931

Photooxidation of Crystalline Ribonuclease in the Presence of Methylene Blue

Leopold Weil and Thomas S. Seibles

From the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania

Introduction

In previous investigations (1, 2) we studied the effect of visible light mediated by traces of methylene blue on lysozyme and chymotrypsin in the presence of oxygen. The present work is an extension of these studies to crystalline ribonuclease in order to correlate the observed changes with enzyme activity.

Experimental

Crystalline ribonuclease purchased from Armour Company[1] and additionally recrystallized by the method of Kunitz (3) from dilute ethyl alcohol was used throughout the experiments. Two additional recrystallizations did not increase the specific activity of the enzyme. The enzyme preparation used was free of proteolytic activity (4), measured by the method of Herriott (5), even after a 24-hr. incubation period.

Irradiation by visible light at 37°C., and the manometric procedure used for measuring oxygen uptake, have been described previously (6). The enzyme concentration was calculated on the basis of a nitrogen content of 16.5% (7).

For determination of ribonuclease activity, the manometric procedure of Bain and Rusch (8) was used. Yeast nucleic acid served as a substrate. Table I shows the correlation between enzyme concentration and activity.

The linear relationship between enzyme concentration and activity

[1] Mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

TABLE I

Relation between Enzyme Concentration and Activity

Enzyme concn., *μg.*	10	20	30	40	50	60
Enzyme act., *cu. mm. CO_2 evolved in 10 min.*	43	84	130	177	212	254

shown in Table I was observed with that purchased originally, as we as with the preparation additionally recrystallized three times. Thi linear relationship was used for the quantitative evaluation of enzy matic activity.

Photochemical Action of Methylene Blue on the Amino Acid Compositio of Ribonuclease and on Its Enzymatic Activity

In order to correlate the chemical changes that take place during th photooxidation of ribonuclease with its activity, the possible decrease o the various amino acids within the enzyme molecule was determined Particular attention was paid to histidine (tryptophan is absent in thi enzyme), tyrosine, methionine, and cystine which, according to ou previous findings (9), are those involved in the photooxidation. Th analytical procedures for the determination of tyrosine, cystine, and methionine have been described before (10). Histidine was determined by the chromatographic procedure of Moore and Stein (11), who used the 15-cm. column, which at the same time gave us values for the sum of tyrosine and phenylalanine and for lysine and arginine. The phenylalanine concentration was calculated by subtracting from the sum of tyrosine and phenylalanine the tyrosine values obtained colorimetrically. Our analytical values for the various amino acids, based on the molecular weight of 14,000, were as follows: histidine, 4.36% (3.93 moles), tyrosine, 8.95% (6.90 moles), phenylalanine, 3.53% (3.00 moles), methionine, 5.11% (4.80 moles), ½ cystine, 6.73% (7.85 moles), lysine, 11.17% (11.11 moles), and arginine, 5.20% (4.18 moles). These analytical values are in fairly good agreement with those reported previously (7).

The amino acid determinations at any given level of oxygen uptake were carried out in the following manner: 1 ml. of solution containing 10 mg. of ribonuclease adjusted to pH 8.0, and 0.5 ml. of 0.2 *M* phosphate buffer of pH 8.0 were placed in the main chamber of the Warburg vessel, while 0.5 ml. of a 0.02% methylene blue solution was put in the side arm. The center well was charged with 0.2 ml. of a 20% solution of KOH for absorption of the simultaneously evolved CO_2. After tem-

perature equilibrium at 37°, the contents of the vessel were mixed and irradiated to various degrees of oxygen uptake. After the desired degree of oxidation was reached, aliquots containing 8.0 mg. of the enzyme were removed, placed in a vial, and dried by lyophilization. Two milliliters of glass-distilled 6 *N* HCl was added to the dry residue, and, after the vial was sealed, it was autoclaved for 16 hr. at 15 lb. pressure. The hydrolyzate thus obtained was rendered dry again in a vacuum desiccator over solid KOH. The dry hydrolyzate then was dissolved in 2 ml. of 0.1 *M* citrate buffer of pH 5.0. Aliquots containing 4 mg. of the original enzyme were then placed on the chromatographic column, and the separation and estimation of the various amino acids were carried out according to the procedure of Moore and Stein (11). Model experiments on mixtures of amino acids gave us a maximum deviation of ± 3% of the theoretical value.

