Inactivation of Enzymatically Modified Trypsin Inhibitors upon Chemical Modification of the α-Amino Group in the Reactive Site†

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ABSTRACT: The reactive amino groups of soybean trypsin inhibitor (STI) were blocked by guanidination of the ϵ-amino groups and carbamylation of the single α-amino group. This blocked inhibitor retained full activity and when incubated with trypsin at pH 3.0 gave a mixture of 84 ± 2% modified STI (Arg^{63}–Ile^{64} peptide bond hydrolyzed) and 16 ± 2% virgin STI (Arg^{63}–Ile^{64} peptide bond intact). Chemical modification of the newly exposed α-amino group of Ile^{64} at the reactive site of modified STI could then be unambiguously carried out. Citraconylation, amidination, and carbamylation (yielding substituents containing a negative, positive, and no charge respectively) of the free α-amino group in modified STI leads to quantitative loss of activity, the virgin inhibitor remaining fully active. The substitution of the single α-amino group could be monitored by high pH gel electrophoresis since the modified STI acquires an additional negative charge upon citraconylation and loses a unit negative charge on amidination. Virgin STI remains unaffected. Selective inactivation of modified STI can be seen on disc gels by addition of excess trypsin to the chemically modified inhibitor mixture at high pH prior to electrophoresis. Decitraconylation of the citraconylated modified STI leads to regain of activity. A general assay was developed whereby loss of inhibitory activity upon citraconylation or carbamylation could be monitored. For virgin-modified mixtures of STI (α- and ϵ-amino groups not blocked), chicken ovomucoid, and bovine secretory inhibitor (Kazal), citraconylation or carbamylation resulted in loss of activity corresponding quantitatively to the fraction of modified inhibitor. This technique is limited to inhibitors whose virgin form is not inactivated by citraconylation or carbamylation. These results suggest that any blockage of the reactive-site α-amino group in modified protein proteinase inhibitors will lead to loss of inhibitory activity. Methods described here can be utilized in searches for inhibitor reactive sites as well as in measurements of the fraction of virgin and modified inhibitor in their mixtures.

A generalized reactive site of protein proteinase inhibitors can be depicted employing the notation of Schechter and Berger (1967) as shown

$$H_2N—P_4P_3P_2P_1—P_1'P_2'P_3'P_4'—COOH$$

where the reactive-site peptide bond (P_1—P_1') is enclosed in a disulfide loop. This localized site on the inhibitor molecule comes in intimate contact with the active site of the enzyme in the enzyme–inhibitor complex. The result of this contact has been shown to be limited proteolysis of the reactive-site peptide bond and equilibration of the virgin (peptide bond intact) with the modified (peptide bond hydrolyzed) inhibitor (Finkenstadt and Laskowski, 1965).

It has been shown that removal of the P_1 residue (Arg or Lys in trypsin inhibitors; Leu in the few chymotrypsin inhibitors thus far studied) in the modified inhibitor by the appropriate carboxypeptidase results in total loss of inhibitory activity. Furthermore, chemical modifications which destroy the positive charge of the Lys or Arg side chain of P_1 have also been shown to render the inhibitor inactive (for a review, see Laskowski and Sealock, 1971).

The P_1' residues have so far been found to be Ile, Ala, Ser, or Leu. Chemical substitution of the α-amino group of P_1' in an enzymatically modified inhibitor was first carried out with trinitrobenzenesulfonic acid by Haynes and Feeney (1968), who concluded that the α-amino group is sometimes essential to activity. Later, Fritz *et al.* (1969), in studies with maleic anhydride, concluded that a free α-amino group was never essential.

In order to resolve this confusion, we felt an unambiguous study of the chemical substitution of the α-amino group of P_1' was necessary. As a result of blockage of the α- and ϵ-amino groups in virgin soybean trypsin inhibitor (S)[1] prior to trypsin modification, chemical substitution was limited solely to the α-amino group of Ile^{64} in S*. Blockage of this functional group with substituents of negative charge (citraconyl or maleyl), no charge (carbamyl), or positive charge (acetimido) led to quantitative loss of activity (see Discussion). Having demonstrated the requirement for a free α-amino group at P_1' in S*, the citraconylation and carbamylation reactions were extended to virgin-modified mixtures of chicken ovomucoid and bovine secretory inhibitor (Kazal) with the same result. In both cases, the virgin forms were not inactivated. The observations reported herein formed the basis for our earlier statement that blockage of the reactive site amino terminal in any modified inhibitor will lead to loss of inhibitory activity (Laskowski and Sealock, 1971; Laskowski *et al.*, 1971). Since then, Fritz *et al.* have come to agreement with our position (Fink *et al.*, 1971).

Experimental Section

Materials. Virgin soybean trypsin inhibitor (C2905) was obtained from Gallard-Schlesinger Chemical Corp., and

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[1] Abbreviations used are: STI, soybean trypsin inhibitor in all of its forms; S, virgin STI (Arg^{63}–Ile^{64} bond intact); S*, modified STI (Arg^{63}–Ile^{64} bond hydrolyzed); S_G, guanidinated S (see Figure 1 for further clarification); cS_G, carbamylated S_G; cS_G*, modified cS_G; Substitutions on the α-amino group of Ile^{64} are written as prefixes to cS_G* (*i.e.*, citraconyl-cS_G*, carbamyl-cS_G*, and acetimido-cS_G*). Other abbreviations are: GdnBzONph, *p*-nitrophenyl *p'*-guanidobenzoate; TNBS, trinitrobenzenesulfonic acid.

further purified by passage through a Sephadex G-75 column (4.7 × 100 cm) equilibrated with 0.01 N Tris–0.1 N NaCl[2] (pH 8.4) to remove what is thought to be STI dimer (D. Kowalski, unpublished data). Bovine trypsin (EC 3.4.4.4) was obtained from Worthington Biochemical Corp. (TRL OFA), and porcine trypsin from NOVO Industries (S-01-3). Virgin chicken ovomucoid (OI 7GA) was purchased from Worthington Biochemical Corp. and further purified by Mr. James Schrode of this laboratory. Bovine secretory inhibitor (Kazal) was a kind gift of Dr. S. Schneider, Roswell Park Memorial Institute, Buffalo, N. Y. Insoluble trypsin was a generous gift of Dr. H. Fritz, Munich University, Germany.

Materials for disc gel electrophoresis were identical with those previously described (Niekamp *et al.*, 1969). *N*-Ethylmorpholine was obtained from Aldrich Chemical Co., Inc., and redistilled from ninhydrin. Technical grade dioxane was redistilled. Methyl acetimidate·HCl was synthesized by the method of McElvain and Nelson (1942), stored *in vacuo*, and showed a melting point of 92–93° (decomposes with gas evolution). Other materials and their sources of supply were *p*-nitrophenyl *p'*-guanidobenzoate·HCl (K-5965), Cyclo Chemical Corp.; *O*-methylisourea·H_2SO_4, Aldrich Chemical Co., Inc.; potassium cyanate, J. T. Baker Chemical Co.; citraconic anhydride, Eastman Organic Chemicals; maleic anhydride, Matheson Coleman & Bell; Sephadex products, Pharmacia Fine Chemicals; sodium barbital, Fisher Scientific Co.

