Modification of the Basic Trypsin Inhibitor of Bovine Pancreas. The ε-Amino Groups of Lysine and the Amino-Terminal Sequence

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ABSTRACT: The four ε-amino groups of lysine and the amino-terminal Arg-Pro-Asp- sequence of the basic trypsin inhibitor of bovine pancreas are not essential for the activity. This was demonstrated by guanidination and amidination of the four lysine residues and removal of the three amino acids from the amino terminal of the guanidinated inhibitor.

Chemical modification of the individual amino acid residues of an *inhibitor* is one approach to identification of the components of the inhibitor molecule that are necessary for the specific activity. When the activity is not affected, the altered residue or residues can then be eliminated from consideration as a functional part of the inhibitor. Alternatively, any modification of the *enzyme*, which neither inactivates it nor affects its reaction with an inhibitor, can be interpreted in the same way in its relation to the particular inhibitor.

Of the many trypsin inhibitors known, the basic inhibitor of bovine pancreas (Kunitz and Northrop, 1936) is the only one for which the sequence of the amino acids has at present been completed. Three laboratories have reported slightly different linear sequences for this protein (Chauvet *et al.*, 1964; Kassell *et al.*, 1965; Kassell and Laskowski, 1965; Dlouhá *et al.*, 1965). Our sequence (Figure 1) has been supported by further experiments (Kassell and Laskowski, 1966) and by the finding that the kallikrein inhibitors of bovine parotid gland and lung both have the same sequence as this trypsin inhibitor (Anderer, 1965; Anderer and Hörnle, 1966). This inhibitor is therefore a logical choice for this type of investigation.

One exclusion-type modification of the basic pancreatic trypsin inhibitor has already been made. Oxidation of the single methionine residue to the sulfoxide did not affect the activity (Kassell, 1964).

The present paper deals with guanidination of the four lysine residues of this protein and subsequent removal of the amino-terminal sequence. Some experiments on amidination are also included.

Materials and Methods

The trypsin inhibitor was prepared in the same manner as previously described (Kassell *et al.*, 1963). Trypsin and α-chymotrypsin were Worthington preparations. Factors were calculated to make the activity for trypsin correspond to the most active preparation previously reported (Kassell *et al.*, 1963) and for chymotrypsin to correspond to the data of Kunitz (1947).

For guanidination, the method of Chervenka and Wilcox (1956) was modified to use a smaller amount of protein. A solution of 2-methylisourea was prepared by dissolving 4.2 g of the sulfate (Fisher Scientific Co.) in about 5 ml of water in an ice bath, and slowly adding hot saturated barium hydroxide until the suspension was blue to thymol blue. The mixture was diluted to 50 ml and centrifuged in the cold; 45 ml of the supernatant solution was added to 100 mg of solid inhibitor, and the pH was adjusted to 10.3 at 0° with 5 N NaOH. The final volume was 50 ml. The solution was kept at 4° for 72 hr, then dialyzed in acetylated 18–100 Visking cellulose casing (Kassell *et al.*, 1963) against two changes of 6 l. each of 0.001 M HCl, and lyophilized.

Amidination followed the "exhaustive" procedure of Wofsy and Singer (1963) with the exception of the protein concentration, which was 6.5 mg/ml at the start of the reaction. Ethyl acetimidate was prepared by the method of McElvain and Nelson (1942), stored in an evacuated desiccator, and used within 2 weeks.

Amino acid analysis was carried out as described by Moore and Stein (1963) with single 24-hr hydrolysates of 1-mg samples. A Beckman-Spinco, Model 120B, amino acid analyzer was used. Homoarginine was detected in the accelerated system in the same relative position after arginine as reported by Habeeb (1960) in the older system. It was determined quantitatively by comparison with a sample of homoarginine obtained from Calbiochem. The amidinated lysine appeared in the same position as described by Wofsy and Singer (1963), but was not determined quantitatively.

