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An ESR Study of Amino Acid and Protein Free Radicals in Solution*

Part VI. Enzymatic Inactivation of Lysozyme in Aqueous Solution Resulting from Exposure to Ti-H_2O_2 System and Gamma-Irradiation.

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Abstract The activity change of lysozyme resulted from its exposure to Ti-H_2O_2 system in aqueous liquid at room temperature and to γ-irradiation in ice at 195°K has been measured at room temperature with a Cary-14 spectrophotometer.

The enzymatic activity of lysozyme which had been added to a previously flow-mixed solution of $TiCl_3$ and H_2O_2 (system *I*) was compared with the activity of a lysozyme-H_2O_2 solution after flow-mixing with $TiCl_3$ (system *II*), considering the differences between these two activity changes as the extent of the enzymatic inactivation by the involvement of OH radical reaction.

The fraction of lysozyme inactivated by OH radical in the system containing 0.0025 M $TiCl_3$-0.1 M H_2O_2 (pH 3.5) was 13%. When the $TiCl_3$ concentration is double (pH 3.0), the fraction of enzyme inactivated increases to 36%. The activity of the system containing 0.025 M $TiCl_3$-0.1 M H_2O_2 (pH 1.5) was essentially zero. The results seem to support the previous view that the production of OH radical should be proportional to $TiCl_3$ concentration when H_2O_2 is present in excess. Increase in the extent of inactivation found in system *I* with increasing $TiCl_3$ concentration may be due to a pH effect. H_2O_2 seems to be less effective than $TiCl_3$ in the inactivation.

1% lysozyme solution, when γ-irradiated with a total dose of 3M rads, loses about 20% of its activity. Lowering of temperature also was found to yield a reduction in enzymatic activity.

Introduction

The first attempt to correlate the ESR results of an irradiated enzyme to its inactivation data was reported by Hunt et al[1,2]. They observed change in the ESR spectrum of γ-irradiated dry

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ribonuclease in every treatment which resulted in a change in the sensitivity of enzymatic activity, suggesting that a correlation exists between the stable free radicals produced in the irradiated enzyme and the effect of radiation on the activity of enzyme. In the presence of oxygen, the primary induced radical is assumed to react with its environment to form a new peroxy-type radical, leading to an increase in the permanent damage to the enzyme molecule by an unknown mechanism[1~3].

Henriksen[3,4], in addition, found a good correlation between production of secondary radicals and inactivation for dry trypsin and suggested that the loss of enzymatic activity is the result of a sequence of events in which free radicals are important intermediates.

It is now generally accepted that in the case of irradiated aqueous solutions the chemical damage in the solute is produced largely by the action of water radicals[5], mainly OH radical[6].

In our previous experiments[7~11] on ESR of lysozyme and its constituent amino acids after exposing these substances to the Ti-H_2O_2 flow-mixing system and γ-irradiation in aqueous solution, we obtained indirect evidence suggesting that OH radical may play a significant role in the production of those solute free radicals.

The purpose of the present experiment was to measure the activity change of lysozyme which has resulted from its exposure to the Ti-H_2O_2 flow-mixing system and γ-irradiation.

Experimental

Lysozyme (Code; LY) and its substrate *Micrococcus Lysodeikticus* were obtained from Worthington Biochemical Corp. Frechold, New Jersey.

The rate of lysis of *Micrococcus Lysodeikticus* was determined as suggested by Shugar[12] except for the preparation of lysozyme solutions.

$TiCl_3$ solution is very acidic and the pH value of the enzyme solution will change according to the amount of added $TiCl_3$. This in turn will affect the stability of the enzyme solution in the Ti-H_2O_2 system. H_2O_2 also will produce significant enzymatic inactivation when added chemically at concentrations greater than 10^{-5} M[13,14]. In addition, the $[Ti^{3+}]/(H_2O_2)$ ratio[7] and temperature[8] of the solution are also assume to affect the enzymatic activity.

Therefore, special considerations were taken to minimize these effects of instability; the enzymatic activity of lysozyme which had been added to a previously flow-mixed solution of Ti^{3+} ion and H_2O_2 was compared with the activity of a lysozyme-H_2O_2 solution after flow-mixing with Ti^{3+} ion. The difference between these two activity changes was considered as the extent of enzymatic inactivation of lysozyme by the involvement of OH radical reaction.

Both solutions were prepared by mixing 0.3 g. lysozyme and 15m*l* each of 0.01 M $TiCl_3$ and 0.2 M H_2O_2, and different only in the sequence of mixing. We chose this $[Ti^{3+}]/(H_2O_2)$ ratio for this pair presented a fairly good result in the production of lysozyme free radical in our previous ESR experiments.[7~9]

Each resulting solution was diluted by one twentieth with water, and the rate of lysis was measured at 450mμ with a Cary-14 spectrophotometer, Applied Physics Corporation, Monrovia, California.