General Methods. Measurements of pH were performed using either an Orion Model 701 digital pH meter coupled to a Fisher microprobe combination electrode or a Radiometer (Model TTT 1A) in conjunction with a Radiometer combination electrode (GK 2302 C). Solutions were made with distilled, deionized water. Trypsin activity was determined by the method of Chase and Shaw (1967). Amino acid analyses were performed using a Spinco Model 120B analyzer coupled to an Infotronics digital readout system (Model CRS-12AB). A single 22-hr hydrolysis with 6 N HCl at 110° was employed. Reduction, carboxymethylation, and separation of the resulting STI fragments was carried out as previously described by Ozawa and Laskowski (1966). When determined spectrophotometrically, concentrations were obtained using the optical factor 1.1 mg ml^{-1} $(ODU)^{-1}$ for STI. Molecular weights used were 20,000 for STI and 24,000 for trypsin. All experiments were performed in an air-conditioned room at 21 ± 1°.

Analytical Gels. Analytical disc gel electrophoresis was performed as described previously (Niekamp *et al.*, 1969) with several alterations. The pH of the unpolymerized gel solution was adjusted to 9.4 to lower the mobility of STI with respect to the tracking dye, allowing for separation of species several unit charges more negative than virgin STI. An upper buffer consisting of 0.025 M Tris titrated to pH 9.3 with glycine gave better stacking than the pH 8.4 Tris–glycine buffer previously employed (R. W. Sealock, unpublished data). Approximately 30–35 μg of STI was used per gel. Stained gels were scanned at 625 nm using a Gilford spectrophotometer (Model 240) equipped with a Gilford linear-transport and the absorbance–distance profile recorded by a Sargent SR recorder. The resultant curves were resolved and integrated using a Dupont 310 curve resolver.

Guanidination of STI. Reaction of STI with *O*-methylisourea was performed essentially by the method of Hughes *et al.* (1949). To a 0.6 M solution of *O*-methylisourea hydrogen sulfate, solid $Ba(OH)_2$ was added to render the solution basic to Thymol Blue. The precipitated $BaSO_4$ was centrifuged and a volume of the supernatant was added to the solid STI to achieve a protein concentration of 5 mg/ml, resulting in a mole ratio of *O*-methylisourea to lysyl residues of about 200 to 1. Reaction was allowed to proceed for 72 hr at 0°. The solution was then dialyzed against 0.05 M acetic acid, then H_2O, and finally lyophilized.

Reaction of Inhibitors with Cyanate. Carbamylation of inhibitors was performed as described by Stark (1965, 1967). A 0.2 M solution of KNCO in 0.2 M *N*-ethylmorpholine acetate (pH 7.0) was added to the solid inhibitor resulting in an inhibitor concentration of about 2 mg/ml. The reaction was allowed to proceed at room temperature (21 ± 1°) and monitored in several ways as described in Results. When used in a preparative manner, *i.e.*, for carbamylation of guanidinated STI, the reagent was removed by dialysis against H_2O and the solution was lyophilized.

Maleylation and Citraconylation of Inhibitors. Both maleic anhydride (Butler *et al.*, 1969) and citraconic anhydride (Dixon and Perham, 1968) were routinely used as 1.0 M solutions in dioxane. Enough of the 1.0 M anhydride solution to produce an approximately 100-fold excess per amino group was added to the inhibitor (1–5 mg/ml) in a nonnucleophilic buffer (*e.g.*, 0.2 M *N*-ethylmorpholine acetate (pH 8.5) or 0.2 M sodium borate (pH 9.0)). Reactions were run at room temperature for about 10 min.

Amidination of STI. A solution of 0.4 M methyl acetimidate was prepared by the addition of enough 2 M NaOH to neutralize the hydrogen chloride followed by 0.3 M sodium borate (pH 9.0) to bring the solution up to volume. This solution at room temperature was added to the solid protein to achieve a concentration of about 2 mg/ml. Over the course of about 1 hr the pH rose from 9.0 to a final value of 9.4.

Preparation of Modified Inhibitors. STI was converted to an equilibrium mixture of virgin and modified inhibitor and the mixture separated from the trypsin–inhibitor complex as described by Ozawa and Laskowski (1966). cS_G was modified with trypsin as described above except that the trypsin incubation was carried out at pH 3.0 due to the insolubility of cS_G at pH 3.75 (see Results).

A pH 3.0 equilibrium mixture of virgin and modified chicken ovomucoid was prepared and isolated by Mr. James Schrode of this laboratory. This preparation contained 94% modified inhibitor.

Bovine secretory inhibitor was incubated in 0.05 M $CaCl_2$ (pH 3.0) with 2% porcine trypsin for 7 hr at room temperature. The entire solution was then kept frozen until needed for the chemical modification experiments.

Inhibitor Assay. GdnBzONph. The activity of inhibitors was assayed using a modified method of Chase and Shaw (1967) to measure the concentration of free trypsin at pH 8.3 in solutions to which inhibitor had been added (Laskowski and Sealock, 1971). The *p*-nitrophenolate bursts were monitored by a Cary 14 spectrophotometer at 410 nm and extrapolated back to the instant of *p*-nitrophenyl *p'*-guanidobenzoate addition. Keeping both the stock bovine trypsin solution and the 0.01 M GdnBzONph on ice throughout the measurements greatly increased the precision of the assay.

The loss of activity upon chemical modification of inhibitors was followed using the above method at one point on the titration curve. Thus a constant amount of both trypsin and inhibitor were used such that the inhibitor did not exceed 50% of the trypsin, thus assuring the stoichiometry of the

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

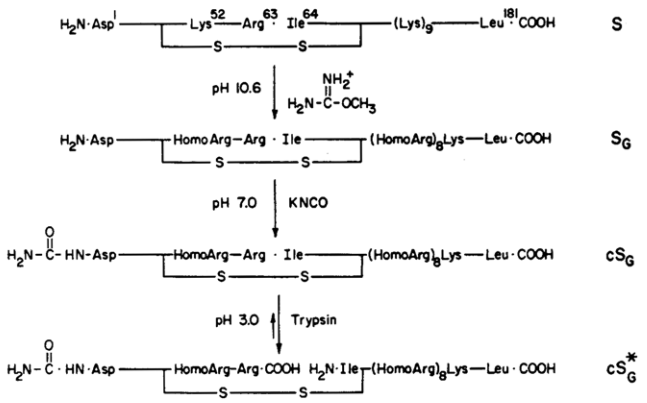


FIGURE 1: Scheme showing the sequence of reactions employed in blockage of the reactive amino groups of STI and subsequent tryptic modification. The positions of various residues are based on the recently determined sequence of STI (Koide *et al.*, 1972). Note that some of these positions differ somewhat from those previously reported by us and by other workers.