The DNP-guanidinated or -amidinated protein was prepared according to Schroeder and LeGette

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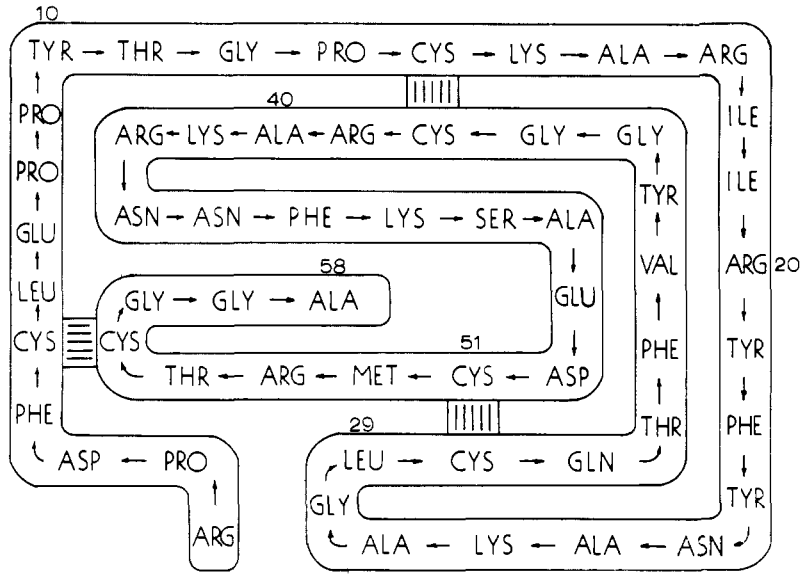

FIGURE 1: A schematic representation of the structure of the trypsin inhibitor. The molecular weight is 6513.

(1953). The procedures of Fraenkel-Conrat *et al.* (1955) were followed for hydrolysis of the DNP-protein, chromatography of the aqueous phase in the t-amyl alcohol–phthalate system, and identification of DNP-arginine by the Sakaguchi reaction.

Activity of the inhibitor against trypsin was determined by the method of Erlanger *et al.* (1961), except that 38° was used as the temperature of incubation. Trypsin and inhibitor were incubated together at pH 8.2 for 5 min before addition to the substrate, benzoyl-arginine *p*-nitroanilide (Nutritional Biochemicals Corp.).

Activity against chymotrypsin was determined by the casein digestion method of Kunitz (1947). Gel filtration on Sephadex G-50 (Fine beads, Pharmacia Fine Chemicals) was carried out in the buffer used by Anderer (1965), 0.02 M sodium phosphate of pH 7.3, containing 0.8% NaCl.

The amino-terminal group was removed from the guanidinated inhibitor by a modified Edman method similar to the procedure described by Konigsberg and Hill (1962). Starting with 15 mg of protein in a 100-ml lyophilizing flask, 2 ml of the morpholine acetate buffer used by the authors cited and 0.1 ml of phenyl isothiocyanate were added; the flask was flushed with nitrogen (Blombäck *et al.*, 1966), stoppered, and kept 2.5 hr at 38°. The contents were lyophilized and the dry residue was extracted with benzene. The benzene extract was decanted (centrifuged if necessary) and discarded. The remaining benzene was removed in a warmed evacuated desiccator. The phenylthiocarbamyl protein still in the flask was treated with 2 ml of anhydrous trifluoroacetic acid in a desiccator over NaOH. After 30 min at room temperature, the desiccator was evacuated with a water pump just until the liquid disappeared. The solid was taken up at once in 0.5 ml of 0.05 M acetic acid. The protein was separated from the phenylthiohydantoin derivative of the terminal group by gel filtration on Sephadex G-25 (Fine beads, Pharmacia) equilibrated with 0.05 M acetic acid. The protein peak was pooled, a sample removed for activity determination, and the remainder lyophilized. A sample was taken from the dried material for amino acid analysis. The subtractive procedure was repeated on the remainder until four steps had been carried out.

Results

Guanidination. Preliminary experiments with smaller quantities, but with the same concentrations of reagents and protein as described above, showed that after 72 hr, essentially all four lysine residues were converted to homoarginine residues (Table I). The α-amino group of the terminal arginine residue did not react. This was demonstrated in two ways. First, the dinitrophenyl (DNP) derivative was prepared, and DNP-arginine was identified qualitatively after paper chromatography by the change in color of the yellow spot to pink on spraying with Sakaguchi reagent. Second, the amino-terminal arginine residue was removed by the Edman reaction; this is described below.

The trypsin-inhibiting activity is shown in Table I. The guanidinated inhibitor had at least as high activity as the native inhibitor. It appeared that the activity of the derivative was actually slightly higher, and an explanation was sought.

To test for the presence of polymers (presumably inactive), both proteins were passed through Sephadex G-50 at pH 7.3. The results are shown in Figure 2. The native inhibitor showed a small peak at the void volume of the column (peak 1). This peak had no activity when tested promptly, but on standing overnight, some activity became apparent (0.66 mg of trypsin inhibited/mg of inhibitor). Peak 2 showed normal activity. The guanidinated inhibitor showed only one

TABLE I: Amino Acid Analyses and Trypsin-Inhibiting Activities during Guanidination of the Inhibitor.

