Effect of Glutathione on Ribonuclease

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GSH, but not GSSG, inhibits the reactivation by phosphate ion of ribonuclease activity inactivated by urea or guanidine. The effects of GSH are rather slow and pretreatment of ribonuclease with urea is a requisite for the inhibitory action of GSH on enzyme reactivation. GSH is more effective in urea than in guanidine and its action is greatly enhanced by EDTA. An optimum pH of about 9.0 was found for the inhibitory effect of GSH. Titration of the thiol groups formed after inactivation of ribonuclease by GSH strongly suggests that the reduction of only one disulphide linkage is involved. The reduction of this bond is sufficient to completely abolish the enzymic activity.

Treatment of ribonuclease with 8M-urea decreases enzymic activity, optical rotatory dispersion and ultraviolet absorption (Sela, Anfinsen & Harrington, 1957a). However, ribonuclease thus treated regains its enzymic activity when urea is removed or when 0.09M-phosphate is added in the presence of urea, and the optical rotatory dispersion and ultraviolet absorption return to their original state, indicating reconformation of the enzyme molecule. However, the presence of reducing agents such as thioglycolic acid (White, 1960; Anfinsen & Haber, 1961) or β -mercaptoethanol prevents the reconformation of ribonuclease. We now report data that demonstrate that GSH also inhibits the recovery of ribonuclease activity, after its loss on urea treatment. Titration of the thiol groups indicates that GSH attacks specifically one disulphide bond to inactivate ribonuclease activity completely.

EXPERIMENTAL

Materials. Crystalline pancreatic ribonuclease (five-times recrystallized), a commercial grade of yeast RNA, *p*-chloromercuribenzoic acid, GSH and GSSG were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Urea was obtained from Fisher Scientific Co. (Philadelphia, Pa., U.S.A.) and was used without recrystallization. Other reagents were obtained from local suppliers.

Assay for the effect of GSH on reactivation of ribonuclease activity. Into a test tube were introduced 0.4 ml. of 10M-urea in 0.1M-tris-HCl buffer, pH 9.0, and 50 μ g. of crystalline ribonuclease in solution in 0.05 ml. of water. The mixture was kept for 3 min. at 30° and 0.05 ml. of GSH solution was added as indicated in the Figures. After the mixture had stood for 15 min. at 30°, 4.5 ml. of ice-cold 0.1M-sodium phosphate buffer, pH 7.3, was added. The tube was kept in ice, usually for less than 5 min., until ribonuclease activity was assayed. A control without GSH was run simultaneously. Assay of ribonuclease activity was essentially as described

by Kalnitsky *et al.* (1959). An 0.1 ml. portion of the reaction product was incubated with 1.9 ml. of 0.1M-sodium phosphate buffer, pH 7.3, and 1.0 ml. of 2% (w/v) RNA for 4 min. at 30°. The reaction was terminated by adding 1.0 ml. of 0.75% uranyl acetate in 25% (w/v) HClO₄. After complete mixing, the mixture was centrifuged, and 0.1 ml. of the supernatant was diluted with 3 ml. of water and E_{260} of the acid-soluble oligonucleotides was determined with a Zeiss PMQII spectrophotometer. Under the conditions described, the full ribonuclease activity (control without GSH) gave E_{260} 0.65 and, as shown below, reactivation was complete.

Titration of thiol groups. Each component listed in Table 1 was dissolved in a final volume of 1 ml. of solution. This solution contained 8m-moles of urea and 0.08m-mole of tris-HCl buffer, pH 9.1. After incubation for a certain time (see Table 1) 0.2 ml. of *N*-acetic acid was added and the mixture dialysed overnight against four changes of 1000 ml. of 0.1N-acetic acid. Acetic acid prevents reoxidation of reduced ribonuclease (Anfinsen & Haber, 1961). About 10–25% of the ribonuclease passed out through the dialysing bag, and this fraction was not included in the later titration procedure. The thiol group content of the ribonuclease remaining in the bag was titrated spectrophotometrically by a modified method of Boyer (Boyer, 1954; Sela, White & Anfinsen, 1959). Ribonuclease activity was measured as described above. After dialysis, the amount of ribonuclease was determined by measuring E_{275} .

