A STUDY OF THE COMPLEX OF TRYPSIN WITH ITS PANCREATIC INHIBITOR

V.Dlouhá, B.Keil, F.Šorm

Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, Prague, Czechoslovakia

Received March 1, 1968

The formation of a complex of trypsin with its basic pancreatic inhibitor (BPTI) represents a rather rare model which offers a chance for investigating the interaction of two biologically active proteins whose primary structures have been known (Mikeš et al., 1966; Walsh and Neurath, 1964; Dlouhá et al., 1965a; Kassell et al., 1965; Chauvet et al., 1964). Numerous earlier studies have produced evidence that the character of the inhibition is competitive (Green,1953) and that the interaction is stoichiometric. The complex is very stable in alkaline media. Its dissociation constant is 2×10^{-10} (Green and Work, 1953) while in acid media the stability of the complex is lower. The dissociation in acid media is reversible.

Recently, after the primary structure of BPTI has been completed, attention has turned to studies designed to elucidate, via chemical modification, the character of those functional groups of BPTI which are responsible for its interaction with trypsin. In our own studies on the primary structure of the inhibitor we found that BPTI is resistant to hydrolysis with proteolytic enzymes if its disulfide bonds are intact. Since the trypsin--trypsin inhibitor complex is stable in alkaline media we tried to split it proteolytically under conditions which did not bring about its dissociation. The analysis of the combined tryptic-chymotryptic digest of the complex leads us to postulate the existence of more than one bond between the two partners involved in the complex. The native state of trypsin is obviously very important for the formation of the complex and of bonds which are so stable that they are retained even when the trypsin portion of the complex has been digested. The resistance of BPTI to hydrolysis with proteolytic enzymes is due to the three-dimensional arrangement of its molecule since the inhibitor remains undigested even in the complex where its inhibitory effect is neutralized.

Material and Methods

Trypsin used for the formation of the complex and for enzymatic digestion was a four-times crystallized preparation. Chymotrypsin was crystallized four times. Both enzymes were purchased from Léčiva, Prague. BPTI was prepared by the method of Dlouhá et al., (1965b).

Quantitative amino acid analyses were performed after hydrolysis of the samples in 6N HCl at 105° for 48 hours.

The homogenity of samples was checked by starch-gel electrophoresis in 4M urea according to Cohen and Porter (1964) and by low-voltage paper electrophoresis in the buffer system formic acid - acetic acid - water (1:3:16) at pH 1.9 and 300 volts, 16 hours.

The conditions of N-terminal end-group analysis and oxidation were described in the preceding paper (Dlouhá, 1965a).

The activity of BPTI was determined according to Nagel et al. (1966) by measuring absorbance at 405 nm after hydrolysis of benzoyl-arginine-p-nitroanilide-HCl (BAPA) wi trypsin. A 100- µl aliquot was withdrawn from each fraction and 2.5 ml of 0.1M Tris-H buffer at pH 8 and 100 µl of a trypsin solution containing 0.04 mg of enzyme in 0.001 M HCl/0.02 M $CaCl_2$ was added. After 5 min. of incubation at 24°C BAPA was added. The reaction mixture was incubated 10 min. at 37°C and then acidified by the addition of 0.5 ml of 35% acetic acid. The absorbance of the solution was measured at 405 nm. I experiments where the dissociation of the complex was brought about by trichloroaceti acid (TCA), 100 µl of 5% TCA was added to 100 µl of the tested sample. After 5 min. the buffer and trypsin were added and the subsequent procedure was the same as descril above. The inhibitory activity was determined as the difference between the tryptic activity of a standard and the tryptic activity of the assayed fraction and then calculated per µg of inhibited trypsin with the aid of a standard curve.

For the determination of the tryptic activity of the complex a solution of the latter was used instead of the trypsin solution.

Preparation of the complex. Trypsin (435 mg) was dissolved in 150 ml of distilled

water and the pH of the solution was adjusted to 8.4 by the addition of $(NH_4)_2CO_3$. To this solution 121 mg of BPTI was added.