Protein (mg)	Reaction Time (hr)	Moles of Amino Acid/Mole of Protein[a]			Activity[b]
		Lysine	Homoarginine	Arginine	
—	0	3.99 ± 0.22	—	5.74 ± 0.21	2.78 ± 0.11
5	22	0.39	3.65	5.89	—
35	48	Lost	3.69	5.77	—
5	72	0.10	3.82	6.11	3.23 ± 0.10
100	72	0.06	3.97	6.12	3.12

[a] Calculated on the basis of six residues of glycine per mole of protein. Other amino acids were unchanged from the values previously reported (Kassell *et al.*, 1963). [b] Expressed as milligrams of trypsin inhibited per milligram of inhibitor.

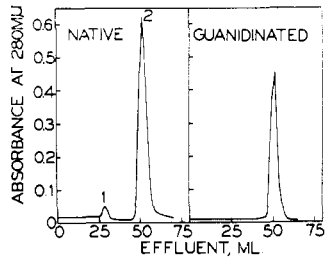

FIGURE 2: Gel filtration of the native and guanidinated inhibitors on Sephadex G-50. Column 0.9 × 102 cm; load, 5 and 4 mg, respectively; fractions, 2 ml; rate, 12 ml/hr; void volume of column, 26 ml.

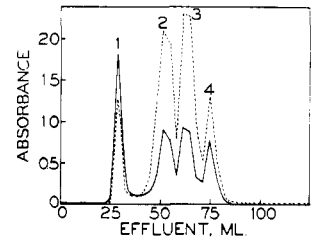

FIGURE 3: Purification of the protein on Sephadex G-25 after the first step of the Edman degradation. Column 1 × 53 cm; load, entire reaction mixture (15 mg of protein); fractions, 2 ml; rate, 12 ml/hr; A_{280}, solid line; A_{263}, dashed line.

peak in the same position as the main peak of the native inhibitor. The separation of a small amount of inactive, reversibly polymerized material from the native and not from the guanidinated inhibitor is an indication that the difference found in activity between the two inhibitors was significant.

Chymotryptic digestion of casein was inhibited to about the same extent by the native and guanidinated inhibitors. In a single experiment, the activity was 2 mg of chymotrypsin inhibited/mg of native inhibitor, and 1.8 mg/mg of guanidinated inhibitor. This is similar to published data for the native inhibitor (Kunitz and Northrop, 1936; Wu and Laskowski, 1955).

Removal of the Amino-Terminal Chain. The guanidinated inhibitor retained only one free amino group which was on the terminal arginine. Four steps of the Edman degradation were carried out as described above with part of the 100-mg preparation of Table I. At each step, the protein was separated by gel filtration from the PTH[1]-amino acids and other products. Figure 3 is a typical effluent curve: the separation of the protein from PTH-arginine. Peak I is the protein peak ($A_{280} > A_{263}$). The other peaks were not investigated, but synthetic PTH-arginine appeared in the same position as peak 3.

Table II shows the composition, with respect to the significant amino acids, and the trypsin-inhibiting activity at each step. The reaction was not quite complete at each step, so that by the third step only about one-half the residue was removed. Nevertheless, it is quite clear that activity was not lost by removal of the first three amino acids. By step 4, there was a significant loss of cystine, a common occurrence when cystinyl peptides are put through several steps of this reaction. Interpretation of the drop in activity after step 3 is therefore not possible at this time.

Amidination. Both the native and the guanidinated inhibitors were amidinated. There was no significant change in the activity.

The amidinated derivative of lysine is only partially stable to acid hydrolysis. To determine whether the reaction was complete, the amidinated proteins were converted to DNP derivatives (Hunter and Ludwig, 1962) as described above.

Hydrolysis and amino acid analysis were carried out on the DNP derivatives. With native, amidinated inhibitor, a large peak of acetamidyllysine, a small peak of lysine, and only a trace of DNP-lysine were found, indicating that all four residues of lysine had

[1] Abbreviation used: PTH, phenylthiohydantoin.

TABLE II: Subtractive Edman Degradation of the Guanidinated Inhibitor. Amino Acid Composition[a] and Trypsin-Inhibiting Activity[b] of the Original and Degraded Proteins.

