Sealock, R. W., and Laskowski, M., Jr. (1969), *Biochemistry 8,* 3703.
Sealock, R. W., and Laskowski, M., Jr. (1973), *Biochemistry 12,* 3144.
Seidl, D. S., and Liener, I. E. (1971), *Biochim. Biophys. Acta 251,* 831.
Seidl, D. S., and Liener, I. E. (1972), *Biochim. Biophys. Acta 258,* 303.
Stevens, F. C., Wuerz, St., and Krahn, J. (1974), *Bayer Symp. 5,* 344.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M. (1974), *Biochemistry 13,* 4212.
Takahashi, H., Iwanaga, S., Kitagawa, T., and Suzuki, T. (1974), *Bayer Symp. 5,* 265.
Tan, C. G. L., and Stevens, F. C. (1971), *Eur. J. Biochem. 18,* 515.
Tschesche, H., and Wachter, E. (1970), *Eur. J. Biochem. 16,* 187.
Tschesche, H., Wachter, E., Kupfer, S., Obermeier, R., Reidel, G., Haenisch, G., and Schneider, M. (1971), *Proc. Int. Res. Conf. Proteinase Inhibitors, 1st, 1970* p 207.
Wang, D., and Laskowski, M., Jr. (1972), Abstracts, 164th National Meeting of the American Chemical Society, New York, N.Y., BIOL-134.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242.
Wilson, K. A., and Laskowski, M., Sr. (1974), *Bayer Symp. 5,* 286.
Wilson, K. A., and Laskowski, M., Sr. (1975), *J. Biol. Chem. 250,* 4261.

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.

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received August 25, 1975.* This paper was taken from the thesis submitted by David Kowalski in partial fulfillment of the requirements for the Ph.D. degree, Purdue University, 1974. This work was presented in part at Bayer Symposium V on Proteinase Inhibitors in Cologne, Germany, 1973 (Kowalski et al., 1974). This work was supported by Grants GM 10831 and GM 11812 from the Institute of General Medical Sciences, National Institutes of Health.

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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).

These studies were performed on Kunitz soybean trypsin inhibitor (STI)[2] which had all reactive amino groups, except that of the P_1' Ile^{64} in the modified inhibitor, protected (see Figure 1). The use of Boc-(amino acid)-ONSu esters in aqueous solution was previously described (Kowalski and Laskowski, 1976) and was one method employed for substitution of a single amino acid on the α-amino group of Ile^{64}. Another method was via the *N*-carboxyanhydride of Glu. The reaction of proteins with *N*-carboxyanhydrides has been described by others (Fraenkel-Conrat, 1953; Virupaksha and Tarver, 1964; Weinert et al., 1971), but in no case were conditions for the addition of a single amino acid to a specific locus sought.

The results are discussed in terms of x-ray crystallographic evidence on the nature of the stable enzyme–inhibitor complex (Ruhlmann et al., 1973; Janin et al., 1974; Sweet et al., 1974) and in terms of the equilibrium constant for peptide bond hydrolysis in the reactive sites of proteinase inhibitors.

Experimental Procedures

Materials. All proteins used in this study and their sources were previously described (Kowalski and Laskowski, 1976). Boc-(amino acid)-ONSu esters were obtained from Fox Chemical Co. Trifluoroacetic acid and *N*-ethylmorpholine, both Sequanal grade, were products of Pierce Chemical Co. and were stored at 4 °C under N_2. Phosgene was obtained from the Matheson Co. Glu-*N*-carboxyanhydride was prepared by the method of Hirschmann et al. (1971) and stored at −20 °C.

General Methods. The methods of analytical disc gel electrophoresis, assay of inhibitory activity, sodium dodecyl sulfate gel electrophoresis, N-terminal amino acid determination, and the test for reactive amino groups by citraconylation were previously described (Kowalski and Laskowski, 1976). Solutions were prepared with distilled, deionized water. Molecular weights used were 24 000 for trypsin and 20 000 for STI. All experiments were performed at 21 ± 1 °C unless otherwise indicated. The preparation of I(63,64) was previously described (Kowalski and Laskowski, 1976) and a mixture of 85% I(63,64) and 15% I was used throughout this work.

Reaction of I(63,64) with Boc-(amino acid)-ONSu Esters and Purification of the endo-Boc-(amino acid^{63A})-I(63,63A). The I(63,64)–I mixture (85%:15%) at 3 mg/ml (ca. 1.5×10^{-4} M) in 0.2 M *N*-ethylmorpholine acetate[3] was treated with either $1/33$ the volume of 0.5 M Boc-Ile-ONSu in CH_3CN (100-fold molar excess, 2.9% (v/v) CH_3CN) or $1/17$ the volume of 0.25 M Boc-Ala-ONSu in CH_3CN (100-fold molar excess, 5.6% (v/v) CH_3CN). After addition of the reagent, the mixture was agitated on a Vortex mixer and kept at 21 °C for 18 h to allow for the reaction and the hydrolysis of the Boc-(amino acid)-ONSu.

In studies of the reaction rate, samples (30 μg of protein) of the reaction mixture were analyzed for the loss of inhibitory activity on pH 9.4 disc gels (7 cm) which were previously charged with a fourfold molar excess of porcine trypsin. Even though the reaction mixture appeared biphasic, reproducible inactivation curves were obtained.

After making it 0.5 M with respect to KCl and Millipore filtering, the reaction mixture was passed through a trypsin-Sepharose column (7.5 ml packed bed in a 10-ml syringe) equilibrated with 0.05 M sodium borate (pH 8.5)–0.5 M KCl–0.01 M $CaCl_2$ to remove any of the remaining active inhibitor. The inactive material, *endo*-Boc-(amino acid^{63A})-I(63,63A), was then dialyzed vs. 10^{-3} M ammonium acetate (pH 9) and lyophilized.

