Enzymatic Replacement of the Arginyl by a Lysyl Residue in the Reactive Site of Soybean Trypsin Inhibitor*

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ABSTRACT: The arginyl residue in the reactive site of soybean trypsin inhibitor was enzymatically replaced by a lysyl residue. The original arginyl residue was removed by treatment of virgin inhibitor (Arg(64)–Ile(65) bond intact) with trypsin to produce modified inhibitor (Arg–Ile bond hydrolyzed) followed by treatment with carboxypeptidase B. Addition of free lysine to inactive des-64-Arg-modified inhibitor was then catalyzed by large concentrations of carboxypeptidase B in the presence of free trypsin. The driving force for this peptide-bond synthesis was supplied by reaction of the trypsin with newly synthesized [64-lysine]-modified inhibitor to form trypsin–inhibitor complex.

Free virgin [64-lysine]-inhibitor was obtained by dissociation of this complex in 6 M guanidine·HCl. This new protein is almost fully active. It is indistinguishable from virgin authentic inhibitor by disc gel electrophoresis. It is converted by catalytic amounts of trypsin into modified [64-lysine]-inhibitor, although considerably more slowly than the authentic inhibitor. Carboxypeptidase B releases about 1 mole/mole of lysine from this new modified inhibitor.

We wish to describe a sequence of enzymatic reactions which lead to the removal of the reactive site arginyl residue in soybean trypsin inhibitor and to its replacement by a lysyl residue. The product, [64-lysine]-soybean trypsin inhibitor is ully active but shows some altered kinetic properties.

Description of the Experiment

The reactive site of soybean trypsin inhibitor (Kunitz)[1] is the Arg(64)–Ile(65) peptide bond (Figure 1). Incubation of the inhibitor with catalytic amounts of trypsin (1–2 mole %) leads to formation of modified inhibitor in which this bond has been hydrolyzed. Intact or virgin inhibitor (designated as S)[2] and modified inhibitor (S*) are in pH-dependent equilibrium (reaction 1, Figure 1). Both forms of the inhibitor are active although the kinetics of their reactions with trypsin are quite different. Arg(64) is easily removed from the modified inhibitor by short treatment with Cpase B (reaction 2, Figure 1); the product, des-64-arginine-modified inhibitor (hereafter, desarginine inhibitor or S_c*), appears to be devoid of inhibitory activity and can be isolated in large quantities. Virgin inhibitor is not affected by carboxypeptidase B treatment (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966; Niekamp *et al.*, 1969).

Although the Cpase B reaction (reaction 2, Figure 1) proceeds essentially to completion, arginine and desarginine inhibitor are necessarily in equilibrium with modified inhibitor. The equilibrium constant for this reaction is not known but it can be roughly estimated from sparse literature data bearing on hydrolysis of COOH-terminal amino acids from peptides with blocked NH_2 terminal. From data listed by Edsall and Wyman (1958) and by Carpenter (1960), we estimate[3] that this equilibrium constant is of the order of 100 M. Thus in the absence of additional driving force the direct addition of arginine to desarginine inhibitor catalyzed by Cpase B should be expected to proceed to only 1% completion in the presence of 1 M arginine. However, an additional driving force can be readily provided, since modified inhibitor, S*, is strongly bound by trypsin at neutral pH, while desarginine inhibitor combines with trypsin weakly, if at all. The equilibrium constants for soybean trypsin inhibitor–trypsin association were measured by Lebowitz and Laskowski (1962). If the resynthesis of the Tyr(63)–Arg(64) peptide bond is conducted in the presence of trypsin the following equilibria should apply:

$$S_c^* + \text{Arg} \rightleftharpoons S^* \qquad K = 10^{-2}\ \text{M}^{-1} \qquad (1)$$

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[1] Dr. T. Ikenaka has kindly sent us extensive partial sequences of soybean trypsin inhibitor (Kunitz). In general his results are in excellent agreement with those of Ozawa and Laskowski (1966). However, he assigns the position of the reactive site to Arg(63)–Ile(64) rather than to Arg(64)–Ile(65). Since his results are still partial we have chosen for the sake of consistency to follow our former and probably erroneous assignment.

[2] Abbreviations used are: STI, soybean trypsin inhibitor in all of its forms; S (or S_A), virgin STI (Arg(64)–Ile(65) bond intact); S_L, virgin [64-lysine]-STI; S* (or S_A*), modified STI (Arg(64)–Ile(65) bond hydrolyzed); S_L*, modified [64-lysine]-STI; S_C*, des-64-arginine-modified STI. (See Figure 1 for further clarification.) The term authentic refers to S_A and S_A* in which the Tyr(63)–Arg(64) bond was never cleaved and to complexes of these inhibitors with trypsin. The term synthetic refers to inhibitors prepared from S_C* by procedures outlined in this paper and to the trypsin–inhibitor complexes which were intermediates in such preparations. Other abbreviations are Cpase B, carboxypeptidase B; TAME, α-*N*-tosyl-L-arginine methyl ester hydrochloride.

[3] This estimate is based on the assumption that Arg(64) does not significantly interact with the remainder of the S_A* molecule and that its removal does not lead to a conformation change. This assumption is supported by the finding that the "melting curves" of S_A* and of S_C* are indistinguishable (C. W. Niekamp and M. Laskowski, unpublished experiments).