The formed complex was digested with trypsin 12 hours at 37°C. Trypsin was added three times at equal intervals. The final enzyme to substrate ratio was 1:50. Immediately afterwards the complex was digested with chymotrypsin under the same conditions.

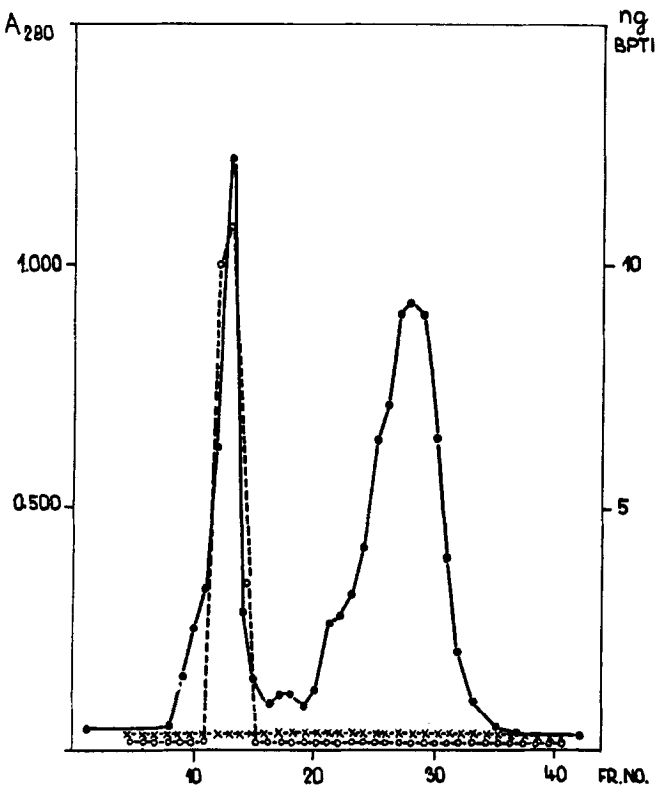

Fig. 1

Fractionation of Tryptic-Chymotryptic Digest of Trypsin-Trypsin Inhibitor Complex (75 ml) on Sephadex G-50.

Dimension of column 6 x 80 cm, eluent 0.01 M $(NH_4)_2CO_3$, flow rate 100 ml/hr., fraction size 100 ml.

x - x - value of inhibitory activity (μg of BPTI/100 μl of sample) without incubation of samples with trichloroaceticacid; o - o - value of inhibitory activity after incubation of aliquots of fractions with trichloroacetic acid (μg BPTI) 100 μl of sample).

Results

The formation of the complex was checked by determination of tryptic and inhibitory activity of the reaction mixture. Neither free inhibitory activity nor tryptic activity were found after 3 min. Similarly, after the completion of combined tryptic-chymotryptic hydrolysis of the complex, the reaction mixture did not show any inhibitory or tryptic activity. On the other hand, full activity of the inhibitor originally added was proved after incubation of the reaction mixture with trichloroacetic acid.

The hydrolysate was subjected to fractionation on Sephadex G-50 (Fig.1). The course of the fractionation was tested by determination of the inhibitory activity of samples before and after incubation with TCA. As can be seen in Fig.1, no inhibitory activity was found in any of the fractions when the tested samples were not incubated with TCA. After incubation of aliquots of samples with trichloroacetic acid the inhibitory activity was detected in the first peak containing the high-molecular weight amount.

When this amount was submitted to rechromatography on Sephadex G-75, only minor impurities were separated and the main peak was homogeneous. Its amino acid composition is shown in Table I.

Dissociation of the high-molecular weight fragment of the complex.

The dissociation of the high-molecular weight fragment of the complex in acid media on an analytical scale (20 mg) was effected in 0.01 M HCl and 1M NaCl during 3 hours at 24°. The dissociation proceeded only to 50%.

The stability of the bond between BPTI and the contact site in trypsin as a function of the ionic strength was examined in an alkaline medium. The results show that the high--molecular weight fragment of the complex does not dissociate even after incubation in 2M NaCl/Tris-buffer at pH 8.0 for 1 hour at 24°C. On the other hand, oxidation by performi acid leads to complete dissociation of the high-molecular weight fragment of the complex.