Deblocking of endo-Boc-(amino acid^{63A})-I(63,63A). The Boc group was removed by treatment of *endo*-Boc-(amino acid^{63A})-I(63,63A) (30 mg/ml) for 30 min with trifluoroacetic acid. The protein was precipitated from the trifluoroacetic acid by the addition of 5 vol of anhydrous ethyl acetate containing a small amount (5–50 μl) of concentrated HCl. After centrifugation (bench top centrifuge) and removal of the supernatant, the precipitate was washed with a smaller volume of ethyl acetate and centrifuged. The supernatant was removed and 10^{-3} M ammonium acetate (pH 9) was added (total protein 5 mg/ml), magnetically stirred for 5 h, and lyophilized. Finally, enough 0.2 M *N*-ethylmorpholine acetate (pH 8.0) was added to achieve a protein concentration of 5 mg/ml and the mixture, after dissolution with stirring, was kept at 21 °C for 6 days.[4]

Reaction of I(63,64) with Glu-N-carboxyanhydride. The procedure used for the reaction of I(63,64) with Glu-*N*-carboxyanhydride (Glu-NCA) was similar to that described by Manning and Moore (1968) for dipeptide synthesis. Sodium borate was not used, however, because at the protein and NCA concentrations used here (10^{-3} M), much lower yields were obtained in this buffer compared to *N*-ethylmorpholine.

The I(63,64)–I mixture (85%:15%) was dissolved in 0.2 M *N*-ethylmorpholine acetate (pH 10.2) to the extent of 20 mg/ml and cooled in an ice bath to approximately 0 °C. While vigorously agitating on a Vortex mixer, enough of freshly prepared 0.2 M Glu-NCA in dioxane was added to give a twofold molar excess over the protein ([protein] ≈ 10^{-3} M, [Glu-NCA] ≈ 2×10^{-3} M). After 1 min, the reaction with the protein was stopped by the addition of 3 vol of cold 0.1 M Tris-HCl (pH 10.2)–0.5 M KCl. It was shown that addition of an excess of a competing nucleophile was equivalent to lowering the pH of the reaction to 5.0, as was done by Manning and Moore (1968). In testing various conditions, the reaction was quenched by addition to the disc gel stacking medium (Sephadex G-200 equilibrated with 0.025 M Tris-HCl–0.005 M glycine (pH 9.4)).

Removal of the unreacted, active inhibitor from the *endo*-Glu^{63A}-I(63,63A) was accomplished by passing the quenched reaction mixture (21 °C) through a trypsin-Sepharose column (25 ml packed bed in a 25-ml syringe) equilibrated with 0.05 M sodium borate (pH 8.5)–0.5 M KCl–0.01 M $CaCl_2$. The isolated *endo*-Glu^{63A}-I(63,63A) was then dialyzed vs. H_2O and lyophilized.

[2] 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 STI; I(63,64), modified I (Arg^{63}-Ile^{64} bond hydrolyzed); AA, an L-amino acid; *endo*-AA^{63A}-I, the product of insertion of an amino acid residue in the reactive site (Arg^{63}-AA^{63A} bond intact); *endo*-AA^{63A}-I(63,63A), the product of the substitution of the α-amino group of Ile^{64} in I(63,64) by an amino acid residue (Arg^{63}-AA^{63A} bond hydrolyzed); Boc, *tert*-butyloxycarbonyl; ONSu, *N*-hydroxysuccinimide ester; NCA, *N*-carboxyanhydride; PTI, bovine pancreatic trypsin inhibitor (Kunitz).

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

[4] Preliminary results show that denaturation in 6 M guanidine-HCl (70 °C, 30 min) and renaturation (Kowalski and Laskowski, 1976) are faster and more efficient than this method.

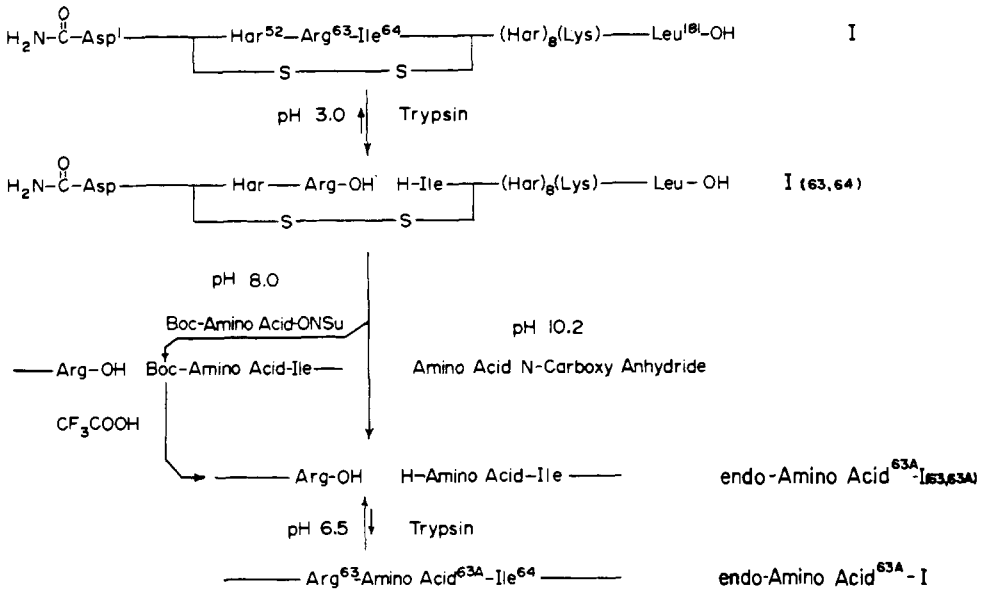

FIGURE 1: Scheme of the reactions used in the insertion of an amino acid residue in the reactive site of I(63,64).[2] Guanidination of 9 of the 10 ϵ-amino groups of STI and carbamoylation of the N-terminal Asp were previously described (Kowalski and Laskowski, 1972). The single ϵ-amino group which is not guanidinated is unreactive toward the activated amino acids shown and is presumably buried in the native protein. The positions of various residues are based on the sequence of STI determined by Koide et al. (1972).

