

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

Part V. An ESR Study of Gamma-Irradiated Lysozyme in Frozen Aqueous Solutions.

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Abstract An electron spin resonance study has been made on lysozyme in frozen aqueous solutions irradiated with ^{60}Co γ -rays in air at 77°K.

Water resonances are dominant when the concentration and the temperature are both below 20% and 130°K respectively. More solute radicals are produced in the solution of higher concentration. Majority of the solute radicals results from direct hit of the radiation. The same types of radicals are induced at 77°K whether the substances are irradiated in the dry state or in frozen aqueous solution. Based on these results, it is assumed that the number of ESR centers produced by the secondary intermolecular radical reactions and stabilized in aqueous solutions may depend on the concentration of the solution, and the presence of water may facilitate the secondary radical reactions occurring in the solute molecules after heat treatment. Majority of the solute radicals above around 193°K are believed to react with oxygen to form peroxy-type radicals. However, when the solution is subjected to heat-treatment at 265°K after irradiation at 195°K the peroxy-type resonance was not observed, suggesting that an appreciable amount of oxygen is condensed into the ice, at 77°K, in addition to the oxygen that has already been dissolved in solution and react with solute free radicals during the process of heat-treatment. When the solution contains H_2O_2 , no water resonance but $\text{HO}_2\cdot$ type resonance was observed probably indicating that the radiation-induced OH radicals are trapped in H_2O_2 aggregates and react readily with H_2O_2 molecules to produce $\text{HO}_2\cdot$ type radicals even at 77°K.

Introduction

Many investigations^{1,2} on the production of

protein free radicals by irradiation of solids have been reported since Gordy et al³ presented, in 1955, the first ESR signals in irradiated proteins. Based on the ESR results it is assumed²

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that the ESR centers induced in irradiated proteins consist of a mixture of positive holes and negative ions formed by the ionization processes, as well as radical fragments formed by the rupture of chemical bonds. In the next phase, these ESR centers are assumed to take part in the secondary reaction with each other, with intact neighbor molecules, or with other groups within the same molecule. The secondary protein radicals are formed in these reactions.

In a recent study⁴ of X-ray-induced gelatin radicals in frozen aqueous solution, the data indicated that a substantial indirect effect may occur even after irradiation at 77°K, largely due to the action of water radicals formed in the small sphere of bound water surrounding each solute molecule. In addition, it was found⁵ that the intermolecular transfer of unpaired spins occur in frozen aqueous mixtures also.

It is assumed, however, that the ESR centers induced depend² strongly on the treatment of the sample. The parameters such as irradiation temperature and oxygen effect will greatly influence the ESR spectra.

In this investigation, lysozyme in frozen aqueous solutions has been irradiated at 77°K with 3M rad γ -ray, and the ESR spectra recorded on the process of their transformation during warming up to room temperature have been studied. The purpose of this experiment was to study the qualitative effect of heat treatment, concentration, oxygen and H₂O₂ on lysozyme free radical produced in frozen aqueous solution.

Experimental

Lysozyme was obtained from Worthington Biochemical Corps., Frechold, New Jersey and used without further purification. Solutions of 0.1, 1, 10 and 20 % (w/v) lysozyme were prepared and examined. Gamma-irradiation and

ESR spectroscopy have been described previously⁶. During the experiments where the temperature was varied from 77°K to room temperature, the samples were annealed steepwise of 10 degree intervals for 3 minutes to assure temperature equilibration. After each annealing, the spectrum was recorded.

Results and Discussion

The spectrum of a 0.1 M lysozyme ice recorded immediately after the exposure at 77°K was not distinguishable from that of pure ice. Spectrum A in Fig. 1 recorded at 113°K however, consists of the water resonance with a little indication of a resonance due to radicals induced in the solute. The spectral changes observed on heat-treatment to approximately 133°K (Fig. 1C) are mainly due to the disappearance of the water radicals and there appear spectra which are quite similar to those⁷ observed at low temperature for the solid irradiated at 77°K. This result supports the previous conclusion^{4,8,9} that the same types of radicals are induced at 77°K, whether the substances are irradiated in the dry state or in frozen aqueous solution.