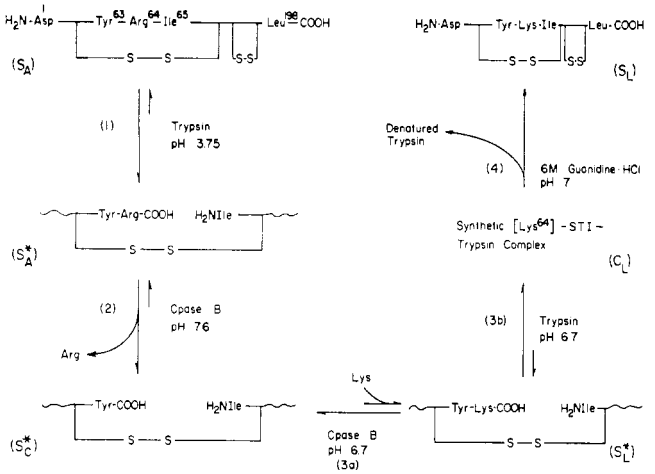

FIGURE 1: The sequence of reactions employed in the "enzymatic mutation" of soybean trypsin inhibitor. For details, see text. The relative lengths of arrows qualitatively indicate equilibrium positions of the individual reactions. The positions of various residues are based on the work of Ozawa and Laskowski (1966) and of other authors cited there, except that the assignment of Tyr preceding reactive site Arg is based on personal communication from Dr. K. Ikenaka.[1]

$$S^* + T \rightleftharpoons C \qquad K = 10^8\ M^{-1} \tag{2}$$

$$S_c^* + Arg + T \rightleftharpoons C \qquad K = 10^6\ M^{-2} \tag{3}$$

where T is trypsin and C is trypsin–trypsin inhibitor complex. By addition of reactions 1 and 2 we obtain reaction 3. It is seen that if the endergonic resynthesis of Tyr(63)–Arg(64) bond is coupled with the highly exergonic trypsin–modified inhibitor complex formation, the over-all reaction is quite favorable. This calculation predicts that at high concentration of free arginine trypsin–inhibitor complex should be synthesized in high yield from desarginine inhibitor and free trypsin.

The product of this reaction is synthetic trypsin–inhibitor complex. It has been shown (Finkenstadt and Laskowski, 1967) that authentic trypsin–inhibitor complex, prepared with *modified* inhibitor, can be dissociated to trypsin and *virgin* inhibitor as follows. At neutral pH a solution of trypsin–modified inhibitor complex contains essentially only complex, although the formal equilibria of eq 4a apply.

$$T + S \rightleftharpoons C \rightleftharpoons T + S^* \text{ (pH 8)} \tag{4a}$$

$$\downarrow$$

$$T + S \xleftarrow{\text{fast}} C \xrightarrow{\text{slow}} T + S^* \text{ (pH 2)} \tag{4b}$$

If this solution is rapidly (<2 sec) titrated to pH 2, the complex immediately dissociates, as one expects from the thermodynamics of the system, but the initial distribution between virgin and modified inhibitor is kinetically controlled (eq 4b, Finkenstadt and Laskowski, 1967). If the inhibitor is quickly isolated by selective ammonium sulfate precipitation, it is found to be >90% virgin. (*At equilibrium* at pH 2 the inhibitor would be ~99% modified.) Synthetic trypsin–inhibitor complex may be similarly treated.

A. Morawiecki has developed a variant of this experiment which is more efficacious although not different in principle. Trypsin–inhibitor complex is dissociated in 6 M guanidine hydrochloride at neutral pH. The products are denatured trypsin and native, *virgin* soybean trypsin inhibitor, which may be readily separated. The ability to obtain *virgin* inhibitor from the synthetic complex completes the reactions required for resynthesis of both the Tyr(63)–Arg(64) and the Arg(64)–Ile(65) bond and thus allows the preparation of virgin inhibitor from desarginine inhibitor.

In the above considerations the only constraints upon the choice of free amino acid are that it satisfy the specificity requirements of trypsin and of Cpase B, and that its addition to desarginine-STI at position 64 restore inhibitory activity. Thus since several trypsin inhibitors have lysyl residues in their reactive sites (Ozawa and Laskowski, 1966; Haynes *et al.*, 1967), lysine may be substituted for arginine in eq 1, as shown in reactions 3a,b and 4 of Figure 1. The complete scheme of reactions leading to the synthesis of [64-lysine]-virgin soybean trypsin inhibitor is shown in Figure 1.

Experimental Section

Virgin soybean trypsin inhibitor (lot B7303 was) obtained from Gallard-Schlesinger Chemical Corp. Chromatographically prepared porcine carboxypeptidase B (EC 3.4.2.2, cat. no. COBC), supplied as concentrated solutions in 0.1 M NaCl, and bovine trypsin (EC 3.4.4.4) (lots TRL 6261 and TRL 71C) were products of Worthington Biochemical Corp. Other materials and their sources of supply were: Tris (primary standard), Fisher Scientific Co.; Sephadex products, Pharmacia Fine Chemicals; *p*-nitrophenyl-*p*-guanidobenzoate·HCl (lot K-5965) and hippuryl-L-arginine, Cyclo Chemical Corp.; Special Grade guanidine·HCl, arginine·HCl, and TAME, Mann Research Laboratories; lysine·HCl, Nutritional Biochemicals.

