KINETICS OF REDUCTION OF THE DISULFIDE BONDS IN RIBONUCLEASE A

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1. Introduction

Breaking the disulphide bonds is one of the steps involved in the complete denaturation of proteins which have such intramolecular crosslinks. This breaking is usually achieved by reduction with a thiol reagent, using a thiol concentration of 0.1 M or above, in the presence of a denaturing agent, at pH values above 8, and for a few hours at 25°C or room temperature [1,2]. These conditions have been shown to indeed lead to a completely reduced protein. We have attempted to follow the kinetics of reduction of the disulphide bonds of ribonuclease in these almost standard conditions, and we have found that this kinetics was too fast to be measured. We have therefore studied the range of conditions under which the kinetics of reduction of disulphide bonds could be observed, and this article reports some results about the kinetics of reduction of ribonuclease by β-mercaptoethanol. It is found that complete reduction of ribonuclease by β-mercaptoethanol can be achieved in milder conditions than those generally used, conditions under which the kinetics of reduction can be observed.

2. Materials and methods

Ribonuclease (lot RAF 54 J 355) was obtained from Worthington, 2',3' CMP > from Sigma, NEM from Aldrich, [^{14}C]NEM (4.1 Ci/mol) from CEA, β-mercaptoethanol from Eastman Kodak, and all other reagents were from Merck. No difference was found when either commercial or purified ribonuclease was used.

Abbreviations: Ribonuclease, bovine pancreatic ribonuclease A (EC 3.1.4.22); 2',3'CMP >, cytidine 2',3' monophosphate; NEM, *N*-ethyl-maleimide; Gdn·HCl, guanidine hydrochloride

2.1. *Activity measurements*

Ribonuclease activity towards 2',3' CMP > was measured in a Zeiss PMQ II equipped with a Sefram recorder by the absorbance change at 292 nm in the following conditions: 1–2 mM 2',3' CMP >, 0.1 M Cacodylate buffer, pH 6, 25°C. This activity assay was linear with respect to ribonuclease concentration in the range from 3–200 nM ribonuclease.

2.2. *Reduction of ribonuclease*

Before reduction, ribonuclease was first unfolded for 0.5–1 h in 6 M Gdn·HCl, 1.5 mM EDTA, 20 mM Tris buffer, pH 8.5, 25°C then β-mercaptoethanol was added to initiate the reduction reaction. The reduction reaction was stopped at various times by alkylating all sulfhydryl groups present by reaction with a 3–5-fold molar excess of NEM in 0.1 M cacodylate buffer at pH 7. At neutral pH, the reaction of NEM with thiol groups is fast and almost stoichiometric [3]. The residual activity of ribonuclease was measured as described above directly on NEM treated samples, after removal of the Gdn·HCl by dilution. All activities are expressed relatively to that of native ribonuclease.

2.3. *Incorporation of [^{14}C]NEM into ribonuclease*

The reaction with [^{14}C]NEM was used to follow the appearance of sulfhydryl groups of ribonuclease upon reduction of the disulphide bonds. After reaction with [^{14}C]NEM, the protein was precipitated

with 10% trichloroacetic acid (TCA), washed once with 10% TCA, then washed three times alternatively with ethanol and 10% TCA. The protein precipitate was dissolved in 0.1 N NaOH, and the amount of radioactivity was measured according to Bray [4] on samples the protein amount of which was determined by amino acid analysis.

3. Results

The treatment with NEM has no effect on ribonuclease activity when applied to either native or Gdn·HCl-unfolded ribonuclease. Therefore the activity measured during the reduction and after alkylation by NEM of the sulfhydryl groups, corresponds at least to the protein which has not been reduced at all, and maybe also to some partially reduced and alkylated species. Figure 1 shows the kinetic of both [^{14}C]NEM incorporation and loss of activity when followed as described above, upon reduction of ribonuclease by 3.5 mM β-mercaptoethanol. The loss of activity follows apparent first order kinetics down to a level lower than 0.1% of the starting activity. The apparent first order rate constant of this activity loss is proportional to the concentration of β-mercaptoethanol used to reduce ribonuclease. This is shown in fig.2 which gives the residual activity measured after 1 min, 2 min and 3 min of reduction performed at various β-mercaptoethanol concentrations. From the data of fig.2, the second order rate constant of the reaction of inactivation of ribonuclease by β-mercaptoethanol can be evaluated to about 5 $M^{-1}s^{-1}$.

