The Reactivity of the Disulphide Bonds of Bovine Pancreatic Ribonuclease with Glutathione

By B. E. DAVIDSON AND F. J. R. HIRD

*The Russell Grimwade School of Biochemistry, University of Melbourne,
Parkville, N.2, Victoria, Australia*

(Received 22 December 1964)

1. Bovine pancreatic ribonuclease is not reduced by GSH at near-physiological concentrations and pH. 2. Disruption of the structure of ribonuclease by proteolytic enzymes leads to products that can be reduced by GSH. 3. At higher temperatures the disulphide bonds of ribonuclease are completely reduced by GSH in a coupled system. The T_m is 51° and this has been found to be lower than the T_m for the abnormal tyrosine residues under the same conditions.

The reaction of GSH with the disulphide bonds of bovine serum albumin and bovine insulin giving GSSG as end product has been found to be very slow at pH values and GSH concentrations approximating to physiological (Hird, 1962). The reaction with serum albumin, presumably only as far as the formation of the mixed disulphide, has been further studied (Frater & Hird, 1963) and again the results indicated that only slight reaction had occurred. Gluten was also examined in this latter investigation and in this case some slight to moderate reaction was observed. However, with all three proteins it was demonstrated that proteolysis to a limited degree gave products that reacted extensively with GSH.

As proteins within the cell are in contact with GSH at concentrations up to 8 mM (Jocelyn, 1959; Isherwood, 1959; Davidson & Hird, 1964) and many proteins with disulphide bonds lose functional activity on reduction, it was decided to investigate the conditions under which the disulphide bonds of the well-defined protein bovine pancreatic ribonuclease became reactive towards GSH. The reduction of this protein by other thiol compounds has been reported (see review by Scheraga & Rupley, 1962), but usually the systems used were at pH values greater than 8 and contained a large excess of reagent.

MATERIAL AND METHODS

Materials. The following materials were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.): NADPH₂ type II (90–96%), glutathione reductase type III [0.25 μmole unit/μl. in (NH₄)₂SO₄ solution], oxidized glutathione, bovine pancreatic ribonuclease (lots R100B–69, R22B–70 and R51B–062, all 'crudely chromatographed'), bovine pancreatic ribonuclease A (lot 33B–781–4), α-chymotrypsin,

pepsin and trypsin. GSH was purchased from S.A.F. Hoffmann-La Roche (Basle, Switzerland), Pronase-P from the Kaken Chemical Co. (Tokyo, Japan), bovine pancreatic ribonuclease (lots BM2470, DN0252 and EB2451) from Armour and Co. (Eastbourne, Sussex) and bovine pancreatic ribonuclease (lot F3238) from Mann Research Laboratories (New York, N.Y., U.S.A.). Other samples of ribonuclease were obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) and the Worthington Chemical Corp. (Freehold, N.J., U.S.A.). Yeast RNA was prepared by the method of Crestfield, Smith & Allen (1955) and methylmercuric iodide by the method of Maynard (1932). Subtilisin A was a gift from Professor M. Ottesen.

The sample of GSSG was subjected to microanalysis for S and N (Dr Zimmermann, C.S.I.R.O. Microanalytical Service) and was also estimated for total disulphide content. These results indicated a purity of 93.5%. This GSSG, with the appropriate correction, was used as a primary standard in the analysis of reactive disulphide bonds.

Assay of total disulphide groups. These were estimated amperometrically by the method of Frater & Hird (1965) given in the Appendix. The values obtained experimentally were corrected for a small amount (approx. 0.1–0.2 μmole) of methylmercuric iodide, which reacted with most samples of ribonuclease in the absence of sulphite.

