THE BASIC TRYPSIN INHIBITOR OF BOVINE PANCREAS

V. THE DISULFIDE LINKAGES*

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Recently we presented the linear sequence of amino acids in the basic pancreatic trypsin inhibitor (Kassell and Laskowski, 1964; Kassell et al., 1965). Chauvet et al. (1964) published a slightly different sequence. The present paper confirms our original results (Fig. 1) and summarizes experiments which locate the S-S linkages to complete the primary structure of the protein.

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 1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16
ARG-PRO-ASP-PHE-CYS-LEU-GLU-PRO-PRO-TYR-THR-GLY-PRO-CYS-LYS-ALA-

 17  18   19   20  21  22  23  24  25  26  27  28  29  30  31  32
ARG-ILEU-ILEU-ARG-TYR-PHE-TYR-ASN-ALA-LYS-ALA-GLY-LEU-CYS-GLN-THR-

 33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48
PHE-VAL-TYR-GLY-GLY-CYS-ARG-ALA-LYS-ARG-ASN-ASN-PHE-LYS-SER-ALA-

 49  50  51  52  53  54  55  56  57  58
GLU-ASP-CYS-MET-ARG-THR-CYS-GLY-GLY-ALA
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Figure 1. Linear Sequence of the Pancreatic Trypsin Inhibitor

Because of the sensitivity of S-S linkages to exchange under alkaline conditions (Ryle and Sanger, 1955; Ryle et al. 1955; Spackman et al. 1960) and the undesirability of using any enzyme containing -SH groups, we were unable to digest the native inhibitor. It is resistant to all the common enzymes (Kassell and Laskowski, 1956, 1965). Two methods were used to obtain S-S peptides: enzymic digestion of the succinylated inhibitor and acid hydrolysis. Methods of analysis were described previously.

Succinylated inhibitor: The inhibitor (88 micromoles) was succinylated by the procedure of Habeeb et al. (1958) at pH 8.0. The product was

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TABLE I

Molar Ratios of Amino Acids in 2 Pairs of Oxidized Peptides from the Enzymic Digests of the Succinyl Inhibitor (Impurities are shown in parentheses)

	Pair 1		Pair 2	
	A	B	A	B
Alanine	(0.11)	1.00	0.94	(0.04)
Aspartic Acid	(0.07)	1.02	(0.21)	1.00
Cysteic Acid	1.00	1.08	1.20	1.07
Glutamic Acid	1.04	1.12	1.24	(0.19)
Glycine	(0.11)	(0.05)	1.00	(0.09)
Leucine	(0.07)	(0.01)	0.91	--
Lysine	(0.02)	0.90	0.73	(0.03)
Methionine Sulfone	(0.04)	0.93	--	0.92
Phenylalanine	(0.32)	(0.02)	0.57	(0.07)
Serine	(0.04)	0.84	(0.19)	(0.06)
Threonine	0.89	(0.12)	1.05	(0.15)
Valine			(0.21)	--

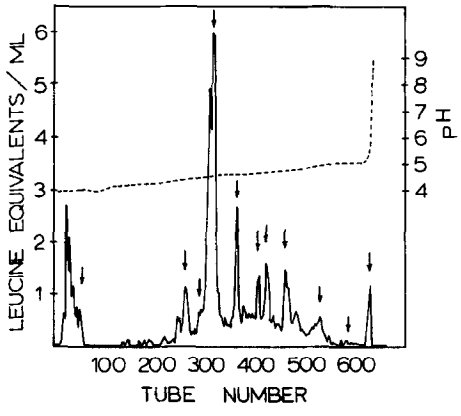

Fig. 2

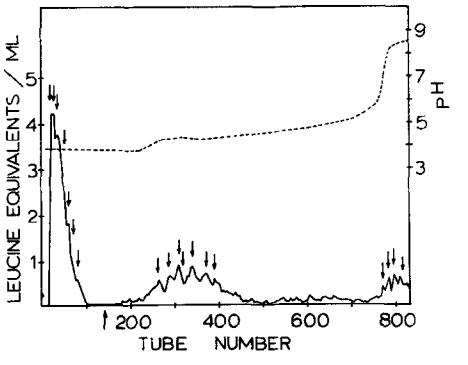

Fig. 3

Figure 2. Chromatography of the peptic-tryptic digest of the succinyl inhibitor on phosphocellulose. Column 2.5 x 24 cm. Fractions 8.5 ml. Rate 60 ml/hr. Starting buffer 0.02 M ammonium acetate, pH 3.95. Gradient started at once. ——— Elution curve. - - - - pH. Arrows above peaks indicate positive test for S-S.