All pH measurements were made using a Sargent Model DR pH meter with a combination electrode, Model S-30070-10. Protein concentrations were determined spectrophotometrically using the optical factors 0.651 and 1.1 mg ml^{-1} $(ODU)^{-1}$ for trypsin and for all forms of STI, respectively. Solutions were made with distilled deionized water. Trypsin activity was measured both by the method of Chase and Shaw (1967), and by monitoring the rate of tryptic hydrolysis of TAME at pH 7 in a Radiometer pH-Stat equipped with an Ole Dich recorder. Cpase B was assayed according to Wolff *et al.* (1962), and always had specific activity greater than 170 units/mg. Analytical disc gel electrophoresis was performed as previously described (Niekamp *et al.*, 1969). A Spinco Model 120B analyzer was used for all amino acid analyses. Sedimentation velocity runs were performed in a Spinco Model E ultracentrifuge and monitored with schlieren optics. Molecular weights used were 22,000 for STI, 24,000 for trypsin, and 34,000 for Cpase B.

Analysis of Reaction Mixtures. In order to monitor synthesis of complex or to harvest the complex, an aliquot or all of a reaction mixture was titrated with HCl to pH 3.0 to precipitate Cpase B, then clarified by centrifugation and retitrated to pH 7.6. This sample was applied to a 90-cm column of Sephadex G-75 equilibrated and eluted with 0.01 M Tris–0.1 M NaCl (pH 8.5). Fractions were read at 230 or at 280 mμ; complex containing fractions were pooled, dialyzed against deionized water, and lyophilized.

TABLE I: Composition of Reaction Mixtures.[a]

Component	Concn (M)
Des-64-Arg-modified inhibitor	1×10^{-4}
Trypsin	2×10^{-4}
Arg or Lys	0.07
Carboxypeptidase B	1×10^{-4}

[a] Solvent was 0.15 M KCl–0.03 M $CaCl_2$ (pH 6.7).

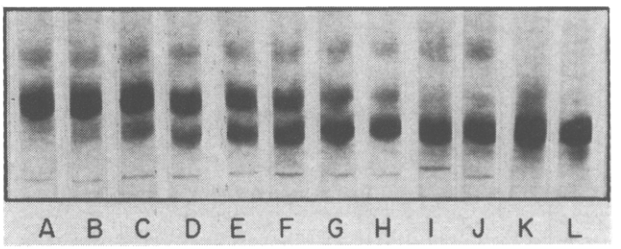

FIGURE 2: Disc gel electrophoresis of aliquots of a desarginine inhibitor preparation. For details, see text. From the top the bands are assigned to virgin, modified, and desarginine inhibitors, respectively. (A) Initial virgin-modified inhibitor equilibrium mixture, prepared at pH 3.75. (B–J) Aliquots removed at 1, 2.5, 4.5, 7.5, 11, 15, 20, 30 and 40 min, respectively, after addition of Cpase B at pH 7.6. (K) Result of second equilibration at pH 3.75. (L) Aliquot removed 2 hr after second addition of Cpase B at pH 7.6. Samples contained ~80 μg of protein.

Results

Preparation of Desarginine Inhibitor. Niekamp *et al.* (1969) noted that the existence of equilibrium between virg n and modified inhibitor slightly complicates the preparation of pure desarginine inhibitor. Therefore, the following procedure was developed.

(1) Equilibrium mixtures of virgin and modified inhibitor are prepared by overnight incubation at room temperature of 150 mg of STI and 3 mg (2 mole %) of trypsin in 150 ml of 0.1 M NaCl–0.05 M $CaCl_2$–0.01 M Tris, adjusted to pH 3.75 with 1 M HCl. The distribution between the two forms can be determined as described by Niekamp *et al.* (1969) (gel A, Figure 2). From densitometer traces of this gel, it was calculated that $K_{hyd} \simeq 10$, in agreement with the value obtained by Niekamp *et al.* (1969).

(2) The pH is raised to 7.6 and 1 wt % Cpase B is added. Because desarginine inhibitor is approximately one charge unit more negative than modified inhibitor its production can easily be followed by disc gel electrophoresis of aliquots removed at various times (gels B–J). The amount of virgin inhibitor remains unchanged. After 40 min the reaction is essentially complete (gel J); the product consists of approximately 90% desarginine inhibitor and 10% unaltered virgin inhibitor.

(3) After 2 hr, the pH is again lowered to 3.75 for another 12-hr incubation to reestablish virgin-modified equilibrium (gel K). The amount of virgin inhibitor remaining, less than 2% of the total protein, is too small to detect on the gels.

(4) Again at pH 7.6, another 1 wt % Cpase B is added. At the end of 2 hr no detectable virgin or modified inhibitor remains (gel L).

(5) The solution is chromatographed on a 5 × 85 cm column of Sephadex G-75 in 0.1 M NaCl–0.1 M Tris (pH 8.5) to remove the Cpase B and the virgin inhibitor as its trypsin complex. The desired fractions are pooled, dialyzed against distilled water, and lyophilized.

Reaction Conditions. Initial experiments were designed to show whether eq 3 in fact represents a feasible chemical reaction and to find conditions suitable for the preparative scale synthesis of trypsin–STI complex according to that reaction. An extensive quantitative study to optimize these conditions was not carried out. Reaction mixtures of 1.2-ml volume contained 2×10^{-4} M desarginine inhibitor, 4×10^{-4} M trypsin, 1×10^{-4} M Cpase B, and varying concentrations of arginine or lysine. The solvent was 0.3 M KCl–0.03 M $CaCl_2$. The pH of the reactions was varied between pH 6 and 8, and was adjusted before addition of Cpase B. Any subsequent changes in pH were neutralized by careful addition of dilute HCl or KOH. The free amino acid concentration was varied between 0.05 and 0.8 M. Reaction temperature was 21 ± 1°. Synthesis of complex was monitored by chromatography of aliquots on 0.5-cm i.d. columns of Sephadex G-75, as described in Experimental Section.

