PREPARATION OF 14,38-BIS-[S-CARBAMIDOMETHYL]-(BASIC TRYPSIN INHIBITOR) POSSESSING FULL BIOLOGICAL ACTIVITY[1]

Wan-Kyng Liu and Johannes Meienhofer

Children's Cancer Research Foundation, The Children's Hospital Medical Center and Harvard Medical School, Boston, Massachusetts 02115

Received April 4, 1968

Summary: Dithiothreitol reduced selectively the disulfide bond linking the half cystine residues 14 and 38 of bovine basic trypsin inhibitor in the absence of urea at pH 8.2. Iodoacetamide treatment afforded 14,38-bis-[S-carbamidomethyl]-(basic trypsin inhibitor) possessing full biological activity.

The bovine basic pancreatic trypsin inhibitor (Kunitz/Northrop inhibitor) or kallikrein inhibitor (called "basic trypsin inhibitor" throughout this communication, abbrev. TI) represents a polypeptide of known structure (Kassell and Laskowski, Sr., 1965), see Fig. 1. It consists of 58 amino acids and contains three intra-chain disulfide bonds linking the half cystine residues at positions 5 and 55, 14 and 38, and 30 and 51.

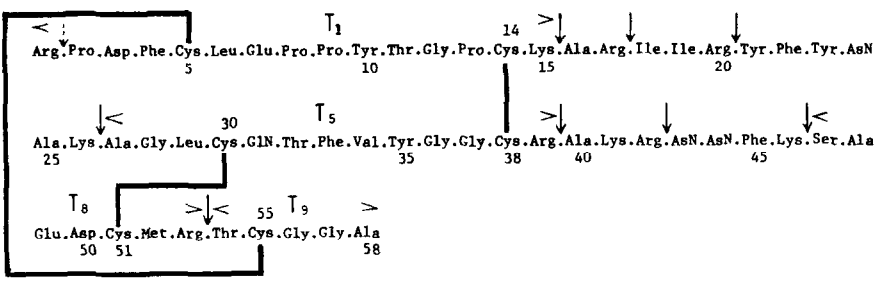

Figure 1. Structure of basic trypsin inhibitor (Kassell and Laskowski, Sr., 1965). ↓ indicates point of tryptic attack. The tryptic peptides are designated as described by Chauvet et al. (1966), [< T_n >].

In this work a selective cleavage of one disulfide bond of the inhibitor (TI) was obtained by treatment with 2,3-dithiothreitol (Cleland, 1964) in the absence of urea using the procedure described by Bewley, Dixon and Li (1968).

(1) Supported in part by Public Health Service Research Grant C-6516 from the National Cancer Institute, and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health, and by The Albert Lasker Foundation, New York, and The Alvan T. and Viola D. Fuller Cancer Research Unit Grant, American Cancer Society (Massachusetts Division) Inc.

Treatment with iodoacetamide gave a bis-[S-carbamidomethyl] derivative which possessed full biological activity, as shown by total inhibition of the cleavage of benzoyl-DL-arginine-p-nitranilide, BAPA, (Erlanger et al., 1961) and of p-toluenesulfonyl-L-arginine-methyl ester, TAME, (Hummel, 1959) by trypsin under standard conditions. However, treatment of the partially reduced TI with iodoacetic acid gave a totally inactive bis-[S-carboxymethyl] derivative(2).

The homogeneity of both bis-[S-alkylated] inhibitor derivatives was demonstrated by correct amino acid analyses (Table 1) and by disc electrophoresis on polyacrylamide gel. Alkylation with α-C^{14}-iodoacetic acid, followed by oxidation, tryptic digestion, and peptide mapping (Katz et al.,1959) gave two radioactive spots, T_1 and T_5 (Fig.2), which provided added evidence for a specific reduction and alkylation of one single disulfide bond. Amino acid analyses of T_1 and T_5 (Table 1) proved unequivocally that the selectively reduced disulfide bond was the 14-38 bond. Thus the biologically active derivative was 14,38-bis-[S-carbamidomethyl]-(basic trypsin inhibitor).

It has been reported (Dixon and Li, 1966; Bewley, Dixon, and Li, 1968) that the complete reduction and S-carbamidomethylation of human growth hormone resulted in a derivative with a biological activity comparable to that of the native hormone. Recently other investigators (Light and Sinha, 1967; Kress and Laskowski, Sr., 1967; Neuman et al., 1967) reported retention of full biological activity after selective reduction of a part of the constituent disulfide bonds without subsequent alkylation in trypsin, trypsin inhibitor, and ribonuclease, respectively, which indicated that the native conformation remained essentially unchanged in each case.