inhibition. The inhibitor stock solution was divided into two equal volumes, one treated with a solution of the chemical modification reagent and the other with an equivalent volume of solvent[3] (0.2 M *N*-ethylmorpholine acetate–0.2 M KCl for carbamylation; dioxane for citraconylation). From the three bursts (trypsin, trypsin plus inhibitor, and trypsin plus chemically modified inhibitor) the fraction inactive inhibitor is simply

$$f_{\text{inactive}} = \frac{(\text{T} + \text{chemically modified I}) - (\text{T} + \text{I})}{\frac{V_{\text{T}}}{V_{\text{T+I}}}(\text{T}) - (\text{T} + \text{I})}$$

where (T + chemically modified I), (T + I), and (T) are the values of the bursts due to trypsin plus chemically modified inhibitor, trypsin plus inhibitor, and trypsin alone, respectively, V_{T} is the volume of solution in the cuvet containing trypsin, and $V_{\text{T+I}}$ is the volume of solution in the cuvet containing trypsin plus inhibitor. The dilution correction for the burst due to trypsin alone is obviated by the addition of a volume of the inhibitor solvent (stock solvent plus modification reagent solvent) equivalent to the volume of the inhibitor solution used in the titration. This is necessary when addition of the solvent either changes the pH in the cuvet or affects the trypsin activity.

DISC GEL. Since both the trypsin–STI complex and free STI migrate in the pH 9.4 gel system, inhibitory activity was easily assessed by the addition of excess (3- to 5-fold) porcine trypsin dissolved in 0.01 M $CaCl_2$ (pH 3.0) to the Sephadex G-200 (equilibrated with pH 9.3 Tris–glycine buffer) atop the gel just prior to the addition of the inhibitor sample. The fraction of active inhibitor then corresponds to the fraction of inhibitor found as complex. Since resolution of the various STI components was of no importance, 7-cm gels were employed. The stained gels were scanned and the peaks integrated as described under Analytical Gels.

Results

Chemical Modification of the Reactive Amino Groups in Virgin STI. In order to assess without ambiguity the results of chemical modification of the α-amino group of Ile^{64} in modified STI (S*), the ϵ-amino groups of virgin STI (S) were blocked by guanidination and the α-amino group blocked by carbamylation. This material was then subjected to low-pH trypsin incubation, producing a mixture of blocked S and S*. The sequence of reactions is shown in Figure 1 and the details are discussed below.

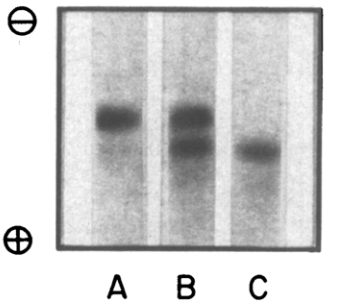


FIGURE 2: Effect of citraconylation on guanidinated S (S_G). (A) S_G. (B) Mixture of A and C. (C) Citraconyl-S_G.

GUANIDINATION OF S. Amino acid analysis showed 1.0 mole of Lys/mole of guanidinated S (S_G), and by difference, 9.0 moles of homo-Arg. This is in agreement with the 93% guanidination achieved by Steiner (1966). Analysis of the reduced, carboxymethylated small fragment (residues 1–63) of S_G* gave 0.07 mole of Lys and 0.94 mole of homo-Arg (based on Arg standard), showing almost complete guanidination of the single Lys in the small fragment. S_G is fully active as shown by titration against trypsin. It has one reactive amino group as shown by citraconylation and subsequent disc gel electrophoresis (see Figure 2). Citraconylation of S_G produces a derivative one charge unit more negative[4] than S_G, *i.e.*, monocitraconyl-S_G. This reactive amino group could be either the nonguanidinated ϵ-amino of a single Lys or α-amino of Asp^1. It is simplest to assume that the nonreactive Lys remains unreactive and it is the α-amino group that is citraconylated. Approximately 7% of the guanidinated material was one charge unit more positive than S_G and did not react with citraconic anhydride. This was assumed to be the result of guanidination of the α-amino group, forming guanyl-(Asp^1)-S_G. Other than the difference in one charge, this material behaved identically with carbamyl-(Asp^1)-S_G (described in the next section).

CARBAMYLATION OF Asp^1 IN S_G. Blockage of the α-amino group of S with a substituent of zero charge produces no change in the mobility on the high-pH disc gel system, since at high pH, the α-amino group is in the basic form. Thus, S_G and carbamyl-S_G (cS_G) are indistinguishable under these conditions. The blockage could be monitored on gels, however, by citraconylation of the free (uncarbamylated) α-amino group. Citraconylation, a very fast reaction, was used to stop the carbamylation reaction and to separate uncarbamylated from carbamylated S_G on gels. The results are shown in Figure 3. A pseudo-first-order half-time for carbamylation of approximately 13 min was observed. The product, cS_G, was shown to be fully active titrations *vs.* trypsin.

As expected, S_G showed dansylaspartic acid upon dansylation and subsequent hydrolysis (Woods and Wang, 1967).

[3] In the case of carbamylation, 0.2 M KCl was substituted in the control for 0.2 M KNCO.

[4] The assignment of charge differences between STI derivatives on the pH 9.4 disc gel system is based on calibration with S* and des-Arg^{63}-S* which differ by one charge unit. In all chemical modification reactions discussed in this work, expected differences in one charge conform to the separation distance between S* and des-Arg^{63}-S*.

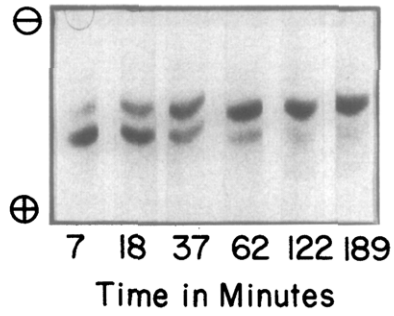


FIGURE 3: Time course of carbamylation of the α-amino group of Asp[1] in guanidinated S as followed by citraconylation of the uncarbamylated α-amino group.

No amino termini were observed when cS_G was treated in similar manner.

CONVERSION OF cS_G TO cS_G*. cS_G was converted by trypsin at pH 3.0 to a mixture of 84 ± 2% cS_G* and 16 ± 2% cS_G as assayed by analytical disc gel electrophoresis (see Figure 4, gel A). Due to the limited solubility of cS_G and cS_G* at low pH, it is difficult to determine whether this composition represents the true equilibrium composition.