Figure 3. Chromatography of the partial acid hydrolysate on phosphocellulose. Column 1.9 x 45 cm. Fractions 4 ml. Rate 35 ml/hr. Starting buffer, 0.02 M ammonium acetate, pH 3.85. Gradient started at arrow on baseline. ——— Elution curve. - - - - pH. Arrows above peaks indicate positive test for S-S.

inactive. It was digested at a concentration of 10 mg/ml with 0.01% pepsin at pH 1.5 and 37° for 40 hours, lyophilized, and then digested with trypsin at pH 3.5 and 37° for 96 hours (inhibitor concentration 17 mg/ml, trypsin concentration 0.15 mg/ml). The digest was diluted to 115 ml, adjusted to pH 3.4 and chromatographed on phosphocellulose (Fig. 2). The procedure and buffers were those used by Canfield and Anfinsen (1963).

The individual peaks which contained cystine were subjected to the diagonal paper electrophoresis and oxidation method of Brown and Hartley (1963). Two pairs of peptides, both containing the same S-S linkage, and both derived from the last part of the break-through peak, were identified. They accounted together on a molar basis for 1.7% of the protein digested. Their structures derived from the analyses in Table I were:

```
                    30
                 CYS-GLN-THR      1A
                  |
LYS-SER-ALA-GLU-ASP-CYS-MET       1B
                    51
                                        30
                                 LYS-ALA-GLY-LEU-CYS-GLN-THR-PHE    2A
                                                 |
                                           ASP-CYS-MET              2B
                                                 51
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The other peaks contained very large peptides with more than one S-S linkage. One which was obtained fairly pure yielded on oxidation three pieces containing the other 2 S-S linkages, and which, by the absence of cystines 30 and 51, confirmed the linkage established above.

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  5                                    14
CYSO3H-LEU-GLU-PRO-PRO-TYR-THR-GLY-PRO-CYSO3H-LYS-ALA-ARG-ILEU      3A
                          38
          VAL-TYR-GLY-GLY-CYSO3H-ARG-ALA-LYS-ARG-ASN-ASN            3B
                    55
          ARG-THR-CYSO3H-GLY-GLY-ALA                                3C
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Partial acid hydrolysis: Our previous experiments (Kassell and Laskowski, 1964) on digestion of the N-terminal pentadecapeptide of the inhibitor with 0.03 M HCl under the conditions of Schultz et al. (1962) showed that partial splitting occurred between cystine 5 and cystine 14. We therefore used the somewhat more drastic conditions of McDowell and Smith (1965): 50 micromoles of inhibitor, conc. 5 mg/ml, 0.03 M HCl saturated with N_2 and containing 1.1×10^{-4} M thioglycolic acid, 110°, 48 hours, sealed evacuated tubes. The digest was lyophilized and chromatographed on phosphocellulose (Fig. 3). From the final peak, which was eluted between pH 6 and 8.5, a reasonably pure, very basic peptide was isolated in a yield, on a molar

TABLE II

Molar Ratio of Amino Acids of an S-S Peptide from the Acid Hydrolysate and of Its Pieces Separated after Oxidation (Impurities are shown in parentheses)

	S-S Peptide	Oxidized piece 4A	Oxidized piece 4B
Alanine	2.57	1.15	0.88
Arginine	3.90	2.02	2.33
Aspartic Acid	(0.29)	(0.08)	(0.11)
Cystine	1.78	--	--
Cysteic Acid	--	1.00	1.00
Glutamic Acid	(0.37)	(0.05)	--
Glycine	2.00	0.85	0.88
Isoleucine	1.07*	1.01	--
Leucine	(0.39)	(0.03)	--
Lysine	2.17	1.06	1.21
Methionine	(0.17)	--	--
Methionine Sulfone	--	--	--
Phenylalanine	1.19	0.80	--
Proline	1.21	1.03	--
Threonine	(0.87)**	(0.38)	(0.08)
Tyrosine	1.39	1.01***	--
Valine	(0.41)	(0.18)	--

* After 24 hours hydrolysis, the usual value for the 2 isoleucine residues of this protein (#18 and 19) is 1.0-1.3.

** The threonine appeared as a whole residue here, but not in the separated pieces. While it might be residue #11, it is more likely to account for 2 residues in the peptide present as impurity.

*** Plus some oxidized tyrosine.

basis, of 12% of the starting material. Analysis (Table II) indicated the structure:

