue need not be conserved. Evidently, STI is a member of such a family in light of the strong inhibitory activity of the semisynthetic variants described in this work.

That the P_1' residue in STI can be altered without affecting the function of the protein is in agreement with the

available x-ray data. Sweet et al. (1974) have shown that the STI–porcine trypsin complex exists as a stable tetrahedral adduct, as was previously shown for the PTI–bovine trypsin complex (Ruhlmann et al., 1973). Furthermore, in the complex, STI and PTI have almost identical conformations in the reactive site region P_3 through P_1' (Sweet et al., 1974). The P_1' Ile in STI is external and does not appear to interact strongly with the remainder of the inhibitor molecule. Thus, since PTI and some PTI homologues have P_1' Ala and P_1' Gly, respectively, one would expect that for STI, having a similar reactive site conformation, P_1' residues *smaller* than Ile would be tolerated. Indeed, as shown here, [Gly^{64}]-I(63,64) and [Ala^{64}]-I(63,64) are fully active as inhibitors. Conversely, that PTI could tolerate *larger* hydrophobic residues than Ala in the P_1' position was suggested by Fersht et al. (1973) from model building studies with the PTI–bovine chymotrypsin complex.

Thus, provided that the backbone conformation of the residues surrounding the reactive site is correct, the nature of the P_1' side chain has little effect upon the formation of the enzyme–inhibitor complex. Why then is the P_1' residue conserved in family 3? One possible answer is that it is not strictly conserved and the impression we get from examining Table I is erroneous, since too few cases are being considered. For example, when only two members of family 2 (PTI and bovine colostrum) were known, P_1' Ala appeared to be conserved. Such an explanation seems to us unlikely for the class of double-headed inhibitors from legumes, since, in all ten known reactive sites, P_1' is Ser. On the other hand, a great number of mutations were accepted in other positions in the reactive sites of these inhibitors. As is seen in Table I, five different amino acids (Lys, Arg, Leu, Phe, and Ala) have been found in the P_1 position; four different amino acids (Ile, Met, Asn, and Tyr) are present at the P_2' position (based on only six reactive site sequences). Furthermore, since this family of inhibitors presumably arose by partial gene duplication (Tan and Stevens, 1971), the amino acid sequences surrounding each of the two reactive sites within a single inhibitor can be compared. Of the 27 residues which can be aligned at the Cys residues and the reactive sites (allowing one single residue deletion in the COOH terminal region), 11, 12, and 13 residues are identical in the inhibitors from soybean (Odani and Ikenaka, 1972), garden bean (Wilson and Laskowski, 1975), and lima bean (Stevens et al., 1974), respectively. Excluding the 6 Cys residues, the P_1' Ser is one of only 5 to 7 residues out of 21 residues which has been conserved. The above interspecific and intraspecific comparisons suggest the presence of a P_1' Ser in the ancestral monovalent inhibitor as well. The simplest explanation for the conservation of the P_1' Ser is that it is required to achieve the proper conformation of the reactive site in the double-headed legume inhibitors. While the x-ray data show that the ϕ, ψ angles of residues surrounding the reactive site peptide bond in STI and in PTI are essentially the same, different interactions are responsible for achieving this conformation in the two inhibitors. For example, in PTI, the P_1' Ala residue is involved in a hydrogen bond with the remainder of the inhibitor molecule (Huber et al., 1971), while in STI, P_1' Ile^{64} appears to be external and not involved in any interaction with the rest of the inhibitor molecule (Sweet et al., 1974). On the basis of this discussion, it appears that replacement of P_1' Ser in inhibitors from legumes should be a particularly interesting experiment.

While the side chain of the P_1' Ile in STI does not appear to be necessary for activity, the backbone *is* essential since des-Ile^{64}-I(63,65) is inactive as a trypsin inhibitor. Restoration of the Ile backbone, in the form of a Gly residue, restores inhibitory activity. There is evidence from our laboratory that P_1' α-amino blocked S(63,64) can form an acyl-enzyme. Incubation of dicarbamoyl-S(63,64) (carbamoylated at P_1' Ile^{64} and Asp^{1}) with trypsin in the presence of glycinamide leads to the formation of an Arg^{63}-Gly-NH_2 peptide bond (Wang and Laskowski, 1972). We assume that des-Ile^{64}-I(63,65) would also be capable of acyl-enzyme formation. Even though these derivatives can interact with the enzyme at the active site, they are inactive as inhibitors.[3] Elimination of the P_1' amino acid or acylation of the P_1' α-amino group (Kowalski and Laskowski, 1972) apparently disrupts a highly cooperative process, geared toward the formation of the stable tetrahedral adduct (with the peptide bond intact) where the enzyme–inhibitor interactions are maximal. In going either to the loose (Michaelis) complex (Luthy et al., 1973) or the acyl complex, the interactions present in the tetrahedral adduct are weakened (Ruhlmann et al., 1973; Robertus et al., 1972). This then accounts for the instability of the trypsin complex with the des-Ile^{64} derivative of STI and also derivatives of STI acylated at the α-amino group of Ile^{64}.