*Chemical Modification of the α-Amino Group of Ile[64] in cS_G**. CITRACONYLATION. The effect of reaction of the α-amino group of Ile[64] in cS_G* with citraconic anhydride is shown in Figure 4 (gel C). The trypsin modified inhibitor (cS_G*) takes on an additional unit negative charge while the virgin inhibitor (cS_G) remains unaltered. Addition of excess porcine trypsin to the citraconylated inhibitor at high-pH complexes out the virgin inhibitor, leaving behind the inactive, citraconylated, modified inhibitor (citraconyl-cS_G*, Figure 4, gel D). Quantitation of the amount of complex *vs.* the amount of inactivated (citraconylated) inhibitor by analytical disc gel electrophoresis showed 83% inactivated. The fact that the virgin inhibitor is unaltered in activity and that the per cent inactivated corresponds to the per cent modified inhibitor proves that citraconylation of the modified inhibitor is responsible for the loss of activity. That the activity loss can only be due to substitution of the α-amino group of Ile[64] is deduced from the fact that the citraconylated modified inhibitor acquires only a single additional negative charge while the virgin inhibitor is not affected. This cannot be due to reaction with any other residue (for example, the Lys which is not guanidinated in S) since there is no conformational difference between virgin and modified STI (Niekamp and Laskowski, 1972).[5]

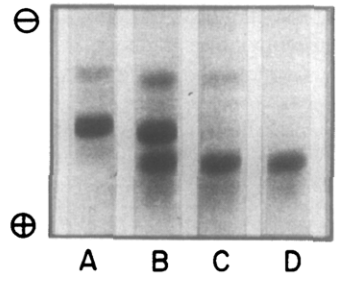


FIGURE 4: Effects of citraconylation on the cS_G-cS_G* mixture. (A) cS_G–cS_G* mixture. The upper band is cS_G and the lower band is cS_G*. (B) Mixture of A and C. (C) Citraconylated cS_G–cS_G*. cS_G* takes on an additional unit negative charge while cS_G is unaffected. (D) C plus excess trypsin added prior to electrophoresis showing selective removal of the virgin inhibitor.

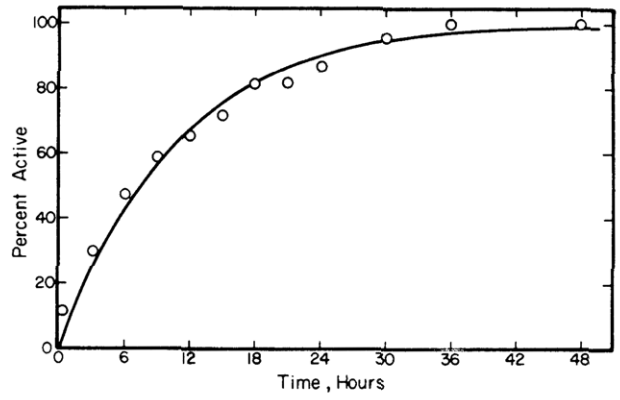


FIGURE 5: Decitraconylation at pH 2.5 of the citraconylated cS_G–cS_G* mixture showing regain of activity. The curve drawn is first order, $t_{1/2}$ = 7.5 hr. The data points are normalized for the per cent cS_G* in the mixture (84%). The protein concentration is 2 mg/ml. Each point represents 15 μl.

The inactivation of cS_G* due to citraconylation was also followed using the GdnBzONph assay. Results showed that an average of 81% of the inhibitor mixture was inactivated by citraconylation, also in good agreement with the amount of cS_G* present as determined by analytical disc gel electrophoresis.

As a final assessment of the activity, the citraconylated inhibitor was passed through an insoluble trypsin column. The bulk of the inhibitor was not retained by the column and eluted with the void volume. Disc gel electrophoresis showed this material to be pure citraconyl-cS_G*.

Decitraconylation (Dixon and Perham, 1968) of citraconyl-cS_G* at pH 2.5 proceeds with regain of activity with a half-time of about 8 hr as monitored by the increase of trypsin–inhibitor complex on disc gels (Figure 5). The half-time at pH 3.0 is approximately 16 hr. These relatively slow rates of deblocking are probably due to interaction of the negative citraconyl group with either Arg[63] or Arg[65] causing a lowering of the pK_a of the citraconyl group.

CARBAMYLATION. As in the case of the carbamylation of Asp[1] of S_G, carbamylation of Ile[64] in cS_G* produces no change in mobility on the high-pH disc gel system. Since it was found that carbamylation also inactivated cS_G*, the reaction could be monitored by complexation of active inhibitor with trypsin prior to disc gel electrophoresis, and subsequent separation on disc gel of complexed (active) and carbamylated (inactive) inhibitor. The results are shown in Figure 6. Note the appearance of a single product (carbamyl-cS_G*) as the carbamylation proceeds and the complete absence of cS_G. The maximum per cent inactivation on completion of the reaction was 83%, again the amount of modified inhibitor in the cS_G–cS_G* mixture.

AMIDINATION. Modification of the α-amino group of Ile[64] with the positively charged acetimido group virtually negates any separation of cS_G and acetimido-cS_G* on the high-pH disc gel system. The amidination was followed by the same method discussed above for carbamylation. Under the conditions employed, the reaction was complete in about 20 min, showing an average of 81% inhibitor inactivated, again cor-

[5] To be published.

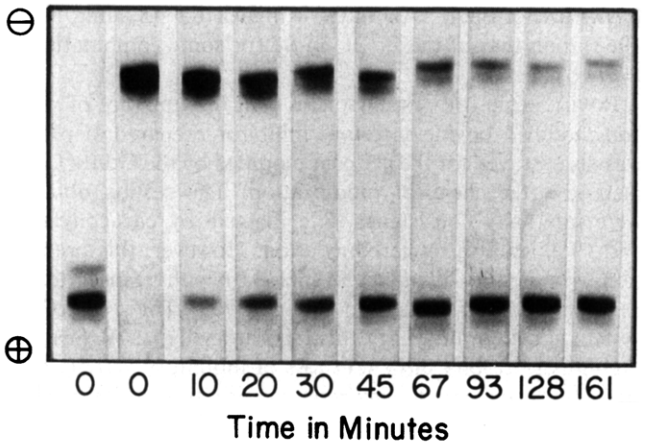
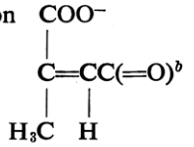


FIGURE 6: Time course of the carbamylation of the α-amino group of Ile^{64} in cS_G* as followed by loss of inhibitory activity. The zero time gels are the cS_G–cS_G* mixture without and with excess porcine trypsin, respectively. The upper bands represent the porcine trypsin–STI complex. The lower bands were described in Figure 4A. Carbamyl-cS_G* migrates identically with cS_G*.

TABLE I: Summary of Chemical Modifications of the N-Terminal Ile^{64} in the Reactive Site of cS_G*.[a]

Modification	Substituent	% Inactive	Inhibitor Assay
Citraconylation		81 ± 2	GdnBzONph
		83 ± 2	Disc gel
Carbamylation	$H_2NC(=O)$	83 ± 2	Disc gel
Amidination	$CH_3C(=NH_2^+)$	81 ± 2	Disc gel

[a] These results are to be compared with the 84 ± 2% cS_G* as determined by analytical disc gel electrophoresis (cS_G* relative to cS_G). [b] The methyl radical is on carbon-2 or -3.

responding to the amount of modified inhibitor. The effect of amidination is shown in Figure 7. As seen in gel D, the addition of trypsin removes the active virgin inhibitor, leaving behind the inactive acetimido-cS_G*. The component in gel D which migrates like cS_G*, but is inactive, is probably due to a side reaction in the amidination procedure previously observed by Hunter and Ludwig (1962). That this component was indeed blocked at the α-amino group of Ile^{64} was shown by its failure to react with citraconic anhydride.