In the γ-irradiation experiments, two aliquots of 1% lysozyme aqueous solution were prepared. One was γ-irradiated at dry ice temperature with a total dose of 3 M rads while the other sample was kept at the same temparature without irradiation for the same period of time. The difference between the activity change of these two aliquots was considered as due to the action of γ-ray. After irradiation and thawing, the

lysis was recorded as in the flow experiment.

Ti-H_2O_2 flow-mixing[7] and γ-irradiation[10] techniques were described in the previous paper.

Results and Discussion

The data shown in *Table* 1*B* indicate that the amount of $TiCl_3$ employed, and accordingly pH change, has significant effect on the loss of enzymatic activity. In agreement with the previous view[12] that the lysozyme activity is not affected by pH variation between 3.6 and 12, the activity change is remarkable at the pH range over 1.5 to 3.5.

$$Ti^{3+}+H_2O_2 \longrightarrow Ti^{4+}+OH^-+\cdot OH \qquad (1)$$

In view of the fact that reaction (1) is the principal one in the Ti–H_2O_2 system, the production of OH radical should be proportional to $TiCl_3$ concentration when H_2O_2 is present in excess[16]. This relation predicts that the OH radical effect on enzymatic activity should also be proportional to Ti^{3+} concentration. In this experiment, the change in activity from system *I* to *II* represents the extent of OH radical effect, and the changes are listed for 0.0025 *M*, 0.005*M* and 0.025*M* $TiCl_3$ with 0.1 *M* H_2O_2. As shown in *Table* 1*B*, the fraction of lysozyme inactivated by the OH radical in the system containing 0.0025*M* $TiCl_3$–0.1 *M* H_2O_2 (pH 3.5) is 13%. When the $TiCl_3$ concentration is double, the fraction of lysozyme inactivated increases to 36% as one would expect. The activity of system *I* for 0.025 *M* $TiCl_3$–0.1 *M* H_2O_2 (pH 1.5) is essentially zero, and thus this fraction of inactivation cannot be computed. Thus the result seems to be in agreement with our expectation, but the data are not sufficient for a quantitative analysis.

The effect of Ti^{3+} or H_2O_2 reaction alone on lysozyme was also investigated. As shown in *Table* 1*C*, the effect of $TiCl_3$ (probably a pH

Table 1. Activity change of lysozyme due to exposure to Ti-H_2O_2 system and gamma-irradiation;

	final conc. (M) Ti^{3+}	final conc. (M) H_2O_2	pH	procedure	measured activity (unit)	inactivated fraction (%)
A	—	—	5.0	H_2O+LY*	420.0	0.0
B	0.0025	0.10	3.5	(Ti+H_2O_2)+LY	240.0	42.9
				Ti+(H_2O_2+LY)	207.5	50.6
	0.0050	〃	3.0	(Ti+H_2O_2)+LY	88.0	79.1
				Ti+(H_2O_2+LY)	56.0	86.7
	0.0250	〃	1.5	(Ti+H_2O_2)+LY	2.5	99.4
				Ti+(H_2O_2+LY)	0.0	100.0
C	—	〃	5.0	H_2O_2+LY	262.5	37.5
	0.0050	—	2.1	Ti^{3+}+LY	170.0	59.5
D	—	—	5.0	no irradiation: kept at 195° K	344.0	18.1
				γ-irradiation at 195°K (3 *M* rad)	275.0	34.6

A. activity of lysozyme aqueous solution.

B. activity change as a function of the concentration of $TiCl_3$ (pH) and/or OH radical in Ti-H_2O_2 system.

C. control experiments to check activity in the absence of Ti^{3+} only and H_2O_2 only.

D. activity change due to gamma-irradiation and/or temperature lowering.

*. lysozyme: all solutions listed contain 1% (w/v) lysozyme as a final concentration.

effect) on the enzymatic activity was found to be considerably greater than that of H_2O_2.

As shown in *Table* 1*D*, 1% lysozyme aqueous solution loses about 20% of its activity when it is irradiated with a tontal dose of 3 *M* rad of γ-rays. The deactivation observed for the aliquot which had been kept at 195°K without irradiation is assumed to be resulted from temperature lowering. This experiment, however, does not indicate to what extent the activity loss is caused by the action of OH radicals produced by the radiolysis of water compared to losses by the direct action of radiation.

Thus, the present experiment seems to indicate that the OH radical generated in the Ti-H_2O_2 system causes a remarkable inactivation of lysozyme. This damage in the flow-mixed solution at room temperature seems to agree with the previous view[17] that the effect of ionizing radiation on aqueous solution systems is in general considerably greater when they are irradiated at temperatures above the freezing point than when they are irradiated in the frozen state. However, the qualitative data obtained here do not suggest any quantitative correlation between the OH radical effect on the enzymatic inactivation produced by chemical methods and by γ-irradiation.

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