REACTION OF ARGININE RESIDUES IN BASIC PANCREATIC TRYPSIN INHIBITOR WITH PHENYLGLYOXAL

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1. Introduction

Many topographical studies have been oriented towards explaining the mechanism of complex formation between basic pancreatic trypsin inhibitor (BPTI)** and trypsin. With respect to the specificity of trypsin, the arginine and lysine residues of BPTI can play an essential role in this interaction. There are six arginine (one of them *N*-terminal) and four lysine residues in the molecule.

The *N*-terminal arginine residue is not important for activity, because after its degradative elimination or substitution the remaining molecule retains inhibitory activity [1, 2]. The four lysine residues can be guanidylated or amidinated without loss of inhibitory activity [1, 2]. In contrast to this kind of substitution, which does not alter the charge of the residues, acetylation or succinylation of lysine residues destroys activity [1–3].

Since trypsin does not hydrolyse peptide bonds involving homoarginine or ε-acetamidyllysine [4–6], it appears that the free ε-amino groups of lysine are not necessary for the activity of the inhibitor. On the other hand, reactions of the trypsin–inhibitor complex and of the free inhibitor with *N*-carboxy-DL-alanine anhydride have shown that lysine residue no. 15 is buried in the complex (fig. 1) [7]. Consequently, this residue has been regarded as the specific site of interaction with trypsin.

The role of arginine residues nos. 17, 20, 39, 42 and 53 is not known. In the alkaline pH range, the inhibitor–trypsin complex is essentially undissociated at pH 10.5 and only partially dissociated at pH 11.8 [8], which seems to indicate that the critical interaction involves a guanidinium rather than an ε-amino group. The three-dimensional model resulting from

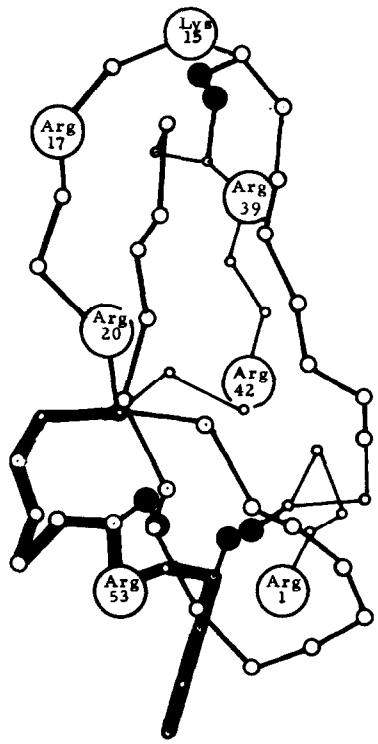

Fig. 1. Positions of the α-carbon atoms of the six arginine residues (black spots) and of lysine residue no. 15 within the polypeptide backbone of BPTI [9]. Thick line: fragment containing the unsubstituted arginine residue.

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** Abbreviation used: BPTI, basic pancreatic trypsin inhibitor.

X-ray analysis [9] shows that arginine residue no. 17 is close to lysine residue no. 15 (fig. 1). If this arginine is the specific site, the inertness of lysine residue no. 15 in the complex can be explained on the basis of steric hindrance.

The aim of this work was to study the relative importance of arginine residues for the activity of BPTI. Their specific reaction with phenylglyoxal was used for this purpose. This reagent preferentially forms a stable derivative with the guanidinium group. The *N*-terminal amino acid is deaminated by this treatment, whereas the ϵ-amino group of lysine reacts very slowly under appropriate conditions [10].

2. Materials and methods

Bovine pancreatic trypsin inhibitor was prepared as described [11]. Phenylglyoxal hydrate was prepared according to Riley and Gray [12].

The substitution reaction was performed in 0.2 N *N*-ethyl morpholine buffer adjusted on pH 7.5 by acetic acid. Forty milligrams of BPTI were dissolved in 2 ml of the morpholine buffer and a solution of 120 mg of phenylglyoxal hydrate in 6 ml of the same buffer was added. The reaction was performed for different intervals at 25°. To stop the reaction, 2 ml of acetic acid was added, the precipitate removed by centrifugation and washed twice with 10 ml portions of water. The combined supernatants were separated on a column of Sephadex G25 stabilized with 0.25% acetic acid, and fractions containing the modified inhibitor were lyophilized.

Activity was assayed by a modification of the method of Nagel et al. [13]; the inhibition of tryptic hydrolysis of benzoyl arginine *p*-nitroanilide was determined [14].

Oxidation of the inhibitor derivatives was performed according to Hirs [15], excess performic acid was removed from the product by dilution with water and passage through a Sephadex G25 column stabilized with 0.25% acetic acid. Amino acid analyses were performed by the method of Spackmann et al. [16] in a Beckmann Spinco amino acid analyzer after a 20 hr hydrolysis period. No effort was made to determine substituted or deaminated arginine derivatives.

Tryptic hydrolysis of the insoluble oxidized inhibitor derivative was performed in a Radiometer pH-stat. The substrate (7 μM) was solubilized at pH 7.8 and 37° to a final concentration of 1%, two 1 mg portions of TPCK–trypsin were added. The total time of digestion was 2 hr 40 min after which the digest was separated on a Sephadex G25 column. Fractions containing arginine peptides (Sakaguchi positive reaction) were pooled and purified further by vertical paper electrophoresis at pH 5.6 [17] followed by paper chromatography in the solvent system butanol–pyridine–acetic acid–water (30:20:6:24).

