The Reversible Reduction of Disulfide Bonds in Trypsin and Ribonuclease Coupled to Carboxymethyl Cellulose

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It now seems very probable that the linear amino acid sequences of proteins are in some way uniquely determined by the sequence of nucleotides in the deoxyribonucleic acids of the chromosomes, or in the ribonucleic acids of some viruses. The simplest hypothesis regarding the control of the subsequent formation of three-dimensional configuration is that the amino acid sequence, alone, is sufficient for directing this process and that no further genetic information is required. It has been suggested (1) that the configuration of a native protein represents the state of lowest free energy attainable and that the unfolded protein will, under favorable circumstances, fold into that structure which is unique to the native molecule. In the simplest case, the process would be a completely spontaneous one. However, for some proteins, this process might require the presence of another protein or of a smaller molecule, such as an enzyme substrate, to induce the proper configuration.

One approach to the "proof" of the above thesis is the demonstration that proteins, converted to randomly coiled polypeptide chains by disruption of disulfide bonds and of noncovalent interactions, can be restored to their native three-dimensional forms by oxidation. Such reversibility has been demonstrated in experiments on bovine pancreatic ribonuclease in which it was shown that the reconstituted protein was identical with the native material as far as could be determined by x-ray, viscosity, optical rotatory and immunological characterization, and measurements of catalytic properties (2-4). The generality of this phenomenon has been similarly investigated with other proteins. Isemura et al. (5) have recently reported the reactivation of reduced Taka-Amylase A and egg white lysozyme, both of which have four disulfide bridges. Extensive studies of the latter protein have also been carried out by White (6). The formation of insulin by oxidation of its reduced chains has also been shown to take place to an extent greater than that predicted on the basis of random pairing of half-cystine residues (7, 8).

The present report concerns work with trypsin, which contains six disulfide bonds (9). Initial experiments demonstrated that reduced trypsin was highly insoluble at hydrogen ion concentrations considered optimal for reoxidation. The ability of active trypsin to digest itself and, particularly, its reduced form was also a source of difficulty. In an attempt to circumvent these problems, insoluble carboxymethyl cellulose-trypsin was prepared. Trypsin molecules in this material are not subject to autolysis and, because of their fixed positions on the CM-cellulose matrix, cannot undergo aggregation. Columns of CM-cellulose-trypsin were extremely stable and, after full reduction of disulfide bonds, could be restored to the active form in reasonably good yields by air oxidation.

EXPERIMENTAL PROCEDURE

1. Preparation of CM-cellulose-Trypsin—The method of Michael and Fawars (10) as modified by Mitzi and Summaria (11) was used. CM-cellulose hydraside was prepared from 200 to 325 mesh CM-cellulose (BioRad, control No. 59-3, 0.6 mEq per g) by esterification with methanol and HCl, followed by treatment of the ester with hydrazine. One gram of the hydraside was converted to the acid with nitrous acid (NaNO₂ in HCl), and was then coupled to 400 mg of trypsin (Worthington, 2 times recrystallized, Lot No. 839) by stirring the mixture at 5° for 2 hours at pH 8.7. After washing with cold 0.001 n HCl and water, and lyophilization, 700 mg of a fine white powder were obtained. The trypsin content of this material was 17.5% by amino acid analysis and 15.5% by nitrogen analysis (see below).

2. Preparation of CM-cellulose-RNase—The above method was employed, using 500 mg of CM-cellulose hydraside and 100 mg of ribonuclease (Sigma, Lot No. R31B-204). A total of 385 mg of CM-cellulose-ribonuclease was obtained.

3. Preparation of CM-cellulose-Trypsin and CM-cellulose-Ribonuclease Columns and Measurement of Enzymic Activity—On the basis of a report by Bar-Eli and Katchalski (12), dealing with the preparation of a trypsin column from an insoluble poly-peptidyl trypsin derivative, columns were prepared from CM-cellulose-trypsin and CM-cellulose-RNase. CM-cellulose-enzyme, 1 to 10 mg, was mixed with 25 to 30 mg of Celite (Johns-Manville Company) and the mixture was suspended in dilute 0.001 M hydrochloric acid or 0.1 M acetic acid and poured into a 0.8 X 10 cm glass column. The lower tip of the column was plugged with glass wool. The narrow bore of the column was necessitated by the small amounts of enzyme material used and the need to minimize agitation at the surface of the stationary phase. The column was perfused with substrate at various rates, the rate being determined by the air pressure applied. A constant pressure of compressed air was maintained by a simple mercury pressure regulator. In general, the perfusion time could be varied from 3 to 120 seconds per ml, the actual time being measured in each instance with a stop watch.

