Effect of Divalent Cations on the Reduction and Re-formation of the Disulfide Bonds of Deoxyribonuclease*

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PAUL A. PRICE,† WILLIAM H. STEIN, AND STANFORD MOORE

From The Rockefeller University, New York, New York 10021

SUMMARY

The availability of stable, purified preparations of bovine pancreatic deoxyribonuclease (mol wt 31,000) has prompted study of the reduction and re-formation of the two disulfide bonds cross-linking its single polypeptide chain. The two disulfide bonds of DNase are quantitatively reduced by mercaptoethanol and similar reagents in minutes at pH 7.2 and room temperature, without need for 8 M urea or any other denaturing agent. The reduced protein is inactive. The unusual ease of reduction of the disulfide bonds is accompanied by remarkable stability of the reduced form of the protein in the absence of divalent cations. After 24 hours of exposure to air oxidation at pH 7.5 in 1 mM EDTA, 95% of the half-cystine residues remain as cysteine. The situation changes if 4 μM Ca++ is added; re-formation of disulfide bonds and full regain of activity occurs in minutes. If Ca++ is present during the reduction, the product contains one disulfide bond and two —SH groups. The molecule is fully active and remains so even after the two —SH groups are carboxymethylated. The same half-reduced, half-oxidized molecule can be obtained by adding Ca++ to the fully reduced protein providing the reducing agent is still present. These experiments, and the requirement for a divalent cation-DNA complex as the substrate for DNase, demonstrate an ion-protein interaction. However, simple gel filtration at neutral pH removes 45Ca* completely from both the native enzyme and the active form containing only one disulfide bond; thus, the divalent metal is not bound strongly in either case.

The primary sequence of DNase is not sufficient to direct a rapid refolding of the reduced protein under conditions which are similar to those that are effective with RNase and a number of other proteins; in order for the information resident in the amino acid sequence of DNase to be fully expressed, and the native three-dimensional structure to be formed, divalent cations are required.

The experiments of White (1) and Anfinsen and Haber (2) on the re-formation of the disulfide bonds in reduced pancreatic ribonuclease led to the stimulating finding that the information necessary for the determination of the structure of refolded ribonuclease resides in the primary sequence of the enzyme. In the following experiments, pancreatic deoxyribonuclease (3), as another single chained enzyme of pancreatic origin, has been submitted to a study of the regain of its activity and disulfide bonds after reduction.

EXPERIMENTAL PROCEDURE

Materials—The bovine pancreatic DNase employed was stabilized and purified (3) from Worthington DP grade DNase. Methods—Amino acid analyses, radioactivity determinations, and DNase assays were carried out as previously described (3). As will be pointed out under “Results,” it was usually desirable to substitute an equimolar amount of Zn(OAc)₂ for MnCl₂ in the assay medium.

Reduction and Re-formation of Disulfide Bonds—It was found that the disulfide bonds of DNase reduce readily without the need for the 8 M urea which is required with many proteins, including RNase (4). In the present procedure, DNase (0.1 to 0.4%) was kept for 15 min under N₂ in Tris-chloride buffer, 0.05 M in Tris, pH 7.2, 5 μM in EDTA, prior to the addition of mercaptoethanol (Eastman) or dithiothreitol (Calbiochem) to give a solution 0.05 M in reducing agent. Samples (5 to 10 μl) were taken for assays of DNase activity just before and at suitable intervals after the addition of the reducing agent. Samples (5 to 10 μl) were taken for assays of DNase activity just before and at suitable intervals after the addition of the reducing agent. The reduced protein was freed of reagents on a column (2 x 30 cm) of Sephadex G 25 equilibrated either with 5% acetic acid at 5° or at 25° with 5 mM Tris-chloride buffer, pH 7.3, 5 mM in EDTA. To bring the protein in 5% acetic acid to pH 7.5 for further work, an equal volume of molar Tris base was added. To measure the sulphydryl content, a 1500-fold molar excess of sodium iodoacetate was added as a 1 M solution. The mixture was allowed to stand in the dark at 25° for 30 min and dialyzed against 3 changes of 1000 volumes of distilled water for 24 hours at 5°. The contents of the dialysis bag were lyophilized, hydrolyzed, and analyzed for S-carboxymethylcysteine.

To study the re-formation of disulfide bonds, DNase (0.05 to 0.1%) was allowed to stand at 25° for various periods of time in the presence of the reagents whose effects were being studied.
substrate.

Inactivation by mercaptoethanol was first order with respect to DNase concentration. The fully reduced enzyme remains inactive after all of the SH groups have been blocked by S-carboxymethylation.

### Table I

**Relationship between inactivation of DNase by mercaptans and reduction of disulfide bonds**

The reaction was carried out at 25° in the presence of 0.05 M Tris-chloride buffer, pH 7.2, and 5 mM EDTA. The extent of reduction was determined from the S-carboxymethylcysteine content after reaction with iodoacetate. Activity was measured against Zn-DNA as substrate.