Digestion of the soluble oxidized inhibitor derivative containing one free arginine residue was performed in 0.1 N ammonium carbonate pH 7.6, 0.002 M $CaCl_2$. 1.2 μmole of substrate was incubated in 2 ml of buffer at 55° with 100 μl of 0.1% thermolysine for 30 min. The temperature was lowered to 37°, 100 μl of 0.1% chymotrypsin added and digestion terminated after a further 2 hr by freezing and lyophilization. The peptide mixture was fractionated by vertical electrophoresis and paper chromatography as described above.

Sakaguchi positive peptides resulting from both insoluble and soluble inhibitor derivatives were analyzed for quantitative amino acid composition.

3. Results and discussion

Reaction of BPTI with phenylglyoxal gave soluble and insoluble products which differed in their composition. The amount of precipitate increased with time (table 1). The soluble and insoluble fractions were treated independently.

Amino acid analyses (table 2) have shown that five out of six arginine residues present in the inhibitor

Table 1
Relative yield of soluble and insoluble substituted BPTI as function of reaction time.

Time (hr)	Yield (%)	
	Soluble	Insoluble
0	100	–
2.5	58	10
7	30	62
21	–	94

disappeared. This is accounted for by deamination of the *N*-terminal arginine by the reagent [10] and substitution of the other four residues. In the soluble products all lysine residues remained intact. Even after a prolonged reaction time one of the six arginines and three of four lysines remain unaltered.

All soluble products remain fully active. Fig. 2 shows that the binding ratio of the inhibitor and trypsin remains equimolar for the derivative with five out of six the arginines substituted. Further substitution of one of the four lysine residues causes a loss of solubility in acidic and neutral media. No inhibitory effect of this insoluble derivative on trypsin could be detected.

Both soluble and insoluble derivatives containing one intact arginine residue were oxidized and subjected to partial enzymatic hydrolysis. Fragments containing unsubstituted arginine were isolated. Two peptides, corresponding to the sequence 47–53 and 47–58 of the inhibitor molecule, were isolated from the tryptic digest of the insoluble product. Fractionation of the

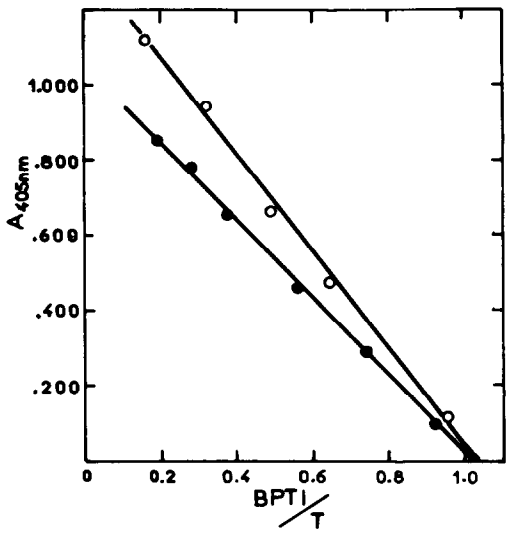

Fig. 2. Inhibition of trypsin by native BPTI (○——○) and by BPTI with five out of six arginines modified (●——●). Dependence of residual trypsin activity on BPTI-trypsin molar ratio.

Table 2
Amino acid content of substituted BPTI.

Reaction time (hr)	0	2.5	7	7	21
Solubility	+	+	+	–	–
Activity	+	+	+	–	–
Amino acids					
Lys	4	4.3	4.2	3.1	3.1
His	–	–	–	–	–
Arg	6	2.9	1.3	0.8	0.7
Asp	5	5.0	5.0	4.8	4.9
Thr	3	2.9	2.9	2.8	2.9
Ser	1	1.0	1.0	1.0	1.0
Glu	3	3.0	3.1	3.0	3.1
Pro	4	3.9	4.0	4.2	4.0
Gly	6	6.0	6.1	6.2	6.1
Ala	6	6.0	5.9	5.8	5.9
Val	1	0.8	1.0	0.9	0.9
Met	1	1.1	0.9	1.0	1.0
Ile	2	1.2	1.2	1.1	1.1
Leu	2	2.0	2.0	1.9	2.0
Tyr	4	3.5	3.8	3.7	3.8
Phe	4	4.3	4.1	4.0	4.0

For zero time, numbers of residues are derived from the structural formula. The low yield of Ile is due to the short time of hydrolysis (20 hr). Cystine content was not determined.

combined thermolysine and chymotryptic digest of the soluble product yielded a Sakaguchi-positive fragment whose analysis fitted the sequence 45–58. The only arginine residue which resisted substitution with phenylglyoxal is therefore arginine residue no. 53 (fig. 1).

The specificity of the substitution reaction with phenylglyoxal corresponds to that described for other proteins [10], namely arginine residues are substituted preferentially. In a small fraction, whose importance increased with a longer reaction time, one of the four lysine residues also reacted. It could be easily separated on the basis of its low solubility.

The lack of reactivity of arginine residue no. 53 cannot be explained from what we at present know about the conformation of BPTI. This residue is situated in a helical segment forming three turns near the *C*-terminus and it should be exposed to the surrounding solvent [9].

From the experimental results, a clear conclusion can be drawn with regard to the relative importance of arginine and lysine residues for the inhibitory activity of BPTI. This inhibitor remains fully active when arginine residues no. 1, 17, 20, 39 and 42 have been modified. The non-involvement of *N*-terminal arginine was shown some time ago [1, 2]. Arginine

no. 53 is spacially remote from lysine no. 15 (fig. 1), which has been shown to be directly involved or shielded in the complex. Thus, the participation of any of the six arginine residues in the activity of BPTI is highly improbable and lysine no. 15 is the only basic residue involved in the complex formation.

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