A summary of the results of specific chemical modification of the N-terminal Ile^{64} of the reactive site of cS_G* is shown in Table I. The results are in very good agreement with the 84 ± 2% modified inhibitor found by analytical disc gel electrophoresis.

Titration of the cS_G–cS_G Mixture with Porcine Trypsin.* Since quantitation of most of the chemical modification reactions presented herein relied on the measurement of the per cent trypsin–inhibitor complex relative to the per cent uncomplexed inhibitor as measured by analytical disc gel electrophoresis, it was desired to compare the per cent complex obtained by disc gel to that calculated from the molarity of trypsin active sites (determined by the GdnBzONph assay) and the concentration of the cS_G–cS_G* mixture. The results are shown in Figure 8 where the straight line represents the per cent complex calculated.

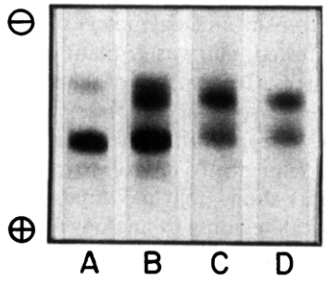


FIGURE 7: Effects of amidination on the cS_G–cS_G* mixture. (A) cS_G–cS_G* mixture. (B) Mixture of A and C. (C) Amidinated cS_G–cS_G*. cS_G* takes on a unit positive charge while cS_G is unaffected. (D) C plus excess trypsin added prior to electrophoresis showing selective removal of the virgin inhibitor.

It is worthy of note that in obtaining the per cent complex by integration of the gel scans, no correction was required for staining of the porcine trypsin–STI complex relative to free STI. On the other hand, bovine trypsin–STI complex requires a correction factor of one-half, the complex staining twice as much as free STI (T. R. Leary, unpublished data). This is probably due to the fact that bovine trypsin is fixed under the staining conditions (7% acetic acid–0.5% Aniline Black, 1 hr) whereas porcine trypsin, which is more stable, is not fixed and can therefore diffuse out of the gel under the acidic staining and destaining conditions.

Comparison of the Per Cent Modified Inhibitor as Determined by Analytical Disc Gel Electrophoresis and Inactivation by Citraconylation. In order to establish a general method for assessing the per cent modified inhibitor, the citraconylation reaction was carried out on various S–S* mixtures (Asp^1 and ϵ-amino groups of lysine *not* blocked) and the per cent inactivated inhibitor measured by the GdnBzONph method. The same S–S* mixtures, without reaction with citraconic anhydride, were analyzed for the per cent S* by analytical disc gel electrophoresis (15-cm gels). As shown in Figure 9, a one to one correspondence was obtained between the per cent inactive inhibitor and the per cent S*. Since inactivation

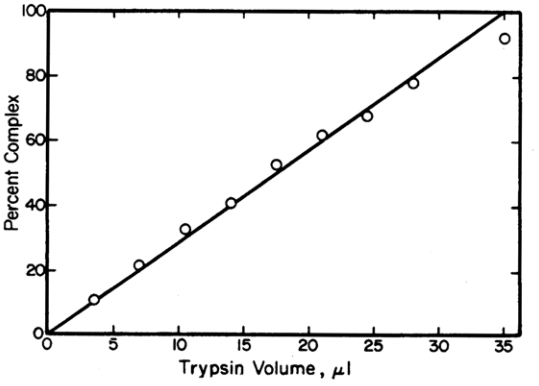


FIGURE 8: Titration of the cS_G–cS_G* mixture with porcine trypsin as determined by analytical disc gel electrophoresis (7-cm gels). The straight line is calculated based on the molarity of trypsin active sites (4.0×10^{-5} M) and the concentration of the inhibitor (4.0×10^{-5} M). Each point represents 35 μl of the inhibitor solution.

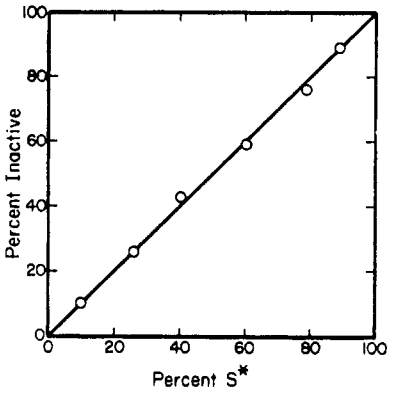


FIGURE 9: Comparison of the per cent modified STI (S*) as determined by inactivation (citraconylation followed by GdnBzONph assay) and by analytical disc gel electrophoresis (S* relative to S).

by citraconylation properly reflects the fraction of S* in S–S* mixtures, it can be applied to other inhibitors, provided the virgin form is not inactivated by the chemical modification.

Chemical Modification of Other Inhibitors. CHICKEN OVOMUCOID. A pH 3.0 equilibrium preparation of 94% modified ovomucoid (O*) lost 93% of its trypsin inhibitory activity upon citraconylation. Virgin ovomucoid, treated in like manner, lost no measurable activity (<1%).

Carbamylation of the pH 3.0 equilibrium preparation of 94% O* resulted in a loss of precisely 94% of the trypsin inhibitory activity, virgin ovomucoid remaining unaffected. The time courses of the inactivation of O* and S* by carbamylation are shown in Figure 10. The curves are normalized for the maximum per cent inactivated (94% for O* and 86% for S*). It is seen in this direct comparison using the same stock solution of potassium cyanate that S* reacts 2.2 times faster than O*. Therefore, either the reactive site α-amino group of the P_1' Ala in O* is less accessible to the reagent,

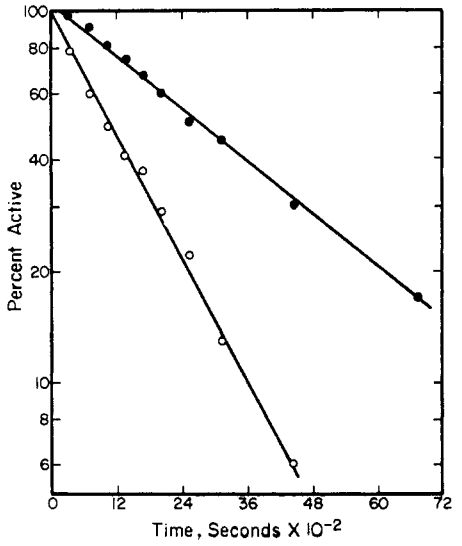


FIGURE 10: Semilogarithmic plot of the inactivation of modified STI (○) and modified chicken ovomucoid (●) by carbamylation. Data points are normalized for the per cent of modified STI (86%) and modified chicken ovomucoid (94%) in the virgin-modified mixture. The concentration of S* is 4 mg/ml and that of O* is 5 mg/ml. Carbamylation conditions are 0.2 M KNCO–0.2 M *N*-ethylmorpholine acetate (pH 7.0).

or the pK_a of the P_1' Ala in O* is higher (0.5 pK_a unit, Stark, 1965) than that of the P_1' Ile in S* (or some combination of the two effects).