These semiquantitative experiments showed clearly that synthesis of a protein of the desired molecular weight is possible in these reactions with either arginine or lysine as the free added amino acid. The very high concentrations of Cpase B employed, 1×10^{-4} M or higher, are essential. Rate of synthesis of complex is roughly proportional to Cpase B concentration and in control experiments run in the absence of this enzyme there was no evidence of reaction. Contrary to expectation, the rate is depressed at higher concentrations of free amino acid. Fragmentary data suggest that this may be due to inhibition of Cpase B by substrate arginine and lysine, but there is some doubt on this point. Higher values of pH also depress the rate of synthesis; this may be due to increased autolysis of trypsin and tryptic attack on Cpase B.

Finally, control experiments in which the free amino acid was omitted were not negative. Complex was, however, synthesized at a much slower rate and with much greater production of low molecular weight protein fragments than in the presence of added arginine or lysine. Because of the mutual specificities of trypsin and Cpase B, the reaction mixtures are ripe for the production of significant amounts (up to 10^{-3} M or so) of endogenous arginine and lysine from these fragments. If the free-energy change associated with eq 2 is sufficiently large (and $K = 10^8$ is, in fact, a minimum number), synthesis of a mixture of [64-arginine]-STI– and [64-lysine]-STI–trypsin complexes is possible. In the normal situation, however, the lower production of protein fragments and the much higher concentration of exogenous arginine or lysine essentially insure that the desired product predominates. By amino acid analysis we have established a probable upper limit for the extent of contamination of [64-lysine]-STI by synthetic [64-arginine]-STI (see below).

The conditions finally selected for the synthesis of large quantities of complex are shown in Table I. In this way a small amount of [64-arginine]-STI–trypsin complex was prepared. Partial characterization of this complex is described below. Most work, however, was directed toward the preparation of [64-lysine]-STI. There are no essential differences between the two syntheses.

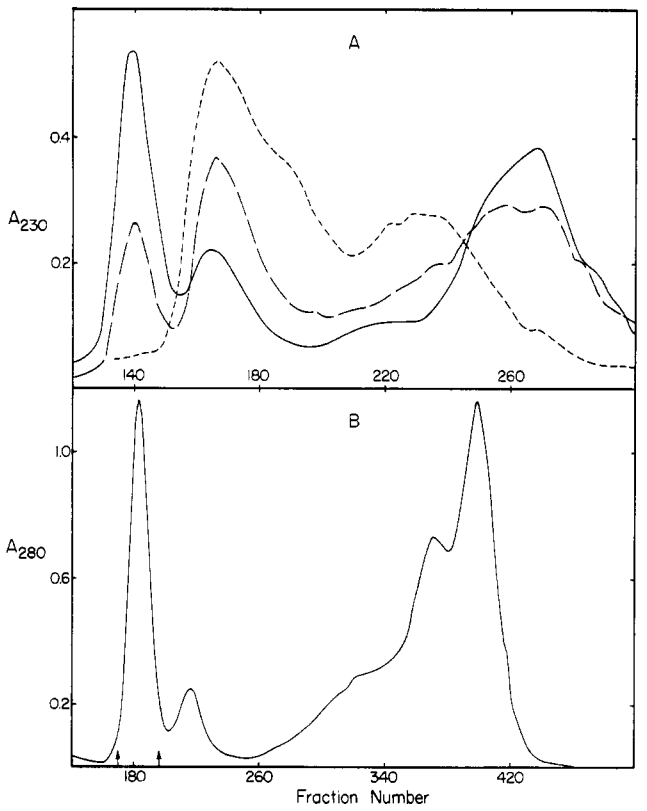

FIGURE 3: Chromatographic analysis and harvest on Sephadex G-75 columns of reaction mixtures prepared according to Table I, with lysine as the free amino acid. See text for details of procedure and for peak assignments. (A) Aliquots (0.5 ml) were removed at 0 hr (----), 15 hr (— — —), and 63 hr (—) for analysis on a 0.5 × 90 cm column. For this experiment the Cpase B concentration was 1.5 times that listed in Table I. Fraction volume was ~0.5 ml. (B) Harvest of synthetic lysine complex on 5 × 90 cm column. The arrows mark pooled complex containing fractions. Fraction volume was ~5 ml.

Time Course of the Synthesis. The progress of a typical reaction mixture, similar to that of Table I, using lysine, was followed by chromatography of aliquots on 2.5-cm i.d. columns of G-75. The results are shown in Figure 3A. Repeated calibrations of these columns confirm that the first, or leftmost peak corresponds to trypsin–inhibitor complex; the second peak, to free trypsin and/or free desarginine inhibitor; and later peaks to mixtures of peptides of molecular weights less than 5000 presumably resulting from autolysis of trypsin and tryptic attack on Cpase B (but not on desarginine inhibitor; see below). Cpase B was removed from each aliquot before analysis. The analysis at 0 hr shows that desarginine inhibitor and trypsin associate only very weakly, if at all. In later analyses the slow synthesis of complex is apparent, as are the concomitant decreases in amounts of trypsin and of desarginine inhibitor and increases in amounts of autolysis products. It should be noted that the activity of Cpase B does decrease significantly during the several days of a reaction. Nonetheless, it is clearly a bit of luck that this enzyme can coexist at all with so much trypsin for such an extended time.