It might be speculated that S-carbamidomethylation of the half cystine residues 14 and 38 of the basic trypsin inhibitor did not affect its conformation,

(2) During the course of this work Kress and Laskowski, Sr., (1967) reported about the observation of full biological activity upon partial reduction of TI with sodium borohydride and loss of activity upon carboxymethylation.

Table 1 Amino Acid Analysis (Spackman et al., 1958) (1)

Amino Acid	Native Inhibitor		TI-Derivatives (2) bis NM	bis CM	hexa NM	hexa CM	Peptide T_1		Peptide T_5	
	Found	Theory					Found	Theory	Found	Theory
Lys	3.9	4.0	4.2	4.2	4.3	4.2	1.0	1.0		
Arg	5.8	6.0	6.2	6.3	5.9	5.8	1.1	1.0	1.0	1.0
CM-Cys			2.0	1.9	5.9	5.7				
Cys-SO_3H							1.6(5)	2.0	1.7(5)	2.0
Asp	5.2	5.0	4.9	4.9	5.0	5.0	1.0	1.0		
Thr	3.0	3.0	2.8	2.7	2.8	2.7	0.9	1.0	1.1	1.0
Ser	1.0	1.0	1.3	0.9	1.0	1.1				
Glu	3.2	3.0	3.2	3.1	3.1	3.1	1.0	1.0	1.4	1.0
Pro	3.8	4.0	4.0	3.7	4.1	3.7	4.2	4.0		
Gly	5.8	6.0	6.0	6.0	6.0	6.0	1.0	1.0	3.0	3.0
Ala	6.0	6.0	6.0	6.0	6.0	6.0			1.0	1.0
1/2 Cys	4.8(3)	6.0	2.6(3)	2.6(3)						
Val	1.0	1.0	0.9	1.0	1.1	1.0			1.1	1.0
Met	0.9	1.0	1.1	1.0	1.0	0.8				
Ile	1.4(4)	2.0	1.7	1.7	1.8	1.7				
Leu	2.1	2.0	2.2	2.1	2.2	2.2	1.0	1.0	1.2	1.0
Tyr	3.8	4.0	4.0	4.1	4.1	4.0	1.0	1.0	1.1	1.0
Phe	4.0	4.0	4.1	4.1	4.1	4.0	1.0	1.0	1.1	1.0

(1) Amino acid analysis was done on 24 hrs hydrolysates in a Phoenix analyzer Model M-6800. The values reported are uncorrected for decomposition or incomplete hydrolysis. (2) bis NM = 14,38-bis-[S-carbamidomethyl]-TI; bis CM = 14,38-bis-[S-carboxymethyl]-TI; hexa NM = hexa-[S-carbamidomethyl]-TI; hexa CM = hexa-[S-carboxymethyl]-TI. (3) Half-cystine values were always low in our material, but 6 moles of cysteic acid were obtained when the oxidized sample was analyzed. (4) Incomplete hydrolysis of Ile-Ile bond. (5) Cysteic acid and S-carboxymethyl-cysteine sulfone emerge as one peak from the medium column (0.9 x 60 cm) and cannot be distinguished.

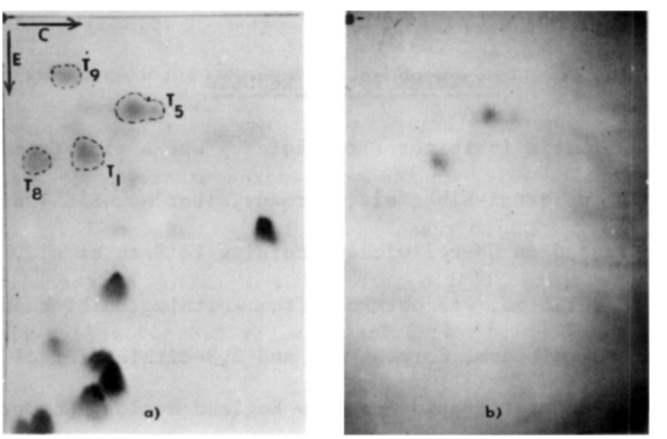

Figure 2. Peptide map of the tryptic digest of oxidized 14,38-bis-[S-C^{14}-carboxymethyl]-TI; chromatography in solvent system, n-BuOH/acetic acid/water (4:1:5 v/v) for 16 hr and electrophoresis in pH 2.1 formic acid-acetic acid buffer (HCOOH/HOAc/H_2O, 25:87:888 v/v) at 2000 volts for 75 min. a) Sprayed with ninhydrin (0.1% in ethanol). ( ) = Cysteic acid containing peptides; the sequences of peptides T_1, T_5, T_8, and T_9 see Fig. 1. b) Autoradiograph of a).