In practice, the enzymic activity was determined by perfusing the column at four or more different rates, and then plotting the change in optical density of the emerging substrate solution as a function of the time of perfusion. The column effluent was generally collected directly into the spectrophotometer cuvettes.
### Table I

<table>
<thead>
<tr>
<th>Material</th>
<th>Trypsin</th>
<th>Method of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-cellulose-trypsin</td>
<td>17.5</td>
<td>Nitrogen analysis</td>
</tr>
<tr>
<td>CM-cellulose-trypsin</td>
<td>15.5</td>
<td>Amino acid analysis*</td>
</tr>
<tr>
<td>Column 11 (after reoxidation)</td>
<td>6.3</td>
<td>Amino acid analysis</td>
</tr>
<tr>
<td>Column 19 (after reoxidation)</td>
<td>6.4</td>
<td>Amino acid analysis</td>
</tr>
</tbody>
</table>

* By amino acid analyzer.

4. Strutures—The substrate for the experiments with CM-cellulose-trypsin was benzoyl-l-arginine ethyl ester (13), at a concentration of 7.2 μg per ml in 0.05 M Tris buffer, pH 7.5. The optical density, measured at 253 nm with a Beckman model DU spectrophotometer, was approximately 0.485 for the hydrolyzed substrate.

For the CM-cellulose-RNase columns, the substrate was the barium salt of uridine 2',3'-cyclic phosphate (Schwarz BioResearch, Inc.), 100 μg per ml in 0.1 M sodium acetate buffer, pH 5.0. The substrate solution had an optical density of approximately 0.510 at 280 nm.

5. Reduction and Reoxidation of Enzyme Columns—The method employed was analogous to that described by Anfinsen and Haber (14) for soluble enzymes. A reducing solution was prepared from 2.4 g of recrystallized urea, 6 ml of 0.05 Tris buffer at pH 8.3, and 1 ml of β-mercaptoethanol (Eastman Kodak). This solution had a final volume of 10 ml and a urea concentration of 8 M. Approximately 5 ml were allowed to flow through the column over a 1/2-hour period. The lower end of the column was then sealed, and the equilibrated enzyme preparation allowed to incubate overnight (16 to 20 hours) at room temperature. The reduced column was then freed of urea and β-mercaptoethanol by flushing with 0.1 M acetic acid. Oxidation of the columns was accomplished by slow perfusion for 24 to 48 hours with 250 to 500 ml of 0.05 M Tris buffer, pH 8.3. In some cases, oxidation was carried out in the presence of 8 M urea, dissolved in the same buffer.

6. Mercurobenzoate Titration—To establish the degree of reduction, sulfhydryl groups were titrated with mercurobenzoate. Sodium "para-chloromercuribenzoate" (1.2 mg of the sodium salt, Nutritional Biochemicals Corporation) was dissolved in a small volume of dilute NaOH, and then was diluted to 50 ml with 0.5 M sodium acetate-0.01 M sodium phosphate buffer, pH 7.5. The solution was filtered before use. Mercurobenzoate solution, 10.0 ml, was allowed to flow through the column by gravity, over a 2-hour period. The column was then flushed with 10 ml of the acetate-phosphate buffer, and the combined effluents were diluted to 25 ml with buffer. For control determination, 10.0 ml of the mercurobenzoate solution were similarly diluted. The optical density of the mercurobenzoate solution was measured at 232 μm, and an extinction coefficient of 1.09 × 10⁶ per mole of mercurobenzoate per liter (15) was used to calculate the quantity of mercurobenzoate that had reacted. The content of —SH groups per mole of trypsin was computed as shown in Table II. Sucessive perfusions with mercurobenzoate showed that the titration of —SH groups was complete within the first 2-hour period. After the analysis, the mercurobenzoate could be removed from the column by perfusion for 10 minutes with 10 ml of 10% β-mercaptoethanol in 0.1 M acetic acid.

