Reduction of Ribonuclease by Glutathione at Elevated Temperatures: the Molecular Mechanism

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1. The mechanism of the reaction between ribonuclease and GSH at elevated temperatures has been studied by using *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide to label the reduced ribonuclease. 2. After incubation for 2 hr. at 35°, enzymically active ribonuclease was recovered; at 50·8° half of the initial ribonuclease was recovered as enzymically active ribonuclease and half as reduced labelled ribonuclease; at 55° all of the initial ribonuclease was recovered in the labelled form. 3. It was inferred that the rate-limiting step was the reduction of the first disulphide bond in any one molecule. This was followed by rapid reduction of the other bonds in the same molecule.

In previous studies (Hird, 1962; Davidson & Hird, 1965, 1967) it was found that the disulphide bonds of ribonuclease and bovine serum albumin that are not reduced by GSH at physiological temperature (37°) became reactive at higher temperatures. Thus the four bonds of ribonuclease are rapidly reduced at 55° whereas none react at 37°.

The susceptibility of individual disulphide bonds to reduction is of interest since there are two major possibilities: the reduction may be on a molecular basis, whereby all four bonds of individual molecules are reduced at the 'same' time, or on a bond-by-bond basis, whereby each particular disulphide bond of all the molecules is reduced at the 'same' time. These two mechanisms may be distinguished at an intermediate temperature when approximately half of the total bonds are reduced. If the former mechanism were operating, a mixture of native and fully reduced molecules would be formed and could be identified. By the latter mechanism, identification of the bond(s) reduced at the lower temperatures would indicate regions of the molecule that are more susceptible to thermal unfolding.

The reaction with ribonuclease has been examined to see which mechanism was operating. To do this, GSH was incubated with ribonuclease at three different temperatures, 35°, 50·8° and 55°, at which no reduction, 50% reduction and complete reduction respectively were known to occur (Davidson & Hird, 1965). The resulting thiol groups were allowed to react with the coloured

reagent DDPM† described by Witter & Tuppy (1960), and the ribonuclease so labelled was separated from unchanged ribonuclease on the basis of its insolubility in water.

MATERIALS AND METHODS

Materials. The source of most reagents has been listed previously (Davidson & Hird, 1965). DDPM was obtained from the Mann Research Laboratories (New York, N.Y., U.S.A.) and cytidine-2',3'-(cyclic)-monophosphate and ribonuclease A, lots 25B-8610 and 63B-8620, were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Reduction of ribonuclease and labelling with DDPM. The experimental system used was a scaled-up version of one previously described (Davidson & Hird, 1965). The volume of the reaction mixture at each temperature was governed by the need for a suitable fall in extinction (less than 2) and the amounts of glutathione reductase and NADPH₂ were greater at elevated temperatures to allow for their progressive destruction. The details are given with the values in parentheses referring to experiments done at 35°, 50·8° and 55° respectively.

The following components were added in 0·1 M-potassium phosphate buffer (pH 7·8 and 10 mM with respect to EDTA) to a water-jacketed vessel: NADPH₂ (8, 28 and 22 mg.), glutathione reductase (12·4, 46 and 42 μmole units), GSH (6 mM final) and ribonuclease A (3·3, 6·6 and 3·2 μmoles) in respective final volumes of 20, 37 and 34·5 ml. A reference solution (1·6 ml.) without ribonuclease was set up in an anaerobic cuvette of the type described previously (Davidson & Hird, 1965).

Both solutions were gassed with N₂ (O₂-free) for 25 min. and the temperature was rapidly raised to the desired level.

† Abbreviations: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; S-DDPS-, *S*-[*N*-(4-dimethylamino-3,5-dinitrophenyl)succinimido]-.

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Scheme 1. Fractionation of ribonuclease reduction mixtures.

The starting time of the reaction was taken as the time immediately before the temperature was raised since ribonuclease A does not react with GSH at room temperature. Samples (approx. 1.5 ml.) of the test solution were removed at 0, 30, 60, 90 and 120 min. and E_{340} was quickly measured against the reference cuvette. This gave an estimation of the extent of the reaction. A sample (0.4 ml.) for determination of ribonuclease activity was also removed at zero time and added to *m*-sodium acetate, pH 4.3 (0.08 ml.), giving a final pH of 4.6.