The acyl complex of enzyme and inhibitor has been described as being unstable due to weakened interactions (compared to the tetrahedral adduct with the peptide bond intact) on the leaving group side of the inhibitor (Ruhlmann et al., 1973) and also due to removal of the carbonyl oxygen of P_1 from the oxyanion hole (Robertus et al., 1972). If des-Ile^{64}-I(63,65) can form this unstable acyl complex but cannot form the stable tetrahedral adduct due to the missing P_1' residue, it would seem that in cases where the formation of the tetrahedral adduct is unnecessary for a stable complex (e.g. S plus anhydrotrypsin; Ako et al., 1974) the P_1' residue also might not be necessary. Preliminary experiments (D. Kowalski, unpublished) with des-Ile^{64}-I(63,65) and porcine anhydrotrypsin show that a stable complex can be seen on disc gels (pH 9.4) identical in appearance with that of I and porcine anhydrotrypsin. Furthermore, citraconylation of the des-Ile^{64}-I(63,65) at Arg^{65} destroys the ability of this inactive inhibitor to complex with the inactive enzyme. Thus, with the *active* enzyme, the P_1' residue of the inhibitor is essential for stable complex formation since the state of maximal intimacy of enzyme and inhibitor is the tetrahedral adduct with peptide bond intact. When the side-chain oxygen of Ser^{195} is removed, the "inhibitor" can achieve maximal interactions with the "enzyme" without forming a tetrahedral adduct and therefore the P_1' residue is unessential.

The results presented above show that the replacement of the P_1' residue (Ile^{64}) in soybean trypsin inhibitor (Kunitz) by several other amino acid residues is feasible. Since Ile^{64} was removed chemically from I(63,64) and since the new amino acids were also added chemically to des-Ile^{64}-I(63,65), the methods employed here are somewhat more general than those used in the P_1 replacements (Sealock and Laskowski, 1969; Leary and Laskowski, 1973, 1976). In the P_1 replacements, both removal and replacement were purely enzymatic, requiring that the carboxypeptidases employed be strictly specific. It appears that the facile hydrolysis and resynthesis of the 63–64 bond in STI can be exploited to produce a large variety of semisynthetic derivatives of this protein. At the present stage of technology in protein chemistry, such semisynthetic derivatives are much

easier to prepare, purify, and characterize than the totally synthetic variants.

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Chemical–Enzymatic Insertion of an Amino Acid Residue in the Reactive Site of Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: *Modified* (Arg^{63}-Ile^{64} reactive-site peptide bond hydrolyzed) soybean trypsin inhibitor (Kunitz) with all reactive amino groups, except that of Ile^{64}, protected was described in the preceding paper (Kowalski, D., and Laskowski, M., Jr. (1976), *Biochemistry,* preceding paper in this issue). Treatment of this inhibitor with *tert*-butyloxycarbonyl-Ala- and *tert*-butyloxycarbonyl-Ile-*N*-hydroxysuccinimide esters yields inactive *endo-tert*-butyloxycarbonyl-Ala^{63A}- and *endo-tert*-butyloxycarbonyl-Ile^{63A}-*modified* inhibitors. The *tert*-butyloxycarbonyl groups were removed by treatment of the proteins with trifluoroacetic acid. After renaturation and purification, the resultant *endo*-Ala^{63A}- and *endo*-Ile^{63A}-*modified* inhibitors co-electrophorese with *modified* inhibitor both on disc gels (pH 9.4) and sodium dodecyl sulfate gels (after reduction of disulfide bonds) and show end groups corresponding to the 63A residue. These derivatives fail to form stable complexes with trypsin, extending the previous observation (Kowalski, D., and Laskowski, M., Jr. (1972), *Biochemistry 11,* 3451) that acylation of the P_1' residue in *modified* inhibitors leads to inactivation. However, the incubation of *endo*-Ala^{63A}- and *endo*-Ile^{63A}-*modified* inhibitors with trypsin at pH 6.5 leads to the synthesis of the Arg^{63}-Ala^{63A} and Arg^{63}-Ile^{63A} peptide bonds in 4% yield. This is very close to the yield anticipated from a semiquantitative theory for the value of the equilibrium constant for reactive-site peptide bond. An alternative chemical method of insertion is also described. Controlled treatment of *modified* inhibitor with the *N*-carboxyanhydride of Glu produced inactive *endo*-Glu^{63A}-*modified* inhibitor. Incubation of this inactive derivative with trypsin at pH 6.5 leads to 16% synthesis of the Arg^{63}-Glu^{63A} peptide bond. The higher yield of single chain protein in this case is attributed to the influence of the negative charge of the Glu^{63A} side chain. Thus, the insertion of an amino acid residue between the P_1 and P_1' residues in soybean trypsin inhibitor (Kunitz) converts a trypsin inhibitor into a trypsin substrate.

It was previously shown that the acylation of the α-amino group of the P_1' residue (notation of Schechter and Berger, 1967) in the reactive site of modified (P_1–P_1' bond hydrolyzed) proteinase inhibitors results in the quantitative loss of inhibitory activity (Kowalski and Laskowski, 1972).[1] Closure of the P_1 to P_1' peptide bond was not possible with the acyl groups previously used. In this paper, the substitution of the α-amino group of P_1' by various amino acid residues with free α-amino groups is described. Inhibitors so altered are inactive; however, it is shown here that trypsin can catalyze the synthesis of the peptide bond between P_1 and the *new* P_1' residue, resulting in an insertion "mutant" of the starting inhibitor.

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[1] The statement that activity is quantitatively lost is merely operational, being defined in terms of the methods applied to detect it. It is indeed true that the modified inhibitors acylated at the P_1' α-amino group can form very weak, fast dissociating complexes with the enzyme (Wang and Laskowski, 1972, and this work).