ROLE OF LYSINE 18 IN ACTIVE CENTER OF COW COLOSTRUM TRYPSIN INHIBITOR

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1. Introduction

The mechanism of formation of complexes of trypsin with naturally-occurring trypsin inhibitors requires the participation of a specific lysine or arginine residue in the inhibitor molecule. With some inhibitors, the specific arginyl or lysyl bond is actually cleaved by trypsin in the process of complex formation [1–4]. It has been shown [5] that a lysine residue of responsible for the activity of cow colostrum trypsin inhibitor (CTI). When the free amino groups are modified, the inhibitor loses its activity. The CTI molecule consists of a single polypeptide chain of 67 amino acids to which a non-protein moiety is bound [6]. There are three free amino groups in the chain, two lysine residues in positions 18 and 66 and one *N*-terminal phenylalanine [7]. The present paper provides evidence that lysine 18 plays a role in the activity of this inhibitor.

2. Materials and methods

The trypsin inhibitor which occurs in large quantities in cow colostrum was used as starting material [8]. Trypsin was a twice recrystallized product of Léčiva, Prague. The trypsin activity was determined by the method of Nagel et al. [9]. The activity of the inhibitor was measured by its reaction with trypsin [10]. The trypsin-trypsin-inhibitor complex was prepared by mixing a solution of the inhibitor (15 mg) with trypsin (30 mg) in distilled water and by adjusting the pH to 8.2 with ammonium carbonate. After the small precipitate had been centrifuged off, the clear solution was applied to a 2.5 × 34 cm column of Sephadex G-100. The column was eluted with 0.01 M ammonium carbonate at a rate of 6 ml/hr. Fractions containing the pure complex were pooled and freeze-dried.

Free CTI (7 mg) was carbamylated [5] by 0.3 M potassium cyanate in 0.05 M tris-buffer at pH 8.5, 20 hr at 37°. The CTI-TR (18 mg) complex was carbamylated by 0.6 M cyanate in 0.01 M tris-buffer at pH 8.5, 20 hr at 37°. Guanidination [11] of both the free inhibitor and of the complex was carried out in 0.5 M *o*-methylisourea – 0.01 M – EDTA at pH 9.5, 70 hr at 25°. Free CTI (15 mg) was dissolved in 3 ml, the complex (12 mg) in 2.4 ml. The modified proteins were freed of the excess of the reagents by desalting on columns of Sephadex G-25 equilibrated either with 0.1 M ammonium carbonate (carbamylated derivatives) or with 0.2 % formic acid (guanidinated derivatives).

The dissociation of the modified complexes was effected by dissolving the complex in distilled water and by adding the same volume of 5% trichloroacetic acid (TCA). The mixture was allowed to stand 15 min at 37°. Precipitated trypsin was centrifuged off and the clear solution of the modified inhibitor was freed from TCA by desalting on a Sephadex G-25 column equilibrated with 0.2% formic acid. The pure inhibitor was freeze-dried.

The quantitative amino acid composition of the

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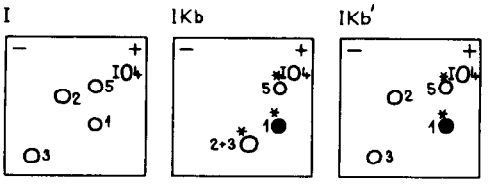

Fig. 1. Peptide maps of tryptic digests of oxidized native inhibitor (I), of inhibitor carbamylated in free form (IKb), and of inhibitor released from carbamylated complex (IKb'). The peptides are numbered with respect to the order of tryptic peptides in the molecule [7]. Lysine 18 is at the *C*-terminus of peptide 2, lysine 66 is at the *N*-terminus of peptide 5. The *N*-terminal peptide marked in black is not stained by ninhydrin in IKb and IKb'. The presence of the peptide was shown by chlorination.