Assay of reactive disulphide groups. These were estimated by a modification of the method of Hird (1962), the reaction:

being done under O₂-free N₂ to prevent aerial oxidation of thiol groups. NADPH₂ (0.4 mg. usually, 0.65 mg. at higher temperatures), glutathione reductase (0.25 μmole unit) and GSH (9.6 μmole) in a total volume of 1.5 ml. of 0.1 M-potassium phosphate buffer, pH 7.8, made 0.01 M with respect to EDTA, were placed in a Pyrex cuvette (10 mm. light-path) fitted with a B14 socket. A glass gassing tube, passing through a B14 cone to which was attached a side arm, was placed into the cuvette. The gassing tube went

down to the bottom of the cuvette and was bent out of the light-path. The unknown sample was placed in the side arm (0.0–3 μ mole of reactive disulphide in 0.1 ml. of buffer), and O_2 -free N_2 was bubbled through the solution in the cuvette and out through the side arm for 20 min. After this time the gas flow was stopped and the apparatus placed in the cell housing of a Beckman DK-2 spectrophotometer for a 5 min. temperature equilibration. The contents of the side arm were then tipped in. The temperature in the cell housing, unless otherwise stated, was maintained at 20–21° during the reaction period.

The extinction at 340 $m\mu$ was in most cases followed for 80 min., by which time the reaction was usually complete. This time was increased to 120 min. when determining the transition temperature. Small blank corrections were made for the decomposition of NADPH₂ and oxidation of GSH by traces of residual O_2 .

Under the conditions described 1 μ mole of GSSG caused a fall in extinction at 340 $m\mu$ of 3.75.

Assay of ribonuclease activity. This was done titrimetrically in a pH-stat (Radiometer, Copenhagen, Denmark), with yeast RNA (5 mg./ml.) as substrate in the presence of 0.3 M-KCl. At pH 7.4 and 30° with NaOH (0.01 N) as titrant, the uptake of alkali from 0.5 min. after the addition of enzyme to 3.5 min. was proportional to added ribonuclease (0–20 μ g.) and was therefore used to calculate activities. Ribonuclease S and the S-protein were estimated by the methods of Richards & Vithayathil (1959).

Enzymic hydrolysis of ribonuclease. (i) Ribonuclease R51B-062. The protein solution (25 mg. in 5.0 ml. of water) was equilibrated at 30° in the pH-stat (pH 3.0 for pepsin and pH 8.0 for other digestions) for 20 min. The proteolytic enzyme was then added (0.25 mg. in 50 μ l. of water) and the digestion followed by the uptake of HCl (0.1 N) at pH 3 and NaOH (0.02 or 0.1 N) at pH 8. When the reaction was complete, or after 15 hr., the mixture was frozen and freeze-dried. Controls were treated in exactly the same way except that no proteolytic enzyme was added.

(ii) Ribonuclease 33-B781-4. Solutions of this protein in water gave pH 9.5, and when adjusted to pH 8.0 there was a slow uptake of protons over 10 hr. (3.5 μ moles/25 mg.). It was therefore necessary to equilibrate samples at pH 8.0 in the pH-stat for 10 hr. with HCl (0.02 N) as titrant before adding the proteolytic enzyme. In other respects the treatment was identical with that described under (i). Samples for peptic digestion did not require this equilibration.

Concentration of ribonuclease A solutions. The concentrations of all ribonuclease A solutions were determined from their extinction coefficients. A sample of ribonuclease A was analysed for N (Dr Zimmermann, C.S.I.R.O. Micro-analytical Service) and found to be 17.35% of the dry wt., compared with 17.5% of N calculated from the formula of Smyth, Stein & Moore (1963). This value (17.35% N) served as the basis for determining the molar extinction coefficient as 9.9×10^3 . Spectra were corrected for light-scattering as outlined by Leach & Hill (1963).

Effect of temperature on absorption spectrum. A solution of ribonuclease A (2 mg./1.6 ml.) in the phosphate-EDTA buffer used in the determination of reactive disulphide groups was placed in a stoppered silica cuvette (10 mm. light-path) in the cell housing of the spectrophotometer. The housing had previously been adjusted to the required temperature, and after 25 min. equilibration the spectrum

was recorded from 360 $m\mu$ to 260 $m\mu$ against a buffer blank. The extinction at 287 $m\mu$ was obtained from these curves and the effect of temperature on E_{287} was then derived by subtracting the value at 20° from that obtained at each separate temperature.

RESULTS

Reaction of GSH with ribonucleases. Fig. 1 shows that different samples of ribonuclease react with GSH at different rates and to different extents. The initial lag period in the reaction is similar to that observed with other disulphides (Pihl, Eldjarn

Fig. 1. Reduction of ribonucleases by GSH. Ribonuclease (0.2 μ mole) was added at the time indicated by the arrow: A, 33B-781-4; B, BM2470; C, Worthington; D, DN0252; E, R100B-69; F, Lights; G, EB 2451. Experimental details are given in the text.