The final composition of a preparative reaction mixture, made up according to Table I, using lysine and 90 mg of desarginine inhibitor, is shown in Figure 3B. Reaction was terminated at 10 days and product was harvested on a 2.5-cm i.d. column of G-75. The fractions of the first peak were pooled, dialyzed, and lyophilized. The white powder (80 mg) was tentatively identified as [64-lysine]-STI–trypsin complex, or synthetic lysine complex.

Characterization of Synthetic Trypsin–Inhibitor Complex. The position of elution on G-75 verified that the product of the synthesis was of approximately correct molecular weight. The two following experiments sufficed to show that this material would undergo a reversible pH-dependent dissociation into equivalent amounts of inhibitor and fully active trypsin.

If into a solution of L-TAME (pH 5) is injected a small amount of trypsin–inhibitor complex, there initially is no tyrptic hydrolysis of the substrate. As the complex slowly dissociates, the rate of hydrolysis increases as a ramp function until all complex is dissociated and the rate of hydrolysis remains constant. The final rate is an index of the quantity of dissociated trypsin, and analysis of the ramp function allows the evaluation of the rate of trypsin–inhibitor complex dissociation (Green, 1953; Duran, 1965; Laskowski and Duran, 1966).

Accordingly, a small volume (<20 μl) of a concentrated solution (pH 5) of authentic arginine complex, synthetic arginine complex or synthetic lysine complex was injected into 7 ml of 0.01 M L-TAME in 0.5 M KCl–0.05 M $CaCl_2$, and the ensuing zero-order hydrolysis was monitored by base uptake in a thermostated (24°) pH-Stat at pH 5. The initial concentrations of complex in the reaction vessel were 1.5×10^{-7} M; control experiments established that under these conditions complete dissociation takes place. Recorder tracings of base uptake are shown in Figure 4. Quite evident in each curve are the first-order production of tryptic activity and the constant rates of hydrolysis at later times. Because these final rates are identical, all three types of complex must yield equal amounts of presumably fully active trypsin. The intersection on the time axis of the extrapolation of the late linear portion of such a curve is equal to the reciprocal of the rate constant for complex dissociation, k_d (Duran, 1965). Thus k_d for synthetic lysine complex is about one-sixth that of authentic arginine complex, while k_d for synthetic arginine complex is only slightly (though consistently) lower. The small difference between k_d's of authentic and synthetic arginine complexes has not been definitively explained.[4]

The pH dependence of complex dissociation was followed in the ultracentrifuge, as previously done by Sheppard and McLaren (1953). Solutions of authentic arginine complex and of synthetic lysine complex were adjusted to the desired pH from above and below the transition pH and the observed sedimentation coefficients were determined (Figure 5). The two complexes are essentially identical, both being fully dissociated below pH 3 and fully associated above pH 5. The dissociation of synthetic lysine complex is reversible, and any possibility that this complex could be a ternary complex of desarginine inhibitor, trypsin, and free lysine is thereby eliminated.

[4] It is probable that the trypsin molecules in synthetic and authentic complex are not the same. Several authors (*e.g.*, Schroeder and Shaw, 1968) have shown that trypsin with some internal peptide bonds cleaved may still be active. The conditions of our synthesis clearly favors formation of such partially autolyzed trypsin molecules, which are likely to be further partially degraded by carboxypeptidase B. Therefore, complete identity of properties of synthetic and authentic complexes should not be anticipated.

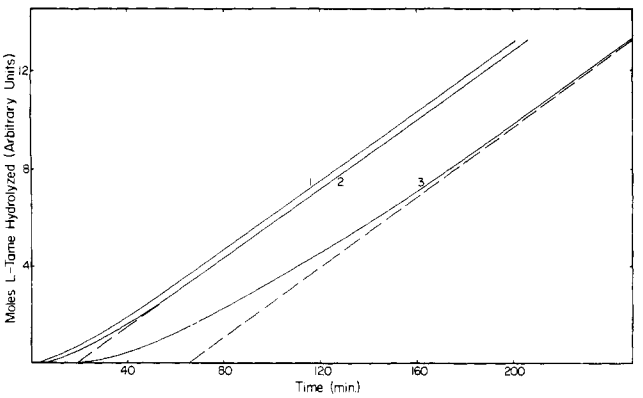

FIGURE 4: Dissociation of trypsin–inhibitor complex as monitored by zero-order tryptic hydrolysis of TAME, at pH 5. See text for details. (1) Authentic trypsin–STI complex. (2) Synthetic trypsin–[Arg^{64}]-STI complex. (3) Synthetic trypsin–[Lys^{64}]-STI complex.

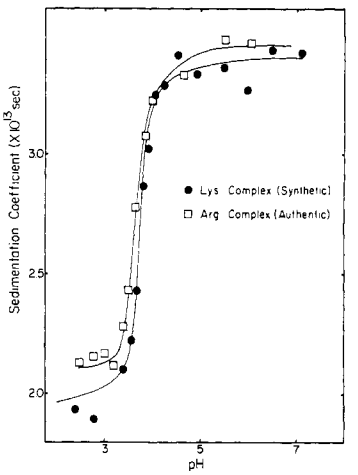

FIGURE 5: Observed sedimentation coefficients (20°, 0.5 M KCl–0.05 M $CaCl_2$) of authentic trypsin–STI complex (□) and synthetic trypsin–[Lys^{64}]-STI complex (●) as a function of pH. Protein concentration in each experiment was 2 mg/ml.