Results

Figure 1 is a scheme of the reactions used in the insertion of an amino acid in the reactive site of I(63,64). The preparation and properties of I(63,64) were previously described (Kowalski and Laskowski, 1972). The amino acid was added to I(63,64) either as a Boc-(amino acid)-*N*-hydroxysuccinimide ester or an (amino acid)-*N*-carboxyanhydride. I(63,64) has but one reactive amino group, that of Ile^{64}. The single ϵ-amino group which is not guanidinated remains unreactive in the native protein toward other reagents.

Reaction of I(63,64) with Boc-(amino acid)-ONSu Esters. The reaction of a mixture of 85% I(63,64) and 15% I with Boc-Ile-ONSu and Boc-Ala-ONSu was monitored by the loss of inhibitory activity of I(63,64). The presence of 15% I in the mixture served as an internal control for inactivation by substitution at sites other than the α-amino group of Ile^{64}. Samples of the reactions were placed on top of pH 9.4 disc gels (7 cm) previously charged with a fourfold molar excess of porcine trypsin and the increase with time of free (inactive) inhibitor relative to the enzyme–inhibitor complex was followed. Over the course of the reaction, I(63,64) was quantitatively inactivated,[1] the I remaining active. Based on previous studies (Kowalski and Laskowski, 1972), the selective inactivation of I(63,64) was attributed to the substitution of the α-amino group of Ile^{64}, resulting in the formation of *endo*-Boc-(amino $acid^{63A}$)-I(63,63A). The time courses of the reactions, normalized for the 85% I(63,64) in the mixture, are shown in Figure 2. With Boc-Ala-ONSu, the reaction was complete in <40 min, while with Boc-Ile-ONSu, the reaction was pseudo first order, $t_{1/2}$ = 36 min. Since the reagents were not completely soluble under the conditions (4% CH_3CN) of the reaction, the difference in reactivity could be attributed to differences in solubility; however, under conditions (10% dioxane) where the Boc-(amino acid)-ONSu esters were soluble, large differences in the reaction rate of the two reagents were also observed suggesting that the Ile side chain might interfere with the reaction (Kowalski and Laskowski, 1976).

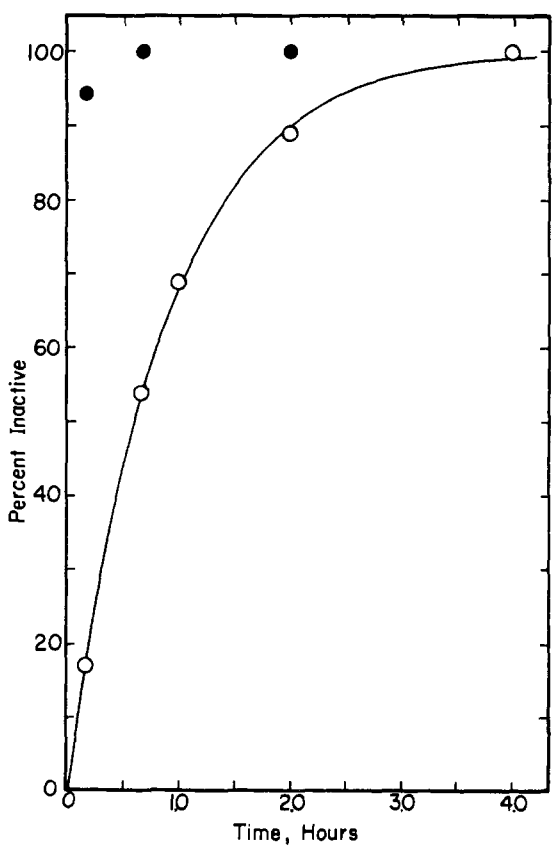

FIGURE 2: Reaction of an I(63,64)–I mixture (85%:15%) with Boc-Ile-ONSu (○) and Boc-Ala-ONSu (●). The data points are normalized for the 85% I(63,64) in the mixture. The curve drawn (—) is first order, $t_{1/2}$ = 36 min. The reaction conditions are: 10^{-4} M I(63,64)–I in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) plus 100-fold molar excess of 0.25 M Boc-(amino acid)-ONSu in acetonitrile.

Removal of I was readily accomplished by passage through a trypsin-Sepharose column at pH 8.5, the pure *endo*-Boc-Ile^{63A}-I(63,63A) and pure *endo*-Boc-Ala^{63A}-I(63,63A) passing through unretarded.

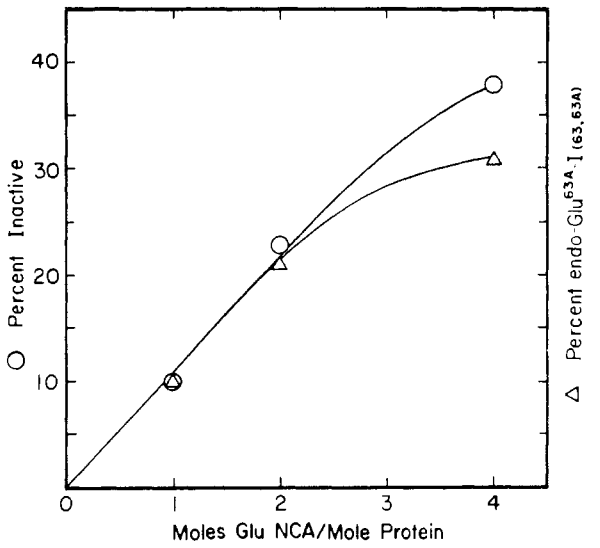

FIGURE 3: Percent inactive inhibitor (O) and the percent *endo*-Glu^{63A}-I(63,63A) (Δ) vs. the molar excess of Glu-NCA over the I(63,64)–I mixture (85%:15%) after 1-min reaction at pH 10.2, 0 °C. The data points are averages of duplicate determinations.

