The Relationship of Disulfide Bonds and Activity in Ribonuclease*

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The RNase molecule consists of a single chain, arranged in a compact, folded structure, cross-linked through 4 disulfide bridges (1). After hydrolysis to determine the amino acid composition of RNase, 8 half-cystine or cysteic acid residues have been identified (2, 3) and the approximate location of these residues in the partial structural formula for oxidized RNase has been determined (4). More recently, the elucidation of the pairings of these 8 cysteic acid residues has been accomplished. Numbered from the N-terminal end of the protein chain, the presence of 4 disulfide bridges has been established, linking half-cystines 1 and 6, 2 and 8, 3 and 7, and 4 and 5 (5, 6).

Total cleavage of the 4 disulfide bridges in RNase, whether by oxidation with performic acid (3) or by reduction with thioglycoic acid (7), leads to complete loss of enzymatic activity (3, 7). Activity still remains high with cleavage of 1 or 2 disulfide bonds in the molecule but decreases rapidly with reduction of more than 2 disulfide bonds (7).

In an extension of previous studies relating secondary structure to activity for the enzyme RNase (8), we attempted to evaluate the influence of the —S—S— bonds on the secondary structure and activity of the enzyme. The amperometric technique for sulfhydryl determinations has been adapted, with modifications, to the measurement of disulfide bonds in proteins (9). With this procedure, 1 of 4 disulfide bridges in RNase was found to be resistant to cleavage. Further modifications such as carrying out the sulfitolysis reaction and titration in various concentrations of urea up to 8 M, have resulted in a relatively simple, rapid and accurate method for the controlled cleavage of all the disulfide bonds in many proteins, including RNase (10). By this method, we have attempted to correlate enzyme activity with the integrity of the disulfide bonds present in RNase. This communication presents evidence that not all of the disulfide bridges are essential for activity of RNase, confirming the results of Sela et al. (7) obtained with a different method. In addition, evidence is presented which strongly suggests that it is not only the cleavage of disulfide bonds, but in addition, a subsequent structural alteration in the molecule, in the denaturation sense, which is responsible for loss in enzymatic activity.

EXPERIMENTAL

Materials and Methods

Crystalline ribonuclease was purchased from Armour and Company, Lot No. 381-059; sodium ribonucleate was obtained from Nutritional Biochemicals Corporation, Lot No. 5557, or was isolated from yeast according to the method of Crestfield et al. (11). The “A” fraction of ribonuclease was prepared according to the method of Hirs et al. (12).

Titration Procedure—The argentimetric, amperometric titration procedure and the reagents used are as given in the preceding paper (10). The titration vessel contained 0 to 8 M urea, 6 × 10⁻³ M ethylenediaminetetraacetate, 2 mg. of RNase (previously dialyzed for 16 hours against cold, redistilled water and the protein content determined by Kjeldahl digestion followed by nesslerization) plus 0.1 ml. of a saturated, freshly prepared Na₂SO₃ solution in a total volume of 30 ml. at 37°C. Titrations were performed at pH 8.3 ± 0.5. Several experiments were carried out at 27°C, after an incubation period in the total titration mixture for 5 minutes.

Aliquots for assay were removed from the above reaction mixtures before the addition of sulfite (zero time). After the addition of Na₂SO₃, sulfitolysis was judged to be complete when the galvanometer registered a steady state, usually about 5 to 10 seconds at 37°C. Additional aliquots then were taken for assay; one sample was diluted immediately in 0.1 M acetate buffer, pH 5.0. The other aliquot was diluted for assay after the completion of the titration with standard 0.001 M AgNO₃ solution. The assays in all cases were begun immediately. The results reported herein were obtained from seven separate experiments, each carried out in duplicate. In addition, each assay was performed in duplicate.

Assay of RNase—The assay method was a modification of those described by Kunitz (10) and by Anfinsen et al. (1). For assay at pH 7.3, 0.01-ml aliquots of ribonuclease (containing 0 to 1.0 mg. for the standard curve, or approximately 0.5 μg. from the titration vessel) were added to 2.0 ml. of 0.1 M phosphate buffer, pH 7.3 and ionic strength 0.254. This was followed by the addition of 1.0 ml. of a 2 per cent solution of sodium ribonucleate prepared in water. After incubation for 4 minutes at 30°C, the reaction was stopped by the addition of 1.0 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid. Precipitated enzyme and substrate were cooled in an ice-water bath and then centrifuged at 0°C. A 0.1-ml aliquot of the supernatant was immediately diluted with 3.0 ml. of water and the solution read in a Beckman spectrophotometer at 260 μm.