	Moles of Amino Acid[c]/Mole of Protein					Activity % of
Step	Arg	Pro	Asp	Phe	½-Cys	Step 0
0	6.12	4.11	5.09	4.14	5.79	100[d]
1	*5.24*	4.06	5.07	4.14	5.85	92
2	4.99	*3.13*	4.98	3.87	5.71	104
3	5.10	3.32	*4.56*	4.08	5.72	83
4	5.16	—	4.43	*3.67*	5.48	67

[a] Amino acids not shown did not differ significantly from the composition previously reported (Kassell *et al.*, 1963) except for lysine and homoarginine, which corresponded to the values of Table I, last line. [b] Because of some variation in absolute values from one set of determinations to another, the activity is compared in each case to the activity of undegraded guanidinated inhibitor run at the same time. [c] Calculated on the basis of six residues of glycine per mole of protein. [d] The starting activity was 3.12 mg of trypsin inhibited/mg of inhibitor (Table I, last line).

been amidinated. When the DNP derivative was formed from *unmodified* inhibitor as a control, most of the lysine appeared as DNP-lysine just after arginine on the amino acid analyzer. With guanidinated, amidinated inhibitor, a very small amount of amidinated lysine could be detected on the amino acid analyzer.

Amidination also affected the amino-terminal group to some extent. This was shown by carrying out one step of the subtractive Edman degradation on the amidinated derivatives (Table III). With the native, amidinated inhibitor, 26% of the amino-terminal group was amidinated. With the guanidinated, amidinated inhibitor, this value was 36%. The presence of the entire six residues of arginine in the hydrolysates of the amidinated native and amidinated guanidinated inhibitors indicates that α-acetamidylarginine is not stable to acid hydrolysis.

Discussion

Since trypsin hydrolyzes at bonds involving lysine and arginine residues, these residues in the inhibitor are possible points at which the inhibitor might attach itself to trypsin. The present experiments were designed to test whether a lysine residue or the amino-terminal arginine–proline linkage of the inhibitor is necessary for the inhibitor–trypsin complex formation.

The two modifications of the lysine residues, guanidination and amidination, both substituted other positively charged groups ($NHC(=NH_2^+)NH_2$ and $NHC(=NH_2^+)CH_3$, respectively) for the ε-amino groups of lysine. Both derivatives retained the activity of the inhibitor.

These substitutions show that attachment of the ε-amino groups of lysine to trypsin is not necessary for the activity of the inhibitor. It is known that trypsin does not hydrolyze peptide bonds involving homoarginine (Weil and Telka, 1957; Shields *et al.*, 1959) or ε-acetamidyllysine (Hunter and Ludwig, 1962).

Positive charges in the areas of these residues are necessary, however, since acetylation decreased the activity of this inhibitor over 75% (Avineri *et al.*, 1963).[2] The loss of activity accompanying removal of the charged groups may result from a change in the conformation of the protein. Another, but less likely, explanation is that a positive charge in the general area of a lysine residue is needed for attachment to trypsin, but this charge does not have to be in the exact position of the ε-amino group. The effect of charge has also been discussed by Avineri *et al.* (1963; M. Rigbi, personal communication).

The guanidination of this protein with 2-methylisourea was similar to that of several other proteins (Chervenka and Wilcox, 1956; Klee and Richards, 1957; Geschwind and Li, 1957) in that the lysine residues were converted to homoarginine without modification of the α-amino group of the protein. In contrast, amidination also partially altered the α-amino group of the terminal arginyl residue. This showed that a free amino group was not needed for activity. The α-amino groups of insulin were also amidinated by this procedure (Hunter and Ludwig, 1962).

The amino-terminal arginyl–proline linkage was suggested as a likely functional region (Chauvet *et al.*, 1964) and previously discussed (Kassell and Laskowski, 1965), but no evidence was available at that time. Having an active guanidinated inhibitor, which retained the amino-terminal group as the only free amino group, provided an unique opportunity to determine the importance of the amino-terminal chain for the activity of the inhibitor. The first three amino acids (arginine, proline, and aspartic acids) were removed from the guanidinated inhibitor by the Edman reaction without loss of activity. The arginyl–proline linkage is, therefore, not the essential group.

These experiments have eliminated the four ε-amino groups of lysine and the arginyl–prolyl–aspartyl sequence of the amino terminal from consideration as functional parts of the inhibitor. The previous study excluded methionine (Kassell, 1964). In all, only eight of the 58 amino acid residues have been studied.