Simultaneous irradiation experiments were carried out for determination of ribonuclease activity by the method of Bain and Rusch (8). Aliquots containing 50 μg. of the enzyme were used. Figure 1 shows the combined results on amino acid composition and activity during the irradiation.

As Fig. 1 shows, elimination of the total enzymatic activity was observed at 3 moles of oxygen uptake per mole of enzyme, at which point the only significant change in amino acid composition of the irradiated enzyme was the photooxidation of 3 moles of histidine out of the total of 4 moles. No alteration in the tyrosine, phenylalanine, lysine, and arginine contents of the enzyme was observed, but a small decrease in methionine was noted. A 50% inactivation of ribonuclease was accomplished when 0.5 mole of oxygen was taken up by 1 mole of enzyme, at which degree of photooxidation the change amounted to only 0.5 mole of histidine. Photooxidation of ribonuclease below 0.5 mole of oxygen uptake resulted in a proportionality between activity and photooxidation of histidine. It has to be pointed out, however, that at this low level of oxidation, the small decreases in histidine concentration (6.5–8.8%) are subject to analytical error. Our values, however, appeared to fit into the expected rate of decrease of histidine concentration. At 1 mole of oxygen uptake per mole of enzyme, the enzymatic activity was found to be reduced by 72%, whereas the decrease in histidine was 1 mole. Only a 4.1% decrease in methionine content could be noted at 3 moles of oxygen uptake (corresponding to 0.2 mole), and a decrease of 16.2% (corresponding to 0.80 mole) was found at 4.9 moles of oxygen uptake per mole of ribonuclease. Irradiation of ribonuclease under identical conditions (as described for Fig. 1) but in a nitrogen atmosphere did not produce any inactivation of the enzyme.

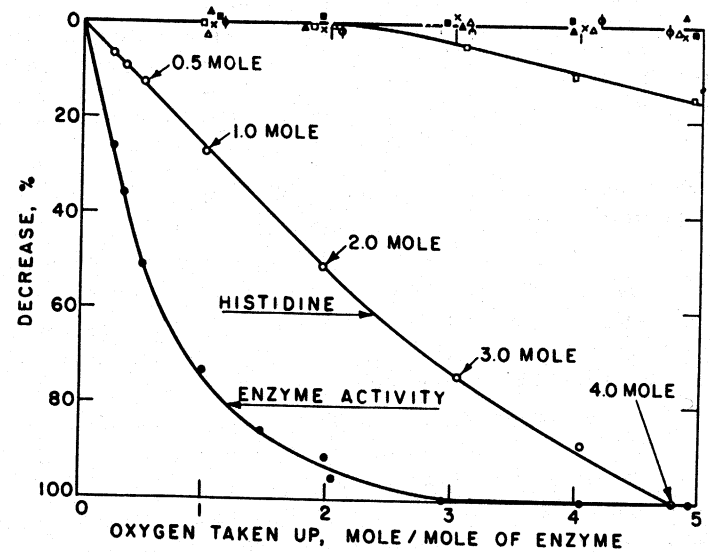

FIG. 1. Changes in amino acid composition and enzymatic activity produced by the photooxidation of ribonuclease.
■ = cystine; ▲ = lysine; × = arginine; △ = tyrosine; ∅ = phenylalanine; □ = methionine.