After 120 min. the solution was rapidly cooled and *m*-sodium acetate, pH 4.3 (0.2 ml./ml. of solution) was added to lower the pH to 4.6. A sample was set aside for ribonuclease assay and then DDPM (150, 315 and 300 μ moles in 4 ml. of acetone) was added to a sample (23, 44 and 41 ml.) of the remaining solution. The mixture was stirred for 45 min. at room temperature to ensure complete reaction.

Fractionation of the labelled reduction mixture (see Scheme

1). The mixture was chilled in ice, made 80% (v/v) with respect to cold acetone and centrifuged (1000*g*) for 15 min. Except for the pellet from the 35° experiment, which was white, both the supernatant solution and the pellet were yellow. Preliminary tests had established that both DDPM and *S*-DDPS-GSH were readily soluble in 80% acetone.

After the supernatant solution had been poured off the pellet was washed by suspending it in 5 ml. of water and adding acetone to 80% and centrifuging. This washing procedure was repeated once. The final supernatant solution was colourless in all three experiments, indicating that excess of DDPM and *S*-DDPS-GSH had been removed.

The pellet from the final wash was suspended in water (7.5 ml.) and centrifuged as before. The colourless supernatant solution containing unchanged ribonuclease was removed, leaving a deep-yellow pellet (*S*-DDPS-ribonuclease). This pellet was washed twice with water (7.5 ml.

each washing) and dried over P_2O_5 . Less than 0.5 mg. of pellet was obtained from the reduction at 35°. The last three supernatant solutions, containing unchanged ribonuclease, were combined and freeze-dried to give white powders, all containing some salts.

Determination of total disulphide bonds and thiol groups. Disulphide bonds were estimated polarographically in the presence of sulphite by the indirect method of Frater & Hird (1965). Thiol groups were determined in the same way, in the absence of sulphite.

Determination of GSH. GSH was estimated by the glyoxalase method as modified for the pH-stat (Radiometer, Copenhagen, Denmark) by Davidson & Hird (1964).

Determination of ribonuclease activity. The method used was based on that of Gundlach, Stein & Moore (1959) with cytidine-2',3'-(cyclic)-monophosphate as substrate. The reaction was done under N_2 in a pH-stat at pH 7.4 and 30° in the presence of 0.25 M- Na_2SO_4 . The enzyme was found not to be saturated at up to 15 mM-substrate, but at 5 mM and with NaOH (10 mM in 0.25 M- Na_2SO_4) as titrant, the uptake of alkali from 0.5 to 3.5 min. after the addition of enzyme bore a linear relationship to added ribonuclease (0–120 μ g.).

Amino acid analysis. Samples were hydrolysed *in vacuo* with 5.7 N-HCl for 20 hr. at 110°. The amino acid content was determined with a Beckman model 120B Amino Acid Analyser by using the standard technique of Moore & Stein (1963). Cysteine, in digests, was not oxidized to cystine before application of samples to the columns.

Preparation of S-DDPS-cysteine and S-DDPS-GSH. DDPM (750 μ moles) in 3 ml. of acetone was added to cysteine or GSH (500 μ moles in 5 ml. of 0.1 M-sodium acetate, pH 4.6). A precipitate formed immediately, consisting of S-DDPS-cysteine (S-DDPS-GSH was soluble in the reaction mixture) and unchanged DDPM. After 1 hr. at room temperature the mixture was extracted with three lots (each 10 ml.) of ether to remove the excess of DDPM. The aqueous phase was dried over P_2O_5 and washed thoroughly with 1 ml. (3 $\times$ 0.3 ml.) of water to remove unchanged cysteine or GSH, then dried again over P_2O_5 .