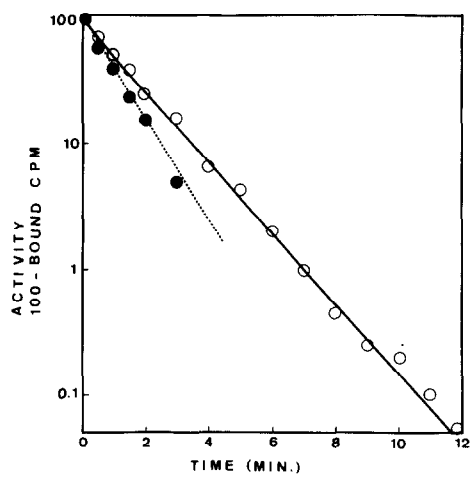

Fig.1. Semi-logarithimic plot of the time dependences of the loss of activity (–○–) and the incorporation of [^{14}C]NEM (–●–) upon reduction of 0.6 mM ribonuclease by 3.5 mM β-mercaptoethanol. Final conditions for reaction with [^{14}C]NEM: ribonuclease 0.4 mM, NEM 50 mM, 10^{11} cpm/mol, 200 mM cacodylate buffer, pH 7.

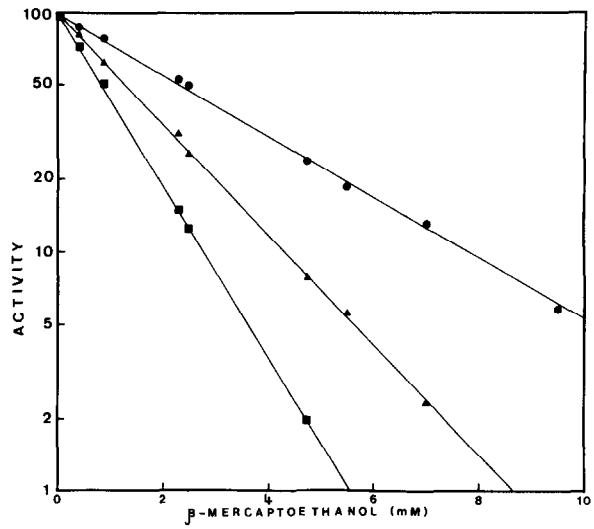

Fig.2. Semi-logarithmic plot of the dependence on β-mercaptoethanol concentration of the residual activity of ribonuclease remaining after 1 min (●), 2 min (▲) and 3 min (■) of reduction. Ribonuclease was at a concentration of 0.6 mM, and the activities were measured after blocking the reduction reaction with NEM.

The incorporation of [^{14}C]NEM into ribonuclease also follows apparent first order kinetics, and seems to be slightly faster than the loss of activity (fig.1), in the same conditions. However, the maximum amount of [^{14}C]NEM incorporated into ribonuclease at the end of the reduction by 3.5 mM β-mercaptoethanol does not correspond to a completely reduced protein, although all the activity has been lost. Only 2–2.5 mol [^{14}C]NEM/mol ribonuclease are found, showing that only about one disulphide bond has been reduced in these conditions. It is difficult to follow the kinetics of incorporation of [^{14}C]NEM when reduction of ribonuclease is performed at higher concentrations of β-mercaptoethanol, because the reaction is too fast. Instead of the rate of reduction, we have therefore measured the maximum extent of reduction of ribonuclease in various concentrations of

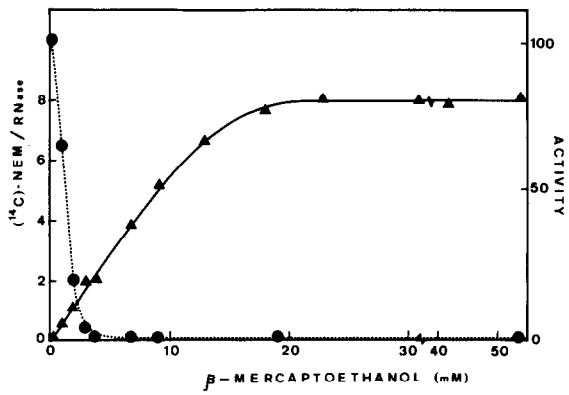

Fig.3. Dependence of the amount of incorporated [^{14}C]NEM (–▲–) and of the residual activity (–●–) on the β-mercaptoethanol concentration used to reduce ribonuclease. In each case reduction was carried out for one hour. Ribonuclease was 0.6–2 mM. Reaction with [^{14}C]NEM was carried out as described in the legend of fig.1, except that NEM was at a concentration of 110 mM and a specific activity of 2×10^{11} cpm/mol. The number of [^{14}C]NEM incorporated/mol ribonuclease was determined from the specific activity of [^{14}C]NEM, the total amount of cpm incorporated, and the amount of protein as measured by amino acid analyses.

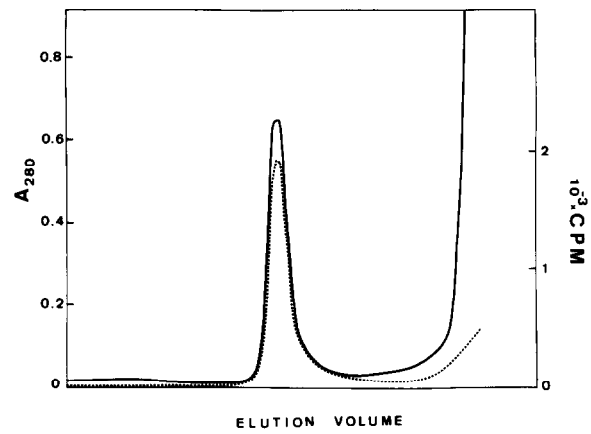

Fig.4. Elution diagram from a Sephadex G-25 Superfine column (120 × 2 cm) of ribonuclease which has been reduced and alkylated by unlabelled NEM once, then reduced with an excess β-mercaptoethanol and alkylated with [^{14}C]NEM (see text) with a specific activity of 4×10^{12} cpm/mol. Radioactivity is measured on 50 μl samples (- - - -); A_{280} nm (——).

