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A STUDY OF THE EFFECT OF IONIZING RADIATIONS ON LACTOPEROXIDASE*

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Ionizing radiation has been used to elaborate upon biological structure[1]. An important assumption in radiation studies of enzymes has been that in the dry state one primary ionization inactivates one enzyme molecule[1,2,3]. More recently, OKADA[4] extended this concept with a kinetic study of X-ray induced inactivation of desoxyribonuclease in aqueous media. This study has shown that although one enzyme molecule is able to react with more than one free radical, only one free radical is responsible for the inactivation of the enzyme molecule.

The free radical, then, can be assumed to inactivate an enzyme by reaction with the active center of the molecule and the rest of the protein would then be considered inert with respect to enzyme activity. The active center is assumed to be the prosthetic group plus whatever portion of the protein is necessary for enzymic activity.

Because of their pronounced spectral properties, the hemoproteins make it possible to study prosthetic group changes independent of enzyme activity. It is thus possible to study the kinetics of inactivation as related to the whole protein, the active center and the prosthetic group.

Irradiation of oxygen-containing aqueous solutions will produce H_2O_2, which can effect enzyme inactivations. For a study of inactivation by free radicals, the hemoprotein, lactoperoxidase, is excellent. Its substrate is H_2O_2, and the amount of H_2O_2 which could accumulate under the experimental conditions can be shown not to cause any inactivation over the test period. The effects of irradiation on the enzyme can thus be attributed to free radical reactions.

The study to be reported will measure the kinetics of the free radical reactions caused by X-irradiation on (a) the whole protein, (b) the active center, and (c) the prosthetic group. This is accomplished by observing the spectral shifts which are due to free radical reactions of the amino acid residues at 250 mμ and those due to reactions of the porphyrin nucleus at 412 mμ. The enzyme activity is used as an assay of the active center.

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EXPERIMENTAL

The enzyme, lactoperoxidase, was isolated from raw skim milk and purified by the method of MORRISON AND HAMILTON[5]*. Based on the ratio 412 mμ/280 mμ absorption[6], the enzyme was about 80% pure and was made up to a concentration of about $3.5 \cdot 10^{-6}$ *M* for the irradiation experiments.

The enzyme activity, assayed by the method of MAEHLY AND CHANCE[7], is expressed in arbitrary units.

A 670 curie cobalt unit was used as a source of gamma rays. The dose rate** was 4,730 roentgens per minute.

Four ml aliquots of the enzyme solution were placed into 10 ml flasks and irradiated for 0, 3, 5, 7, 10, 15 and 20 minutes. After irradiation, spectra of samples were taken in a DU Beckman spectrophotometer in cuvettes with a 10 mm light path. The enzyme activity of all the samples was also measured.

RESULTS

Fig. 1 shows the spectrum of the original enzyme solution as well as the spectrum of the enzyme after 10 and 20 minutes of irradiation. The most marked changes in spectral

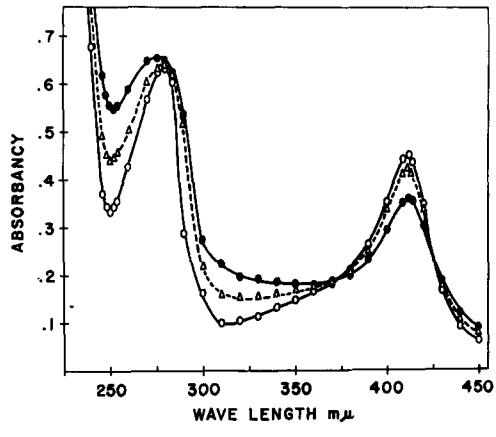

Fig. 1.

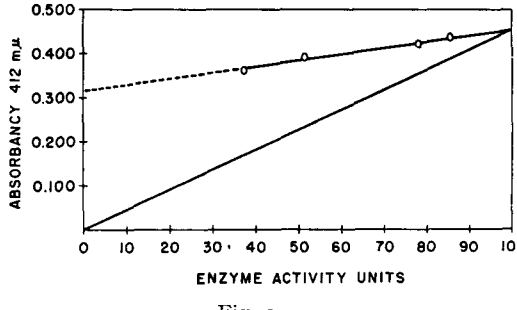

Fig. 3.

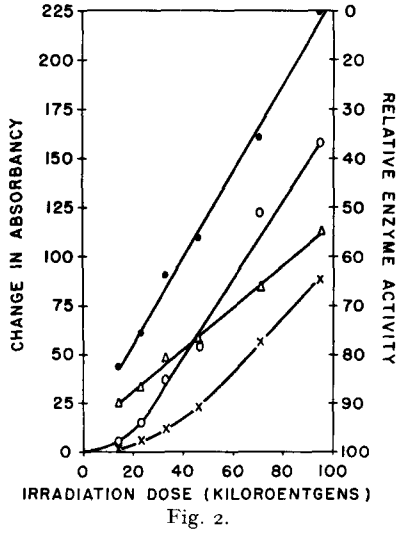

Fig. 2.

Fig. 1. Absorption spectra of lactoperoxidase before and after irradiation. ○ before irradiation; △ after irradiation (10 minutes) 47,300 r; ● after irradiation (20 minutes) 94,600 r.