Table 1. Reactivity of disulphide bonds of ribonuclease with GSH

Experimental details are given in the text.

Ribonuclease sample	Reactive disulphide bonds (μ moles/ μ mole of ribonuclease)	Total disulphide bonds (μ moles/ μ mole of ribonuclease) (determined polarographically)
Sigma 33B-781-4	0	3.5
Armour BM 2470	0.11	3.7
Worthington	0.25*	4.3
Armour DN 0252	0.45	—
Sigma R51B-062	0.45	4.0
Sigma R100B-69	0.52	—
Lights	0.58	—
Armour EB 2451	0.60	—
Mann F3238	0.65	3.9
Sigma R22B-70	1.05	3.6

* Not complete after 1 hr. (see Fig. 1).

& Bremer, 1957; Hird, 1962). Table 1 gives the total number of disulphide bonds measured polarographically and the number of bonds reacting with GSH under the conditions used. Some of the samples do not appear to contain the expected total of 4 disulphide bonds. Moreover, the samples differed in their reactivity with GSH and in practically all cases this was not represented by a whole number of bonds. As this reaction was complete with all samples except for the Worthington one (Fig. 1), these observations suggest a lack of

homogeneity in the samples. This conclusion is consistent with the results of Leach & Springell (1962), who used tritium exchange.

Of the samples studied only Sigma 33B-781-4 was listed as chromatographically pure ribonuclease A. The nitrogen content of this sample indicated a purity of 98.5% on a dry weight basis, and amino acid analysis by the method of Moore & Stein (1963) agreed well with that to be expected from the known composition (Smyth *et al.* 1963). However, this sample did give a low value for total disulphide content. The results suggest that pure and perhaps native ribonuclease has no disulphide bond that reacts with GSH under the conditions used.

Effect of proteolysis on the reactivity of disulphide bonds. Samples 33B-781-4 and R51B-062 were each treated with various proteolytic enzymes and the reactive disulphide content was then determined. The results in Table 2 show that, as the peptide structure is broken, disulphide bonds that were previously unreactive now react with GSH. As expected, the number of disulphide bonds reduced increases with the extent of hydrolysis. Whole numbers of bonds reacting were not found, probably because of heterogeneity in the hydrolysis products.

By using the method of Richards & Vithayathil (1959) ribonuclease A was partly digested with subtilisin A. The reactive disulphides present after such limited digestion are shown in Table 3. Three separate digestions were done with varying yields of ribonuclease S, and the preparation having the largest amount of ribonuclease S has only 0.2 μ mole of reactive disulphide/ μ mole of ribonuclease S. The conclusion has therefore been reached that the disulphide bonds of ribonuclease S are also unreactive. Either 3 or all of the bonds are reactive in the S-protein after removal of the S-peptide.

Effect of increasing temperature on the reactivity of the disulphide bonds. Fig. 2 shows that at about 45° the reactivity of the disulphide bonds starts to

Table 2. *Effect of proteolysis of ribonuclease on the reactivity of the disulphide bonds*

Experimental details are given in the text.

Ribonuclease sample	Proteolytic enzyme	Extent of proteolysis (μ moles of titrant/ μ mole of ribonuclease) (corrected for control)	Reactive disulphide bonds after proteolysis (μ moles/ μ mole of ribonuclease)
R51B-062	—	—	0.45
	Pronase*	24	3.5
	Chymotrypsin*	2.6	0.5
	Trypsin	1.6	0.3
	pH 8 control	—	0
	Pepsin*	10	2.9
33B-781-4 (ribonuclease A)	pH 3 control	—	0
	—	—	0
	Pronase*	22	3.5
	Chymotrypsin	1.9	0.3
	pH 8 control	—	0
	Pepsin	11	3.5
	pH 3 control	—	0

* Digestion still proceeding at 15 hr.

Table 3. *Reactivity of the disulphide bonds of ribonuclease S and the S-protein*

Experimental details are given in the text.