When the concentrations are increased, to 10% for example, the resonance due to the solute radical become more dominant in each spectrum recorded at the corresponding temperature (Fig. 2). The similar result was reported by Sanner⁴ for the X-irradiated thiogel frozen aqueous solutions. It apparently indicates that there is a higher production of solute radicals in the solution of higher concentration, and that the majority of the solute radicals, at this specific temperature level, results from direct hits in the parent molecules. Recent work in irradiated sephadex suspensions, however, has demonstrated that a large portion of the radicals present were due to some type of indirect

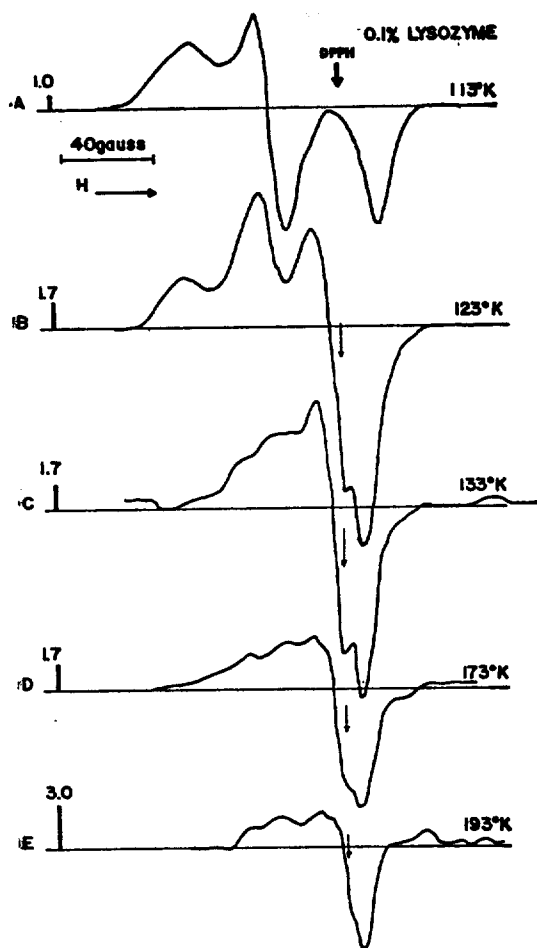


Fig. 1. Qualitative spectra of 0.1% lysozyme ice observed as a function of temperature in the range 113–193°K after irradiation at 77°K with γ -rays in air:

The relative spectrometer sensitivity is otherwise given by the sticks to the left.

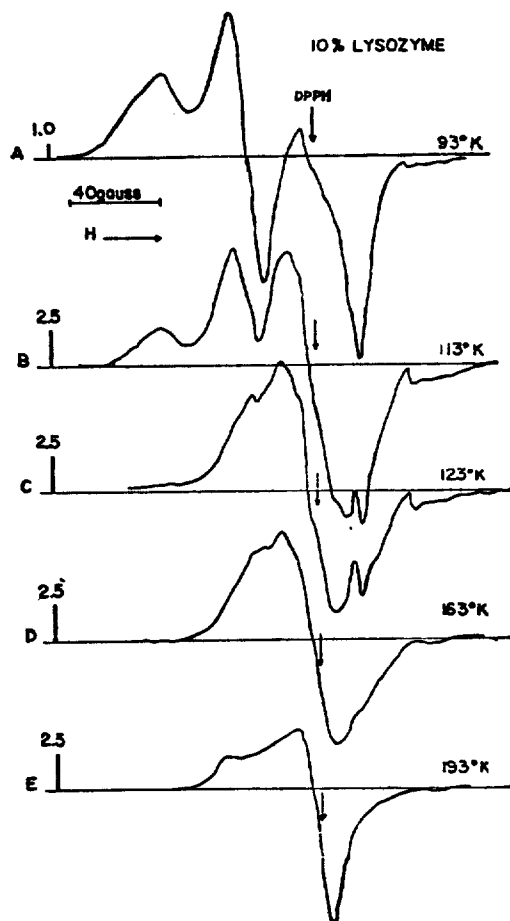


Fig. 2. Qualitative spectra of 10% lysozyme ice observed as a function of temperature in the range 93–193°K after irradiation at 77°K with γ -rays in air.