<table>
<thead>
<tr>
<th>Concentration of reactants</th>
<th>Time of reaction with mercaptan</th>
<th>Loss of activity</th>
<th>Protein-SH groups formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M cysteine, 0.1% DNase</td>
<td>subs</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>0.05 M mercaptoethanol, 0.2%</td>
<td>30</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>DNase</td>
<td>20</td>
<td>78</td>
<td>79</td>
</tr>
<tr>
<td>0.05 M mercaptoethanol, 0.4%</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

At suitable intervals, 5- to 10-μl samples were removed for assay of DNase activity, and 1-ml samples for determination of the remaining cysteine as S-carboxymethylcysteine, as described above.

**RESULTS**

**Effect of Reducing Agents on DNase**—DNase rapidly loses activity in the presence of mercaptoethanol in simple buffered solutions and in the absence of divalent cations. From Fig. 1, the time for the loss of half of the DNase activity is about 4 min. At the same pH and temperature, a comparable inactivation is brought about by 0.05 M dithiothreitol in 5 min. In no case is the presence of urea or any other denaturing agent required. The inactivation by mercaptoethanol was first order with respect to DNase concentration. The fully reduced enzyme remains inactive after all of the -SH groups have been blocked by carboxymethylation.

The degree of inactivation of DNase by mercaptoethanol (Table I) is directly proportional to the number of protein —SH groups formed. When a given reaction mixture has lost half of its enzymatic activity, half of the disulfide bonds have been reduced.

In all of these experiments on the disulfide bonds of DNase, enzymatic activity against DNA has been measured with Zn++ (added as the acetate) as the cation rather than Mn++, as was used previously (3), except with dithiothreitol which forms a firm complex with Zn++; Mg-DNA was used in that instance. Unless this is done, total inactivation on reduction is not observed. In early experiments with Mn-DNA as the substrate, significant activity could be detected even after reduction was complete (4 eq of carboxymethylcysteine formed on reaction with iodoacetate). When it was found (as noted below) that divalent cations can promote the reactivation of DNase, it seemed likely that the activity exhibited by fully reduced DNase was a consequence of some cation-induced reactivation which occurred during assay in the presence of Mn++. This same effect may account for previous failures to bring about complete inactivation of DNase by reduction, although in Melzer's (5) experiments the 38% inhibition observed with 0.01 M mercaptoethanol was probably mainly a result of the lower concentration of mercaptan. A survey of the ability of divalent cations to promote reactivation showed that Mg++ and Zn++ were essentially devoid of this capacity. Since they both can serve effectively as cations in the assay of DNase, and since the specific activity of DNase against Zn-DNA is 6 times higher than against Mg-DNA, Zn-DNA was employed as substrate in most of the assays reported in this communication.

Some of these same considerations entered into the choice of Ca++ for most of the studies summarized below on the specific effect of divalent cations on refolding of the enzyme; Ca-DNA is a relatively ineffective substrate but the previous studies (3) on the marked effect of Ca++ in stabilizing DNase against proteolysis indicated that Ca++ participates in a significant ion-protein interaction.

**Effect of Ca++ upon Reactivation of Reduced DNase**—When completely reduced DNase (0.05%), freed of reagents by passage through Sephadex, is allowed to stand at pH 7.5 for 24 hours at 25°, about 20 to 30% of the initial enzymatic activity is regenerated. In the presence of 1 mM EDTA, the regain of activity is less than 5% and upon subsequent treatment with iodoacetate, it is found that 95% of the original 4 sulfhydryl groups per molecule are still present. If, however, the reduced enzyme is allowed to stand in the presence of 4 mM CaCl₂, regain of activity occurs in minutes (Fig. 2). The same rate of reactivation is observed whether Ca++ is added at once or the reduced enzyme has first stood at 25° for 24 hours. Reactivation is paralleled exactly by a loss of protein —SH groups. For example, in one experiment (not included in Fig. 2), 80% of the enzyme activity had been regained in 28 min and 20% of the SH groups remained (Ca++ concentration 0.05 M). After 90 min, when reactivation was complete, no —SH groups remained. In order to show that reactivation is probably facilitated by Ca++-DNase interaction and not by traces of other divalent cations, such as Cu++, that are known to catalyze the oxidation of disulfide bonds by oxygen, reactivation was allowed to occur in the presence of Ca++ and EDTA. The stability constant (6) of EDTA for Cu++ is 10⁵ M⁻¹ liter, which is far greater than the constant for Ca++ (10⁸ M⁻¹); therefore, if traces of Cu++ were responsible for the observed effects, reactivation in the presence of 1 mM EDTA plus 5 mM Ca++ should be lower than in the presence of
and therefore disulfide bond formation and reactivation must be a consequence of the action of Ca++. Other divalent cations, such as Cu++, Mn++, and Mg++, also are capable in 0.05 M concentration of reactivating DNase to the extent of 70%, 100%, and 60%, respectively, in 3 hours.

Information as to the manner in which divalent cations act was provided by an experiment in which 0.01 M Ca++ (100-fold molar excess) was added directly to completely reduced, inactive DNase with the mercaptoethanol used for reduction still present. Activity was completely regained in 1 hour (Fig. 3) even in the presence of a 1000-fold molar excess of mercaptan.