as judged by the full activity of the derivative. It has been argued by Chauvet and Acher (1967) that the lysine residue in position 15 is involved in the binding of the inhibitor with trypsin. Auhagen (1967) postulated a hydrophobic binding region, comprising the amino acid residues 16-23 and 33-35, by which the inhibitor binds to trypsin, thereby masking and inactivating the active site of the enzyme. In either case, the S-carbamidomethyl groups would be in the immediate neighborhood of the binding area and might then either (a) participate in the binding or (b) not interfere with the binding by pointing away from the binding area of the inhibitor molecule. The isoteric S-carboxymethyl groups in positions 14 and 38 however do inactivate the inhibitor. Electrostatic repulsion of the two negatively charged carboxylates might either directly interfere with the binding to trypsin or change the conformation of the inhibitor. Similar findings with fully active tetra-[S-carbamidomethyl]-(human growth hormone), (Bewley, Dixon, and Li, 1968) and with totally inactive tetra-[S-carboxymethyl]-(human growth hormone), (Li, Dixon, and Bewley, unpublished) suggest the importance of the nature of S-alkylating agents for obtaining biologically active derivatives.

Complete reduction with dithiothreitol of all three disulfide bonds of TI was obtained in the presence of 8 M urea at pH 8.2. Total alkylation with both iodoacetamide and iodoacetic acid gave inactive products.

EXPERIMENTS AND RESULTS

Materials: Kallikrein inhibitor (Trasylol®) was a gift from Farbenfabriken Bayer AG, Wuppertal-Elberfeld, Germany, (Lot No. S5617 dated 2/3/67) and was chromatographed on CM-cellulose according to Sach et al., (1965). Trypsin, 3 x cryst., TRL-50, was obtained from Worthington Biochem. Corp., BAPA from Nutritional Biochem. Corp., TAME and 2,3-dithiothreitol from Calbiochem. Co., and C^{14}-iodoacetic acid from New England Nuclear Corporation.

14,38-Bis-[S-carbamidomethyl]-(basic trypsin inhibitor) (I).-Basic trypsin inhibitor (2 μ moles) was dissolved in 0.1 M phosphate buffer (3 ml) at pH 8.2.

2,3-Dithiothreitol (24 μ moles) was added and the reduction was allowed to proceed for 1.5 hr at room temperature under nitrogen. Iodoacetamide (120 μ moles) was then added and the solution kept at room temperature under nitrogen for 10 min (NaOH being added if necessary to maintain pH 8.2). Acetic acid was then added to adjust the pH to 3. Excess reagents were removed simultaneously with desalting on Sephadex G-25 with 0.1 M HOAc as eluant. The eluate containing the protein peak was lyophilized. 14,38-Bis-[S-carbamidomethyl]-inhibitor was obtained in 76% yield. The biological activity was identical with native TI in BAPA and TAME assays. Gel electrophoresis (pH 4.9) was homogeneous. Table 1 gives the data of the amino acid analysis showing the presence of 2.0 moles of S-carboxymethyl-cysteine.

14,38-Bis-[S-carboxymethyl]-(basic trypsin inhibitor (II)).-Preparation was as described for I. 12 mg of inhibitor and 24 mg of iodoacetic acid gave 9.0 mg (75%) of II. BAPA and TAME assays showed complete lack of activity. II was homogeneous by gel electrophoresis and by amino acid analysis (Table 1).

Reduction and alkylation in the presence of 8 M urea.-These preparations were carried out in 0.1 M phosphate buffer, at ph 8.2, which was 8 molar in urea[3]. The yields of both the hexa-(S-carbamidomethyl) and the hexa-(S-carboxymethyl) derivatives were about 70-80%. Both products were biologically inactive in BAPA and TAME assays. Amino acid analysis gave 6.0 moles of S-carboxymethyl-cysteine for each sample (Table 1).