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action⁵. On the other hand, it was indicated that the presence of one component in a frozen solution may affect the formation and/or stabilization of radicals in other components.⁴

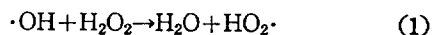
It is therefore reasonable to assume that the number of ESR centers produced by the secondary intermolecular radical reactions and stabilized in aqueous solutions may depend on

the concentration of the solution. In addition, the presence of water may facilitate the secondary radical reactions occurring in the solute molecules after heat-treatment.⁴

The spectrum observed at 193°K, an asymmetrical doublet with approximately 31 G splitting in Fig. 2 as well as in Fig. 1, is found to be quite similar to that for the peroxy-type solute

radical¹⁰, RO_2^\cdot , in polyalanine and polyphenylalanine where the splittings are about 33 G. Similar spectra have been observed during the process of heat-treatment in ices of amino acids, DNA and its constituents¹¹ when they are irradiated in air. The asymmetric doublet type of resonances in protein ices changed to an asymmetrical singlet with smearing of the low field peak at around 253°K with no further change up to the melting point of the ice. Theoretically it is reasonable to expect an asymmetric singlet for an RO_2 type radical for the unpaired electron is localized on oxygen and no proton couplings are probable. In dry lysozyme, when irradiated in vacuum at room temperature and then open to air for 21 hours, a similar singlet was observed² but with an appreciable amount of a low field peak which is believed to be from the sulfur resonance. However, it is doubtful whether the low field resonance in the spectrum observed at 193°K (Fig. 2 E) is due to sulfur radical. The reason is that 1) the formation of considerable amount of sulfur radicals at 193°K after less than one hour from the first measurement at 77°K may not be possible, and 2) polyalanine as well as polyphenylalanine, in spite of no sulfur contained in their molecules, exhibits this peak with appreciable intensity.

When the solution contains H_2O_2 (0.12M with respect to H_2O_2), no water resonance, but only the solute radical was observed (Fig. 3 A) at the temperature from 77°K to around the melting point of the ice. This spectrum seems to simulate the HO_2 resonance observed after irradiation of H_2O_2 ice with β -rays at 77°K.¹² One possibility is, therefore, that the formation of the HO_2 radical occurs by the reaction (1).



Hydrated electrons, e_{aq}^- , produced by the radiolysis of water seems to be very diffusible

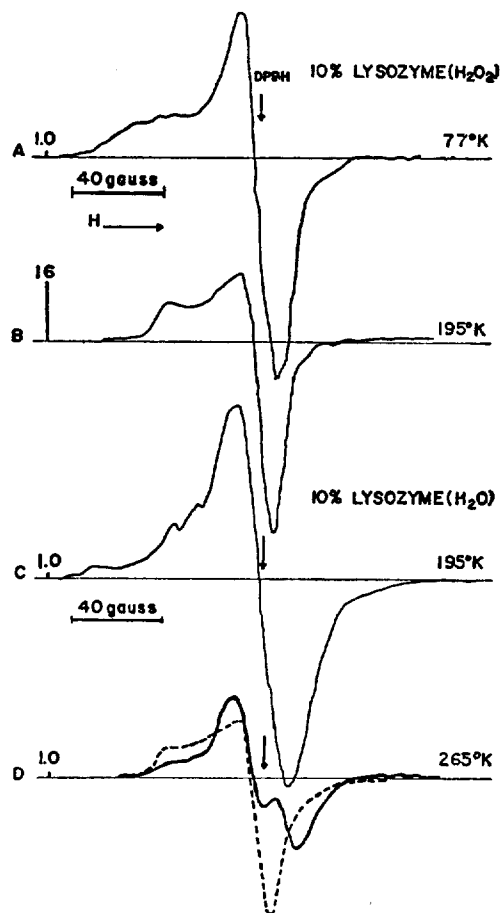
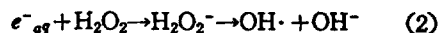