Fig. 2. The effect of irradiation dose on the absorbancy of lactoperoxidase ($3.5 \cdot 10^{-6}$ *M*). ● at 250 mμ; △ at 310 mμ; × at 412 mμ. Enzyme activities are plotted on the right hand axis and the corresponding curve indicated by ○——○.

Fig. 3. The variation in absorbancy at 412 mμ with enzyme activity. —— before irradiation; ○—○ after irradiation.

* Fresh unpasteurized skim milk was supplied through the courtesy of the Genesee Valley Cooperative, Inc., Rochester, New York, U.S.A

** The dose rate was determined by Drs. L. W. TUTTLE and R. BAXTER of the University of Rochester employing a ferrous sulfate dosimeter.

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properties of the enzyme occur at the 310 and 250 mμ regions of the spectra. In both regions there is a marked increase in absorption. At 280 mμ there is a smaller increase in absorption, while at 412 mμ there is a considerable decrease. Isobestic points are observed at about 373 and 427 mμ. POLIS AND SHMUKLER[6], using photo-oxidation, showed similar changes in the enzymes with isobestic points at about the same positions. Fig. 2 shows a plot of the changes of absorbancy at three different wavelengths which occur when the enzyme is subjected to increasing doses of irradiation. When the radiation dosage is much larger than 95,000 r, the enzyme precipitates and consequently the values for high doses cannot be obtained. It should be noted that the absorbancy changes at 250 and 310 mμ represent increases, while at 412 mμ they represent decreases. On the same graph, the axis on the right shows the enzyme activity plotted against irradiation dose. The enzyme activity increases from the top of the graph to the bottom to make comparison clearer.

Fig. 3 shows a plot of enzyme activity against the optical density at 412 mμ. The upper curve represents the irradiated enzyme, the lower curve, the non-irradiated enzyme.

DISCUSSION

It has been noted that irradiation of proteins generally causes an increased absorption in the ultraviolet region of their spectra. The absorption at 280 mμ can be primarily attributed to the aromatic amino acids in the protein. On irradiation at 280 mμ, however, the amino acid residues, tyrosine and tryptophan, display different effects, with tyrosine increasing in absorption and tryptophan decreasing[8]. Lactoperoxidase shows a small but significant increase at this wavelength, suggesting that the tyrosine–tryptophane ratio is in the region of 0.5. The increased absorption at 250 mμ is directly proportional to the irradiation dose (Fig. 2) and can be attributed to oxidation products of amino acid residues of proteins.

The effects of ionizing radiations on hemoproteins have been studied by a number of workers[3,8–13]. There is general agreement that in the presence of air and at relatively high irradiation doses there are alterations in the spectral properties of these compounds[8,13]. The absorption at 412 mμ can be attributed to the intact tetrapyrrole nucleus which is the prosthetic or functional group of the enzyme. The drop in optical density at 412 mμ is due to the destruction of the intact tetrapyrrole nucleus[13]. Fig. 3 shows that the decrease in enzyme activity is related to the destruction of the tetrapyrrole. When the enzyme activity is extrapolated to zero, however, it can be seen that there would still be considerable absorption at 412 mμ. This suggests that the product of irradiation has about three-fourths the extinction of the original lactoperoxidase and this high extinction in this region of the spectra as well as the position of the maxima indicates that a large percentage of the tetrapyrrole is intact. The remainder of the enzymic inactivation, then, is due to destruction of the other part of the active center, namely, the protein required for activity, and not the destruction of the tetrapyrrole directly.

The fact that the relative enzyme activity is not related either exponentially or linearly to irradiation dose suggests that more than two free radical reactions with the protein are necessary to destroy enzyme activity. These results are comparable to those of DALE[14] who has shown that irradiation of only the protein moiety of the

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enzyme D-amino acid oxidase, does cause a marked decrease in the activity of the reconstituted enzyme.

Under our experimental conditions when 63% of the enzyme is inactivated the G value is approximately 0.04. This is a high value when compared to the G value 0.009 reported[8] for catalase which is also a hemoprotein.

The similarity between the spectra (Fig. 1) of the present experiment with X-ray induced inactivation and those of previous workers with photo inactivation [6] suggests that the same mechanisms of inactivation are functioning.

The red protein which accompanies the lactoperoxidase isolated by three different procedures[5,6,15] has been suggested as the degradation product of the lactoperoxidase. Our results show increased absorption in the entire visible region. The difference spectra of the irradiated and non-irradiated product, however, failed to show any peaks in the product of irradiation in the regions such as 470 mμ where the red protein has an absorption peak. This indicates that the red protein is not a major product formed by irradiation of the lactoperoxidase.

SUMMARY

The effects of X-irradiation on lactoperoxidase were studied by means of spectra and enzyme activity. Changes in the absorption spectra of the enzyme on irradiation were most prominent at 250, 310 and 412 mμ and were similar to the changes observed on photo-irradiation of the enzyme. The inactivation of the enzyme is not related exponentially or linearly to the irradiation dose. However, a linear relationship was observed between enzyme activity and changes at 412 mμ.

Inactivation was probably due to two reactions: (1) free radical reactions which destroyed the tetrapyrrole nucleus and (2) reactions which destroyed that portion of the protein which is part of the active center of the molecule. A G value of approximately 0.04 was obtained for the lactoperoxidase.

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