Photochemical Action of Methylene Blue on the Ultraviolet Absorption Spectrum of Ribonuclease

The experimental conditions for the irradiation of ribonuclease in these experiments were identical with those described for experiments presented in Fig. 1. After the desired oxygen uptake was reached, the dye was removed by the addition of a small amount of charcoal, the solutions were filtered through sintered glass, and the absorption spectra were measured after appropriate dilution with water. Total nitrogen determinations were carried out on the filtrate to check for slight losses during the charcoal treatment.

Figure 2 shows the changes observed during the photooxidation. The absorption maximum of 278 mμ, which is due in this case to tyrosine and phenylalanine (tryptophan is absent in ribonuclease), was essentially not altered; this is in agreement with the findings presented in Fig. 1, which showed that these two amino acids were not involved during the photooxidation. The over-all increase in absorption within the range of about 230–270 mμ might be due to the photooxidation of histidine,

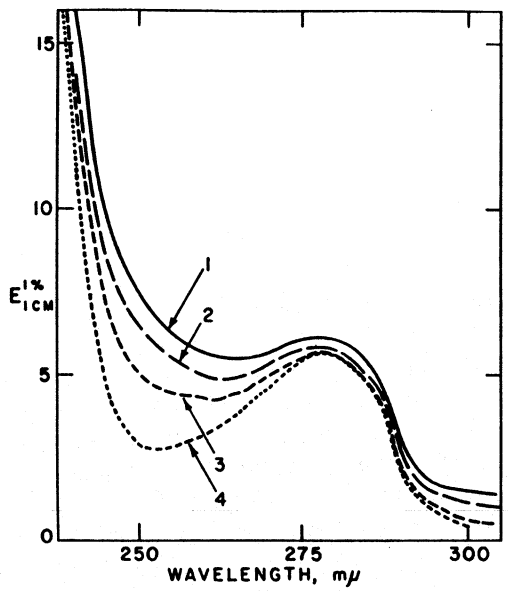

FIG. 2. Changes in the ultraviolet absorption spectrum of ribonuclease produced by photooxidation. *1* = 3.3 moles of O_2 per mole of enzyme; *2* = 2.4 moles of O_2 per mole of enzyme; *3* = 1.4 moles of O_2 per mole of enzyme; *4* = original enzyme.

as shown in Fig. 3, which compares the absorption spectrum of histidine with that of photooxidized histidine [1 mole oxygen/mole histidine (9)].

Photochemical Action of Methylene Blue on the Viscosity and Optical Rotation of Ribonuclease

Inasmuch as denaturation of a protein is usually accompanied by increases in viscosity and optical rotation, the possible changes in these physical properties during the photooxidation of ribonuclease were studied. For the viscosimetric studies, 1.5 ml. of ribonuclease solution (containing 30 mg., adjusted to pH 8.0) was placed in the main chamber of the Warburg vessel and 0.5 ml. of a 0.02% solution of methylene blue was put in the side arm. After temperature equilibrium, the two were mixed and the irradiation carried out to 0.8, 1.1, 1.5, 2.0, 2.5, 3.0, and 4.9 moles of oxygen uptake per mole of enzyme. The irradiation times required for these uptakes were 5, 7, 12, 17, 20, 23, and 40 min., respectively. The relative viscosity was determined as described previously (10). Results obtained and presented in Fig. 4 indicate that for up to 2.5 moles of oxygen uptake per mole of enzyme no changes in relative viscosities occurred. Beyond this point, however, an increase in this property was observed. Irradiation experiments for the measurement of optical rotation were identical with those described for the study of viscosity. After the desired oxidation was reached, the dye was removed by the addition of a small amount of charcoal, the solutions were filtered, and the optical rotation of the clear filtrates was measured in a 20-cm. tube. Slight losses due to the charcoal adsorption were checked by total nitrogen determinations. The results obtained in this way are incorporated in Fig. 4, which shows that, in agreement with our viscosity studies, no changes in optical rotation took place up to about 2.5 moles of oxygen uptake, but a gradual increase could be noted after passing this mark.

