Studies on the disulfide bridges in ribonuclease

The approximate locations of the eight half-cystine residues (numbered, in the following discussion, from 1 to 8, beginning with the one nearest the N-terminal end of the protein chain) along the single polypeptide chain of ribonuclease may be deduced from the available information on its amino acid sequence[1,2]. The elucidation of their exact position and pairings in disulfide linkage is clearly essential for an understanding of the structural basis of the physico-chemical and catalytic behavior of the protein[3,4].

Native bovine pancreatic ribonuclease (Armour Lot No. 381-059) was dissolved in water and brought to pH 8.0 in the Coleman Autotitrator with 0.1 *M* NaOH. Following the addition of N-ethylmaleimide (NEMI, final concentration $10^{-3}M$), the protein was digested with subtilisin (generously given by Dr. M. OTTESEN and Prof. K. LINDERSTRØM-LANG) at a level of 2 mg/100 mg ribonuclease at 37° C under nitrogen, until the uptake of alkali had ceased. After digestion, which required approximately 3 h with 1% solutions of ribonuclease, the total contents of the digestion vessel were lyophilized. 10 mg aliquots of the dried material were dissolved in water, streaked on washed Whatman No. 3 filter paper, and subjected to filter paper electrophoresis at pH 6, as described by RYLE *et al.*[5] for insulin fragments, using the Michl high-voltage electrophoresis apparatus[6]. A typical pattern, which was the same in the presence or absence of NEMI in the electrophoresis buffer, is shown in Fig. 1. The location of the disulfide-containing peptide bands, as revealed by the cyanide-nitroprusside method of TOENNIES AND KOLB[7] is shown on the figure. These bands, A, B, C, D, and E were eluted at 5° C with 10% acetic acid or with pyridine-acetic acid buffer, pH 3.6[5], at room temperature and the eluates lyophilized. The eluted peptides were further purified by a second electrophoresis run at pH 3.6 in pyridine-acetic acid buffer and eluted as above.

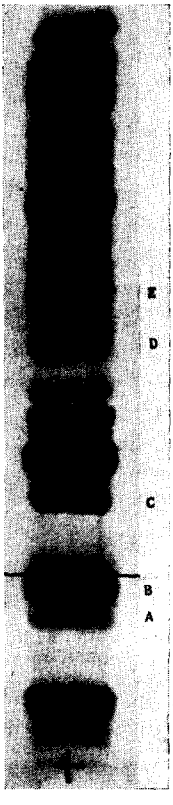


Fig. 1. Ninhydrin-positive bands obtained upon filter paper electrophoresis[5] of subtilisin digest of ribonuclease. Pyridine-acetic acid buffer, pH 6, 2 h, 2000 volts. Letters refer to disulfide-containing components. Length of strip – 55 cm.

The eluates were oxidized with performic acid at 0° C[5] and the resulting cysteic acid-containing peptides separated by electrophoresis at either pH 6 or pH 3.6. The amino acid compositions, and in some experiments the N-terminal residues as well, were determined by application of the LEVY chromatographic technique[8] to hydrolysates of the peptides and the dinitrophenylated peptides. These analyses, when considered together with the known portions of the ribonuclease sequence[1,2], sufficed in most cases for the identification of the half-cystine residue in question.

The compositions of the two "half-bridges" resulting from the oxidation of the disulfide bond in the peptide occupying band D are given in Table I, and agree with the structure to be expected from a cystine peptide containing half-cystine No. 2 joined to one of the three half-cystine residues No. 7, No. 8, or No. 1. The peptide, ($CySO_3$,Ala,Tyr), was shown, however, to contain N-terminal cysteic acid and may thus be written $CySO_3$-(Ala,Tyr). Furthermore, partial acid hydrolysis of the dinitrophenylated peptide yielded a peptide with the sequence, DNP-$CySO_3$-Ala, the presence of which suggests that this tripeptide must have the sequence $CySO_3$-Ala-Tyr. Since HIRS[9] has shown, in his studies on the detailed sequence of the ribonuclease chain, that this sequence cannot occur in the neighborhood of half-cystines No. 1 or No. 7, the tripeptide above must include half-cystine No. 8. The other half-cystine-containing peptide had a composition attributable only to the portion of the chain including half-cystine No. 2 and establishes the disulfide bridge, No. 2–No. 8.

Band C (Fig. 1) yielded two cysteic acid-containing components, following oxidation. The compositions of these are given in Table I. These fragments can only have arisen from those areas of the protein chain containing half-cystines No. 3 and No. 7 and indicates the presence of an –S–S– bridge between these residues. The tyrosine and lysine contents of the peptide were considerably less than stoichiometric in comparison with the other amino acids present and this probably reflects the fact that these two amino acids suffer some degradation during the dinitrophenylation procedure[1]. Some tyrosine may also be lost during performic acid oxidation. Careful analysis of the peptides derived from band C by ion exchange chromatographic methods is clearly desirable.