RESULTS

Relation between the amount of GSH and the degree of inhibition of reactivation of denatured ribonuclease. Fig. 1 shows the relation between the amount of GSH and the degree of inhibition of reactivation by phosphate of ribonuclease denatured by 8M-urea. There is a wide scattering of values without EDTA. Though not indicated in Fig. 1, ribonuclease in 0.1M-phosphate buffer without treatment with urea and with or without GSH gave E_{260} 0.65.

Fig. 1. Effect of GSH concentration on reactivation of denatured ribonuclease. The final concentration of EDTA (when present) during the incubation with GSH was 2 mM. The rest of the procedure was as described in the Experimental section except that incubation with GSH was carried out for 10 min. instead of the usual 15 min. ○, EDTA absent; ●, EDTA present.

Fig. 2. Relation between the incubation period and the inhibitory activity. Ribonuclease was preincubated with 15 μ g. of GSH for various times and the reaction mixture was assayed for ribonuclease activity for 4 min. The rest of the procedure was as described in the Experimental section.

Since 8 M-urea abolishes ribonuclease activity (Sela *et al.* 1957a), the value without GSH indicates that reactivation of the denatured ribonuclease activity by phosphate is complete when no GSH is present.

Fig. 1 also shows that EDTA greatly enhances the degree of inhibition by GSH, suggesting that a metal may interfere with the inhibition by GSH.

Fig. 3. Effect of pH on the inhibitory activity. GSH (35 μ g.) was used in 0.01 M-tris-HCl buffer at all pH values. The rest of the procedure was as described in the Experimental section. ○, Percentage inhibition; ●, reactivated ribonuclease activity.

Fig. 4. Effect of concentration of urea and guanidine on the inhibitory activity of GSH. The amounts of GSH in urea solution (○) and guanidine solution (●) were 35 μ g. and 750 μ g. respectively. The Experimental section describes the assay details.

However, the mechanism cannot be explained at present.

Time-activity curve. The results in Fig. 2 were obtained by treating ribonuclease with a sub-

Table 1. *Estimation of thiol groups in ribonuclease at various stages of reduction by GSH*

Components	Time of incubation at 30°	Remaining ribonuclease activity (%)	SH groups/mol. of ribonuclease
Ribonuclease (20.4 mg.), no GSH	4 hr.	95.0	0
Ribonuclease (20.3 mg.) + β -mercaptoethanol (0.1 ml.)	4 hr.	0	7.52
Ribonuclease (20.7 mg.) + GSH (2 mg.)	5 min	33.5	0.89
Ribonuclease (43.0 mg.) + GSH (5.9 mg.)	15 min.	2.3	1.32
Ribonuclease (20.5 mg.) + GSH (2 mg.)	20 min.	0.76	1.66
Ribonuclease (20.6 mg.) + GSH (9.6 mg.)	4 hr.	0	1.89

maximal amount of GSH (15 μ g.) for various times before reactivation with 0.1M-phosphate. The zero-time value was obtained by introducing GSH after addition of phosphate to the urea-treated ribonuclease. Since the original enzyme activity was 0.65 (Fig. 1), this result indicates that GSH is ineffective when once-denatured ribonuclease has been fully reformed. Inhibition is relatively slow in onset and the degree of inhibition is linear with time up to about 30 min.

pH-activity curve. When ribonuclease was treated with 35 μ g. of GSH at various pH values in the presence of 8M-urea and assayed at pH 7.3 as described above, optimum pH about 9.2 was observed for the inhibition of reactivation of the denatured ribonuclease activity (Fig. 3).

Effect of urea concentration on the inhibition of denatured ribonuclease reactivation. The inhibitory effect of GSH on the denatured ribonuclease reactivation was investigated at various concentrations of urea (Fig. 4). Maximum inhibition occurred when ribonuclease was completely denatured in 8M-urea. GSH was not effective when the concentration of urea or guanidine was lower than a critical level, indicating an absolute requirement of breakage of hydrogen bonds for the action of GSH. Fig. 4 shows that GSH was more effective in urea than in guanidine, since 750 μ g. of GSH was required for effective inhibition in guanidine solution and only 35 μ g. in urea solution.

Thiol groups of reduced ribonuclease. Table 1 lists the results on the estimation of formed thiol groups in ribonuclease at various stages of reduction by GSH. No thiol group was detected in the ribonuclease molecule after incubation for 4 hr. without GSH. However, incubation of the enzyme with β -mercaptoethanol for 4 hr. broke all the disulphide bonds, as indicated by the value of 7.52 thiol groups/mol. of ribonuclease. Table 1 shows that a gradient relationship exists between the period of incubation with GSH and the remaining enzymic activity. Ribonuclease incubated with GSH for 20 min. had only 0.76% of the original activity; in this time 1.66 thiol groups/mol. of ribonuclease appeared, i.e. approximately one disulphide bond was broken. Further treatment of ribonuclease with a greater amount of GSH and a

longer period of incubation did not break further disulphide bonds.