Test for Possible Peptide Bond Cleavage During the Photooxidation of Ribonuclease

For determination of a possible photolysis of ribonuclease during photooxidation, 40 mg. of the enzyme was irradiated as described for

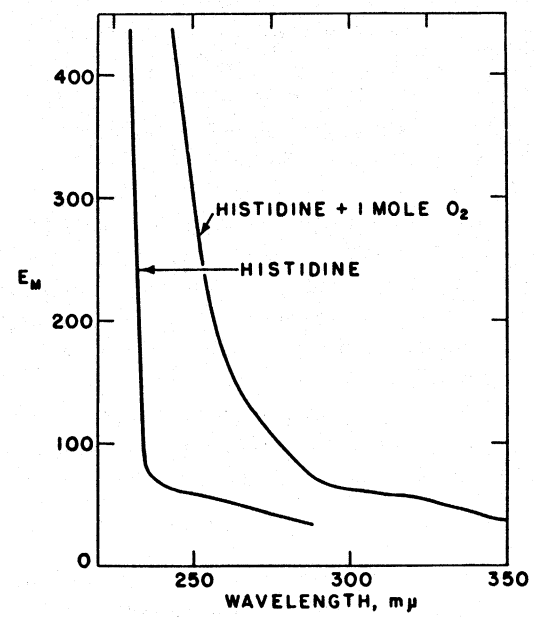

FIG. 3. Changes in the ultraviolet absorption spectrum of histidine produced by photooxidation.

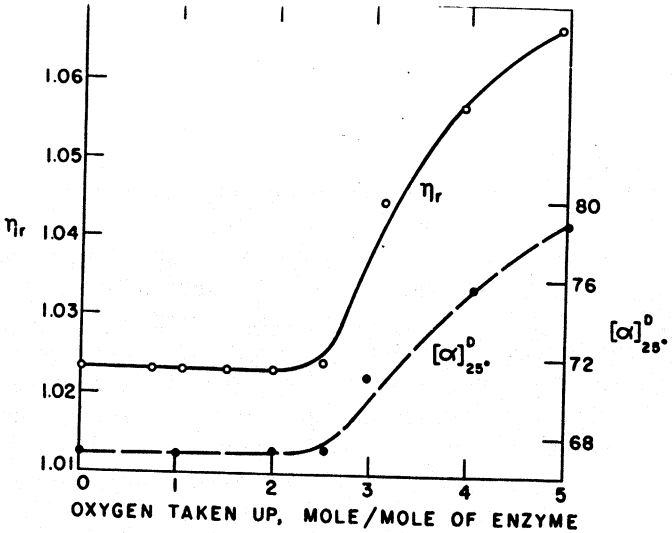

FIG. 4. Changes in relative viscosity and optical rotation of ribonuclease produced by photooxidation.

experiments presented in Fig. 1, to the extent of 1.2, 1.9, 2.9, 4.6, and 7.3 moles of oxygen per mole of enzyme. The α-amino nitrogen was measured by the manometric Van Slyke procedure. No increase in α-amino nitrogen content was observed during the photooxidation of ribonuclease at any of these oxidation levels, indicating the absence of photolysis.

Test for the Possible Presence of Sulfhydryl Groups in Ribonuclease

Recent work of Ledoux (12) advanced the possibility that sulfhydryl groups of ribonuclease might be associated with enzymatic activity. Although previous work of Barron *et al.* (13) has indicated the absence of these groups in ribonuclease, it was found necessary to reinvestigate this possibility. The possible presence of these groups was tested in a 3% solution of the enzyme; the nitroprusside spot-test was used (14). Although this test gave a strongly positive reaction even with 4 μg. cysteine, it was negative with the enzyme. Similarly negative results were obtained if the enzyme was dissolved in saturated guanidine·HCl solution.