Fig. 3 Qualitative spectra of 10% lysozyme ice with 0.2 M H_2O_2 and 10% lysozyme ice without H_2O_2 irradiated with γ -rays in air at 77°K and 195°K, respectively:

(A) spectrum of 10% lysozyme ice containing 0.2M H_2O_2 observed at 77°K after irradiation at this temperature; (B) spectrum of the above sample observed after annealing at 195°K; (C) spectrum of 10% lysozyme ice observed at 195°K after irradiation at this temperature; (D) spectrum of the above sample observed after annealing at 265°K. Dashed line in D is the superimposed spectrum of 10% lysozyme ice observed at 193°K after irradiation with γ -rays in air at 77°K

even at temperatures of liquid nitrogen and may be captured by the electrophilic H_2O_2

molecule to produce H_2O_2^- which in turn splits into OH radical and OH^- ion.



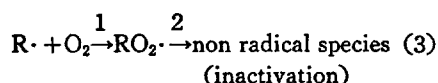
Altogether, the OH radical produced by this reaction and the radiolysis of water may then react with neighboring H_2O_2 molecules. It has been suggested that in ices, the solute molecules may not be homogeneously distributed but may be present as aggregates.^{4,13} If the assumption is made that the OH radicals are formed in H_2O_2 aggregates it may be possible for the trapped OH radical to react readily with neighboring H_2O_2 molecules even at the low temperature. This reaction is assumed to be faster than that with solute molecules, and appreciable amount of HO_2 radicals could be formed.

However, in 10% lysozyme solutions at 77°K the resonance is predominantly induced by direct action and therefore the HO_2 type resonance is not seen in the spectrum. Upon annealing this sample to 195°K, a RO_2 type resonance was also observed (Fig. 3 B). The shape of this spectrum is closely similar to that of the HO_2 radical obtained by irradiation of H_2O_2 ice at 77°K. However, the radicals should not be confused since most of the HO_2 radicals are believed to have decayed at this temperature.¹²

If the 10% lysozyme aqueous solution is subjected to heat-treatment at 265°K after irradiation at 195°K in air, a doublet instead of the RO_2 type resonance (Fig. 3 D) was observed. This spectrum is essentially the same as that² observed after irradiation of solid lysozyme in vacuum and keeping it in air for 10 minutes at 295°K. This result therefore seems to indicate that fewer oxygen molecules are available at 195°K than at 77°K. At the temperature of liquid nitrogen an appreciable amount of oxygen is assumed to be condensed into the ice in addition to the oxygen that has already been dissolved in solution. This oxygen is able to

react with solute free radicals during the process of heat-treatment. The spectrum from Fig. 2 E (dashed line in Fig. 3 D) was observed at 193°K after irradiation of 10% lysozyme at 77°K. When this spectrum is superimposed on spectrum D in Fig. 3, it indicates that a RO_2 -type resonance also appears after irradiation at 195°K but with lesser intensity.

Biological damage during irradiation with oxygen present is considered to be due to the formation of a peroxy-type radical, $\text{RO}_2\cdot$, by a two-step reaction scheme



Whether this RO_2 radical can be observed depends on the rate of peroxy radical formation compared with the rate of radical decay.^{10,14} If the second reaction is fast, compared with the first one, a decay with no spectral changes will be observed. On the other hand, if this second process is slow the ESR pattern will change to a singlet and decay slowly.

It then means that the present results at low temperatures belong to the latter category.

It was previously reported¹⁵ that the doublet resonance which has been attributed to the backbone type radical is very sensitive to oxygen, whereas the sulfur radicals are unlikely to take part in the oxygen effect. The assumption was made² thereafter that the doublet type radicals lead to inactivation when they react with oxygen.

Thus, a lysozyme ice irradiated in air was found to produce a peroxy-type solute radical which in turn resulted in an increase in inactivation.

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