Deblocking of endo-Boc-(amino acid^{63A})-I(63,63A). Pure *endo*-Boc-Ile^{63A}-I(63,63A) did not react with citraconic anhydride (see General Methods section), showing that the α-amino group of Ile^{64} had indeed reacted with Boc-Ile-ONSu. The product, after treatment of *endo*-Boc-Ile^{63A}-I(63,63A) with trifluoroacetic acid for varying amounts of time, was renatured in 0.2 M *N*-ethylmorpholine acetate for 1 day and then citraconylated. After addition of excess porcine trypsin to digest any of the protein which had not refolded, the samples were subjected to pH 9.4 disc gel electrophoresis. As evidenced by the reaction with citraconic anhydride, a new amino group, presumably that of Ile^{63A}, was revealed in <30 min.

In preparations of *endo*-Ile^{63A}-I(63,63A) and *endo*-Ala^{63A}-I(63,63A), the proteins, after 30 min of trifluoroacetic acid treatment and precipitation, were dissolved in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) and renaturation was allowed to proceed over a period of 6 days at 21 °C.[4] After similar trifluoroacetic acid treatment of the I–I(63,64) mixture, the regain of inhibitory activity at pH 8.0 followed a first-order time course, $t_{1/2} \approx 6$ h. The maximum amount of active, native inhibitor recovered by this procedure was 55% of the total.

Purification and Properties of endo-Ile^{63A}-I(63,63A) and endo-Ala^{63A}-I(63,63A). After renaturation, the *endo*-(amino acid^{63A})-I(63,63A) derivatives were chromatographed on Sephadex G-75 to remove the unfolded material. The chromatograms for *endo*-Ala^{63A}-I(63,63A) and *endo*-Ile^{63A}-I(63,63A) are similar to those obtained for trifluoroacetic acid treated phenylthiocarbamoyl-I(63,64) (Kowalski and Laskowski, 1976) and I(63,64). The material contained in the peak eluting in the position of native I(63,64) was not digested in the presence of excess porcine trypsin and was assumed to be refolded *endo*-(amino acid^{63A})-I(63,63A). The yield of refolded *endo*-(amino acid^{63A})-I(63,63A) by this method was 30% of the total Boc-(amino acid^{63A})-I(63,63A).

Besides co-chromatographing with native I(63,64), refolded *endo*-Ala^{63A}-I(63,63A) and refolded *endo*-Ile^{63A}-I(63,63A) co-electrophorese with I(63,64) on pH 9.4 gels. When reduced and subjected to sodium dodecyl sulfate gel electrophoresis, both of the refolded semisynthetic proteins show two fragments (residues 1–63 and 63A–181) identical in molecular weight with the fragments of I(63,64). The refolded *endo*-(amino acid^{63A})-I(63,63A) derivatives both react with citraconic anhydride giving a new species on pH 9.4 disc gels, one charge unit more negative than the starting material. Thus, these derivatives have but one reactive amino group, that of amino acid^{63A}, and the addition of the amino acid to functional groups other than the α-amino group of Ile^{64} was minimal. On the order of 8% of the total refolded *endo*-(amino acid^{63A})-I(63,63A) did not react with citraconic anhydride. This was assumed to be the result of incomplete removal of the Boc group, a problem which has been encountered for other Boc-proteins (Levy and Carpenter, 1967; Robinson et al., 1973).

Dansyl end group analysis of the refolded *endo*-(amino acid^{63A})-I(63,63A) derivatives gave the expected N-terminal (Ala or Ile) as well as ε-dansyl-Lys and an intense *O*-dansyl-Tyr spot (STI has 4 Tyr; Koide et al., 1972). It is significant that, after 6 h of acid hydrolysis at 110 °C, dansylated *endo*-Ile^{63A}-I(63,63A) showed spots of approximately equal intensity corresponding to dansyl-Ile and dansyl-Ile-Ile (for the position of the dansyl-dipeptide on the chromatogram, see Johnson and Smillie, 1971). Thus, a large fraction of the added Ile was shown directly to be linked to Ile^{64} The appearance of ε-dansyl-Lys showed that the one free (not guanidinated; see Figure 1) ε-amino group was unreactive toward the Boc-(amino acid)-ONSu esters. The large amount of *O*-dansyl-Tyr observed leads to a similar conclusion concerning the four Tyr side chains.