DISCUSSION

In the last decade, several publications have appeared on the reduction of protein disulphide bonds: lysozyme (Anfinsen, 1958), ribonuclease S protein (Haber & Anfinsen, 1961) and ribonuclease (White, 1960; Sela *et al.* 1959) by treatment of the native proteins in 8M-urea with reducing agents such as thioglycollic acid or β -mercaptoethanol. Hydrogen bonds of protein molecules break in the presence of urea, thus rendering them more easily accessible to the action of reducing agents. Therefore the action of GSH in the present study can be best explained on the basis that GSH reduced the disulphide bond in ribonuclease when the hydrogen bonds were broken. This prevents the reactivation of ribonuclease activity by phosphate. Indeed, results on the titration of the thiol groups formed during reduction substantiated this hypothesis, and further revealed that only one sulphide bond is specifically reduced. This bond appears to be most essential for the enzymic activity. Several workers have reported that not all of the disulphide bonds are essential for full enzymic activity of ribonuclease (Sela, White & Anfinsen, 1957b; Resnick, Carter & Kalnitsky, 1959; White & Sandoval, 1962). The fact that only one of the four disulphide bonds of ribonuclease was broken by GSH could be a very important finding in studying the structure-activity relationship. No specificity towards disulphide bonds in ribonuclease was reported with sodium sulphite in urea (Resnick *et al.* 1959) or with thioglycollic acid (Sela *et al.* 1957b). Further study will be required to determine the exact location of the disulphide bond in the ribonuclease molecule that is susceptible to GSH.

Goldberger, Epstein & Anfinsen (1964) reported a rat liver system that reactivates reduced ribonuclease. This reactivation requires a microsomal and a soluble fraction, and GSSG can replace the soluble fraction. It is also worth noting that glutathione-insulin transhydrogenase was shown to be capable of catalysing the reductive cleavage of disulphide bonds of insulin in the presence of excess of GSH (Katzen & Tietze, 1966). Since

glutathione is widely distributed in various tissues (e.g. 100mg. of glutathione is present in 100g. of rat liver; Waelsch, 1952), it is possible that GSSG and GSH may play an important role in the regulation of enzyme activity *in vivo*. However, a more important and physiological significance of the present finding may be that non-folded nascent protein, freshly released from the ribosomal protein-synthesizing apparatus, is highly susceptible to the action of GSH.

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REFERENCES

- Anfinsen, C. B. (1958). In *Symposium on Protein Structure*, p. 223. Ed. by Neuberger, A. New York: John Wiley and Sons Inc.
- Anfinsen, C. B. & Haber, E. (1961). *J. biol. Chem.* **236**, 1361.
- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
- Goldberger, R. F., Epstein, C. J. & Anfinsen, C. B. (1964). *J. biol. Chem.* **239**, 1406.
- Haber, E. & Anfinsen, C. B. (1961). *J. biol. Chem.* **236**, 422.
- Kalnitsky, C., Hummel, J. P., Resnick, H., Carter, J. R., Barnett, L. B. & Dierks, C. (1959). *Ann. N.Y. Acad. Sci.* **81**, 542.
- Katzen, H. M. & Tietze, F. (1966). *J. biol. Chem.* **241**, 3561.
- Resnick, H., Carter, J. R. & Kalnitsky, G. (1959). *J. biol. Chem.* **234**, 1711.
- Sela, M., Anfinsen, C. B. & Harrington, W. F. (1957a). *Biochim. biophys. Acta*, **26**, 502.
- Sela, M., White, F. H., jun. & Anfinsen, C. B. (1957b). *Science*, **125**, 691.
- Sela, M., White, F. H., jun. & Anfinsen, C. B. (1959). *Biochim. biophys. Acta*, **31**, 417.
- Waelsch, H. (1952). *Advanc. Enzymol.* **13**, 273.
- White, F. H., jun. (1960). *J. biol. Chem.* **235**, 383.
- White, F. H., jun. & Sandoval, A. (1962). *Biochemistry*, **1**, 938.