Isolation of [64-lysine]-STI. The above results appeared to demonstrate the chemical reality and synthetic utility of eq 3. To complete the demonstration it was necessary to isolate synthetic lysine inhibitor, preferably in virgin form, and to quantitate the amount of lysine incorporated.

Isolation of virgin inhibitor through dissociation of complex is complicated by the difficulty of separating *active* trypsin and the inhibitor. Because of trypsin–STI association in neutral dilute salt solutions, such a separation must be affected under some unusual dissociating solvent conditions. The most obvious such condition is low pH. However, STI and trypsin have closely similar molecular weights and, at low pH, closely similar net charges. Thus, the wide difference in low pH solubilities of the two proteins was used by Finkenstadt and Laskowski (1967), when they first demonstrated that trypsin–modified inhibitor complex could be dissociated to yield virgin inhibitor (*vide supra*). Their method consistently gives low yields and is unsuitable for preparative work. Nonetheless, some synthetic lysine complex was dissociated in this manner, and a small amount of synthetic lysine inhibitor was isolated. This inhibitor appeared to be identical with that isolated by the more efficacious method described below.

A. Morawiecki, in our laboratory, has found that in 6 M G·HCl at room temperature and neutral pH trypsin–inhibitor complex is fully dissociated and further that trypsin is fully denatured, but STI is only slightly disturbed, if not actually native. The resulting difference in apparent molecular sizes allows easy separation of the two proteins on Sephadex G-100.

Authentic trypsin–modified inhibitor complex (20 mg) was dissolved in 1 ml of 6 M Gu·HCl–0.01 M Tris (pH 7) and chromatographed on a 1.5 × 90 cm column of G-100 equilibrated and eluted with the same solvent. The fractions of the second peak were pooled, dialyzed against distilled water at 3°, and lyophilized. By criterion of disc gel electrophoresis this protein was indistinguishable from authentic virgin STI. The yield was 90%.

Accordingly, 120 mg of *synthetic lysine complex* in 20-mg batches were treated in the same way. A representative elution pattern is shown in Figure 6. The over-all yield was 45 mg of white powder, tentatively identified as virgin [64-lysine]-STI.

Characterization of Virgin [64-lysine]-Inhibitor. In order to establish our claim that this material is, in fact, native virgin [64-lysine]-STI, it is sufficient to show that (1) the material is active as a trypsin inhibitor, (2) it is indistinguishable from authentic virgin inhibitor by disc gel electrophoresis, (3) it is converted by catalytic amounts of trypsin into a form indistinguishable from modified authentic inhibitor, and (4) lysine can be removed from this modified inhibitor by Cpase B and quantitatively analyzed. All four of these requirements have been met.

The inhibitory capability of synthetic lysine-STI was assessed by titration of 1 ml of 5 μM (active) trypsin with aliquots of 50 μM inhibitor, both in 0.5 M KCl–0.05 M $CaCl_2$ (pH 7). The stepwise decrease in tryptic activity was monitored in a pH-Stat at pH 7, using 0.001 M TAME as substrate. The observed activities were corrected for volume changes. The [64-lysine]-STI isolated by Gu·HCl dissociation was 85% active as a trypsin inhibitor, while authentic STI treated similarly was 90% active. Desarginine inhibitor is completely inactive in this assay.

Results of disc gel electrophoresis are shown in Figure 7. In gel A is shown again the distinction between modified and virgin forms of authentic STI. In gel B synthetic lysine inhibitor alone was examined; it appears to consist of only one major component. If the only difference between authentic STI and synthetic lysine-STI is the transformation shown in Figure 1, the two proteins should be electrophoretically indistinguishable at pH values much below the pK of the ϵ-NH_2 of lysine. This expectation is realized in gel C. This finding suggests in particular that desarginine inhibitor suffered no random tryptic cleavages during the synthetic reaction. However, the possibility of removal of neutral amino acids from desarginine inhibitor by Cpase B cannot be discounted on this evidence. Finally, the incubation of the inhibitor (0.1 mM) with 4 mole % trypsin in 0.5 M KCl–0.05 M $CaCl_2$ at pH 3.75 and room temperature leads to slow formation of a second protein which is indistinguishable from modified authentic inhibitor (gel D). On this basis, synthetic lysine inhibitor is considered virgin.

To determine the identity and quantity of the C terminus created during the reaction of lysine-STI with catalytic amounts

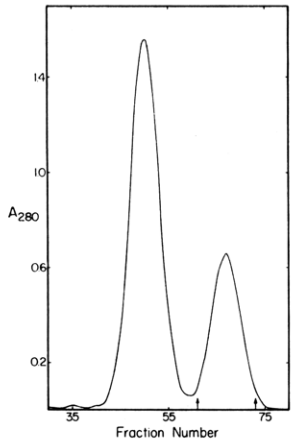

FIGURE 6: Sephadex G-100 chromatography of synthetic lysine complex in 6 M Gu·HCl. Sample (25 mg) was dissolved in 1 ml of 6 M Gu·HCl–0.01 M Tris (pH 7) and eluted from the column (1.5 × 90 cm) with the same solvent. Arrows indicate pooled fractions (S_L). Fraction volume was ~1.2 ml.