RESULTS

Amino acid analysis of S-DDPS derivatives. To interpret accurately the amino acid content of the S-DDPS-ribonuclease after hydrolysis, it was necessary to observe the positions of ninhydrin-positive products of hydrolysis of S-DDPS-cysteine and S-DDPS-GSH. Amino acid analyses of the adducts, after hydrolysis, gave the expected results (Table 1). There was no formation of cysteine, cystine, cysteic acid or any other amino acids that could interfere with the amino acid analyses of ribonuclease. The unknown basic component was possibly ethylamine, even though it emerged closer to ammonia than previously reported (Smyth, Blumenfeld & Konigsberg, 1964).

Samples of ribonuclease A (lot 63B-8620; approx. 8 mg.) were also hydrolysed in the presence or absence of S-DDPS-cysteine (approx. 1.5 mg.) to determine its effect on the recoveries of the constituent amino acids of ribonuclease. The ratio of S-DDPS-cysteine to ribonuclease used approxi-

Table 1. *Amino acid analysis of S-DDPS-cysteine and S-DDPS-GSH after acid hydrolysis*

Amino acid	S-DDPS-cysteine (relative area of peak)	S-DDPS-GSH (μ mole/ μ mole of glutamic acid)
Unknown*	0.08	0.10†
S-Succinylcysteine	1.0	0.72†
Glutamic acid	—	1.0
Glycine	—	0.96

* This peak follows ammonia.

† These results were calculated with the average peak integration constant of the amino acid analyser.

Table 2. *Recovery of amino acids from ribonuclease hydrolysed in the presence of S-DDPS-cysteine*

Amino acid	% recovered in the absence of S-DDPS-cysteine
Cystine (half)	91.5
Proline	94
Methionine	81.5
Tyrosine	43.5
Phenylalanine	82

mates to the ratio in S-DDPS-ribonuclease where all the cysteine residues are labelled. Table 2 gives the recovery of the amino acids where these have been less than 96%. Tyrosine, methionine, phenylalanine and cystine were destroyed to various extents, but most of the amino acids were unaffected.

In the results presented it has been assumed that similar losses also occurred during hydrolysis of S-DDPS-ribonuclease.

The reaction causing the large loss of tyrosine is unknown and did not result in the appearance of any new peaks on the chromatogram.

Reduction of ribonuclease. Table 3 shows, at the three temperatures used, the extent of reduction of ribonuclease after 120 min. The number of disulphide bonds reduced was obtained from the fall in E_{340} and ribonuclease activity was estimated on samples taken at 0 and 120 min.

The amount of reduction in this preparative experiment is in general agreement with the results obtained under analytical conditions (Davidson & Hird, 1965), except that reduction previously was more complete at 55°. Because of technical difficulties involved in measuring the amount of reduction in large-scale experiments the value of 84% may be 5–10% low.

Table 3 also shows that progressive reduction of the disulphide bonds leads to progressive loss of enzymic activity. Since fully reduced ribonuclease has no enzymic activity (Sela, White & Anfinsen,

1957) the experimental result of half the activity lost at 50.8° is to be expected if half of the molecules are fully reduced. However, if only two disulphide bonds are reduced in all of the molecules the result obtained would require that such partly reduced molecules possess 50% of the native activity. This coincidence is a possibility, but the lack of colour in one fraction and the colour in the other (see

Scheme 1 and below) indicates that half of the ribonuclease molecules were intact and the other half fully reduced.

Analysis of reduction products. (a) Composition of the two fractions: the yellow fraction, insoluble in water, tentatively referred to as 'S-DDPS-ribonuclease', and the colourless fraction, soluble in water, tentatively referred to as 'ribonuclease'.