The possible reactivation of the photoinactivated ribonuclease by a reducing agent was also tested. Ribonuclease was photooxidized to the extent of 3 moles of oxygen per mole of enzyme, as described for experiments presented in Fig. 1. To 0.4-ml. aliquots of the photoinactivated enzyme containing 4.0 mg. of the original enzyme, 0.5 ml. of HCN, glutathione, and Na thioglycolate solutions containing 1.0 mg. of each were added. After 2 hr. incubation at 37°, aliquots containing 50 μg. of the enzyme were assayed for possible reactivation (8). In no instance was any restoration of the enzymatic activity observed.

DISCUSSION

A thermostable enzyme, present in pancreas and capable of digesting ribonucleic acid, was recognized by Jones (15) and Dubos and Thompson (16), and isolated in crystalline form by Kunitz (3). The physicochemical findings on this enzyme (3, 17) have fulfilled the criteria of purity. Additional evidence of homogeneity of ribonuclease was presented recently by Anfinsen *et al.* (18) concerning the terminal amino acids of this enzyme, which led them to the conclusion that ribonuclease is a single-chain protein. On the other hand, one has to mention the chromatographic studies of Hirs *et al.* (19), and Martin and Porter (20), which indicate that this enzyme might be composed of two very closely related proteins with identical specific activities. Although there is some question as to the homogeneity of ribonuclease, the difference might be only a minor one. We have extended our studies (1, 2) on the photochemical action of methylene blue, therefore, to ribonuclease in order to correlate the observed changes with catalytic activity.

Our results show that progressive photooxidation of ribonuclease resulted in a rapid inactivation of the enzyme, reaching zero activity at the level of 3 moles of oxygen uptake per mole of enzyme. At this point, the only chemical change observed in the amino acid composition was the oxidation of 3 moles of histidine out of the total of 4. At 1 mole of oxygen uptake, the decrease in histidine content amounted to 1 mole, with a simultaneous 74% decrease in enzymatic activity. A proportionality between the decrease in enzymatic activity and photooxidation of histidine could be observed up to 52% inactivation, inasmuch as this degree of photooxidation resulted in a final decrease of 0.5 mole of this amino acid.

Similar experiments reported by Shugar (21) on the photochemical action of riboflavin on ribonuclease showed no inactivating effect. These findings are difficult to interpret, inasmuch as no data can be found as to whether a photochemical action actually took place. Inactivation of

ribonuclease by ultraviolet light as shown by McLaren *et al.* (22) was interpreted as indicating that the primary process involved in the inactivation was the modification of the aromatic residues of the enzyme, a postulate that would be in agreement with our findings.

The fact that no changes in viscosity and optical rotation occurred up to 2.5 moles of oxygen uptake per mole of enzyme (at which level about 95% inactivation resulted) indicates that the photoinactivation of ribonuclease was not due to possible denaturation but was related in some way to the observed chemical alteration of histidine.

The possible relationship of sulfhydryl groups of ribonuclease to activity as suggested by Ledoux (12) does not appear probable, since our experiments failed to demonstrate the presence of such groups. Addition of reducing agents such as HCN, glutathione, and thioglycolic acid did not restore the activity of the photoinactivated enzyme. The previously mentioned proportionality between decrease of histidine and enzyme activity (a 52% inactivation of enzyme when 0.5 mole of histidine was photooxidized) might be interpreted to mean that the photooxidation of only 1 mole of histidine was sufficient to eliminate the catalytic activity; the observed disproportionality beyond that point might be due to the participation of the two additional histidine molecules not associated with the activity. Because of insufficient evidence at present, however, consideration has to be given to the other two histidine units as possible contributing factors for the catalytic activity of ribonuclease.

These and previous findings (2, 23–25) support the contention that histidine may represent an important factor, which in some yet unknown fashion is related to the activities of the hydrolytic enzymes.

