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THE INACTIVATION OF RIBONUCLEASE A BY $Fe^{2+} + H_2O_2$ (FENTON'S REAGENT)

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SUMMARY

Ribonuclease A (ribonucleate pyrimidine-nucleotido-2'-transferase (cyclizing), EC 2.7.7.16) was treated with Fe^{2+} and H_2O_2 (Fenton's Reagent), a chemical source of hydroxyl radicals ($OH\cdot$). H_2O_2 alone had little or no effect on ribonuclease A activity at low H_2O_2/ribonuclease A molar excesses. As the molar ratio increased (H_2O_2/ribonuclease A > 100) ribonuclease A was inactivated by H_2O_2; the magnitude of this effect increased with increase in H_2O_2 concentration and was enhanced with time. Fe^{2+} and Fe^{3+} alone had no effect on ribonuclease A activity.

In an attempt to isolate the $OH\cdot$ effect, ribonuclease A was inactivated by Fe^{2+} and H_2O_2 using a minimum excess of H_2O_2 and very short reaction time. Inactivation was a function of Fe^{2+} concentration and was attributed to hydroxyl radicals. There was a nonlinear but reproducible relationship between molar ratio Fe^{2+}/ribonuclease A and degree of enzymic inactivation.

INTRODUCTION

On the basis of numerous studies of the inactivation of enzymes by ionizing radiation, it has been concluded that inactivation in dilute solutions is brought about by the "indirect" mechanism, that is, by the intermediate formation of free radicals from water[1–6]. In an attempt to mimic the effects of ionizing radiation, several experiments have been conducted using chemically generated free radicals to inactivate enzymes[1,7–9].

Collinson *et al.*[8] and Slobodian *et al.*[9] have used Fenton's reagent ($Fe^{2+} + H_2O_2$), a chemical source of hydroxyl radicals[10], to inactivate ribonuclease. Reactions A, B and C illustrate the production of $OH\cdot$ and two of the side reactions which can influence the availability of $OH\cdot$ for reaction with a substrate S:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (A)$$
$$Fe^{2+} + OH\cdot \rightarrow Fe^{3+} + OH^- \quad (B)$$
$$H_2O_2 + OH\cdot \rightarrow H_2O + HO_2\cdot \quad (C)$$

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At low H_2O_2/Fe^{2+} ratios only steps A and B occur; high H_2O_2/Fe^{2+} ratios favor Step C[11]. Thus, the concentration of reactants determines the efficiency of OH· capture by a substrate *S*. Depending on conditions, some effects on *S* may not be due to OH· but to other reactive species, such as $HO_2\cdot$ and H_2O_2.

Accordingly, in order to establish reproducible and optimum conditions for the reaction of OH· with ribonuclease A, a study was made of the effects of different concentrations of Fe^{2+} and H_2O_2 on ribonuclease A activity.

MATERIALS AND METHODS

Ribonuclease A (ribonucleate pyrimidine nucleotido-2'-transferase (cyclizing), EC 2.7.7.16) was supplied by Worthington Biochemicals as a 1% solution in phosphate buffer. Ribonuclease A was desalted and lyophilized[12]; water solutions of the enzyme were adjusted to pH 6. The ε_M at 278 nm was 9600 and the $A_{255\ nm}/A_{278\ nm}$ ratio was 0.395. Yeast ribonucleic acid was obtained from Nutritional Biochemicals and was dialyzed before use. $FeSO_4$ and 30% H_2O_2 were obtained from Merck; solutions were freshly prepared using glass-distilled water. Bio-Gel P-2 was obtained from Bio-Rad Co.; the resin was swelled in 50 mM NaCl and a column 2.5 cm × 12 cm was poured from a slurry of the resin. Ribonuclease A activity was measured by a modified acid soluble nucleotide assay[12]. Protein concentration was determined by the Biuret method.

RESULTS

The data in Table I show that H_2O_2 alone had little or no effect on ribonuclease A activity, at H_2O_2/ribonuclease A molar excesses of 100 or less, during the first hour of incubation. Higher H_2O_2/ribonuclease A molar ratios caused inactivation and the effect was enhanced with time. A 63% active protein sample, isolated after a 24-h incubation period with an H_2O_2/ribonuclease A molar ratio of 100, had an elevated absorption spectrum: ε_M at 278 nm was 11 250, and the ratio $A_{255\ nm}/A_{278\ nm}$ was 0.560.

TABLE I

EFFECT OF H_2O_2 AND INCUBATION TIME ON RIBONUCLEASE A ACTIVITY

Ribonuclease A (750 μM) was treated with 10 to 1000 times molar excesses of H_2O_2 and stored in the dark; at intervals, aliquots were taken and diluted 500-fold for assay.