In Table II the data on S—S bond formation and activity regain under these conditions are given. The regain of activity is 100% when only one S—S bond is formed (45% regain of the two S—S bonds), as judged by carboxymethylcysteine forma-

<table>
<thead>
<tr>
<th>Time in presence of Ca++</th>
<th>Protein —SH groups remaining</th>
<th>Regain of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>72</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>100</td>
</tr>
</tbody>
</table>

Reduction was carried out at 25° in 5 mM Tris-chloride buffer, pH 7.2, 0.10 M mercaptoethanol, 0.1% DNase. After 30 min, the solution was brought to 0.01 M Ca++ by the addition of 1.0 M CaCl₂ solution. At the indicated times 1.0-ml samples were withdrawn for measurement of activity and residual protein —SH groups. The latter were determined by adding enough iodoacetate (1.0 M solution) to render the mixture 0.15 M and sufficient Tris base to bring the pH to 8. After 1 hour, in order to chelate Ca++, 0.3 M EDTA was added to give a concentration of 0.1 M and the alkylation reaction was continued for an additional hour. In the presence of mercaptoethanol, 0.05 M; DNase, 0.1%; 0.05 M Tris-chloride buffer at pH 7.2.

A 10-fold molar excess of EDTA over Ca++ was used to reduce the possibility that Ca++ might be bound to the two —SH groups and block the alkylation. Final proof that Ca++ is not being strongly bound under these conditions came from a repetition of the experiment with ⁴⁰Ca++ (cf. Reference 3). There was no detectable radioactivity in the active DNase obtained after simple gel filtration on a Sephadex G-25 column (2 X 7 cm) at pH 7.5 and 4°.

The dependence upon Ca++ concentration of this reactivation in the presence of mercaptoethanol is shown in Fig. 4. By the same type of calculation used in the preceding communication (7) in studying the effect of Mn++ on the alkylation of histidine, the slope of 1.0 in Fig. 4 indicates that reactivation by the forma-
tion of a single S–S bond is effected by the binding of one Ca++ per molecule of DNase. The molecule which contains the one re-formed S–S bond and the other two —SH groups as the carboxymethyl derivatives is also fully active.

Ca++ not only promotes the reactivation of fully reduced DNase; it also protects native DNase against full reduction and consequent inactivation. If Ca++ is added to DNase (final concentration, 0.01 M), followed 30 min later by mercaptoethanol (final concentration, 0.05 M), there is no loss of enzymatic activity.

**DISCUSSION**

One of the principal conclusions that can be drawn from these experiments is that one of the two disulfide bonds in DNase is not needed for catalytic activity. The presence of a divalent cation such as Ca++ is essential to protect one disulfide bond from reduction or to re-form it from the totally reduced enzyme. Thus, the monodisulfide form of the enzyme can be approached from either direction; Ca++ can be added to the fully reduced enzyme with the reducting agent still present, or reduction with mercaptoethanol can be performed in the presence of Ca++. A corollary of this conclusion, which is suggested by the data in Table I, is that when reduction is carried out in the absence of metal ions, the splitting of the first disulfide bond is rate-limiting; the second one is then split much more rapidly. Otherwise the loss of activity would not parallel the formation of —SH groups; under these conditions there is no detectable concentration of half-reduced DNase molecules. The reduction process follows an all-or-none course. Conversely, during air oxidation in the presence of Ca++ and in the absence of a mercaptoan, the formation of the first disulfide bond is followed rapidly by formation of the second, since the percentage regain of activity parallels the percentage loss of —SH groups.

An unusual feature of the reduction of DNase is the ease with which the change from S–S to —SH is accomplished; no denaturing agent is required. Apparently the S–S bonds are readily available. Equally unusual, particularly in view of the ease of —SH formation, is the fact that the reduced enzyme stays 95% in the —SH form after exposure to the air for 24 hours at neutral pH in the absence of Ca++. Neither intramolecular nor intermolecular S–S bonds are formed, as proved by the yield of carboxymethylcysteine after reaction with iodoacetate.

The presence of Ca++ changes the regain of activity from 5% in 24 hours to 50% in 20 min. We conclude from this result that a specific component of the environment, namely Ca++, is essential for the full expression of the information resident in the amino acid sequence, information that can lead under the proper conditions to the generation of the tertiary structure of the active enzyme.

The closest parallel to those results on reoxidation is in the experiments of Takagi and Isemura (8) and Friedmann and Epstein (9) on the role of calcium in the reactivation of reduced Taka-amylase, a Ca++-containing enzyme. In that case Ca++ is needed for the closing of the fourth disulfide bond but not for the first three (9). A further difference is that the protein binds Ca++ tightly whereas DNase does not. From these results on the effect of Ca++ in the refolding of reduced proteins, it may be expected that small molecules will be found to exert similar specific effects upon other proteins.

A full interpretation of the behavior of the S–S bonds of DNase will depend upon knowledge of both the sequence and the crystallographic structure of this pancreatic enzyme.

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