7. Determination of Trypsin Content of CM-cellulose-Trypsin—Since there was evidence that there was some loss of enzyme from the column during reduction and reoxidation (see below), it was considered necessary to determine the amount of trypsin present after these operations in order to establish the degree of reduction, as measured by mercurobenzoate. The content of trypsin was determined by direct amino acid analysis of the column contents by the following method. After reoxidation, the contents of the column, including the plug of glass wool, were quantitatively transferred to a thick-walled hydrolysis tube with 1.5 ml of constant boiling hydrochloric acid. The tube was evacuated, sealed, and heated for 20 hours at 108°C. The hydrolysate was then removed and the residual solids were carefully washed with small amounts of dilute hydrochloric acid. The washings were combined with the hydrolysate. This solution was reduced to dryness in a vacuum desiccator over sodium hydroxide. Amino acid analysis was performed on the Beckman/Spinco amino acid analyzer, and the quantity of trypsin present was calculated from the amount of aspartic acid found, using the figure of 21.5 residues of aspartic acid per molecule of trypsin (9). The internal consistency of the analyses, as determined by comparing the observed and the expected ratios of different amino acids, was, in general, quite good.

Direct nitrogen analysis was performed on the original CM-cellulose-trypsin as a further check on the validity of the amino acid analyses. The trypsin content was calculated from nitrogen value, using the figure of 16.13% for the nitrogen in trypsin (9).

**RESULTS**

A. Properties of CM-cellulose-Trypsin and CM-cellulose-Ribonuclease—The enzyme derivatives were conveniently stored as dry preparations. The enzyme activity of columns was approximately proportional to the amount of CM-cellulose-enzyme added and remained constant for periods of at least 2 weeks, regardless of whether the columns were stored at pH 3 or pH 8. The flow characteristics of columns also remained constant until they had been reduced and reoxidized. Such treatment resulted in a slight discoloration and a markedly increased resistance to flow. Another factor that occasionally resulted in increased resistance to flow was the accumulation of insoluble material on the top surface of the column. Filtration of all solutions alleviated this difficulty.

Treatment of columns of CM-cellulose-trypsin by the procedures for reduction and reoxidation resulted in a loss of 60% of the trypsin content (Table I). To further investigate this loss, columns of CM-cellulose-trypsin were prepared and perfused, over a 1-hour period, with 500 ml of pH 8.3 buffer. There was no demonstrable change in activity (Fig. 1). However, the buffer-washed material was observed to yield nonlinear activity curves, a phenomenon that has also been observed with CM-cellulose-RNase. Treatment of the CM-cellulose-trypsin columns with 5 ml of 8 M urea for 30 minutes resulted in a diminution of enzymic activity but yielded a linear activity curve. Further treatment with urea solution produced no further significant change. Similar changes were observed with urea-treated CM-cellulose-RNase. These findings indicate that there are either enzyme-CM-cellulose bonds which are dissociable by urea, although not by buffer (e.g. strong H bonds), or that there

* We wish to thank Mrs. Juanita Cooke for her performance of the amino acid analyses.
are CM-cellulose-enzyme particles which are soluble in 8 M urea but not in buffer. The latter possibility is supported by the observation of Mita and Summaria (11) that any individual lot of CM-cellulose contains particles of varying degrees of solubility in water-alcohol mixtures.

Mita and Summaria also discuss the fact that the insoluble CM-cellulose-trypsin was more active against smaller than larger substrates. This observation has been confirmed by the finding that CM-cellulose-trypsin attacked only a few of the available bonds in oxidized ribonuclease, as determined by calculation of base uptake during proteolysis in an autotitrator.

The exact estimation of the relation of the enzymic activity (against benzoyl-L-arginine ethyl ester) of CM-cellulose-trypsin to the activity of native trypsin is difficult to calculate since the former is determined from measurements on a column and the latter in solution. Nevertheless, a crude comparison may be obtained from the following data. The activity of three urea-washed columns averaged 0.089 optical density unit per second per mg of trypsin at pH 7.4 (assuming a value of 6.4% trypsin after urea washing), whereas the activity of native soluble trypsin was 0.43 optical density unit per second per mg at the same pH. Therefore, the CM-cellulose trypsin shows approximately 20% of native trypsin activity. A direct comparison of the enzymic activity of CM-cellulose-RNase with native RNase in solution has not yet been carried out.