β-mercaptoethanol. Figure 3 shows the dependence on β-mercaptoethanol concentration of the amount of [^{14}C]NEM which can be incorporated into ribonuclease after completion of the reduction reaction. For β-mercaptoethanol concentrations of 20 mM and above, about 8 mol [^{14}C]NEM/mol ribonuclease can be incorporated, showing that 8 sulfhydryl groups are able to react with NEM, i.e., that all the 4 disulphide bonds of ribonuclease have been reduced. Comparatively, much lower β-mercaptoethanol concentrations are needed to give an inactive protein after alkylation of its sulfhydryl groups. All activity has disappeared when about only 2 mol [^{14}C]NEM have been incorporated into ribonuclease (fig.3), suggesting that ribonuclease species with three disulphide bonds and two alkylated sulfhydryl groups are inactive.

Another experiment confirms that ribonuclease can be completely reduced by 20 mM β-mercaptoethanol. Ribonuclease was reduced a first time for one hour by 20 mM β-mercaptoethanol as described above, then all sulfhydryl groups present were alkylated by non-radioactive NEM. The protein was separated from low molecular weight compounds by gel filtration, lyophilized and submitted to a second reduction by 50 mM β-mercaptoethanol, in the same conditions as above. After one hour, [^{14}C]NEM was added, and the protein was separated from the excess of [^{14}C]NEM by gel filtration on a column (120 × 2 cm) of Sephadex G-25 Superfine, the elution diagram of which is given in fig.4. The amount of [^{14}C]NEM incorporated into ribonuclease is only 0.15–0.2 mol/mol protein showing that only 0.15–0.2 sulfhydryl groups, out of a total of 8, were not able to react with non-radioactive NEM after the first reduction by 20 mM β-mercaptoethanol and became available to [^{14}C]NEM only after the second reduction with 50 mM β-mercaptoethanol. It is even possible that part of these 0.15–0.2 sulfhydryl groups did not react with NEM after the first reduction because they were engaged in mixed disulphide bonds between the reduced protein and β-mercaptoethanol [5]. We can therefore conclude that the first reduction by 20 mM β-mercaptoethanol, in 6 M Gdn·HCl, at pH 8.5, 25°C, has indeed reduced at least 3.9 disulphide bonds, out of 4, in ribonuclease.

4. Discussion

The results presented above shows that complete reduction of ribonuclease can be achieved by reaction

with β-mercaptoethanol concentrations as low as about 20 mM. Below 20 mM β-mercaptoethanol only a partial reduction of ribonuclease occurs, albeit accompanied by a total loss of activity (fig.3). The activity is abolished when about 2 mol [^{14}C]NEM are incorporated/mol ribonuclease, i.e., when about one disulphide bond has been reduced and the two newly liberated sulfhydryl groups have been alkylated. Since the reduction is performed on ribonuclease unfolded by 6 M Gdn·HCl, in which all disulphide bonds should be equally reactive towards β-mercaptoethanol, the kinetics of loss of activity probably reflect the kinetics of reduction of one disulphide bond. At pH 8.5, 25°C, the bimolecular rate constant of the reaction of β-mercaptoethanol with the disulphide bonds of ribonuclease unfolded by 6 M Gdn·HCl, is found to be about 5 $M^{-1}s^{-1}$, when measured from the enzyme loss of activity (fig.2). If this value corresponds to the reduction of one disulphide bond, the rate constant pertaining to the formation of completely reduced ribonuclease should be 4-times lower, i.e., of the order of 1 $M^{-1}s^{-1}$. With such a value, complete reduction by a β-mercaptoethanol concentration of the order of 20 mM is achieved in a few minutes; this justifies the use of one hour as the time during which the reaction of reduction goes to completion (fig.3,4).

The conditions which are found here to lead to complete reduction of ribonuclease, are milder than those used generally for the same purpose, and even though reduction of the disulphide bonds by β-mercaptoethanol appears to be a rather fast reaction when the disulphide bonds are fully accessible as in Gdn·HCl-unfolded ribonuclease. In native proteins, disulphide bonds are often at least partly buried inside the protein molecule. Therefore, measuring the reactivity of disulphide bonds towards β-mercaptoethanol in various conditions may give some information about the state of exposure of these bonds, and therefore be used as local probes of protein conformation, as proposed by Creighton [6].

Acknowledgements

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