Reaction of I(63,64) with Glu-N-carboxyanhydride. The reaction of I(63,64) with Glu-NCA could be monitored directly on pH 9.4 disc gels since, for each Glu added, the protein takes on one additional negative charge. As with the chemical modifications discussed in this work and elsewhere (Kowalski and Laskowski, 1972, 1976), Glu NCA reacted with and inactivated I(63,64) while the virgin inhibitor, I, was unaffected. Under many conditions (pH 7–8, large excess of Glu NCA, or long (>2 min) reaction times), multiple addition of Glu to the Ile^{64} locus, i.e. "overreaction", was observed in agreement with the observations of Hirschmann et al. (1967). Because it must be run under conditions where it is second order ([(amino acid)-NCA] ~ [nucleophile]), the reaction is highly dependent on the absolute concentrations of the reactants (second order, $t_{1/2} \propto$ concentration^{-1}). Thus, at 10^{-3} M protein and NCA, the rate of reaction was expected to be at least 100 times slower than the rate under the conditions (10^{-1} M reactants) of Hirschmann et al. (1967). Furthermore, at 10^{-3} M reactants, the concentration of hydroxyl ions (pH 10.2) is no longer negligible and the side reactions of the NCA discussed by Hirschmann et al. (1967) become even more significant. In Figure 3 are plotted the percent inactive (*at least* one Glu added) and the percent *endo*-Glu^{63A}-I(63,63A) (*only one* Glu added) vs. the molar excess of Glu-NCA over the protein (I–I(63,64) mixture). The percent inactive was assessed on pH 9.4 disc gels (7 cm) with prior addition of excess porcine trypsin and the percent *endo*-Glu^{63A}-I(63,63A) was estimated on the same gels by the relative areas of the peaks on the gel scans corresponding to the mono-Glu and the di-Glu derivatives of I(63,64). The results (Figure 3) at equimolar reactants show that relatively low yields of product were obtained as was anticipated from the considerations of reactant concentrations and side reactions. The conditions chosen were at twofold

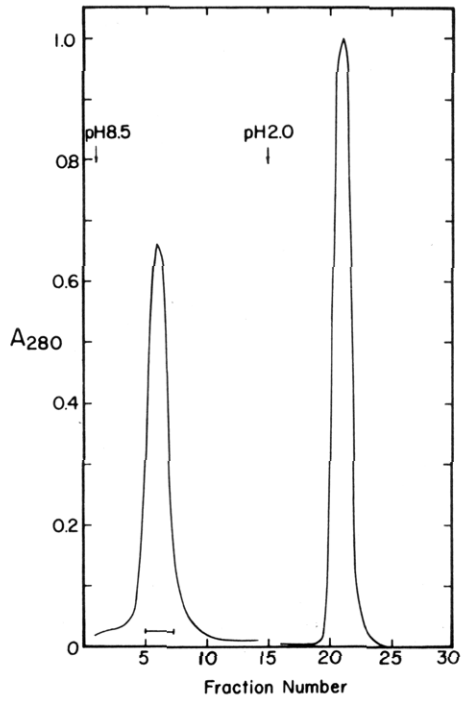

FIGURE 4: Separation of the inactive *endo*-Glu^{63A}-I(63,63A) (first peak) from the active inhibitor (second peak) on a bovine trypsin-Sepharose column (25 ml packed bed in a 25-ml syringe). The fraction volume is 6 ml. Solvents used are: pH 8.5, 0.05 M sodium borate, 0.5 M KCl, and 0.01 M $CaCl_2$, and pH 2.0, 0.05 M glycine.

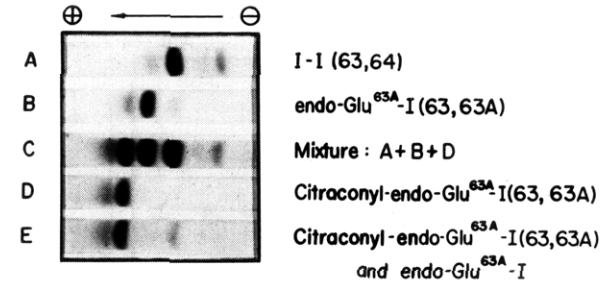

FIGURE 5: Disc gels (pH 9.4) (15 cm): (A) I–I(63,64) mixture (15%: 85%); (B) *endo*-Glu^{63A}-I(63,63A); *endo*-Glu^{63A}-I(63,63A) is one charge unit more negative than I(63,64); (C) mixture of A, B, and D; (D) citraconylated *endo*-Glu^{63A}-I(63,63A); after citraconylation, *endo*-Glu^{63A}-I(63,63A) takes on one additional negative charge; (E) *endo*-Glu^{63A}-I(63,63A) incubated with 5 mol % porcine trypsin at pH 6.5 for 24 h and then citraconylated (see text). The new component (compare D and E) is *endo*-Glu^{63A}-I.

excess Glu-NCA, where the maximal yield of *endo*-Glu^{63A}-I(63,63A) with negligible overreaction was obtained.

Purification and Properties of endo-Glu^{63A}-I(63,63A). After quenching of the reaction of Glu-NCA with 85% I(63,64) and 15% I, the inactive *endo*-Glu^{63A}-I(63,63A) was isolated by passage of the mixture through a trypsin-Sepharose column at pH 8.5 as shown in Figure 4 (first peak). The I and unreacted I(63,64) were then eluted by lowering the pH to 2.0 (Figure 4, second peak). This active material could be recycled after first incubating with trypsin-Sepharose (10 mol %; batchwise with agitation) at pH 3.0 for 1 day which converted the active mixture to largely I(63,64).

In Figure 5, the mobility of I(63,64) on pH 9.4 disc gels (15 cm) is compared with that of purified *endo*-Glu^{63A}-I(63,63A). The *endo*-Glu^{63A}-I(63,63A) (gel B) has one additional negative charge (corresponding to the separation distance between S(63,64) and des-Arg^{63}-S(62,64)) compared to I(63,64) (gel A), due to the addition of a single Glu. After citraconylation, *endo*-Glu^{63A}-I(63,63A) takes on another negative charge (gel D) showing that it contains only one reactive amino group, that of Glu^{63A}. These results, coupled with the fact that I does not react with Glu-NCA, show that a single Glu has been substituted on the α-amino group of Ile^{64}.