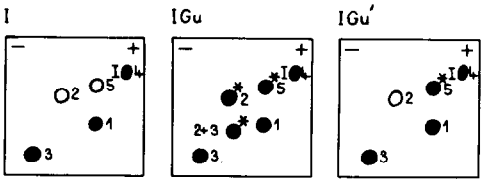

Fig. 2. Peptide maps of tryptic digest of oxidized native inhibitor (I), of inhibitor guanidinated in free form (IGv), and of inhibitor released form the guanidinated complex (IGv'). The peptides marked black gave a positive Sakaguchi reaction. In peptides marked by an asterisk, the presence of homoarginine was determined by quantitative amino acid analysis. The peptide (2-3) was formed by incomplete cleavage of the bond at the carboxyl side of homoarginine 18.

native modified inhibitors was measured on a Beckman-Spinco amino acid analyzer.

The disulfide bonds of the native or modified inhibitors were cleaved by performic acid oxidation [13]. The oxidized proteins were digested with trypsin in distilled water. The pH of the solution was adjusted to 8.4 with ammonium carbonate. The digestion was allowed to proceed 2 hr at 37°. Peptide maps of the tryptic digests of the oxidized proteins were obtained by a combination of electrophoresis (60 V/cm, pH 1.7, formic acid-acetic acid-water, 1:3:16) and descending chromatography (butanol-pyridine-acetic acid-water, 30:20:6:24). The spots of individual peptides were detected by 0.2% ninhydrin and by chlorination [14]. Peptides containing arginine or homoarginine were stained by the Sakaguchi reaction [15]. The presence of peptides bearing a carbamyl group was revealed by the Ehrlich reagent by dipping the chromatogram into 0.5% benzaldehyde in hydrochloric acid-ethanol(1:4 v/v). In preparative-scale experiments, the peptide maps were dipped into 0.01% ninhydrin in acetone, the spots cut out, the products eluted with distilled water and subjected to quantitative amino acid analysis.

Table 1
Analytical values of amino acid residues in native inhibitor (I), in inhibitor carbamylated in free form (IKb) or in complex (IKb'), and in inhibitor guanidinated in free form (IGv) and in complex (IGv').

Amino acid	I	IKb	IKb'	IGv	IGv'
Lysine	2.0	0.6*	1.6*	–	1.0
Arginine	3.0	3.1	3.0	3.2	3.1
Homocitrulline	–	1.4*	0.3*	–	–
Homoarginine	–	–	–	1.8	0.9

* The higher values of lysine and the lower values of homocitrulline in IKb and IKb' can be accounted for by partial decomposition of homocitrulline to lysine during the hydrolysis.

3. Results

The peptide maps of the tryptic digests of the oxidized native cow colostrum inhibitor were compared with the peptide maps obtained from the digests of the inhibitor modified by carbamylation or guanidination, either in the free form or as a complex with trypsin. As seen in fig. 1 and table 1, both lysine residues of the inhibitor which had been carbamylated in the free form (IKb) were converted to homocitrulline residues. The free *N*-terminal amino group was also carbamylated. When, however, the inhibitor was bound to trypsin as a complex during carbamylation (IKb'), then lysine 18 remained intact. The fully carbamylated derivative lost its ability to inhibit trypsin (as assayed with benzoyl-arginine *p*-nitroanilide). In contrast, the activity of the derivative with intact lysine 18 was practically the same as the activity of the native protein. Guanidination of free CTI converts lysine residues into homo-

arginines (fig. 2, table 1). After guanidination of the CTI-trypsin complex, lysine 18 again remained intact. Both modified proteins retained their activity toward trypsin.

The results presented here show that for CTI to inhibit trypsin, a basic amino acid residue at position 18 toward which trypsin is sensitive is essential. When the inhibitor is bound to trypsin as a complex, lysine 18 is resistant to the modifying agents. The modification of lysine 66 and of the *N*-terminal amino acid residue does not affect the inhibitory activity of CTI towards trypsin.

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