BOVINE SECRETORY INHIBITOR (KAZAL). A mixture of virgin and modified bovine secretory inhibitor prepared at pH 3.0 was also assayed for the per cent modified by the GdnBzONph method after chemical modification. The results obtained were internally consistent: 18% inactive by citraconylation and 19% inactive by carbamylation. However, this preparation was not at equilibrium (pH 3.0) with respect to the fraction modified (Rigbi and Greene, 1968). The study of this system is being pursued in this laboratory by R. W. Sealock. Preliminary results show that loss of inhibitory activity after citraconylation is an excellent quantitative technique for measuring the fraction of molecules with the reactive site Arg^{18}–Ile^{19} bond hydrolyzed.

Discussion

The observations listed in the body of this paper tempt us towards a rather sweeping generalization—any blockage of the α-amino group on the P_1' residue of a modified (reactive-site peptide bond hydrolyzed) protein proteinase inhibitor leads to loss of inhibitory activity. We believe that this generalization is consistent with all of the presently available data, but we caution the reader to regard it only as a working hypothesis. The statement that activity is quantitatively lost is merely operational, defined in terms of the methods applied to detect it. Both disc gel electrophoresis and active-site titration with GdnBzONph regard very rapidly dissociating complexes (dissociation half-time of about 10 sec or less) as simply a mixture of free inhibitor and enzyme. It is indeed likely that the P_1' α-amino-blocked modified inhibitors form loose, noncovalent complexes, similar to the intermediate L* defined and described by Luthy *et al.* (1972).[6] Furthermore, a remote possibility exists that considerably stronger complexes are formed between trypsin and P_1' α-amino-blocked modified inhibitors but that these form so slowly that sufficient time was not allowed for their formation. However, since quantitative loss of activity was observed even with preincubations as long as 1 hr, such association reactions (if they exist) must be very slow indeed.

The generalization consists of two parts. The first asserts that blockage by any reagent will inactivate. To make this plausible, we have shown that substituents which introduce a negative charge (citraconyl or maleyl), no charge (carbamyl), or positive charge (acetimido) all lead to quantitative loss of activity. The extension to *all* protein proteinase inhibitors suffers from the fact that only trypsin inhibitors were tested. It would be surprising indeed if the results were different for modified chymotrypsin inhibitors in light of the similarities in behavior of trypsin and chymotrypsin inhibitors. Of the three inhibitors tested, one was of plant origin, and two were from animal sources. Comparison of the amino acid sequences of bovine pancreatic secretory inhibitor (Greene and Bartelt, 1969) and soybean trypsin inhibitor (Koide *et al.*, 1972) and the reactive site sequence of chicken ovomucoid (J. Schrode, unpublished data) shows that all three inhibitors belong to separate classes, related only by analogy and not by homology. It should be added that the P_1' residues of two more modified inhibitors were blocked by other workers—an Arg–Ile reactive-site trypsin-specific inhibitor from guinea pig seminal vesicles (Fink *et al.*, 1971) and an Arg–X reactive-site broad

[6] J. A. Luthy, M. Praissman, M. Laskowski, Jr., to be published.

specificity proteinase inhibitor from sea anemone (Fritz *et al.*, 1972). In both cases, maleylation led to loss of inhibitory activity.

The only published work which is contrary to our generalization is that of Haynes and Feeney (1968) on modified chicken ovomucoid and of Uy and Feeney (1971) on "chymotrypsin-modified" turkey and penguin ovomucoids. In the first investigation, modified soybean trypsin inhibitor (S*) and modified chicken ovomucoid (O*) were reacted with TNBS. In the case of S*, activity was rapidly lost. Activity loss was also observed with O*, but only very slowly. On this basis alone the authors conclude that the loss occurred too slowly to be due to blockage of the P_1' α-amino group and must have arisen from unidentified secondary reactions (note that virgin chicken ovomucoid is not inactivated, Haynes *et al.*, 1967). No evidence was given to show that the rate of substitution of the critical α-amino group of the P_1' alanyl residue was in fact faster than the rate of inactivation. Slow substitution of the P_1' α-amino group in O* by the bulky TNBS seems quite plausible since the reactive site of O* appears to be sterically more hindered than that of S*. As shown in this paper, the rate of carbamylation of the P_1' Ala amino group in O* is two times slower than the rate of carbamylation of the P_1' Ile amino group in S*. Furthermore, release of the P_1 arginine residue from S* by carboxypeptidase B is fast while from O* it is slow. This is not primarily due to an unfavorable amino acid sequence, since release of the carboxy-terminal Arg from the amino-terminal fragments (obtained after reduction and carboxymethylation of the disulfide bridges in the modified inhibitors) is fast for both. We defer the criticism of the Uy and Feeney paper to a later part of the discussion.

The finding that activity is lost on blockage of the α-amino group of P_1' in modified inhibitors sheds little light on the nature of the stable enzyme–inhibitor complex. The possibility that the complex is a tetrahedral intermediate is attractive since it clearly explains the requirement for the unblocked α-amino group of P_1'. However, the loss of inhibitory activity can equally well be explained either as a result of steric hindrance by the blocking group toward intimate formation of an acyl complex or by the loss of at least one of the two possible hydrogen bonds formed by the amino group of P_1' in the acyl complex. Blow *et al.* (1972) suggest in their model of an acyl complex of α-chymotrypsin and of Kunitz pancreatic trypsin inhibitor that the α-amino group of P_1' Ala^{16} of the inhibitor is hydrogen bonded both to the carbonyl of Gly^{36} of the inhibitor and to the catalytic His^{57} of the enzyme. If this model, based on the X-ray crystallographic structure determination of both partner proteins, is a correct representation of the true complex, then both steric hindrance and loss of a hydrogen bond are a sufficient explanation of our results. At the moment, we must adopt an agnostic position, although we favor the steric hindrance/loss of hydrogen-bonding explanation. This problem will presumably be resolved by X-ray crystallographic determination of the structure of several enzyme–inhibitor complexes now in progress.

Although the finding that activity is lost on blockage of the P_1' α-amino group of modified inhibitors provides at best ambiguous information concerning the nature of stable complex, it is of considerable methodological advantage in proteinase inhibitor research. It provides a new, relatively easy and quantitative method of estimating the amount of modified inhibitor in an inhibitor preparation.