**B. Completeness of Reduction and of Reoxidation**—Table II presents the results obtained from the mercuribenzoate titration of several columns after reduction and after reoxidation in either 8 M urea or buffer alone. The values of 13 to 15 —SH groups after reduction agree, as well as can be expected, with the theoretical value of 19 (9), if one considers the errors inherent in the various procedures employed. 2 The data also demonstrate the completeness of reoxidation of the enzyme at pH 8.3 in the presence or absence of 8 M urea.

**C. Enzyme Activity after Reduction and Reoxidation**—Approximately 30 columns with CM-cellulose-trypsin were reduced and reoxidized. Those columns contained between 1 and 10 mg of CM-cellulose-trypsin, the usual amount being approximately 5 mg. In nearly all columns reduced for 4 or more hours, the activity of the columns was zero, and in the remainder the residual activity was negligible. After reoxidation for 24 hours in buffer, pH 8.3, there was a significant return in activity in all columns so treated except for three in which <1 mg of CM-cellulose-trypsin was used. The activity curves obtained were, in all cases, linear. Although it was possible to estimate the recovery of enzymic activity by comparing the reoxidized column activity with the activity of calibration columns (see below), it was felt that the comparisons should preferably be made of the activity of the same column before and after reduction-reoxidation. This direct comparison was carried out in two columns which were first washed with urea and then assayed for “native” CM-cellulose-trypsin activity. The columns were then reduced, reoxidized in buffer, and reassayed. The enzymic activity after reoxidation was 3.7% (Column No. 33) and 3.3% (Column No. 34) of native activity (Fig. 2). By indirect comparison of eight reduced-reoxidized columns and three calibration columns (the latter giving a value of 0.0053 ± 0.00006 optical density unit per mg per second) (Tables III, IV), the value obtained was 4.1 ± 1.4%.

**D. Effect of Reoxidation in 8 M Urea and of Subsequent Treatment with β-Mercaptoethanol**—Previous work (16) suggests that reoxidation of reduced ribonuclease in the presence of 8 M urea is random and leads to the formation of incorrect disulphide bonds. The amount of enzymic activity obtained may, therefore, be a function of the probability of forming the correct bridges during a random reoxidation process. It was also found that the incorrect linkages could be induced to rearrange to the correct pairing by exposure to small amounts of β-mercaptoethanol.

**TABLE II**

<table>
<thead>
<tr>
<th>Column</th>
<th>Treatment</th>
<th>ΔOD. mer</th>
<th>Weight trypsin</th>
<th>Sulfhydryl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>curbenzoate</td>
<td>mg/wt. trypsin</td>
<td>per mole</td>
</tr>
<tr>
<td>17</td>
<td>Reoxidized in urea</td>
<td>0.006</td>
<td>0.38</td>
<td>&lt;1</td>
</tr>
<tr>
<td>18</td>
<td>Reoxidized in buffer</td>
<td>0.020</td>
<td>0.31</td>
<td>2.3</td>
</tr>
<tr>
<td>19</td>
<td>Reduced</td>
<td>0.100</td>
<td>0.39</td>
<td>15.2</td>
</tr>
<tr>
<td>20</td>
<td>Reduced</td>
<td>0.119</td>
<td>0.32</td>
<td>13.1</td>
</tr>
<tr>
<td>21</td>
<td>Reoxidized in urea</td>
<td>0.003</td>
<td>0.32</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>0.150</td>
<td>0.41</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>Reoxidized in urea</td>
<td>0.006</td>
<td>0.41</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Difference between optical density of control and perfusing mercuribenzoate solutions diluted to 25.0 ml and measured at 522 mμ.
† Calculated from weight of CM-cellulose-trypsin, using 6.4% as amount of trypsin actually present.
‡ Formula used: SH = ΔOD./1.60 × 103 × 25/103 × 23,800/ wt. trypsin in grams.

Fig. 1. The effect of washing CM-cellulose-trypsin columns with buffer and with 8 M urea. The activity curve of a column washed for 18 hours with 500 ml of Tris buffer, pH 8.3, showed no change from its initial values and remained nonlinear. After a 1-hour perfusion with 8 M of 8 M urea, there was a loss of activity and conversion of the curve to linearity. Perfusion with an additional 5 ml of 8 M urea (total time, 1 hour) had no additional effect. CMC = carboxymethyl cellulose.
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Recovery of activity after reduction and reoxidation of CM-cellulose-trypsin columns. The activity of the column after treatment with 8 M urea was 0.0066 optical density unit per second at 253 mμ. After reduction in urea and β-mercaptoethanol and reoxidation in buffer, the activity was 0.00022 optical density unit per second giving a recovery of 3.3%.