Sample	Ribonuclease (%)	Ribonuclease S (%)	S-protein (%)	Reactive disulphide bonds	
				(μ moles/ μ mole of sample)	(% of total disulphide bonds)†
Ribonuclease 33B-781-4	100	0	0	0	0
Subtilisin digest	14, 7, 11	54, 67, 71	—	0.8, 0.5, 0.2	22, 14, 8
S-protein*	5, 4		76, 49	3.2, 3.0	92, 86

* The S-protein was prepared from the first two subtilisin digests shown above.

† Calculations based on a total disulphide content of 3.5 (see Table 2).

Fig. 2. Effect of temperature on the reactivity of the disulphide bonds of ribonuclease. ●, Reaction complete before 2 hr.; ○, reaction not quite complete after 2 hr. Experimental details are given in the text.

Fig. 3. Effect of temperature on the abnormal tyrosine residues of ribonuclease. Experimental details are given in the text.

Fig. 4. Effect of GSH on the normalization of the tyrosine residues of ribonuclease. ○, Ribonuclease alone at 53°; □, ribonuclease + GSH (6mM) at 53°; △, ribonuclease alone at 63°. Experimental details are given in the text.

increase and by 56° all the bonds have reacted. The temperature at which half the total bonds react, the transition temperature, T_{tr} , is 51°. The data for this value were obtained from reduction periods of 2 hr. and in a number of cases (indicated) the reaction was not quite complete at this time. The rate, however, had declined to 10–30% of the maximum rate.

It is not yet known whether the reaction at intermediate temperatures involves all the disulphide bonds of part of the molecules or a fraction of the bonds of all of the molecules.

The effect of pH on the temperature of the normalization of the buried tyrosyl residues has been examined in detail by Hermans & Scheraga (1961). However, as their studies extended upwards only as far as pH 7, we examined this parameter at pH 7.8 and found a value of 63° (Fig. 3).

Fig. 4 shows the effect on the extinction at 287 mμ when ribonuclease is incubated with GSH (6mM) at 53°, this temperature being intermediate between those required for the reduction of disulphide bonds and the normalization of the buried tyrosine residues. The presence of GSH and presumably the reduction of disulphide bonds leads to the normalization of the buried tyrosine residues at a lower temperature.

DISCUSSION

The differing reactivities of the disulphide bonds in the different samples of ribonuclease is probably due to small amounts of impurities of other proteins or denatured material or both. Variability among samples was reported by Leach & Springell (1962) with respect to tritium exchange, and Crestfield, Stein & Moore (1963) found that various commercial preparations of ribonuclease were heterogeneous when chromatographed on Amberlite IRC-50. The samples contained at least 15% of impurities. The sample of chromatographed ribonuclease A (Sigma 33B-781-4), which was the purest obtainable, had no reactive disulphide bonds. It seems therefore that native ribonuclease does not react with GSH under conditions that are close to physiological pH, temperature and GSH concentration.

Previous work on the reactivity of the disulphide bonds of ribonuclease has indicated that they do not readily undergo sulphytolysis at 37° and pH 8.3 (Resnick, Carter & Kalnitsky, 1959). However, Cecil & Wake (1962) found that 0.5 disulphide bond in ribonuclease reacts at pH 7 and 37° with a 200-fold molar excess of sulphite. Reaction of the disulphide bonds with thiols such as 2-mercaptoethanol (Neumann, Goldberger & Sela, 1964) and thioglycollate (Sela, White & Anfinsen, 1957) only proceeds partly, and high relative concentrations of thiol and pH values in the vicinity of 8.5 are

required. Thus the lack of reaction with GSH under the conditions used does not differ from published observations with other reagents.

The breakdown of the peptide structure of bovine serum albumin and insulin leads to increased reactivity of the disulphide bonds of these proteins (Hird, 1962; Frater & Hird, 1963), and this has now been found to be true for ribonuclease. Even after hydrolysis by pepsin of about 20 peptide bonds of sample R51B-062 there is still 1 disulphide bond that is unreactive. Ginsburg & Schachman (1960) have reported that in the peptic digestion of ribonuclease the molecules are all degraded simultaneously. They have also shown that 1 disulphide bond could react with 2-mercaptoethanol at pH 6.8 after only about 1.4 peptide bonds were hydrolysed, the native protein showing no reactivity. In the present investigation the hydrolysis of 3 bonds by chymotrypsin does not seem to open up the structure sufficiently to enable the GSH molecule to react.