The fractionation of the oxidized dissociated higs-molecular weight amount of the complex was effected on SE-Sephadex (Fig.2) and four fractions were obtained. Fraction I was desalted on Sephadex G-25 and was found electrophoretically homogeneous. Its N-terminal-group was alanine. The amino acid composition of the fraction is given in Table I.

Table I

Amino Acid Composition of High-Molecular Weight Fragment of Complex and of Its Individual Components after Dissociation

Amino Acid	I (Fig. 2) Peptide Containing Active Serine of Trypsin	IV - 2 (Fig. 3) BPTI Isolated After Dissociation of Complex	High-Molecular Weight Fragment of Complex	Amino-Acid Composition of High-Molecular Weight Fragment of Complex minus BPTI	Amino Acid Composition of BPTI	Amino Acid Composition of Trypsin
Lys	3.16	4.3	10.05	6	4	14
His	-	-	2.00	2	-	3
Arg	-	6.-	6.04	-	6	2
$CySO_3H$	3.65	5.8	12.15	6	6	12
Asp	4.33	5.1	16.00	11	5	22
$MeSO_2$	0.86	0.6	1.70	1	1	2
Thr	2.26	3.0	7.20	4	3	10
Ser	7.80	1.02	19.80	19	1	34
Glu	4.30	3.17	10.00	7	3	14
Pro	3.14	4.02	9.18	5	4	8
Gly	8.80	5.84	20.20	14	6	25
Ala	3.12	5.89	11.20	5	6	14
Val	3.20	0.94	8.00	7	1	17
i-Leu	2.86	2.00	9.00	7	2	15
Leu	4.00	2.07	9.20	7	2	14
Tyr	1.90	3.56	8.00	4	4	10
Phe	0.98	3.78	5.96	2	4	3
Trp	0.98	3.78	5.96	2	-	4
	54	58	166	108	58	223