Summary

The photochemical action of methylene blue on ribonuclease resulted in a complete inactivation when 3 moles of oxygen was taken up per mole of enzyme. At this level of oxidation, the only chemical change observed was the photooxidation of 3 moles of histidine out of the total of 4. Up to 50% inactivation, the decrease of histidine was found to be proportional to the decrease in enzymatic activity, inasmuch as the photooxidation of 0.5 mole of histidine resulted finally in a 52% inactivation.

No changes in relative viscosity and optical rotation of ribonuclease could be noted up to 2.5 moles of oxygen uptake per mole of enzyme, whereas the decrease in enzymatic activity at this point amounted to 95%.

Changes in ultraviolet absorption spectrum during the photooxidation of ribonuclease were in agreement with the changes observed in amino acid composition.

Ribonuclease does not possess any sulfhydryl groups. Addition of reducing agents to the photoinactivated enzyme did not restore its activity.

The possible significance of histidine to the catalytic activity of ribonuclease is discussed.

References

1. Weil, L., Buchert, A. R., and Maher, J., *Arch. Biochem. and Biophys.* **40,** 245 (1952).
2. Weil, L., James, S., and Buchert, A. R., *Arch. Biochem. and Biophys.* **46,** 266 (1953).
3. Kunitz, M., *J. Gen. Physiol.* **24,** 15 (1940).
4. McDonald, M. R., *J. Gen. Physiol.* **32,** 39 (1948).
5. Herriott, R. M., *Proc. Soc. Exptl. Biol. Med.* **46,** 642 (1941).
6. Weil, L., and Maher, J., *Arch. Biochem.* **29,** 241 (1950).
7. Brand, E., *in* "Crystalline Enzymes" (Northrop, J. H., Kunitz, M., and Herriott, R. M.), p. 26. Columbia University Press, New York, 1948.
8. Bain, J. A., and Rusch, H. P., *J. Biol. Chem.* **153,** 659 (1944).
9. Weil, L., Gordon, W. G., and Buchert, A. R., *Arch. Biochem. and Biophys.* **33,** 90 (1951).
10. Weil, L., and Buchert, A. R., *Arch. Biochem. and Biophys.* **34,** 1 (1951).
11. Moore, S., and Stein, W. H., *J. Biol. Chem.* **192,** 663 (1951).
12. Ledoux, L., *Biochem. et Biophys. Acta* **11,** 517 (1953).
13. Barron, E. S. G., Dickman, S., Muntz, J. A., and Singer, T. P., *J. Gen. Physiol.* **32,** 537 (1949).
14. MacDonnell, L. R., Silva, R. B., and Feeney, R. E., *Arch. Biochem. and Biophys.* **32,** 288 (1951).
15. Jones, W., *Am. J. Physiol.* **52,** 203 (1920).
16. Dubos, R. J., and Thompson, R. H. S., *J. Biol. Chem.* **124,** 501 (1938).
17. Rothen, A., *J. Gen. Physiol.* **24,** 203 (1940).
18. Anfinsen, C. B., Flavin, M., and Farnsworth, J., *Biochem. et Biophys. Acta* **9,** 468 (1952).
19. Hirs, C. H. W., Stein, W. H., and Moore, S., *J. Am. Chem. Soc.* **73,** 1893 (1951).
20. Martin, A. J. P., and Porter, R. R., *Biochem. J.* **49,** 215 (1951).
21. Shugar, D., *Bull. soc. chim. biol.* **33,** 710 (1951).
22. McLaren, A. D., Gentile, P., Kirk, D. C., Jr., and Levin, N. A., *J. Polymer Sci.* **10,** 333 (1953).
23. Wilson, I. B., and Bergmann, F., *J. Biol. Chem.* **186,** 683 (1950).
24. Doherty, D. G., and Vaslow, F., *J. Am. Chem. Soc.* **74,** 931 (1952).
25. Wagner-Jauregg, T., and Hackley, B. E., *J. Am. Chem. Soc.* **75,** 2125 (1953).