**TABLE III**

<table>
<thead>
<tr>
<th>Column</th>
<th>Weight CM-cellulose-trypsin (mg)</th>
<th>Activity (ΔO.D./sec)</th>
<th>Activity per mg of CM-cellulose-trypsin (ΔO.D./mg)</th>
<th>% of return</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5.2</td>
<td>0.00073</td>
<td>0.00014</td>
<td>2.6</td>
</tr>
<tr>
<td>13</td>
<td>5.3</td>
<td>0.0013</td>
<td>0.00025</td>
<td>4.7</td>
</tr>
<tr>
<td>15</td>
<td>5.0</td>
<td>0.00182</td>
<td>0.00036</td>
<td>6.8</td>
</tr>
<tr>
<td>17</td>
<td>6.0</td>
<td>0.00118</td>
<td>0.00020</td>
<td>3.8</td>
</tr>
<tr>
<td>18</td>
<td>4.8</td>
<td>0.00153</td>
<td>0.00052</td>
<td>6.0</td>
</tr>
<tr>
<td>19</td>
<td>6.1</td>
<td>0.00118</td>
<td>0.00019</td>
<td>3.5</td>
</tr>
<tr>
<td>20</td>
<td>5.0</td>
<td>0.0007</td>
<td>0.00014</td>
<td>2.6</td>
</tr>
<tr>
<td>21</td>
<td>6.4</td>
<td>0.0010</td>
<td>0.00016</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Calculated on basis of calibration columns, Table IV. Average, 4.1 ± 1.4%.

**TABLE IV**

<table>
<thead>
<tr>
<th>Column</th>
<th>Weight CM-cellulose-ribonuclease (mg)</th>
<th>Activity (ΔO.D./sec)</th>
<th>Activity per mg of CM-cellulose-ribonuclease (ΔO.D./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>0.40</td>
<td>0.002</td>
<td>0.0050</td>
</tr>
<tr>
<td>33</td>
<td>1.15</td>
<td>0.0056</td>
<td>0.0049</td>
</tr>
<tr>
<td>34</td>
<td>1.10</td>
<td>0.0066</td>
<td>0.0060</td>
</tr>
</tbody>
</table>

* Average, 0.0053 ± 0.006.

These observations were used to estimate the amount of activity resulting from random reoxidation of the CM-cellulose-trypsin columns. Four columns (Nos. 12, 17, 20, and 21) were oxidized by perfusion with 8 M urea at pH 8.3 for 40 to 44 hours. No enzymic activity was detectable in these columns. As shown in Table II, reoxidation was complete. Treatment of these columns with 500 ml of 0.2% (volume for volume) β-mercaptoethanol in pH 8.3 buffer for 24 hours resulted in restoration of activity to levels comparable with those of columns oxidized in buffer (Fig. 3).

**E. Effect of Two Successive Cycles of Reduction and Reoxidation**

To ascertain the reproducibility of the reduction-reoxidation process on any single column, a few columns were subjected to two successive cycles of reduction and reoxidation (Columns 20, 21, 29, and 30). The return in activity after the second reoxidation ranged from 20 to 61% of the activity present after the first reoxidation, but there was evidence from mercuribenzoate titrations that additional enzyme had been lost from the columns.

**F. Reduction and Reoxidation of CM-cellulose-Ribonuclease Columns**

Because of the previously demonstrated ability of reduced ribonuclease to regain almost all enzymic activity after reoxidation (4, 14), two CM-cellulose-ribonuclease columns were treated as described for CM-cellulose-trypsin to test the efficiency of the process. The results were calculated by the indirect method, with urea-washed calibration columns, and the recoveries were 34 and 42.5% of native activity.