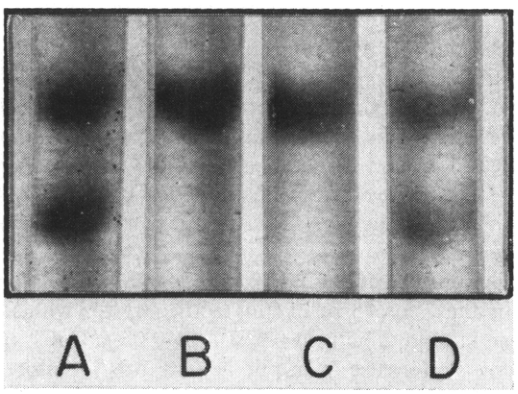

FIGURE 7: Electrophoretic comparison of [64-lysine]-STI and authentic STI. (A) Virgin (top) and modified forms of authentic STI. (B) [64-lysine]-STI. (C) Equimolar mixture of virgin authentic STI and [64-lysine]-STI. (D) Virgin (top) and modified forms of [64-lysine]-STI. Samples contained 20–50 μg of protein.

of trypsin, it is necessary to prepare equilibrium mixtures of virgin and modified inhibitor, to remove the trypsin as complex by Sephadex chromatography, and to quantitate the fraction of modified inhibitor before treatment with Cpase B. Significant amounts of occluded free lysine in untreated inhibitor preparation must be taken into account by a suitable control experiment. For these analyses, 8.6 mg (4 μmoles) of inhibitor was combined with 4 mole % of trypsin in 5.5 ml of 0.1 M KCl–0.02 M $CaCl_2$ (pH 3.75) and allowed to incubate for 1 week at room temperature. Aliquots were removed at various times and subjected to disc gel electrophoresis. Integration of densitometer traces of these gels yielded the relative amounts of virgin and modified inhibitor. The equilibrium position for this peptide-bond cleavage is similar to that of authentic STI, *viz.*, 85% modified, 15% virgin inhibitor.[5]

[5] It was assumed that the system had come to equilibrium when the fraction of S_L* attained a constant value. This was not been proved, however, by showing that the same composition is reached when pure S_L* is employed as a starting material.

TABLE II: Reactive Site COOH Terminus of [64-lysine]-STI in Moles of Amino Acid per Mole of S_L*.

Experiment	Arg	Lys
S_L* → S_c* + Lys	0.25	1.03
S_L* (control)	0.01	0.16
Net	0.24	0.87

However, the rate of attainment of this equilibrium is about 10 times slower than that of authentic STI (Niekamp *et al.*, 1969). When equilibrium was reached (1 week), the sample was chromatographed on Sephadex G-75 in Tris-NaCl buffer to remove the trypsin as complex. Inhibitor-containing fractions were pooled, dialyzed, and lyophilized. This mixture of modified and virgin inhibitor was dissolved in 0.1 M NaCl and divided into two portions, one of which was treated with 1 wt % Cpase B at pH 7.6 for 2 hr; the other was similarly treated at pH 3.0, where Cpase B is inactive. Both portions were analyzed for arginine and lysine; the results, corrected to the amount of modified inhibitor, are presented in Table II. This appears to be substantial evidence that the result of the complete cycle of reactions is indeed the transformation shown in Figure 1. The source of the arginine is not known. It may result from synthesis of [64-arginine]-STI–trypsin complex made possible by the *in situ* production of free arginine as discussed above. From the data of Table II we can estimate an upper limit of 20% for the contamination of [64-lysine]-STI by synthetic [64-arginine]-STI.

Discussion

A primary objective of this research was to demonstrate that eq 3 indeed represents a feasible chemical reaction and to use that reaction in a synthetically meaningful way. The high concentration of enzymes and the long incubation of the synthetic reaction made highly probable such side reactions as extra tryptic cleavages and the removal of additional amino acids from desarginine-modified inhibitor. However, the electrophoretic identity of S_L and S_A clearly demonstrates the absence of additional internal cleavages (see Results and Niekamp *et al.*, 1969) and shows that no extra charged amino acids were removed or added by Cpase B. Therefore, we conclude that eq 3 and Figure 1 correctly represent the series of reactions carried out in this paper. It is most pleasing to show that detailed thermodynamic reasoning can be applied even to a system of such great complexity.

It appears that naturally occurring trypsin inhibitors can be divided into two classes: arginyl inhibitors with an Arg–X peptide bond in the reactive site, and lysyl inhibitors with a Lys–X bond in the reactive site. This hypothesis was suggested by Ozawa and Laskowski (1966), and extensively confirmed by elegant experiments on chemical substitution of lysyl and arginyl side chains by Feeney and collaborators (Haynes *et al.*, 1967; Liu *et al.*, 1968). It is of interest to know to what extent the kinetics and thermodynamics of association between trypsin and a particular inhibitor depend upon that inhibitor's reactive site amino acid. Analogy can be drawn to the chemical modification of subtilisin to form thiolsubtilisin (Polgar

and Bender, 1966, 1967, 1969; Neet and Koshland, 1966; Neet *et al.*, 1968) where the latter has litte remaining activity. Thus, it is likely that in cysteinyl and seryl proteases the active site amino acid and the remainder of the protein sequence must be matched to produce an efficient enzyme. Such an argument does not apply to STI, since [64-lysine]-STI has activity comparable to that of [64-arginine]-STI (Figure 5). Thus, with respect to the thermodynamics of trypsin–inhibitor association, it appears that the remainder of the protein is tolerant of either possible reactive site amino acid.