Table 4 shows the amino acid analyses of the reduction products of ribonuclease. The ribonuclease fraction isolated from the experiments conducted at 35° and at 50.8° shows a close similarity to the parent ribonuclease except for the glycine value. The high value for glycine is probably due to the conversion of adenine from NADPH₂ into glycine during acid hydrolysis of the preparation (Smith & Markham, 1950). The value for isoleucine is low in comparison with the published value of 3 residues/mol. (Smyth, Stein &

Table 3. *Preparative reduction of ribonuclease by GSH*

Temp. of incubation with GSH	Extent of reduction (% of total S-S bonds)	Final ribonuclease activity (% of initial activity)
35°	1.2	99
50.8	51	52
55	84	1.2

Table 4. *Amino acid analysis of reduction products of ribonuclease*

The results are expressed as the molar ratio that is the best fit for the known formula of ribonuclease, i.e. the sum of the deviation of each amino acid from its theoretical value is zero. In some cases certain amino acids were not considered when deriving the ratio of best fit. These were: tyrosine, methionine and phenylalanine in hydrolysates of S-DDPS-ribonuclease (see footnote †); glycine when it could have arisen from NADP(H₂) decomposition; and isoleucine and half-cystine in all cases.

Amino acid	Ratio of amino acids				
	Ribonuclease A (lot 25B-8610)	Ribonuclease fraction from reduction at 35°	Ribonuclease fraction from reduction at 50.8°	S-DDPS-ribonuclease fraction from reduction at 50.8°	S-DDPS-ribonuclease fraction from reduction at 55°
Lysine	9.7	9.2	9.3	9.3	9.5
Histidine	3.8	3.5	3.5	3.5	3.6
Arginine	3.7	3.5	3.6	3.6	3.5
S-Succinylcysteine	0	0	0	6.8	6.4
Aspartic acid	14.9	15.6	15.9	15.9	15.8
Threonine*	10.6	10.6	10.5	10.3	10.4
Serine*	14.5	15.6	15.0	14.8	14.2
Glutamic acid	12.6	12.6	13.0	12.8	12.6
Proline	4.0	4.2	4.0	4.0	4.0
Glycine	3.0	4.0	5.7	3.3	3.4
Alanine	12.3	12.4	12.8	12.7	12.9
Cystine (half)*†	8.3	7.6	7.6	0.51	1.05
Valine	9.1	9.2	9.4	9.4	9.3
Methionine†	4.0	4.0	4.1	3.9	4.1
Isoleucine	2.10	2.25	2.20	2.10	2.25
Leucine	2.05	2.05	2.15	2.05	2.10
Tyrosine*†	6.0	4.5	3.4	6.1	6.9
Phenylalanine†	3.0	3.1	3.0	3.6	3.5
E_{570}/E_{440} for proline‡	5.2	5.0	4.9	5.2	5.0

* Corrections have been applied to these amino acids to allow for their destruction, which always occurs during hydrolysis. The values used were 5% for half-cystine, threonine and tyrosine, and 10% for serine (Moore & Stein, 1963).

† Corrections have been applied to these amino acids to allow for their destruction during hydrolysis in the presence of S-DDPS-cysteinyl residues (see Table 2).

‡ The E_{570}/E_{440} ratio for proline has been included in the Table because cysteine, if present in the acid hydrolysates, was not oxidized to cystine. Cysteine is eluted with proline (Moore & Stein, 1963) and it may be detected by a decrease in the E_{570}/E_{440} ratio. With the analyser and the quantities used, a decrease from 5.2 to 5.0 corresponds to less than 0.04 μ mole of cysteine/ μ mole of ribonuclease. This value, even if real, is small enough to be ignored.

Moore, 1963). For the present analysis the hydrolysis time was 20hr. and under these conditions the isoleucylisoleucyl bond (position 106–107) is not completely hydrolysed (Moore & Stein, 1963). The amino acid analyses therefore are consistent with this fraction being ribonuclease.

In addition, the specific activity of this fraction (compare columns 4 and 5 of Table 5) is identical with that of native ribonuclease.

Table 4 also shows the amino acid analysis of the *S*-DDPS-ribonuclease fraction. These analyses show the presence of substantial amounts of *S*-succinylcysteine and a marked decrease in the value for half-cystine. The value of one half-cystine residue is equivalent to the disulphide bond remaining in 50% of the molecule, i.e. 87% of the total disulphide bonds have been reduced and coupled with DDPM. The values for the other amino acids agree well with the values for the parent ribonuclease except that they are somewhat high for tyrosine and phenylalanine. These high values are due to uncertainties in the correction values used for these amino acids. It is therefore apparent that, after reduction, DDPM reacted with the thiol groups of the cysteine residues to form the adduct *S*-DDPS-ribonuclease.