**DISCUSSION**

The use of insoluble enzymes fixed to a supporting material, in the form of columns, presents certain theoretical difficulties. The process of attaching the enzyme to the CM-cellulose involves the modification of chemical groups of the enzyme and the introduction of restrictions on the freedom of movement of various parts of the molecule. These restrictions might make the proper reoxidation of —SH groups difficult. On the other hand, if it is assumed that no structural strain is involved, fixation of the enzyme may confer a certain degree of stability to the enzyme configuration; this would favor proper reoxidation. In addition, the flow characteristics of a column cannot be assumed to remain constant after drastic treatment of the material. The greatly increased resistance to flow observed in the present studies would be compatible with intermolecular aggregation or cross-
linking through \(-\text{S-S-}\) bonds during experimental manipulations. There is no way of predicting the net effect of these factors on the ability of a CM-cellulose-enzyme to correctly reoxidize after reduction, but the data obtained from the CM-cellulose-ribonucleic columns suggests that the maximal efficiency by this method is less than half of the efficiency in solution. Although the recovery of activity by oxidation of reduced CM-cellulose-trypsin columns is only approximately 4%, random reoxidation, such as takes place in urea, results in no return of activity. The exposure of incorrectly oxidized material to a small quantity of \(\beta\)-mercaptoethanol resulted in a significant return of activity; buffer alone did not have this effect. The most reasonable explanation is that disulfide interchange occurred, with the formation of the correct disulfide pairs. The driving force for such a rearrangement is presumed to be a lower free energy of the molecule, when it is the correct configuration, with \(\beta\)-mercaptoethanol acting as a catalyst. Urea, by interfering with intramolecular interactions, allows the molecule to reach a configuration which is metastable and which does not spontaneously, within a short time, alter to the more stable form in the absence of urea. A direct study of these hypotheses is not possible because of the inaccessibility of the trypsin on the column to degradation studies. However, work with soluble ribonuclease, involving a conversion of incorrectly to correctly paired disulfide bridges by disulfide interchange, is compatible with these suggestions (1).

The significance of a value of 4% may also be evaluated in terms of the return of enzymic activity that would be expected from a random reoxidation process. Sela and Lifson (17) and Kauzmann (18) give a general formula for calculating the probability of reforming a given set of disulfide bonds, assuming no special restrictions. For the case of reduced trypsin, with 12 \(-\text{SH}\) groups, there are 10,395 unique pairings possible, or in other words, a probability of only 0.01% of randomly producing a unique pairing of \(-\text{SH}\) groups. For ribonuclease, with eight \(-\text{SH}\) groups and four \(-\text{S-S-}\) bonds, there are only 105 possibilities, or a probability of 1% for any given set of pairs. If it is assumed that only one unique pairing can result in enzymic activity, and, as is suggested by the CM-cellulose-RNase columns, that fixation of the enzyme does not favor proper reoxidation, the figure of 4% appears highly significant when compared with 0.01% expected from a random process.

The fact that reoxidation, after a second reduction, gives an activity as high as 60% of that obtained after one cycle of reduction and reoxidation suggests another possible explanation for the low over-all return in activity. (The value of 60% may, indeed, be a low estimate in the light of possible further loss of enzyme from the column.) It appears likely that only a fraction of the enzyme molecules in the original CM-cellulose preparation is capable of reoxidation. The size of this population would be determined by such factors as the mode of attachment of the enzyme molecule to CM-cellulose, the spatial relation of 1 enzyme molecule to another, the physical characteristics of the CM-cellulose particles, and the heterogeneity of the trypsin preparation itself.

**SUMMARY**

Insoluble carboxymethyl cellulose-trypsin was used to prepare stable, easily assayed, small columns. Complete reduction of the columns with 8 M urea and \(\beta\)-mercaptoethanol, followed by reoxidation at pH 8.3, resulted in a recovery of 4% of the original activity. Reoxidation in 8 M urea produced no return in activity, but the enzyme could then be activated by treatment with 0.2% \(\beta\)-mercaptoethanol. Since the return in activity was much greater than would be expected, on theoretical grounds, for a random reoxidation process (0.01%), and greater than was actually observed for columns randomly reoxidized in 8 M urea, these findings are compatible with the hypothesis that the three-dimensional configuration of at least the catalytically active portion of the protein is determined by the amino acid sequence alone. A second cycle of reduction and reoxidation resulted in a recovery of up to 60% of the activity found after the first reoxidation. These results suggest that there existed a small, but relatively constant, fraction of trypsin molecules capable of fully reversible reduction and reoxidation. Reduction and reoxidation of carboxymethyl cellulose-ribonuclease resulted in a recovery of approximately 40% of the original activity.

**REFERENCES**

The Reversible Reduction of Disulfide Bonds in Trypsin and Ribonuclease
Coupled to Carboxymethyl Cellulose
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