Dansyl end group analysis of *endo*-Glu^{63A}-I(63,63A) gave dansyl-Glu, ϵ-dansyl-Lys, and a large amount of *O*-dansyl-Tyr. Hence, as with the Boc-(amino acid)-ONSu procedure, neither the single unguanidinated Lys nor the bulk of the Tyr hydroxyl groups have reacted with Glu-NCA.

Synthesis of the Arg^{63} to Amino Acid^{63A} Peptide Bond: endo-(Amino acid^{63A})-I(63,63A) → endo-(Amino acid^{63A})-I. All of the *endo*-(amino acid^{63A})-I(63,63A) derivatives (Ala, Ile, and Glu) described herein are inactive[1] as trypsin inhibitors. It was found, however, that these derivatives can act as trypsin *substrates* as shown by the ability of trypsin to catalyze the peptide bond formation between Arg^{63} and amino acid^{63A} (described below). As substrates, the *endo*-(amino acid^{63A})-I(63,63A) derivatives form very weak, fast dissociating complexes with trypsin, and these derivatives are termed "inactive" only in an operational sense, i.e. a *stable* (large association equilibrium constant, small dissociation rate constant) complex is not formed.

Incubation of *endo*-(amino acid^{63A})-I(63,63A) derivatives with trypsin at pH 6.5 leads to the formation of a new component which is more positive on pH 9.4 disc gels and does not react with citraconic anhydride (see Figure 5, gel E). The formation of this component at pH 6.5 reaches a steady-state value which is independent of the amount of trypsin (5–20 mol %) used in the incubation. This new component was tentatively identified as *endo*-(amino acid^{63})-I since on pH 9.4 disc gels it is more positive than *endo*-(amino acid^{63A})-I(63,63A) due to the loss of the negatively charged carboxylate of Arg^{63}, and since it has no reactive amino group, as shown by its failure to react with citraconic anhydride.

If peptide bond synthesis had indeed occurred, the result would be the formation of a single polypeptide chain from the disulfide bridged fragments (residues 1–63 and 63A–181). After incubation with 10 mol % bovine α-trypsin at pH 6.5 for 24 h and subsequent inactivation of the trypsin with a tenfold molar excess of *p*-nitrophenyl *p'*-guanidinobenzoate, the *endo*-(amino acid^{63A})-I(63,63A) derivatives were analyzed on sodium dodecyl sulfate gels following reduction of the disulfide bonds. The results, before and after incubation with trypsin, are shown in Figure 6. A new band of molecular weight 20 000 appears (gels B and D) in the trypsin-treated samples, confirming the formation of *endo*-(amino acid^{63A})-I. Furthermore, the relative amounts of *endo*-Glu^{63A}-I and *endo*-Ala^{63A}-I observed on the sodium dodecyl sulfate gels (Figure 6) are qualitatively in agreement with the relative amounts observed on pH 9.4 disc gels (discussed below).

The fraction of *endo*-(amino acid^{63A})-I synthesized was measured on pH 9.4 disc gels (15 cm). The gels were scanned and the peaks integrated as previously described (Kowalski and Laskowski, 1972). The *endo*-(amino acid^{63A})-I(63,63A) derivatives were incubated with 5 mol % porcine trypsin at pH 6.5 in 0.2 M *N*-ethylmorpholine

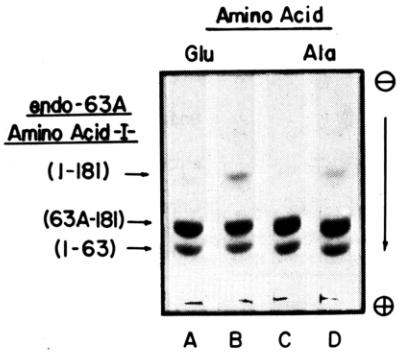

FIGURE 6: Sodium dodecyl sulfate gels before and after incubation of *endo*-(amino acid^{63A})-I(63,63A) with 10 mol % bovine α-trypsin at pH 6.5 for 24 h. The numbers on the left indicate the intact protein (1–181) and the large (63A–181) and small (1–63) fragments: (A) *endo*-Glu^{63A}-I(63,63A); (B) *endo*-Glu^{63A}-I(63,63A) after incubation with trypsin; (C) *endo*-Ala^{63A}-I(63,63A); (D) *endo*-Ala^{63A}-I(63,63A) after incubation with trypsin.

acetate–0.5 M KCl. The pH of 6.5 was chosen since this corresponds to the minimum in the pH-dependent equilibrium constant for hydrolysis (K_{hyd}) of the Arg^{63}-Ile^{64} peptide bond in STI (Mattis and Laskowski, 1973). Prior to the disc gel analysis it was necessary to inactivate the trypsin in the sample using *p*-nitrophenyl *p'*-guanidinobenzoate (tenfold molar excess over trypsin) since the pH in the Sephadex G-200 stacking medium is 9.3; failure to inactivate the trypsin would give a value for the fraction of *endo*-(amino acid^{63A})-I approaching that at pH 9.3 (for example, for STI, at pH 6.5, K_{hyd} = 1.85, while at pH 9.3, $K_{hyd} > 18$; Mattis and Laskowski, 1973). Finally, because approximately 7% of the α-amino group of Asp^1 in I(63,64) is guanidinated (and thus this component is more positive than I(63,64); Kowalski and Laskowski, 1972), samples were raised to pH 8.5 and citraconylated in order to better separate *endo*-(amino acid^{63A})-I, which does not react with citraconic anhydride, from the α-guanyl component of *endo*-(amino acid^{63A})-I(63,64), which does. The results of such an analysis at steady state are shown in Table I and compared with the values of S(63,64) and I(63,64). The value of I(63,64) is shown to agree within experimental error (±0.01) with that of S(63,64). Insertion of an amino acid into the reactive site *decreases* the fraction of protein with peptide bond intact observed at steady state. This is in accord with the theory of the equilibrium constant for peptide bond hydrolysis in reactive sites of proteinase inhibitors (see Discussion). The values for *endo*-Ala^{63A}-I and *endo*-Ile^{63A}-I are in agreement (Table I), while the higher value of *endo*-Glu^{63A}-I is believed to be due to charge effects.