Five of those remaining are arginyl residues. The inhibition of trypsin by synthetic amidines (Mares-Guia and Shaw, 1965) and by agmatine derivatives (Rule and Lorand, 1964) supports the possibility that the arginine residues of the inhibitor may be involved. However, the binding of the pancreatic inhibitor to trypsin ($K_I = 2 \times 10^{-10}$ M at pH 7.0, Green and

[2] We have confirmed this.

TABLE III: Subtractive Edman Degradation of the Amidinated Inhibitors. Arginine Content

	Moles of Arginine/ Mole of Protein[a]			% of the Terminal Amino Group	
	Step			Remaining	
	0	1	Difference from 6.00	Free[b]	Amidinated[c]
Amidinated, native inhibitor	5.99	5.41	0.59	74	26
Amidinated, guanidinated inhibitor	6.12	5.49	0.51	64	36

[a] Calculated on the basis of six residues of glycine per mole of protein. [b] Assuming 80% removal of the terminal residue as with the guanidinated inhibitor. [c] By difference from 100%.

Work, 1953) is tighter by several orders of magnitude. For example, the K_I for *p*-aminobenzamidine, the best of the benzamidine inhibitors, is 8.25×10^{-6} M. This would indicate a more complex binding site for the protein inhibitors. Basic residues are also implicated by a theory of inhibitor action involving tryptic cleavage of a linkage in the inhibitor (Finkenstadt and Laskowski, 1965). The functions of the arginyl residues and of some of the other groups are under investigation.

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References

Anderer, F. A. (1965), *Z. Naturforsch. 20b*, 462, 499.
Anderer, F. A., and Hörnle, S. (1966), *J. Biol. Chem. 241*, 1568.
Avineri, R., Blauer, G., and Ribgi, M. (1963), *Israel J. Chem. 1*, 199.
Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966), *Biochim. Biophys. Acta 115*, 371.
Chauvet, J., Nouvel, G., and Acher, R. (1964), *Biochim. Biophys. Acta 92*, 200.
Chervenka, C. H., and Wilcox, P. E. (1956), *J. Biol. Chem. 222*, 635.
Dlouhá, V., Pospišivlová, D., Meloun, B., and Šorm, F. (1965), *Collection Czech. Chem. Commun. 30*, 1311.
Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.
Finkenstadt, W. R., and Laskowski, M., Jr. (1965), *J. Biol. Chem., 240*, PC963.
Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Analy. 2*, 360.
Geschwind, I. I., and Li, C. H. (1957), *Biochim. Biophys. Acta 25*, 171.
Green, N. M., and Work, E. (1953), *Biochem. J. 54*, 257.
Habeeb, A. F. S. A. (1960), *Can. J. Biochem. Physiol. 38*, 493.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Am. Chem. Soc. 84*, 3491.
Kassell, B. (1964), *Biochemistry 3*, 152.
Kassell, B., and Laskowski, M., Sr. (1964), *Biochem. Biophys. Res. Commun. 17*, 792.
Kassell, B., and Laskowski, M., Sr. (1965), *Biochem. Biophys. Res. Commun. 20*, 463.
Kassell, B., and Laskowski, M., Sr. (1966), *Acta Biochim. Polon.* (in press).
Kassell, B., Radicevic, M., Ansfield, M. J., and Laskowski, M., Sr. (1965), *Biochem. Biophys. Res. Commun. 18*, 255.
Kassell, B., Radicevic, M., Berlow, S., Peanasky, R. J., and Laskowski, M., Sr. (1963), *J. Biol. Chem. 238*, 3274.
Klee, W. A., and Richards, F. M. (1957), *J. Biol. Chem. 229*, 489.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.
Kunitz, M., and Northrop, J. H. (1936), *J. Gen. Physiol. 19*, 991.
Mares-Guia, M., and Shaw, E. (1965), *J. Biol. Chem. 240*, 1579.
McElvain, S. M., and Nelson, J. W. (1942), *J. Am. Chem. Soc. 64*, 1825.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Rule, N. G., and Lorand, L. (1964), *Biochim. Biophys. Acta 81*, 130.
Schroeder, W. A., and LeGette, J. (1953), *J. Am. Chem. Soc. 75*, 4612.
Shields, G. S., Hill, R. L., and Smith, E. L. (1959), *J. Biol. Chem. 234*, 1747.
Weil, L., and Telka, M. (1957), *Arch. Biochem. Biophys. 71*, 473.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 104.
Wu, F. C., and Laskowski, M., Sr. (1955), *J. Biol. Chem. 213*, 609.