There are actually two distinct and important problems in proteinase inhibitor research where the method may prove helpful. The first is in determination of reactive sites of inhibitors. In such a search, the inhibitor is incubated in the presence of the enzyme it inhibits at a variety of pH values until conditions are found where appreciable hydrolysis of an inhibitor peptide bond (or bonds) is observed. Considerable data can be cited to show that in several systems, bonds other than the reactive-site peptide bond are hydrolyzed. Thus, proof that the hydrolyzed bond is at the reactive site is clearly required. We feel strongly (Hixson and Laskowski, 1970a; Bidlingmeyer *et al.*, 1972) that the best proof is formation of complexes of the enzyme with the intact inhibitor and with the enzymatically "modified" inhibitor, followed by kinetic control dissociation of these complexes (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b; Laskowski *et al.*, 1971). In such an experiment, the complexes are caused to dissociate in a manner such that once dissociation takes place the inhibitor can no longer recombine with the enzyme. This can be accomplished in several ways: rapidly dropping the pH of the solution of complex to a very low value (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b; Frattali and Steiner, 1969), rapidly introducing a high concentration of denaturant such as guanidine hydrochloride (Sealock and Laskowski, 1969), or adding a large excess of a very rapidly reacting active-site titrant (Bidlingmeyer *et al.*, 1972). On completion of the dissociation the product inhibitor is isolated in such a manner that it is not allowed to come in contact again with the active enzyme. If the distribution of products (virgin and modified inhibitor) in each preparation is the same, the hydrolyzed peptide bond of the inhibitor must have been in intimate contact with the catalytic site of the enzyme in the enzyme–inhibitor complex, and is therefore unquestionably the inhibitor reactive site. In all systems thus far tested, the distribution largely favors the virgin form of the inhibitor, making the result of kinetic control dissociation of complex prepared from the modified inhibitor particularly striking. We cannot overemphasize the importance of segregation of inhibitor and active enzyme, since failure to do so will tend toward an equilibrium (rather than a kinetic) distribution of products.

Most workers at present resort to less theoretically convincing procedures but ones which are easier to explain and carry out experimentally. The most obvious, but least convincing, of these tests is based on the general observation that the modified form of inhibitors reacts with the enzyme considerably more slowly than the virgin form. However, no strong evidence can be presented to show that all modified inhibitors will react with the enzyme more slowly than virgin inhibitors and further that hydrolysis of a bond other than the reactive site will not affect the rate of the inhibitor–enzyme reaction.

Considerably better than the above, but still less rigorous than kinetic control dissociation, is the removal of the newly formed carboxy-terminal residue (P_1) by the appropriate carboxypeptidase accompanied by total loss in activity of the modified inhibitor (Finkenstadt and Laskowski, 1965). We submit, that insofar as the generalization concerning α-amino group blockage in modified inhibitors is correct, loss of inhibitory activity on citraconylation or carbamylation is equivalent to the loss of activity on removal of P_1 by a carboxypeptidase. In some instances, however, the blockage experiments are much easier than the carboxypeptidase experiments, such as when the release of P_1 is slow or nonexistent.

We feel that such a lengthy discussion on the determination of reactive sites is necessary to reconcile the results reported here with the work of Feinstein *et al.* (1966) and of Uy and Feeney (1971). These investigators incubated several ovomu-

coids with catalytic quantities of trypsin and chymotrypsin, respectively, at low-pH values and provided clear-cut evidence that appreciable proteolysis took place. In the first investigation, the products were submitted to carboxypeptidase B action, and in the second they were blocked by reductive alkylation. In neither case was inhibitory activity lost, even though in the case of carboxypeptidase B treatment, sizeable quantities of arginine and lysine were released, and in the chemical modification experiments, a large fraction of the newly formed amino terminals was blocked. The authors concluded that neither the presence of the P_1 residue nor of the unblocked α-amino group of P_1' of the reactive site are required for inhibition. In the case of P_1, this interpretation leads to an internal paradox. In an elegant experiment, Haynes *et al.* (1967) have shown that one lysyl residue in turkey ovomucoid is essential to activity and that its blockage by TNBS completely eliminates inhibitory activity; yet Feinstein *et al.* (1966) argue that removal of this "reactive-site" lysyl residue by carboxypeptidase B is without effect. We prefer a much simpler explanation. Since no proof was provided either by Feinstein *et al.* (1966) or by Uy and Feeney (1971) that the bonds hydrolyzed by trypsin and chymotrypsin at low pH in various ovomucoids were indeed at the reactive site, the reactive-site bonds were in fact not hydrolyzed. Viewed in this light, the data of these two papers are simply irrelevant to problems at hand.

Another important application of the reactive-site α-amino blockage procedure is its use in rapid, quantitative assessment of the fraction of modified inhibitor in a virgin-modified inhibitor mixture. Such measurements are important in the determination of the equilibrium constant, K_{hyd} (Neikamp *et al.*, 1969; Laskowski *et al.*, 1971; Tscheshe and Obermeier, 1971), for following the kinetics of virgin-modified inhibitor interconversion (Rigbi and Greene, 1968; Niekamp *et al.*, 1969; J. Mattis, unpublished experiments), and for monitoring the products of kinetic control dissociation. In our first paper on this subject we suggested that disc gel electrophoresis at relatively high pH is an excellent quantitative method and that it should be general, since at high pH modified inhibitors will always have one additional negative charge (the carboxylate anion of the newly formed carboxy terminal of P_1) compared to virgin inhibitors. We still feel that when applicable, disc gel electrophoresis is a technique of choice for routine quantitative assay, however, we were quite wrong about its generality. Common problems encountered are fixing the very stable inhibitors in the gel matrix, quantitative staining, and destaining without loss of protein.

The best currently available alternative to disc gel electrophoresis was quantitative evaluation of the loss of activity due to removal of the carboxy-terminal P_1 of the modified inhibitor employed by Rigbi and Greene (1968) in monitoring of virgin to modified bovine pancreatic secretory inhibitor (Kazal). However, the release of P_1 is often slow, making the assay inconvenient and leading to errors due to incomplete release.

Citraconylation (or maleylation) is in effect exactly equivalent to the carboxypeptidase removal of P_1, but it is much more rapid and quantitative. Schrode and Laskowski (1971) have already applied this technique for monitoring virgin to modified chicken ovomucoid conversion and were able to obtain self-consistent and reliable values of K_{hyd} for this system over a broad pH range.

The major disadvantage of the α-amino-terminal blockage as a method of distinguishing modified from virgin inhibitors is that among trypsin inhibitors it can be applied only to P_1 arginine inhibitors. Citraconylation of P_1 lysine inhibitors will of course inactivate both the virgin and modified forms by substitution on the ϵ-amino group of the reactive-site lysine. The extension of the method reported here to P_1 lysine inhibitors is hampered only by the lack of availability of a fast-reacting reagent, highly specific for α-amino groups.

References

Bidlingmeyer, U. D., Leary, T. R., and Laskowski, M., Jr. (1972), *Biochemistry 11*, 3303.

Blow, D. M., Wright, C. S., Kukla, D., Rühlman, A., Steigemann, W., and Huber, R. (1972), *J. Mol. Biol.* (in press).

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112*, 679.

Chase, T., Jr., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.

Dixon, H. B. F., and Perham, R. N. (1968), *Biochem. J. 109*, 312.