That the peptide bond synthesis observed is reversible was shown by incubation of a mixture of *endo*-(amino acid^{63A})-I and citraconyl-*endo*-(amino acid^{63A})-I(63,63A) (for example, the material in gel E, Figure 5) with trypsin at pH 9.3. This resulted in the formation of a band on disc gels in the position of *endo*-(amino acid^{63A})-I(63,63A) and the disappearance of the band corresponding to *endo*-(amino acid^{63A})-I. Isolation of *endo*-(amino acid^{63A})-I will be necessary for measurement of this steady-state value and for a convincing proof of equilibrium.

Table I: The Fraction of Protein with Peptide Bond Intact at Steady State[a] after Incubation with Trypsin at pH 6.5.

Protein	Fraction Intact
S(63,64)	0.35[b]
I(63,64)	0.36
endo-Glu^{63A}-I(63,63A)	0.16
endo-Ala^{63A}-I(63,63A)	0.04
endo-Ile^{63A}-I(63,63A)	0.04

[a] Values for I(63,64) and S(63,64) are equilibrium values, i.e. obtained from both directions. [b] Mattis and Laskowski (1973).

Discussion

The initial objective of this research was the investigation of reactions for the introduction of a single amino acid at a specific locus in a protein in aqueous solutions. Both the Boc-(amino acid)-ONSu esters (Anderson et al., 1964) and Glu-NCA (with proper control of conditions; Hirschmann et al., 1967) fulfill these requirements. Neither of these reagents appears to show significant side reactions with STI. Both reactions are reasonably gentle toward STI and, while the NCA reaction must be carried out at high pH (pH 10.2), the reaction time is only ca. 1 min and the optimal temperature is low. The advantage of the Boc-(amino acid)-ONSu esters is selectivity, i.e. only one amino acid is introduced at a single reactive locus. With the NCA's, the reaction must be carefully monitored to avoid multiple hits of the amino acid at one site. For the selectivity of the Boc-(amino acid)-ONSu esters, however, one must pay the price of Boc removal in trifluoroacetic acid. The relatively low recovery (ca. 50%) of I(63,64) from trifluoroacetic acid treatment may be due to peculiarities of STI itself. While STI is a stable protein and very slow to denature, it is also very slow to *renature* under the conditions described here. The failure to recover all of the original inhibitor after trifluoroacetic acid treatment is probably due to side reactions occurring during the slow renaturation. According to Previero et al. (1972), the biological activity of a number of enzymes after trifluoroacetic acid treatment is completely restored when they are returned to aqueous solution. Clearly, however, a blocking group for the activated amino acid removable under gentle conditions in aqueous solution would be advantageous.

Given that reactive amino groups not of interest can be protected, both the Boc-(amino acid)-ONSu esters and the (amino acid)-NCA's allow for the introduction of isotopically labeled amino acid residues (e.g. ^{13}C for nuclear magnetic resonance studies) at a preselected locus. Using Boc-[1-^{13}C]-Gly-ONSu, Garner and Gurd (1975) have labeled the NH_2 terminal of myoglobin.

The inactivation of I(63,64) upon acylation of the α-amino group of P_1' with an amino acid residue was expected in agreement with previous work (Kowalski and Laskowski, 1972). That trypsin would catalyze the synthesis between the P_1 Arg^{63} and the extraneous amino acid in *endo*-(amino acid^{63A})-I(63,63A), however, was not all obvious. Thus, the inactivation of I(63,64) upon insertion of an amino acid residue in the reactive site is not due to the failure of the enzyme and inhibitor to interact. Since catalysis of peptide bond synthesis and hydrolysis between P_1 Arg^{63} and the inserted amino acid is possible with trypsin, the intermediates on the pathway of peptide bond synthesis [the hypothetical tetrahedral intermediate with the peptide bond hydrolyzed; acyl intermediate (Fastrez and Fersht, 1973); tetrahedral intermediate with peptide bond intact (Caplow, 1969; Fersht and Requena, 1971)] can form. It is now known that the stable complex of STI–porcine trypsin is a tetrahedral

intermediate with peptide bond intact (Sweet et al., 1974) as was shown previously for PTI–bovine trypsin (Ruhlmann et al., 1973). The instability of the tetrahedral adduct of *endo*-(amino acid^{63A})-I and trypsin must in large part be due to the incorrect conformation of the new leaving group since it is now out of register, as shown:

	P_3	P_2	P_1	P_1'	P_2'	P_3'
I			-Ser-	Tyr-	Arg-	Ile-Arg-Phe-
endo-AA^{63A}-I	-Ser-	Tyr-	Arg-	AA-	Ile-	Arg-

where AA represents an amino acid (Ile, Ala, or Glu in this work). In the stable trypsin complexes of PTI (Ruhlmann et al., 1973) and STI (Janin et al., 1974), the set of torsion angles around the scissile bond (P_1 ψ and P_1' ϕ) is identical. In the *endo*-(amino acid^{63A})-I–trypsin complex, it would be difficult to match this set of angles while preserving other important interactions necessary to stabilize the tetrahedral adduct. Hence, the insertion of a single amino acid in the reactive site of STI abolishes inhibitory activity and converts a trypsin inhibitor into a trypsin *substrate*. The distinction between an inhibitor and a substrate lies primarily in the rate of complex dissociation, inhibitors dissociating extremely slowly.