The finding that virgin [64-lysine]-inhibitor is converted into modified inhibitor about ten times more slowly than the natural virgin inhibitor is a distinct surprise since the rates of hydrolysis by trypsin of α-N-substituted arginin- and lysinamides are closely comparable except at very high pH, where the lysyl side chain loses a proton (*e.g.*, Wang and Carpenter, 1968). The slow hydrolysis of S_L may shed some light on the apparent difficulty of obtaining modified forms of lysyl inhibitors compared with the relative ease of obtaining such modified forms of arginyl inhibitors. Clearly defined modified forms of soybean trypsin inhibitor (Kunitz) (Arg–Ile at the reactive site), of chicken ovomucoid (Arg–Ala) (Ozawa and Laskowski, 1966), and of bovine pancreatic secretory inhibitor (Arg–Ile) (Rigbi and Greene, 1968) were readily obtained. It is quite probable that the cleavages observed by Hochstrasser *et al.* (1967) and Hochstrasser and Werle (1969) in trypsin inhibitors from corn (Arg–Leu), wheat (Arg–Ala), and rye (Arg–Ala) are at the reactive sites. Progress was much slower in the lysyl inhibitor family. The cleavage of the reactive site bond (Lys–Ala) (Chauvet and Acher, 1967; Kress and Laskowski, Sr., 1968) in bovine basic pancreatic trypsin inhibitor (Kunitz) was achieved with great difficulty and only after reduction of the Cys(14)–Cys(38) disulfide bridge (Kress and Laskowski, Sr., 1968). Feinstein *et al.* (1966), failed to prepare modified forms of turkey and of cassowary ovomucoids, which are known to be lysyl inhibitors (Haynes *et al.*, 1967), by methods which led to success in the case of chicken ovomucoid, an arginyl inhibitor (Ozawa and Laskowski, Jr., 1966; Haynes *et al.*, 1967; Liu *et al.*, 1968). However, there is an indication that a modified form of porcine pancreatic secretory inhibitor (Lys–X reactive site) was obtained by Tschesche (1967).

Enzymatic replacements are operations on the peptide backbone, *i.e.*, unlike in chemical replacements the entire amino acid—not just the side chain or part of a side chain—is replaced. Quite apart from the intrinsic interest of such modifications they may allow for introduction at *specific sites* of isotopically labeled amino acid residues. The label thus introduced could serve as a chemical marker (*e.g.*, ^{14}C or ^{3}H) or as an environmental probe, especially for nuclear magnetic resonance studies (*e.g.*, ^{13}C or ^{15}N).

Since enzymatic replacements open up some new possibilities in protein chemistry, it is of interest to speculate on possible generality of this phenomenon. In the present paper we are describing a method of placing only two amino acid residues (Arg or Lys) in only one position (64) of one protein (soybean trypsin inhibitor of Kunitz). However, the extension to other trypsin inhibitors whose modified and des (reactive site arginyl) derivatives have been prepared seems obvious. The extension to other specific positions near the reactive site of soybean trypsin inhibitor (and by inference in other inhibitors) is also intellectually straightforward, but it requires the ability to carry out some very specific limited proteolyses. For example, if one could prepare pure des-[65-isoleucine]-modified STI and if it were inactive, a variation of the present scheme of reactions would allow insertion of any amino acid which would restore activity. On the other hand, if it were active, one could attempt to close the Arg(64)–X(66) peptide-bond *via* formation of complex with trypsin, followed by dissociation at low pH or in Gu·HCl. To our knowledge, the aminopeptidases of the necessary specificities are not available.

The above discussion makes it clear that in order to carry out enzymatic mutations by methods similar to those used here, on any protein system, two conditions must be met: (a) removal of one specific amino acid sufficiently alters the thermodynamic properties of the protein (*e.g.*, stability or ability to bind ligands) that a sufficient driving force is available to replace it; and (b) proteolytic enzymes which are capable of removing this one amino acid, and only this one, are found. In our opinion, condition a is likely to be frequently encountered. It is condition b that severely limits the generality of the present approach.

A comment on appropriate nomenclature is in order. Proteins in which one or more of the amino acid residues at specific positions are replaced by other amino acid residues, which are coded for by the genetic code, are called mutant proteins *provided* that the alteration occurs by means of a change in the genetic material. The virgin [64-lysine]-STI reported in this paper is presumably indistinguishable from a mutant which might arise if the Arg(64) codon were transformed into a Lys(64) codon. A similar argument can be put forth for thiosubtilisin. Indeed Neet and Koshland (1966) have called the subtilisin → thiosubtilisin conversion a "chemical mutation" but this term does not appear to enjoy wide popularity among protein chemists. We feel, however, that if two materials are indistinguishable they deserve the same name no matter what processes have produced them. Thus, we feel that thiosubtilisin should be called a mutant of subtilisin and although this is somewhat more debatable the process of obtaining it may be called a "chemical mutation." In the same spirit [64-lysine]-STI is a mutant of STI and was produced by an "enzymatic mutation."

Finally, since the mutation described here utilized only common enzymes and no unusual organic reagents, it is hard to avoid speculation on the possible occurrence and biological importance of such a phenomenon. It should be noted that considerable *coding* was shown by this system composed of pure proteins (and *no nucleic acids*). The system is specific for lysine and for arginine and of the two available positions for carboxypeptidase B action—the new COOH terminal (tyrosine (63)) of desArg(64) inhibitor and the original COOH terminal (leucine (198)); only one is significantly utilized, because addition of Arg or Lys at only this position restores activity. This choice of available positions is not a result of enzyme specificity but rather of thermodynamic *complimentarity* of the inhibitor and trypsin molecules required to make a complex. Thus, at least in these special circumstances, proteins can act both as codes and as enzymes. This demonstration may be of some interest to those engaged in speculation about the sequence of events involved in the origin of life.

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