```
          14
GLY-PRO-CYS-LYS-ALA-ARG-ILEU-ILEU-ARG-TYR-PHE-TYR      4A
    GLY-CYS-ARG-ALA-LYS-ARG                            4B
          38
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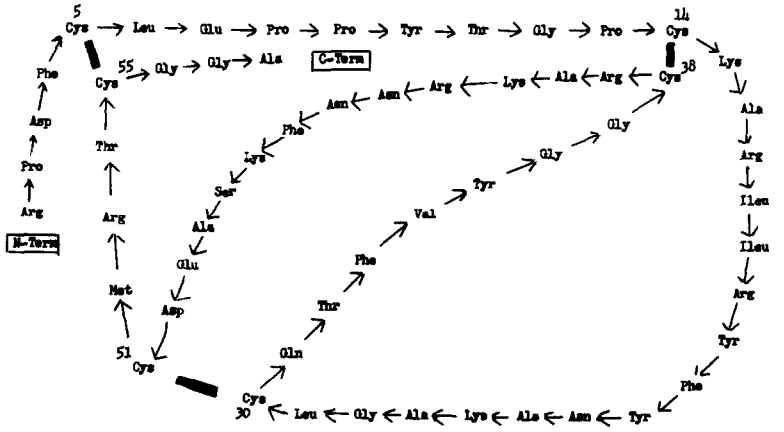
The peptide was oxidized with performic acid according to Hirs (1956) and the pieces separated by paper electrophoresis. The individual pieces had the expected amino acid composition (Table II). Fortunately, exposure to alkaline conditions during elution could not have caused S-S interchange because this was the only peptide remaining on the column.

The linkages split by 0.03 M HCl to form this peptide were of the

type expected from previous studies of this method (Partridge and Davis (1950) and loc. cit.): THR-GLY (11-12), TYR-ASN (23-24), GLY-GLY (36-37) and ARG-ASN (42-43).

The remaining S-S linkage is therefore 5-55 and the complete structure of the inhibitor is:



The location of the S-S linkages indicates a compact, interlocked structure, which is in agreement with the properties of this protein, especially its great resistance to enzymic digestion.

Sequence confirmation: Chauvet et al. (1964) placed ARG-42 in position 21, reversed GLN-31 and THR-32 and tentatively misplaced 3 amide groups. Detailed degradation of the tryptic peptide, 40-42, was already presented (Kassell and Laskowski, 1964). Our sequence was confirmed by the isolation of peptides 3B and 4B above. Table III shows the subtractive Edman degrada-

TABLE III

Subtractive Edman Degradation of Peptide 30-33 (CYS-GLN-THR-PHE)

Step	Molar Ratio of Amino Acids Remaining					Free Amino Acid from PTH
	CM-CyS	GLU	THR	PHE	NH_3	
0	0.74	0.93	0.92	1.00	1.02	---
1	0.05	0.97	0.92	1.00	--	---
2	--	0.13	0.92	1.00	--	GLU*
3	--	--	0.16	1.00	--	---

* 48% recovery as free glutamic acid by acid hydrolysis of the PTH compound extracted.

tion of the chymotryptic peptide, 30-33, to confirm our 31-32 sequence. In the tryptic peptide 18-20, the molar ratio of isoleucine to arginine was 0.89 to 1.00 after 24 hours hydrolysis and 1.85 to 1.00 after 72 hours hydrolysis. This indicates only 1 arginine in this position preceded by 2 isoleucines. Analysis of the overlapping peptides previously shown (Kassell et al. 1965) for ammonia gave at least 2 and as many as 7 separate and unequivocal determinations of the presence or absence of amide groups for every aspartic or glutamic acid in the hydrolysates.

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