For assay at pH 5.0, 0.1-ml aliquots of RNase (containing 0 to 10.0 μg. for the standard curve, or approximately 5.0 μg. from the titration vessel) were added to 1.0 ml. of 0.1 M acetate buffer, pH 5.0, and ionic strength 0.065. This was followed by the addition of 1.0 ml. of a 2 per cent solution of sodium ribonucleate prepared in buffer. After incubation for 4 minutes...
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at 37°, the reaction was stopped by the addition of 1.0 ml. of the uranyl acetate-perchloric acid reagent and the procedure continued as described above.

A standard curve was run with each of the seven experiments, and aliquots were taken from the titration beakers which were calculated to fall approximately on the center portion of the linear curve.

RESULTS

Fig. 1 shows the relationship between the disulfide bonds cleaved per mole of RNase, and the percentage of activity remaining. It seems that one disulfide bond may be cleaved with little, if any, loss in activity; with the cleavage of two disulfide bridges, enzymatic activity is retained up to approximately 80 per cent of the original activity. With 3 \(-S-S-\) linkages broken, about 60 per cent of the activity is still retained. With the cleavage of 3.5 or more \(-S-S-\) bonds per mole of RNase, activity decreases to zero, sharply.

From kinetic studies on insulin, for example (see Cecil and Loening (14)), it would seem that in the range of pH 7.2 to 9.0, the thiol groups formed in the sulfitolysis reaction begin to ionize and the reaction becomes reversible. The reaction is largely reversible at pH 9.0 and not at 7.2. It is for this reason that subsequent assays were carried out at pH 5.0, as well as at pH 7.3. The assays at pH 5.0 and at pH 7.3 are in good agreement

Table I: Effect of urea concentration on disulfide bonds cleaved and activity of RNase

<table>
<thead>
<tr>
<th>Urea concentration</th>
<th>Moles of (-S-S-) cleaved/mole of RNase</th>
<th>Activity remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.34</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>91</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>3.9</td>
<td>27</td>
</tr>
<tr>
<td>6.5</td>
<td>3.9</td>
<td>13</td>
</tr>
<tr>
<td>7.0</td>
<td>3.9</td>
<td>12</td>
</tr>
<tr>
<td>7.5</td>
<td>3.9</td>
<td>11</td>
</tr>
<tr>
<td>8.0</td>
<td>4.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Assays carried out at pH 7.3.

Table II: Ribonuclease “A” activity remaining before and after amperometric titration for \(-S-S-\) bonds

<table>
<thead>
<tr>
<th>Urea concentration</th>
<th>Moles of (-S-S-) cleaved/mole of RNase</th>
<th>Activity remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.2</td>
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<tr>
<td>15</td>
<td>8</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* These assays were carried out at pH 5.0.
† Aliquots were withdrawn from the titration vessel and immediately diluted for assay when the sulfitolysis reaction was complete as judged by the stabilization of the galvanometer.
‡ Aliquots were taken under the same conditions as for Column I, but were diluted for assay after completion of the titration with AgNO₃.

Table III: Effect of time of exposures to the urea-\(Na₂SO₃\) mixture on the activity of RNase

The sulfitolysis reaction was carried out at 27° and allowed to proceed for 5 minutes or 15 minutes, as indicated. The assays were performed at pH 7.3.

<table>
<thead>
<tr>
<th>Time</th>
<th>Urea concentration</th>
<th>Moles of (-S-S-) cleaved/mole of RNase</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>M</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.4</td>
<td>89</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>0.7</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.7</td>
<td>97</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>1.2</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.2</td>
<td>90</td>
</tr>
</tbody>
</table>

* These assays were carried out at pH 5.0.
throughout (Fig. 1) indicating that under our experimental conditions, the sulfitolysis reaction was not reversible.

In Table I we have summarized the effect of various concentrations of urea on the cleavage of disulfide bridges and the resulting activity of the enzyme RNase. With no urea present, no \(-S-S-\) bonds are cleaved; with increasing concentrations of urea, more cystine bonds become susceptible to sulfitolysis. Under these experimental conditions, the 4 disulfide bridges in RNase are susceptible to cleavage only in the presence of 6.5 to 8 m urea. The results obtained are almost identical for both the native and the “A” fraction of RNase. The experimental evidence strongly suggests the functional importance of the secondary structure in RNase. In 6 M urea, despite the cleavage of 3.9 disulfide bonds per mole of RNase, approximately one-third of the total enzymatic activity was retained. In several experiments, where all 4 disulfide bridges were split in 6.5 m urea, some activity still remained. Complete loss of activity invariably occurred at higher concentrations of urea, such as 8 M. In the absence of Na$_2$S$_2$O$_3$, no inactivation occurred when RNase was exposed to urea and then diluted for assay.

Aliquots removed for assay usually were diluted immediately to a large volume during which time the titration was completed in the presence of undiluted concentrations of urea. To check the possibility that a further decrease in activity might occur during the actual amperometric titration, assays were carried out with aliquots diluted immediately, as above, and similar aliquots held and diluted only after completion of the titration with AgNO$_3$. From the results in Table II it is clear that both procedures give virtually identical results, demonstrating that changes in activity did not take place during the short period of 2 to 3 minutes involved in the titration with AgNO$_3$. Furthermore, the stabilization of the galvanometer within 10 seconds after the addition of the Na$_2$S$_2$O$_3$, plus the fact that neither the diffusion current nor the slope of the titration curve changed during the titration procedure, indicates the the sulfitolysis reaction was complete before actual titration was begun.

If the sulfitolysis reaction is carried out at 27° instead of 37°, the reaction is slowed down considerably, fewer disulfide bonds are cleaved at comparable concentrations of urea, and total cleavage of all the 4 disulfide bonds is not always achieved, even in 8 m urea (Table III). At this temperature 5 minutes is optimal for the sulfitolysis reaction. Longer exposure of the enzyme does not result in any further cleavage of \(-S-S-\) bonds in the RNase; on the contrary, the yield of \(-SH\) groups resulting from the sulfitolysis is diminished, presumably due to oxidation (10). In addition, exposure of the enzyme (containing cleaved \(-S-S-\) bridges) to the sulfite-urea mixture for longer than 5 minutes results in an appreciable decrease in enzymatic activity, especially at higher concentrations of urea (Table III). Here again, with 3.9 moles of \(-S-S-\) bonds cleaved per mole of RNase, approximately one-third of the original enzymatic activity still remained.

**DISCUSSION**

In the presence of 6 m urea, appreciable activity remained when 3.9 of the 4 disulfide bridges in RNase were cleaved. In the presence of 8 m urea, at 37° complete inactivation occurred. This can only mean that more than \(-S-S-\) bonds are concerned here; that inactivation occurred as a result of subsequent alterations in the structurally weakened molecule following the cleavage of the disulfide bonds. Similar conclusions have been suggested by Gawron et al. (15) in interpreting the action of cyanide on RNase. Furthermore, it has been demonstrated experimentally that some portion of the secondary, cooperative structure of the RNase molecule is essential for its catalytic activity (8). It would seem then that one of the roles of the \(-S-S-\) bonds is to provide additional stability to the secondary structure. This is in agreement with the conclusions reached concerning the essentiality of the disulfide groups of trypsin (16)

where rupture of the \(-S-S-\) bridges leads to a more extensive unfolding of the molecule and inactivation.

**SUMMARY**

An amperometric technique for the determination of disulfide bonds in ribonuclease has been used. The cleavage of the four cystine bridges proceeds only in the presence of urea at 37°. Cleavage of one disulfide bond results in little, if any, loss in activity; with the breaking of 2 disulfide linkages, activity remains high. With the cleavage of 3.9 disulfide bonds, in 6 m urea, 27 to 37 per cent of the original activity can still be detected. In the presence of 8 m urea, complete inactivation occurs. The significance of these structural alterations is discussed in relation to activity for the enzyme ribonuclease.

**REFERENCES**

The Relationship of Disulfide Bonds and Activity in Ribonuclease
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