H_2O_2/ribonuclease A molar ratio	*Residual enzymic activity (% of original)*					
	15 min	*30 min*	*60 min*	*100 min*	*225 min*	*24 h*
10	100		100		100	
20	100			100		
100	96				70	63
150		85	75			
390	88			65		
500	80			54		
780	70			36		8
1000	79				34	0

Since Fe^{2+} and Fe^{3+} alone have no effect on ribonuclease A activity, attempts were made to isolate the OH· effect by using a minimal excess of H_2O_2 and limiting the incubation time to 10 min in the presence of changing Fe^{2+}/ribonuclease A molar excesses.

Fig. 1 shows the inactivation of ribonuclease A by $FeSO_4$ and H_2O_2. About a 9-fold molar excess of Fe^{2+} to ribonuclease A was needed to cause 90–100% loss of activity. Curves I and II refer to the same purified enzyme preparation, but in Curve II the sample was treated with alternate equimolar aliquots of $FeSO_4$ and H_2O_2 in a stepwise fashion, instead of the usual one-step mode.

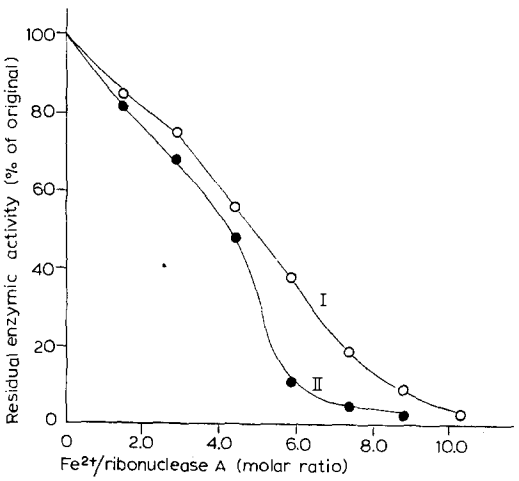

Fig. 1. Inactivation of ribonuclease A by Fe^{2+} + H_2O_2. Residual enzymic activity (per centage of original) is plotted against the molar ratio Fe^{2+}/ribonuclease A. The enzyme was treated with 2–10 times molar excesses of $FeSO_4$, followed by a 2-fold molar excess of H_2O_2 to Fe^{2+}, and stored in the dark. After 10 min, the sample was purified by rapid gel filtration using Bio-Gel P-2 (50 mM NaCl eluant) and the isolated protein fraction assayed. I (300 μM) and II (250 μM) were Worthington Ribonuclease A; I was inactivated using the usual procedure but II was treated with alternate equimolar aliquots of $FeSO_4$ and H_2O_2, in a step-wise fashion.

TABLE II

EFFECT OF H_2O_2 CONCENTRATION ON THE INACTIVATION OF RIBONUCLEASE A BY Fe^{2+} + H_2O_2

Fe^{2+}/ribonuclease A molar ratio*	*H_2O_2/ribonuclease A* molar ratio*	*Reaction time (min)*	*Residual enzymic activity (% of original)*
1.7	3.3	10	89
1.7	150.0	1080	47
2.0	60.0	10	83
2.0	60.0	200	76
2.6	5.0	10	82
2.6	5.0	200	81
5.0	10.0	18	30
5.0	100.0	18	17
5.0	500.0	18	10

* Ribonuclease A concentration was 700 μM.

Under the conditions of limited H_2O_2 excess used, ribonuclease A inactivation was a function of the amount of Fe^{2+} used. However, as expected, use of much higher H_2O_2 concentrations and longer incubation periods, enhanced inactivation as shown in Table II.

DISCUSSION

The finding of little or no H_2O_2 effect at low H_2O_2 concentrations is in accord with earlier findings on ribonuclease A[8,9]. The H_2O_2 effect observed at higher molar excesses of H_2O_2/ribonuclease A is a slow, time-dependent reaction, in contrast to the hydroxyl radical effect, which is rapid and completed within a few minutes. It is concluded that under the conditions used (minimum H_2O_2 excess and 10 min reaction time), inactivation by Fe^{2+} and H_2O_2 was primarily due to hydroxyl radicals. The probability of $HO_2\cdot$ participation was minimal, since $HO_2\cdot$ is formed only at high H_2O_2/Fe^{2+} ratios (Eqn. C).

Use of excessive H_2O_2 enhanced inactivation; the effect was time dependent (Table II) and was thus probably due to the additive effects of H_2O_2 and hydroxyl radicals. The step-wise reaction (Curve II, Fig. 1), which minimized scavenging of $OH\cdot$ by excess Fe^{2+} or H_2O_2, was more efficient.

The reaction curves in Fig. 1 were generally reproducible; the survey of a broad range of activities made it possible, in subsequent experiments with different ribonuclease A samples, to approximate any desired degree of inactivation by referring to Fig. 1.

In the course of purification, it was noted that inactivated ribonuclease A complexed iron. Iron binding has also been observed with γ-irradiated ribonuclease A[13]. The details of these findings will be presented in a separate report.

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