Identification of the reduced disulfide bond.-In order to reproduce the peptide map as described by Chauvet et al., (1966), labeled α-C^{14}-carboxymethyl-trypsin inhibitor was used and, therefore, a batch of partially reduced inhibitor (5 μ moles TI treated with 20 μ moles dithiothreitol) was divided into two portions. One (1 μ mole) was treated with iodoacetamide to give the fully active bis-[S-carbamidomethyl]-TI, the other (4 μ moles) was reacted with α-C^{14}-

(3) Urea was freshly purified by passing through a mixture of Dowex 50 x 8 and Dowex 1 x 8 resin at 50°C just before use.

iodoacetic acid (80 μ moles, spec. activity 0.125 μ C/μ mole) and processed for peptide mapping. The labeled 14,38-bis-[S-C^{14}-carboxymethyl]-inhibitor (16 mg) was dissolved in 5.0 ml of performic acid, prepared according to Li (1957), and the reaction was carried out at 4°C for 1 hr. The solution was diluted 10 times with dist. H_2O and was lyophilized to yield 16 mg. It was dissolved in 3 ml of 0.1 M NH_4HCO_3 at pH 8.2. 240 μ g TPCK-treated trypsin (Kostka and Carpenter, 1964), (E:S = 1:66 w/w) were added and the digestion was carried out at 37°C for 5 hr. The solution was then lyophilized. 2 mg of the lyophilized product was subjected to two-dimensional paper chromatography and paper electrophoresis (Katz et al., 1959). After the paper was dried a sheet of X-ray film was placed over it and exposed for 3 days. The X-ray film was then developed and showed 2 radioactive spots. A peptide map and its corresponding autoradiograph are shown in Fig. 2. The radioactive spots were cut out and eluted with 2% acetic acid. The eluates were dried and subjected to amino acid analysis (Table 1).

Tryptic digestion of the oxidized inhibitor yielded 4 cysteic acid containing peptides (described as T_1, T_5, T_8, T_9, by Chauvet et al., 1966) as shown in Fig. 2a. According to Fig. 1 the 5-55 disulfide bond corresponds to T_1 and T_9, the 14-38 bond corresponds to T_1 and T_5, and the 30-51 bond corresponds to T_5 and T_8. The autoradiograph (Fig. 2b) revealed T_1 and T_5 to be radioactive. The amino acid analyses of T_1 and T_5 (Table 1) further corroborated their identity and proved that the selectively reduced disulfide was the 14 to 38 disulfide bond.

Acknowledgements. The authors wish to thank Dr. S. Farber for his encouragement, Dr. C. H. Li for valuable advice and private communications, Dr. E. Auhagen, Farbenfabriken Bayer AG, Germany, for the generous gift of kallikrein inhibitor, Dr. A. Weeds for helpful discussions, and Mrs. Helene Trzeciak for able technical assistance.

REFERENCES

Auhagen, E., in Drugs of Animal Origin, Proc. 1st Int. Symp. on Drugs of Animal Origin, Milan, 1966, A. Leonardi, J. Walsh, Eds, Ferro Edizioni, Milan, 1967, p. 69-80.

Bewley, T. A., Dixon, J. S., and Li, C. H., Biochim. Biophys. Acta, 154, 420 (1968).
Chauvet, J., Nouvel, G., and Acher, R., Biochim. Biophys. Acta, 115, 130 (1966).
Chauvet, J. and Acher, R., J. Biol. Chem., 242, 4274 (1967).
Cleland, W. W., Biochemistry, 3, 480 (1964).
Dixon, J. S. and Li, C. H., Science, 154, 785 (1966).
Erlanger, B. F., Kokowsky, N. and Cohen, W., Arch. Biochem. Biophys., 95, 271 (1961).
Hummel, B. C. W., Can. J. Biochem. Physiol., 37, 1393 (1959).
Kassell, B. and Laskowski, Sr., M., Biochem. Biophys. Res. Comm., 20, 463 (1965).
Katz, A. M., Dreyer, W. J., and Anfinsen, C. B., J. Biol Chem., 234, 2897 (1959).
Kostka, V. and Carpenter, F. H., J. Biol. Chem., 239, 1799 (1964).
Kress, L. F. and Laskowski, Sr., M., J. Biol. Chem., 242, 4925 (1967).
Li, C. H., J. Biol. Chem., 229, 157 (1957).
Light, A. and Sinha, N. K., J. Biol. Chem., 242, 1358 (1967).
Neuman, H., Steinberg, I. Z., Brown, J. R., Goldberger, R. F., and Sela, M., European J. Biochem., 3, 171 (1967).
Sach, E., Thely, M., and Choay, J., Compt. Rend. Acad. Sci., 260, 3491 (1965).
Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem., 30, 1190 (1958).