Thus, in order for a modified (P_1–P_1' bond hydrolyzed) inhibitor to be active, the P_1' residue must have both a free amino group (or possibly another functional group which would allow bond formation to the P_1 carbonyl carbon) and must be in the proper conformation. Alteration of either of these requirements leads to inactivation[1] of the modified inhibitor. For the formation of a strong complex with *inactive* enzymes, e.g. anhydrotrypsin, the restrictions on the P_1' residue appear to be less severe (Kowalski and Laskowski, 1976).

The fraction of *endo*-(amino acid^{63A})-I at steady state formed by incubation of *endo*-(amino acid^{63A})-I(63,63A) with trypsin (see Table I) was predicted using a theory of the equilibrium constant for reactive-site peptide bond hydrolysis, K_{hyd} (Niekamp et al., 1969; Mattis and Laskowski, 1973; Sealock and Laskowski, 1973; Schrode, 1974; Finkenstadt et al., 1974). For proteinase inhibitors, which are generally very rigid molecules, no significant interactions are gained or lost upon hydrolysis of the reactive-site peptide bonds. The value of K_{hyd} depends primarily on the increase in rotational freedom of the reactive-site amino acid residues upon peptide bond hydrolysis. The equilibrium constant increases by one order of magnitude for each amino acid residue which gains complete rotational freedom. This factor is not accurately known and is only approximate. By insertion of one amino acid in the reactive site of I, we would expect that K_{hyd} would increase by a factor of 10; therefore, since $K_{hyd(I)}$ = 1.8 (pH 6.5), $K_{hyd(endo\text{-}AA^{63A}\text{-}I)}$ should be equal to 18 and the fraction of *endo*-(amino acid^{63A})-I = 0.05. This is in agreement with the value for *endo*-(amino acid^{63A})-I of 0.04 observed for the amino acid residues Ala and Ile. The higher fraction of *endo*-Glu^{63A}-I (= 0.16) is assumed to be the result of a charge effect. While the prediction of the fraction of protein with peptide bond intact as steady state is surprisingly good, proof of equilibrium is lacking.

The study of the interaction of such insertion "mutants" of STI with trypsin should prove valuable in obtaining a deeper understanding of peptide bond hydrolysis equilibria in native proteins as well as the detailed mechanism of proteolysis.

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Am. Chem. Soc. 86,* 1839.

Caplow, M. (1969), *J. Am. Chem. Soc. 91,* 3639.

Fastrez, J., and Fersht, A. R. (1973), *Biochemistry 12,* 2025.

Fersht, A. R., and Requena, Y. (1971), *J. Am. Chem. Soc. 93,* 7079.

Finkenstadt, W. R., Hamid, M. A., Mattis, J. A., Schrode, J., Sealock, R. W., Wang, D., and Laskowski, M., Jr. (1974), *Bayer Symp. 5,* 389.

Fraenkel-Conrat, H. (1953), *Biochim. Biophys. Acta 10,* 181.

Garner, W. H., and Gurd, F. R. N. (1975), *Biochem. Biophys. Res. Commun. 63,* 262.

Hirschmann, R., Schwam, H., Strachan, R. G., Schoenewaldt, E. F., Barkemeyer, H., Miller, S. M., Conn, J. B., Garsky, V., Veber, D. F., and Denkewalter, R. G. (1971), *J. Am. Chem. Soc. 93,* 2746.

Hirschmann, R., Strachan, R. G., Schwam, H., Schoenewaldt, E. F., Joshua, H., Barkemeyer, B., Veber, D. F., Paleveda, W. J., Jr., Jacob, T. A., Beesley, T. E., and Denkewalter, R. G. (1967), *J. Am. Chem. Soc. 32,* 3415.

Janin, J., Sweet, R. M., and Blow, D. M. (1974), *Bayer Symp. 5,* 513.

Johnson, P., and Smillie, L. B. (1971), *Can. J. Biochem. 49,* 1083.

Koide, T., Tsunasawa, S., and Ikenaka, T. (1972), *J. Biochem. (Tokyo) 71,* 165.

Kowalski, D., and Laskowski, M., Jr. (1972), *Biochemistry 11,* 3451.

Kowalski, D., and Laskowski, M., Jr. (1976), *Biochemistry,* preceding paper in this issue.

Kowalski, D., Leary, T. R., McKee, R. E., Sealock, R. W., Wang, D., and Laskowski, M., Jr. (1974), *Bayer Symp. 5,* 311.

Laskowski, M., Jr., and Sealock, R. W. (1971), *Enzymes, 3rd Ed.,* 375.

Levy, D., and Carpenter, F. H. (1967), *Biochemistry 6,* 3559.

Manning, J. M., and Moore, S. (1968), *J. Biol. Chem. 243,* 5591.

Mattis, J. A., and Laskowski, M., Jr. (1973), *Biochemistry 12,* 2239.

Niekamp, C. W., Hixson, H. F., Jr., and Laskowski, M., Jr. (1969), *Biochemistry 8,* 16.

Previero, A., Barry, L. G., and Colletti-Previero, M. A. (1972), *Biochim. Biophys. Acta 263,* 7.

Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 420.

Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77,* 417.

Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27,* 157.

Schrode, J. (1974), Ph.D. Thesis, Purdue University.

Sealock, R. W., and Laskowski, M., Jr. (1973), *Biochemistry 12,* 3144.

Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M. (1974), *Biochemistry 13,* 4212.

Virupaksha, T. K., and Tarver, H. (1964), *Biochemistry 3,* 1507.

Wang, D., and Laskowski, M., Jr. (1972), Abstracts, 164th National Meeting of the American Chemical Society, New York, N.Y., BIOL-134.

Weinert, M., Kircher, K., Bradenburg, D., and Zahn, H. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352,* 719.