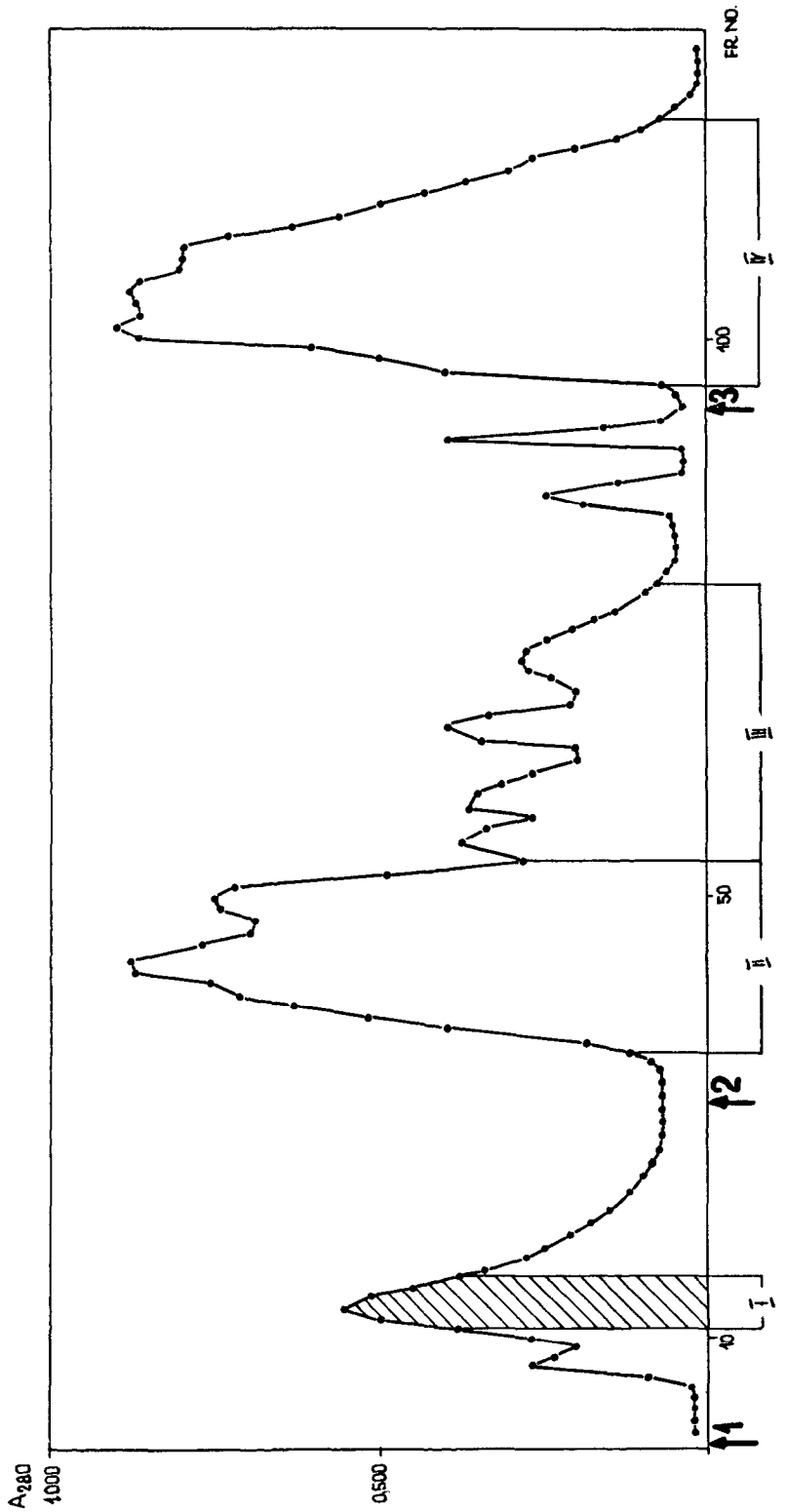

Fig. 2

Fractionation of Oxidized High-Molecular Weight Amount of Complex on SE-Sephadex

Dimensions of column 2.5 x 25 cm, flow rate 50 ml/hr 1st eluent: 0.01 M potassium formate in 2M urea, pH 2.6.2nd eluent: linear gradient of 0.01M - 0.5M KCl . Volume of mixer 500 ml. 3rd eluent: 1M KCl adjusted to pH 9.0 with NaOH.

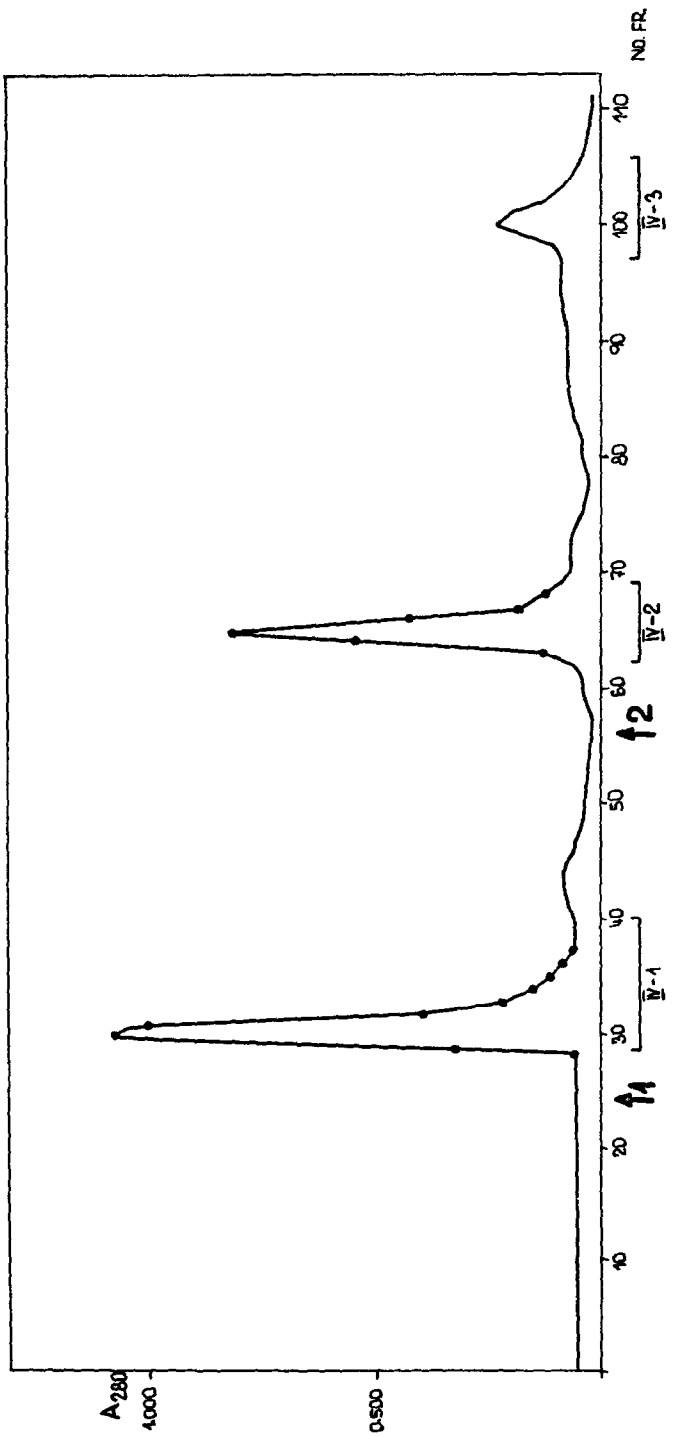

Fig. 3

Rechromatography of Peak IV on DEAE-Sephadex

Dimension of column 2.5 x 10 cm, flow rate 40 ml/hr, fraction size 10 ml - 1st eluent: 0.005 M phosphate buffer at pH 6.5, 2nd eluent: linear gradient of 0.005M-0.5M KCl at pH 6.5. Volume of mixer 500 ml.

Fraction IV was subjected to rechromatography on DEAE-Sephadex and afforded 3 peaks (Fig.3). Peak IV-2 corresponds in its amino acid composition and N-terminal group (Arg) to the parent trypsin inhibitor (Table I).

Discussion

The results of our investigation of the complex of trypsin with BPTI imply that hydrogen bonds are not the main type of bonds holding together the two components. While the stability of hydrogen bonds depends on the ionic strength of the medium the complex studied by us was stable at pH 8.0 even in 2M NaCl. Only partial dissociation was observed in an acid medium (pH 1.9).

The irreversible dissociation of the high-molecular weight amount of the complex, an amount which contains both the inhibitor and the contact site of trypsin for binding of the inhibitor, indicates that these two proteins can interact only when the three-dimensional structures of their molecules are intact. The bonds which hold together the complex, however, are so stable that they are not interrupted even when the complex is subjected to profound proteolytical cleavage.

The results of amino acid analysis of BPTI isolated from the proteolyzed complex (Table I) and of end-group analysis indicate that the inhibitor is not split in the process of complex formation. Therefore the mechanism of inhibition of trypsin by BPTI is obviously different from the mechanism proposed for the interaction of soybean inhibitor with trypsin by Firkenstadt and Laskowski (1965). The resistance of BPTI to proteolytic enzymes is due to the rigid three-dimensional structure of its molecule since the inhibitor remains intact even when it is bound in the complex and its inhibitory effect is therefore neutralized.

In view of the fact that the inhibitor remains intact, the amino acid composition of the high-molecular weight fragment of the complex (Table I) can provide first data on the amino acid composition of that portion of trypsin which is bound to the inhibitor. This portion represents approximately 48% of the original molecule of trypsin. Obviously, the regions of the molecule which are not digested at all when trypsin is bound in the complex are those which are sterically protected by the inhibitor or buried in the interior (Fig.5).

The analysis of peptide I shows that this fragment is derived from the region of the

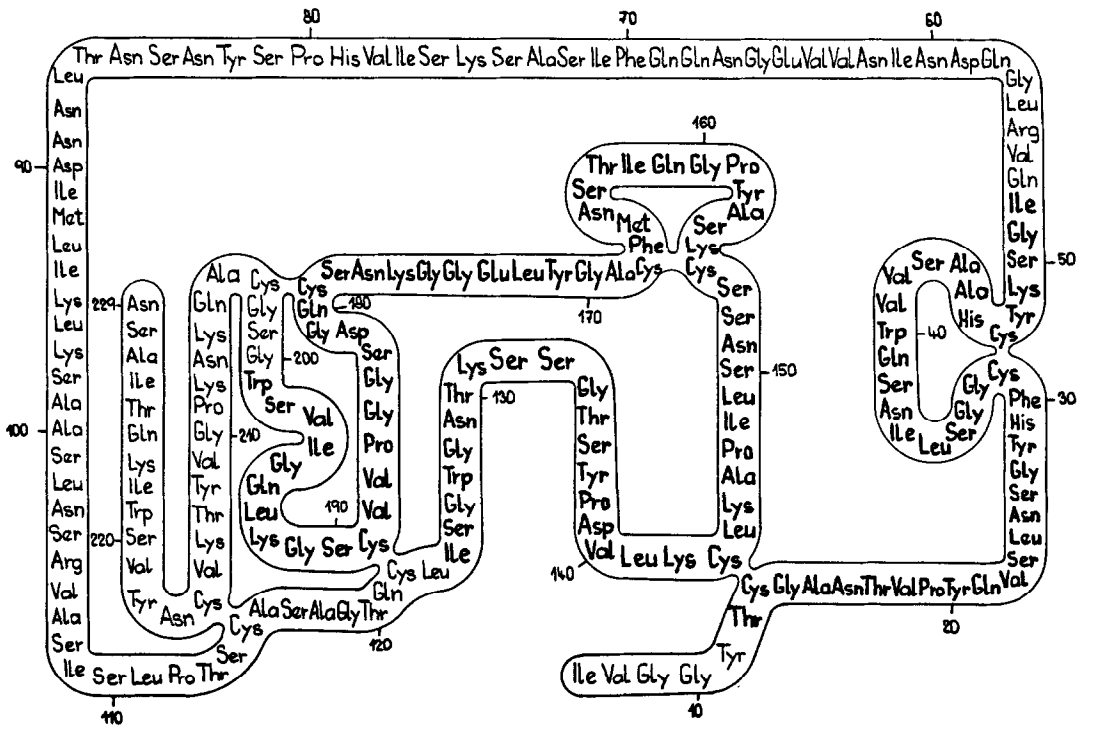

Fig. 4

Covalent Structure of Trypsin (Mikeš et al., 1966)
The numbering system for amino acid residues of trypsin is that used for trypsinogen.

molecule of trypsin between residues Nos 146 and 198, i.e. that it involves also the active serine (No 183) and most likely tryptophan No 199 which was probably present as the C-terminal amino acid of this chymotryptic fragment and then destroyed by oxidation (Fig.4).

From the amino acid composition of the high-molecular weight fragment of the complex we can assume that the latter contains in addition to BPTI and peptide I also other fragments in which histidine is present. Since BPTI does not contain histidine this portion of the complex is derived from trypsin.

As we expected, the fractionation of the dissociated high-molecular weight fragment of the complex (Fig.2) revealed the presence of additional minor peptides which were all derived from the same region split at different sites.

Fractions IV - I and IV - III (Fig.3) contained even after rechromatography a mixture