Feinstein, G., Osuga, D. T., and Feeney, R. E. (1966), *Biochem. Biophys. Res. Commun. 24*, 495.

Fink, E., Klein, G., Hammer, F., Muller-Bardoff, G., and Fritz, H. (1971), *in* Proceedings of the International Research Conference on Proteinase Inhibitors, Fritz, H., and Tschesche, H., Ed., Berlin, Walter de Gruyter and Co., p 225.

Finkenstadt, W. R., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, PC 962.

Finkenstadt, W. R., and Laskowski, M., Jr. (1967), *J. Biol. Chem. 242*, 771.

Frattali, V., and Steiner, R. F. (1969), *Biochem. Biophys. Res. Commun. 34*, 480.

Fritz, H., Brey, B., and Beress, L. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353*, 19.

Fritz, H., Fink, E., Gebhart, M., Hochstrasser, K., and Werle, E. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 933.

Greene, L. J., and Bartelt, D. C. (1969), *J. Biol. Chem. 244*, 2646.

Haynes, R., and Feeney, R. E. (1968), *Biochim. Biophys. Acta 159*, 209.

Haynes, R., Osuga, D. T., and Feeney, R. E. (1967), *Biochemistry 6*, 541.

Hixson, H. F., Jr., and Laskowski, M., Jr. (1970a), *Biochemistry 9*, 166.

Hixson, H. F., Jr., and Laskowski, M., Jr. (1970b), *J. Biol. Chem. 245*, 2027.

Hughes, W. L., Jr., Saroff, H. A., and Carney, A. L. (1949), *J. Amer. Chem. Soc. 71*, 2476.

Hunter, M. J., and Ludwig, M. L. (1962), *J. Amer. Chem. Soc. 84*, 3491.

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

Kowalski, D., and Laskowski, M., Jr. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 876 Abs.

Laskowski, M., Jr., Duran, R. W., Finkenstadt, W. R., Herbert, S., Hixson, H. F., Jr., Kowalski, D., Luthy, J. A., Mattis, J. A., McKee, R. E., and Neikamp, C. W. (1971), *in* Proceedings of the International Research Conference on Proteinase Inhibitors, Fritz, H., and Tscheche, H., Ed., Berlin, Walter de Gruyter and Co., p 117.

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

McElvain, S. M., and Nelson, J. W. (1942), *J. Amer. Chem. Soc. 64*, 1825.

Neikamp, C. W., Hixson, H. F., Jr., and Laskowski, M., Jr. (1969), *Biochemistry 8*, 16.
Ozawa, K., and Laskowski, M., Jr. (1966), *J. Biol. Chem. 241*, 3955.
Rigbi, M., and Greene, L. J. (1968), *J. Biol. Chem. 243*, 5457.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157.
Schrode, J., and Laskowski, M., Jr. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1320 Abs.
Sealock, R. W., and Laskowski, M., Jr. (1969), *Biochemistry 8*, 3703.
Stark, G. R. (1965), *Biochemistry 4*, 1031.
Stark, G. R. (1967), *Methods Enzymol. 11*, 590.
Steiner, R. F. (1966), *Arch. Biochem. Biophys. 115*, 257.
Tschesche, H., and Obermeier, R. (1971), *in* Proceedings of the International Research Conference on Proteinase Inhibitors, Fritz, H., and Tschesche, H., Ed., Berlin, Walter de Gruyter and Co., p 135.
Uy, R. L., and Feeney, R. E. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1130 Abs.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.

Adrenocorticotropin. Synthesis of [6-Phenylalanine]-α^{1-19}-adrenocorticotropic Hormone and Its Steroidogenic, Melanocyte-Stimulating, and Lipolytic Activity†

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ABSTRACT: [6-Phenylalanine]-α^{1-19}-adrenocorticotropic hormone has been synthesized by the solid-phase method. A comparison of its steroidogenic, melanocyte-stimulating, and lipolytic activity to that of α^{1-19}-adrenocorticotropic hormone indicates that the replacement of histidine by phenylalanine results in a marked lowering of the biological activity.

A continuing effort in the study of structure and function of adrenocorticotropin has been directed toward determining the relative importance of each of the amino acid residues in the ACTH structure (Figure 1) for high steroidogenic activity. The results thus far indicate: (1) the carboxyl-half of the molecule plays no vital role in steroidogenic activity, and 50–100% activity can be obtained from the amino-terminal nonadecapeptide amide (Ramachandran *et al.*, 1965); (2) the basic core at positions 15–18 is important for full steroidogenic response, and a partial deletion of the basic residues (Ramachandran *et al.*, 1965) or their substitution with neutral residues (Brugger *et al.*, 1970) lowers activity; (3) an intact Lys-Pro-Val sequence is important for high activity (Blake and Li, 1967). Of the nine amino-terminal residues only arginine (Chung and Li, 1967; Tesser and Rittel, 1969) and tryptophan (Hofmann *et al.*, 1970) seem to be specifically required for high steroidogenic potency. Replacement of any of the first seven amino acid residues by amino acids with similar side chains gives peptides of comparable biological activity. Of particular interest to us was the report that the replacement of histidine with β-(pyrazolyl-3)-alanine in the amino-terminal eicosapeptide of ACTH[1] gave a peptide possessing 40–70% of the steroidogenic activity of the histidine peptide (Hofmann *et al.*, 1967, 1970). To gain further insight into the contribution of the histidine residue toward the steroidogenic and other activities of ACTH, the synthesis and biological assay of [6-phenylalanine]-α^{1-19}-ACTH were carried out; results of these studies are herein reported.

[6-Phenylalanine]-α^{1-19}-ACTH (I) was synthesized by the solid-phase method (Merrifield, 1964) as described for the synthesis (Blake *et al.*, 1972) of α^{1-19}-ACTH (II). Amino acids were coupled as their N^{α}-Boc derivatives throughout the synthesis; side-chain protecting groups were as reported previously. Treatment of the peptide-resin with liquid HF (Sakakibara *et al.*, 1967; Lenard and Robinson, 1967), followed by chromatography of the crude peptide on Sephadex G-25 and carboxymethylcellulose (Peterson and Sober, 1956), gave peptide I (Figure 2). The analytical data of peptide I were as expected for [6-phenylalanine]-α^{1-19}-ACTH (see Experimental Section).

The biological properties of peptides I and II are listed in Table I. The data indicate that replacement of histidine by phenylalanine has a profound effect on the biological activity. The steroidogenic and melanocyte-stimulating activities of peptide I are reduced to approximately 1 and 3%, respectively, of the activities of peptide II. It is of interest that the lipolytic activity of the phenylalanine analog in isolated fat cells of rats and rabbits is very much lower than that expected from its steroidogenic potency. Thus, the histidine residue plays an important role for the function of ACTH. The chemical differences between these amino acids are that the aromatic ring in histidine is smaller than the aromatic ring in phenylalanine and that histidine contains the imidazole ring which may form a hydrogen bond with the receptor in the membrane of the adrenal gland. Whether such an interaction exists remains to be determined.

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl. All the asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration.