Regeneration of Enzyme Activity by Air Oxidation of Reduced Subtilisin-Modified Ribonuclease

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Fully reduced bovine ribonuclease, a molecule which probably exists in a random coil configuration (1, 2), will, under the influence of molecular oxygen alone, oxidize to form an organized, enzymatically active product in high yield (3, 4). This material seems indistinguishable on the basis of physical properties from native ribonuclease (RNase). These observations suggest that the information determining not only the covalent structure, but also the secondary and tertiary structure of the molecule, is contained in the amino acid sequence itself. It is of interest to determine which portions of the sequence are most necessary for correct refolding. The reduction and subsequent oxidation of subtilisin-modified RNase is, therefore, been studied in an attempt to obtain at least a partial answer to this question. The results described below indicate that after removal of 20 of the 124 residues which form the RNase chain, an enzymatically active product may be produced by oxidation of the fully reduced derivative.

EXPERIMENTAL PROCEDURE

Bovine pancreatic RNase (Pentes, Inc.) was subjected to chromatography on carboxymethylcellulose in a manner similar to that employed by Åqvist and Anfinsen (6). The "A" peak was treated with subtilisin and RNase-S was isolated and separated into its protein and peptide components (5).

Reduction—RNase-S or S-protein in 1% solution in 8 M urea (recrystallized) were treated with an excess of mercaptoethanol (The Matheson Company, Inc.) 100 times the molar concentration of protein, at room temperature (22–25°C). After an appropriate period of reduction, the protein was precipitated with 10 volumes of ice-cold acetone-1 M HCl (39:1), washed three times with this solvent, and then twice with acetone (4).

Determination of Sulphydryl Content—The precipitated, reduced protein was dissolved promptly in water (at 0°C) through which nitrogen had been bubbled. Sulphydryl groups were then titrated spectrophotometrically with p-chloromercuribenzoate (7). An alternate sulphydryl determination was performed by alkylating the sulphydryl groups with 1-C14-iodoacetate under conditions in which alkylation of other functional groups of the molecule is minimal. In this procedure, the mixture of protein, mercaptoethanol, and urea is diluted five-fold with water, and a three-fold molar excess of 1-C14-iodoacetate of low specific activity is added, the pH of the mixture being maintained at 8.2.

The reaction is allowed to continue for 15 minutes, at the end of which time the protein is precipitated and washed as described previously, except that the number of washings with each solvent is increased to five, and the final acetone wash is followed by two other washes. The precipitate is then dissolved in water and the protein concentration estimated spectrophotometrically at 280 mμ. Counting is performed in Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Inc.) with anthracene crystals as the phosphor (8). A comparison of the specific radioactivity of the 1-C14-iodoacetate with that of the alkylated protein permits calculation of the molar ratio of carboxymethyl groups to protein. Although it is well known that iodoacetate may react with functional groups other than the free sulphydryls of reduced RNase (9), side reactions appear to be minimal under the conditions described above. As controls on the occurrence of such side reactions, RNase or S-protein subjected to this procedure without prior reduction react with only 0.1 to 0.2 mole of iodoacetate per mole of protein. Furthermore, determinations by the alklylation procedure correspond closely to those obtained by p-chloromercuribenzoate titration. For example, samples of RNase, subjected to conditions known to result in complete reduction (4, 10), give values of 8.0 to 8.3 by the radioactive method.

Oxidation—Solutions of the precipitated, reduced protein were lyophilized in order to remove traces of organic solvent. The lyophilized material was then dissolved in a volume of water at 0°C sufficient to achieve a concentration of 2 mg per ml. An equal volume of 0.2 M K2HPO4 was added and the solution was brought to pH 8.0 with KOH. Gentle agitation in a round bottom flask, stoppered with a loosely packed cotton plug, was then allowed to proceed for 20 hours.

Assay of Enzymatic Activity—Enzymatic activity was assayed with RNA as substrate (11). S-Protein preparations were routinely assayed both in the presence and absence of S-peptide.

Chromatography—S-Protein and reduced, oxidized S-protein were chromatographed on carboxymethylcellulose in 25- × 1-cm columns, with a gradient elution from 0.01 M potassium phosphate at pH 5.8 to 0.1 M potassium phosphate at pH 7.5. A four-bottle variable gradient system (12) was used, the initial buffer being contained in the first three bottles and the final buffer in the fourth; total elution volume was 2 liters.

RESULTS

The data in Table I indicate that reduction of S-protein appears to be complete in 1 minute. At the end of this time, enzymatic activity is also completely lost. The rate of reduc-
tion of S-protein appears to be much more rapid than that of RNase (the detailed kinetics of which is now under investigation). Sulfhydryl determinations by p-chloromercuribenzoate titration and by the radioactivity method described above correspond closely.

The results of oxidation are shown in Table II. Each sample was reduced at least 2 hours and was completely inactive after reduction. It is apparent that, although reduced S-protein will oxidize to form an active product, it does so with lower yield than RNase-S, which in turn is less efficiently oxidized than RNase. Considerable precipitation, with low recovery of soluble products during oxidation, is at least in part responsible for the low yields in the instance of reduced S-protein oxidation. This precipitate is partially soluble in 8 M urea solutions and completely soluble in the presence of both 8 M urea and mercaptoethanol, suggesting that aggregation and the formation of intermolecular disulfide bridges are responsible for its formation.

Chromatographic separation of S-protein is accomplished readily on carboxymethylcellulose, a result which is of interest since Richards (13) has been unable to chromatograph this material on Amberlite IRC-50 (XE-64). The chromatographic mobility of S-protein and the reduced, oxidized product appear identical, as indicated in Fig. 1. Such chromatographically purified material shows, upon addition of S-peptide, the same specific enzyme activity as unmodified RNase-S.

**DISCUSSION**

Random reoxidation of the 8 sulfhydryl groups in reduced RNase would reform the native configuration with a probability of less than 1% (14, 15). Material which can be activated by S-peptide is produced by the oxidation of S-protein in an average yield which is 15 times greater than that expected by random recombination. If one assumes that most of the precipitated material produced during the oxidation procedure is the result of intermolecular bonding and that isomers of differing intramolecular disulfide bridging would remain soluble, the more impressive figure of 35 times the expected yield is obtained. These findings probably indicate that the "information" for the formation of a secondary and tertiary structure compatible with enzymatic activity resides in the 104 amino acid S-protein. The presence of the peptide moiety results in increased efficiency of oxidation, but since most of this increase is associated with a greater yield of soluble product, it may be related only to diminished aggregation.

Evidence has been presented that the location of disulfide

### Table I

**Reduction of S-protein by mercaptoethanol**

<table>
<thead>
<tr>
<th>Method of sulfhydryl determination</th>
<th>Time of reduction</th>
<th>Sulfhydryl/mole of S-protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chloromercuribenzoate titration</td>
<td>min</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Chloromercuribenzoate titration</td>
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<tr>
<td>p-Chloromercuribenzoate titration</td>
<td>5</td>
<td>8.1</td>
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<tr>
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<td>15</td>
<td>8.2</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate titration</td>
<td>60</td>
<td>8.2</td>
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<tr>
<td>p-Chloromercuribenzoate titration</td>
<td>120</td>
<td>8.0</td>
</tr>
<tr>
<td>C4-acetate determination</td>
<td>120</td>
<td>8.1</td>
</tr>
</tbody>
</table>

### Table II

**Enzymatic activity of S-protein, RNase-S, and RNase, fully reduced and then oxidized with air**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Experiment No.</th>
<th>Enzymatic activity</th>
<th>Yield of soluble protein</th>
<th>Regeneration of original activity after oxidation</th>
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<tbody>
<tr>
<td>S-Protein</td>
<td>1</td>
<td>48</td>
<td>70</td>
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<tr>
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<td>31</td>
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</tr>
<tr>
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<td>21</td>
<td>43</td>
<td>9</td>
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<tr>
<td>Mean</td>
<td></td>
<td>35</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>RNase-S</td>
<td>1</td>
<td>47</td>
<td>56</td>
<td>26</td>
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<td>39</td>
<td>100</td>
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<td>3</td>
<td>44</td>
<td>76</td>
<td>35</td>
</tr>
<tr>
<td>Mean</td>
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<td>43</td>
<td>77</td>
<td>33</td>
</tr>
<tr>
<td>RNase</td>
<td>From White (4)</td>
<td>84</td>
<td>52</td>
<td>44</td>
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* All samples were enzymatically inactive after reduction.
* All samples were completely inactive after reduction.
* Expressed as a percentage of activity of equal concentration of native RNase "A" peak.
* Column 3 times column 4.
bridges in reduced, oxidized RNase is identical to that in native RNase (16). This kind of evidence is as yet not available for S-protein, although identical chromatographic mobility and specific enzyme activity suggest similar structure. The observation that reduced RNase does not oxidize to form an active product in 8 M urea solution strongly suggests that the correct matching of half-cystine residues is dependent upon a unique set of hydrogen bond interactions which contribute to the spatial orientation of the reacting SH groups.

SUMMARY

Complete reduction, followed by reoxidation, of the 104 amino acid component of subtilisin-modified ribonuclease (S-protein) results in significant regain of enzyme activity when the material is assayed in the presence of the 20 amino acid peptide component (S-peptide). This finding suggests that “information” determining secondary and tertiary structure of ribonuclease is contained in the amino acid sequence of the above mentioned protein moiety.

REFERENCES

Regeneration of Enzyme Activity by Air Oxidation of Reduced Subtilisin-Modified Ribonuclease
Edgar Haber and Christian B. Anfinsen


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