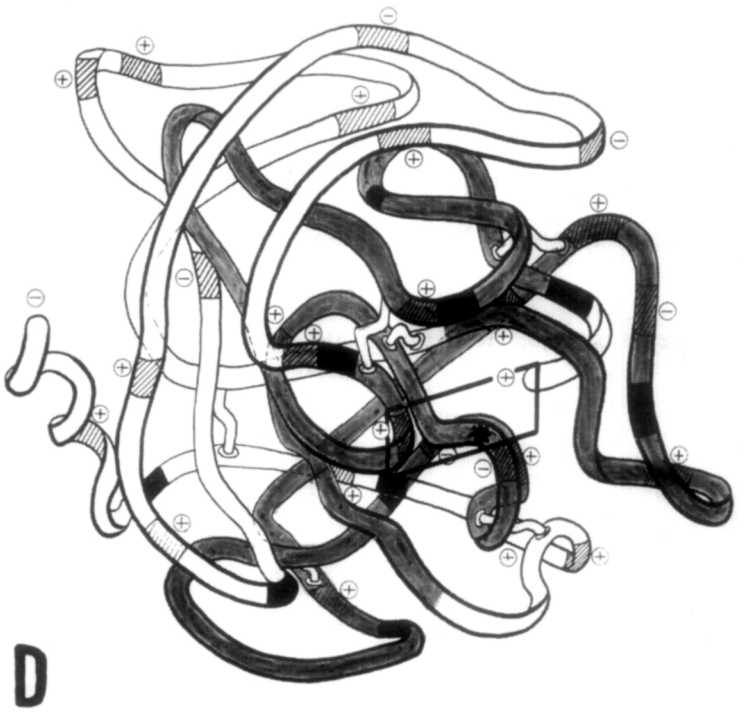

Fig. 5

Three-Dimensional Structure of Trypsin (Keil et al., in press)

The trypsin moiety of the high-molecular weight amount of the complex, the active center is outlined by a rectangle. Aspartic acid residues Nos 59, 139 and 182 are marked ⊖

of peptides in which two histidine residues were present. For lack of material these peptides could not be isolated in completely pure state.

As can be seen in the three-dimensional model of trypsin (Keil et al., in press), which has resulted from our studies on similarities in tertiary structures of chymotrypsin and trypsin (Fig.5), the segment involving active histidine No 46 is in immediate vicinity of the active serine, in agreement with our finding of histidine-containing fragments. It has been also known that lysine residue No 15 of BPTI plays an important role in its interaction with trypsin (Chauvet and Acher, 1967) and that the conversion of half - cystine residue No 14 into the S-carboxymethyl-cysteine residue strongly influences the bond between the inhibitor and trypsin (Meloun et al., in press). From these facts and the

scheme shown in Fig.5, the site of contact in the trypsin molecule can be allocated to the region of the active center outlined by three negatively charged aspartic acid residues (Nos 59, 139, and 182). According to our concept lysine No 15 in BPTI can be bound to aspartic acid No 182. An analogous ionic interaction of aspartic acid No 194 with isoleucine No 16, an interaction which stabilizes the active configuration of the enzyme, exists in chymotrypsin (Matthews et al., 1967).

We wish to thank Dr D.Pospíšilová for kindly supplying us with BPTI.

References

Mikeš O., Holeyšovský V., Tomášek V., Šorm F., Bioch.Bioph.Res.Comm. 24, 346 (1966).

Walsh K.A., Neurath H., Proc.Natl.Acad.Sc.U.S., 52, 884 (1964).

Dlouhá V., Pospíšilová D., Meloun B., Šorm F., Collection Czecholsov.Chem.Comm. 30, 1311 (1965 a).

Kassell B., Radicevic M., Ansfield M.J., Laskowski M.,Sr., Bioch.Bioph.Res.Comm. 18, 255 (1965).

Chauvet J., Nouvel G., Acher R.: Bioch.Bioph.Acta 92, 200 (1964).

Green N.M., J.Biol.Chem. 205, 535 (1953).

Laskowski M., Laskowski M.Jr.: Adv.in Prot.Chem. IX, 203 (1954).

Green N.M., Work E.: Biochem.J. 54, 347 (1953).

Dlouhá V., Neuwirthová J., Meloun B., Šorm E, Collection Czechoslov.Chem.Comm. 30, 1705 (1965 b).

Cohen S., Porter R.R.: Biochem.J. 90, 278 (1964).

Nagel W., Willig F., Peschke W., Schmidt F.N.: Z.Physiol.Chem. 340, 1 (1966).

Firkenstadt W.R., Laskowski M.Jr., J.Biol.Chem. 240, PC962 (1965).

Chauvet J., Acher R., J.Biol.Chem. 242, 4274 (1967).

Meloun B., Frič I., Šorm F., Collection Czechoslov.Chem.Comm. in press.

Matthews B.W., Sigler P.B., Henderson R., Blow D.M., Nature, 214, 652 (1967).

Keil B., Dlouhá V., Holeyšovský V., Šorm F., Collection Czechoslov.Chem.Comm. in press.