Oxidation of band A yielded two peptides with the compositions shown in the table. Peptide A1 is clearly derived from the portion of the chain containing half-cystine No. 6. The cysteic

TABLE I

THE LOCATION OF DISULFIDE BRIDGES IN RIBONUCLEASE

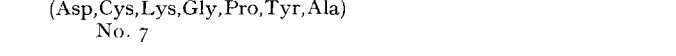
Disulfide-positive band	*Composition of cysteic acid-containing peptides produced by oxidation*	*Half-cystine residues implicated*	*Disulfide bridge*
A	(1) $CySO_3$-Asp-Arg-Glu-(Ser_2,Thr,Gly)	No. 6	No. 1–No. 6*
	(2) ($CySO_3$,Asp,Glu)	No. 1, 3, 5, 8	
C	(1) (Ser,Glu,$CySO_3$)-Lys	No. 3	No. 3–No. 7
	(2) (Gly,Asp,$CySO_3$,Pro,Ala,Lys,Tyr)	No. 7	
D	(1) $CySO_3$-[Ala,Tyr]	No. 8	No. 2–No. 8
	(2) Asp-Arg-$CySO_3$-(Pro,Lys,Asp,Val,Thr)-Phe	No. 2	
E	(1) (Gly,Asp,$CySO_3$,Pro,Ala,Lys,Tyr,Ser)	No. 7	No. 4–No. 5*
	(2) ($Ser_{1.1}$,$Glu_{2.0}$,$Asp_{2.7}$,$Lys_{1.8}$,$Tyr_{0.9}$, $CySO_{3\ 3.2}$,$Gly_{0.7}$,$Thr_{0.6}$,$Ala_{1.0}$,$Val_{1.0}$)	No. 3, 4 and 5 together	(see text)

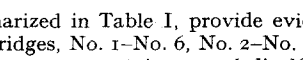
Residues within brackets and separated by commas are in an undetermined order and represent qualitative analyses[8] for constituency. Residues separated by dashes are in known sequence and are quantitatively (± 10%) accurate. Amide nitrogen atoms[2] have been omitted throughout.

* Bridges No. 4–No. 5 and No. 1–No. 6 have also been established by SPACKMAN, MOORE, AND STEIN[10].

acid residue in peptide A2, however, could be derived from either of half-cystines No. 1, No. 3, No. 5, or No. 8, and is only assignable to No. 1 on the basis of the other data shown in Table I.

Band E yielded, upon oxidation, two cysteic acid-containing components. The analysis for one of these was essentially identical with that for peptide C1, indicating the presence of half-cystine residue No. 7. The quantitative analysis[7] of the second component is given in Table I and indicates a peptide sequence containing *three* equivalents of cysteic acid. On the basis of the known distribution of half-cystine residues along the chain and in view of the disulfide bridge assignments already established (No. 3–No. 7, No. 2–No. 8), this component must represent the, oxidized form of a bridge joining half-cystines No. 4 and No. 5, still linked in the same peptide chain to the peptide (Ser, Glu, $CySO_3$)-Lys, the latter representing one side (No. 3) of the bridge No. 3–No. 7. The structure of the peptide forming electrophoretic band E may thus be reconstructed as follows (amide nitrogen atoms[2] have been omitted):





The data, summarized in Table I, provide evidence for the presence in bovine ribonuclease of the 4 disulfide bridges, No. 1–No. 6, No. 2–No. 8, No. 3–No. 7 and No. 4–No. 5.

Band B appears to contain several disulfide bridges joined through bonds which were incompletely hydrolyzed under the present conditions, and has not yielded useful information to date.

The characterization of these disulfide bridges is also being carried out by SPACKMAN, MOORE AND STEIN and evidence for disulfide linkages between half-cystine residues No. 1 and No. 6 and between No. 4 and No. 5 has also been obtained by them. Independent identification of bridges No. 2–No. 8 and No. 3–No. 7 will clearly be invaluable in eliminating the possibilities of error in methodology or interpretation in the results presented here. Such confirmation is particularly important since the possibilities of some disulfide interchange cannot be absolutely excluded.

It is clear that disulfide bridges severely restrict the ribonuclease molecule in terms of its ability to unfold in solution. The considerable change in intrinsic viscosity observed upon exposure of ribonuclease to 8 *M* urea solutions[9] is, therefore, most likely attributable to the spatial arrangements of the non-cross-linked N-terminal and C-terminal "tail" portions and to accordion-like expansion and unfolding of the portions of the sequence situated between the disulfide bridges. Optical rotatory and spectral studies of ribonuclease and its various derivatives under conditions chosen to modify secondary and tertiary structure are consistent with the above interpretation[11].