(b) Relative proportions of ribonuclease and *S*-DDPS-ribonuclease after reduction at different temperatures. Table 5 shows that most of the amino acid residues of the ribonuclease added to the system were recovered in the ribonuclease fraction after incubation with GSH at 35°, i.e. little reduction occurred. Table 5 also shows that at 55° most of the ribonuclease was reduced and recovered as *S*-DDPS-ribonuclease. However, after reduction at the intermediate temperature of 50·8°,

where half of the disulphide bonds were reduced, equal amounts of the two fractions were isolated.

Assay of ribonuclease activity indicated that, after incubation at 35°, 50·8° and 55° respectively, all, half and no activity remained. Polarographic estimation of disulphide bonds in the ribonuclease fraction indicated a corresponding fall in the percentage of the total disulphide bonds recovered in the ribonuclease fraction. As a check on the analyses, the amount of GSH remaining in the ribonuclease isolated was estimated by the glyoxalase method. The results show that the thiol present (3% of the GSH added) could reasonably be accounted for as GSH that failed to react with DDPM.

DISCUSSION

The results presented indicate that reduction of ribonuclease by GSH at elevated temperatures proceeds on a molecule-by-molecule basis. No evidence was obtained that specific individual bonds were selectively reduced. The rate-limiting step in the reduction of the complete molecule therefore is the reduction of the first disulphide bond. The reaction may be pictured as resulting from a thermally induced conformational change in the vicinity of a disulphide bond. This change in geometry or charge or both permits an attack by the glutathione anion (GS⁻) on the disulphide bond, thereby initiating its ultimate reduction to two thiol groups by a second thiol–disulphide exchange reaction.

Since hydrogen bonds and electrostatic forces are weaker at the elevated temperature, the stabilizing effect of a disulphide bond is proportionately

Table 5. Analysis of the reduction products of ribonuclease A

Temp. of incubation with GSH	% reduction (from Table 3)	Fraction	% of initial amino N recovered as ribonuclease by amino acid analysis	% of initial ribonuclease recovered as ribonuclease by enzymic assay	Polarographic estimations: % of initial disulphide as		GSH content (% of total thiol)
					S·S	SH	
35°	1	Ribonuclease	96	96	83	11	110
		<i>S</i> -DDPS-ribonuclease	1*	0	—†	—†	—
50·8	51	Ribonuclease	44	44	30	12	64
		<i>S</i> -DDPS-ribonuclease	48	0	—†	—†	—
55	84	Ribonuclease	1·7	0	2·5	3·3	100
		<i>S</i> -DDPS-ribonuclease	90	0	—†	—†	—

* The amino acid content was not determined since less than 0·5 mg. was obtained. The result given here is calculated from this value.

† Polarographic estimations could not be done on these fractions because of the presence of a substance that carried substantial amounts of current (voltage approx. –0·7 v) and caused erratic behaviour of the dropping-mercury electrode. The nature of the substance is unknown.

greater than at lower temperatures. Thus reduction of any one bond will probably result in the loss of a major constraining force, and this in turn will lead to further molecular unfolding. Unfolding and reduction will therefore occur synergistically, resulting in rapid and complete reduction. That unfolding does occur when the disulphide bonds of ribonuclease are reduced is demonstrated by normalization of the abnormal tyrosine residues in the presence of GSH (Davidson & Hird, 1965).

It is not known if one specific disulphide bond of the ribonuclease molecule is reduced first or if the initial attack is random. The small proportion of partly reduced intermediates present would make a decision between these alternatives difficult.

It could be inferred from the present results that at lower temperatures the relative importance of disulphide bonds in maintaining structure is less than at higher temperatures, and further that unfolding may not follow from the reduction of a particular bond. It is possible therefore that certain proteins have particular disulphide bonds that are susceptible to reduction and are independent from

the rest of the molecule in maintaining configuration.

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