Ribonuclease S, the product of the action of subtilisin on ribonuclease under carefully controlled conditions, has been shown chemically to differ from ribonuclease only in the absence of the peptide bond between residues 20 and 21 (Richards & Vithayathil, 1959). The relatively unchanged properties of the parent and modified protein noted by these workers is also apparent from the present study, where the modification did not allow the reduction of disulphide bonds. However, the removal of the peptide consisting of residues 1-20 to give the S-protein exposed the molecular structure sufficiently to allow the reduction of all or nearly all of the disulphide bonds to take place.

The occurrence of reactivity of the disulphide bonds with increase in temperature is almost certainly related to disruption of the tertiary structure of the molecule. Values for T_m of 62° for optical rotation (Hermans & Scheraga, 1961), and normalization of tyrosine residues (Hermans & Scheraga, 1961; this paper), indicate a similarity with the T_m for disulphide bond reactivity. Loss of enzymic activity also begins at 50-55° (Kalnitsky & Resnick, 1959). There is, of course, the possibility that the increase in reactivity is simply due to an increase in that of the disulphide bonds as such. However, the reaction of these bonds in partial digests at lower temperature seems to preclude this explanation of the results.

At about 50° certain unfolding of the tertiary structure must occur, allowing the successful approach of the negatively charged GS⁻ to the disulphide bond(s). The details of the part of the molecule unfolding at this temperature are not known, but it does not involve the three tyrosine residues, which are not normalized until 63°. The reduction of the disulphide bonds assists in the normalization of the tyrosine residues at a lower temperature.

REFERENCES

- Cecil, R. & Wake, R. G. (1962). *Biochem. J.* **82**, 401.
 Crestfield, A. M., Smith, K. C. & Allen, F. W. (1955). *J. biol. Chem.* **216**, 185.
 Crestfield, A. M., Stein, W. H. & Moore, S. (1963). *J. biol. Chem.* **238**, 618.
 Davidson, B. E. & Hird, F. J. R. (1964). *Biochem. J.* **93**, 232.
 Frater, R. & Hird, F. J. R. (1963). *Biochem. J.* **88**, 100.
 Frater, R. & Hird, F. J. R. (1965). *Biochem. J.* **96**, 895.
 Ginsburg, A. & Schachman, H. K. (1960). *J. biol. Chem.* **235**, 115.
 Hermans, J. & Scheraga, H. A. (1961). *J. Amer. chem. Soc.* **83**, 3283.
 Hird, F. J. R. (1962). *Biochem. J.* **85**, 320.
 Isherwood, F. A. (1959). *Symp. biochem. Soc.* **17**, 3.
 Jocelyn, P. C. (1959). *Symp. biochem. Soc.* **17**, 43.
 Kalnitsky, G. & Resnick, H. (1959). *J. biol. Chem.* **234**, 1714.
 Leach, S. J. & Hill, J. (1963). *Biochemistry*, **2**, 807.
 Leach, S. J. & Springell, P. H. (1962). *Aust. J. Chem.* **15**, 350.
 Maynard, J. L. (1932). *J. Amer. chem. Soc.* **54**, 2108.
 Moore, S. & Stein, W. H. (1963). In *Methods in Enzymology*, vol. 6, p. 819. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Neumann, H., Goldberger, R. F. & Sela, M. (1964). *J. biol. Chem.* **239**, 1536.
 Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
 Resnick, H., Carter, J. R. & Kalnitsky, G. (1959). *J. biol. Chem.* **234**, 1711.
 Richards, F. M. & Vithayathil, P. J. (1959). *J. biol. Chem.* **234**, 1459.
 Scheraga, H. A. & Rupley, J. A. (1962). *Advanc. Enzymol.* **24**, 161.
 Sela, M., White, F. H. & Anfinsen, C. B. (1957). *Science*, **125**, 691.
 Smyth, D. G., Stein, W. H. & Moore, S. (1963). *J. biol. Chem.* **238**, 227.