We would like to express our sincere appreciation to Mr. F. WHITE, Miss J. COOKE, and Mr. H. TRITCH, for their expert assistance in much of this work.

ANDREW P. RYLE §
CHRISTIAN B. ANFINSEN

Laboratory of Cellular Physiology and Metabolism, National Heart Institute, Bethesda, Md. (U.S.A.)

1 R. R. REDFIELD AND C. B. ANFINSEN, *J. Biol. Chem.*, 221 (1956) 385.
2 C. H. W. HIRS, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 221 (1956) 151.
3 W. F. HARRINGTON AND J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1955) 15.
4 C. B. ANFINSEN AND R. R. REDFIELD, *Advances in Protein Chem.*, 11 (1956) 1.
5 A. P. RYLE, F. SANGER, L. F. SMITH AND R. KITAI, *Biochem. J.*, 60 (1955) 541.
6 H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
7 G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823.
8 A. L. LEVY, *Nature*, 174 (1954) 126.
9 C. H. W. HIRS, *Federation Proc.*, 16 (1957) 196.
10 D. H. SPACKMAN, S. MOORE AND W. H. STEIN, *Federation Proc.*, 16 (1957) 252.
11 M. SELA AND C. B. ANFINSEN, *Biochim. Biophys. Acta*, 24 (1957) 229.

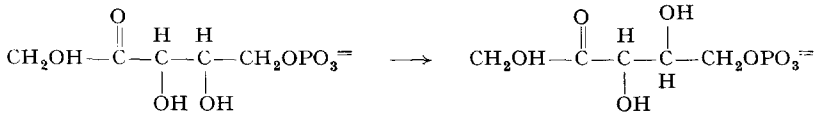
Received March 15th, 1957

§ Commonwealth Fund Fellow. Present address: Medical Research Council, National Institute for Medical Research, The Ridgeway, Mill Hill, London N.W. 7, England.

L-Ribulose-5-phosphate – D-xylulose-5-phosphate stereoisomerase and its role in L-arabinose fermentation*

L-Arabinose fermentation in *Lactobacillus pentosus*[1] and *Aerobacter aerogenes*[2] is initiated by the action of L-arabinose – L-ribulose isomerase. L-Ribulose is phosphorylated by ATP** in the presence of a kinase which has been purified from *A. aerogenes*[3]. L-Ru-5-P has been identified as the phosphorylation product[3]. L-Arabinose, or L-ribulose plus ATP are converted by crude extracts of *A. aerogenes*[2] and *Propionibacterium pentosaceum*[4] to arabinose, ribulose, xylulose, ribose, heptulose, fructose, and glucose phosphates. In addition, D-G-3-P is formed from L-ribulose plus ATP, but not from L-G-3-P[2]. Hence, it has been postulated[2,5] that L-arabinose is transformed into a D-pentose phosphate prior to metabolism via the transaldolase–transketolase and glycolytic pathways[6].

We now wish to report the finding of an enzyme which catalyzes the reaction:



L-Ru-5-P D-Xu-5-P (1)

This enzyme, termed L-Ru-5-P – D-Xu-5-P (C-4) stereoisomerase***, has been purified by treating extracts with protamine, followed by precipitation with ammonium sulfate and by calcium phosphate gel adsorption and elution. An inability to assay crude extracts has prevented an estimate of the purification achieved; however, the L-ribulokinase obtained in another ammonium sulfate fraction in the final step of the same procedure was purified 200-fold[3]. The stereoisomerase was free of phosphoketopentoepimerase (C-3 epimerase)[7,8], L-ribulose – L-arabinose isomerase, transketolase and D-G-3-P dehydrogenase, but contained L-ribulokinase.

* Supported in part by a Grant-in-Aid from the National Science Foundation and from the Rockefeller Foundation. The Authors gratefully acknowledge the generosity of Dr. B. L. HORECKER who made available unpublished manuscripts and experimental materials.

** Abbreviations: ATP = adenosine triphosphate; Ru-5-P = ribulose-5-phosphate; G-3-P = glyceraldehyde-3-phosphate; D-Xu-5-P = D-xylulose-5-phosphate; D-R-5-P = D-ribose-5-phosphate; DPN and DPN·H = oxidized and reduced diphosphopyridine nucleotide; C-3 epimerase = D-phosphoketopentoepimerase; C-4 stereoisomerase = L-ribulose-5-phosphate — D-xylulose-5-phosphate stereoisomerase; TCA = trichloroacetic acid.

*** Until suitable nomenclature can be established, stereoisomerase is provisionally employed to describe the ability to convert the substrate into its stereoisomer.