Electron Spin Resonance Studies on the Damage of Ribonuclease Exposed to Ultra-violet Light

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ABSTRACT

Primary damage in RNase caused by exposure to UV-light was studied by ESR. Frozen aqueous solution of RNase was irradiated by 254 m\( \mu \) light and ESR spectra and remaining enzymatic activity were measured. ESR spectra showed that the free radicals formed in RNase were localized in chromophores of RNase; namely tyrosine, phenylalanine and cystine. Effect of warming on the spectra supported the assignments. The amount of radicals in RNase had linear relationship with the extent of enzyme inactivation.

INTRODUCTION

The process of radiation inactivation of enzymes can be divided into two stages; unstable electronic damage and stable chemical damage. Radical formation, detectable with ESR, would be one of the states in the electronic stage which bring about enzyme inactivation. In fact, free radicals were detected in amino acids\textsuperscript{1,2} and in bovine serum albumin\textsuperscript{3} irradiated with UV-light. Brustad et al.\textsuperscript{4,5} reported colinearity between radical formation and inactivation of UV-irradiated trypsin. Present study was carried out to assign the radicals formed in ribonuclease (RNase) irradiated with UV-light, and to know whether the radical formation is one of the intermediate steps of the inactivation process.

MATERIALS AND METHODS

Crystalline pancreatic RNase (Sigma Chemical Co.) was purified by the method...
of Tabolsky, and fraction D was obtained. Amino acids, tyrosine (Tyr), phenylalanine (Phe), histidine (His) and glycine (Gly) (Wako Pure Chemical Industries, LTD.), oxidized glutathione (GSSG) and reduced glutathione (GSH) (Sigma Chemical Co.) were used without purification. GSSG was used for a model substance of cystine, because of high solubility of the former, and similarity of ESR spectra after UV-irradiation and absorption spectra of the two compounds.

RNase, amino acids, GSSG and GSH were dissolved in distilled water at the concentration of \(10^{-5}\) to \(10^{-2}\) M, and the solutions were sealed in quartz tubes, diameter of which were about 4 mm, in air or in vacuum. The sealed tubes were placed in a quartz Dewar vessel filled with liquid nitrogen, and were irradiated for various periods (0.5--9 hours) with a low pressure mercury lamp (Toshiba GL 15). Approximately 90% of the energy was radiated at the 254 m\(\mu\) line. Under the conditions adopted, only the surface layer of the frozen sample was exposed to UV-light, because the frozen state of the solution was not glass, so it scattered so much of light.

The ESR spectra of irradiated samples were measured at 77\(^{\circ}\)K, in a Varian V-4500 X-band spectrometer with 100 Kc/s field modulation. The microwave power was about 5 mW, when signal saturation did not occur. The irradiated samples were then annealed in dry-ice-ethanol mixture (about 195\(^{\circ}\)K) for a definite period and again the ESR spectra were measured at 77\(^{\circ}\)K. In every case the spectrum was stable at 77\(^{\circ}\)K.

Spin concentrations were determined using a \(\alpha, \alpha'-\text{diphenil-\(\beta\)-picrylhydrazyl (DPPH) solution in benzen as the reference. After ESR measurement, the RNase solution was thawed at room temperature and the enzymatic activity was assayed by using RNA as a substrate according to Anfinsen.}

The amount of sulfhydryl (SH) groups in irradiated RNase was determined by p-chloromercuribenzoate (pCMB) titration.

RESULTS

Fig. 1-I shows the first derivative ESR spectra of frozen aqueous solutions of RNase, Tyr, Phe, GSSG and GSH which were irradiated in air. His and Gly do not give ESR signal by irradiation. Fig. 1-II and -III show the spectra after annealing for 2 minutes and 20 hours in dry-ice-ethanol mixture, respectively. The dotted lines in Fig. 1 indicate the reference position of the DPPH spectrum, and the figures indicate the g-values for the respective peaks. As is shown in Fig. 1-I, the spectrum of irradiated RNase consists of main singlet spectrum and four small peaks in lower magnetic fields. Phe and Tyr also give singlet spectra too, whereas GSSG show a complicated broad spectrum. After 2 minutes annealing, the spectra of RNase, Tyr and Phe become sharper (Fig. 1-II), but the spectrum of GSSG does not change even after 20 hours annealing. After 20 hours annealing, the spectrum of RNase becomes similar to that of GSSG, and that of Tyr or Phe considerably diminishes in its intensity (Fig. 1-III). By comparing these spectra, it is concluded
that the spectrum of RNase is made up of the superposition of spectra of the constituent chromophores, Tyr, Phe and cystine, which absorb 254 m\(\mu\) light.

In the case of irradiation in vacuum, the effect of annealing was different from that of irradiation in air, though the spectra were the same so far as they were stored at 77°K. Once annealed at 195°K, intensity of the spectra of Tyr and Phe decreased very rapidly, whereas that of GSSG remained unchanged. The spectrum of RNase generally decreased in its intensity very rapidly, except in the region similar to GSSG. The result supports the assignment of radicals in RNase to Tyr, Phe and cystine.

The spin concentration of RNase irradiated in air was proportional to the degree of inactivation as it was shown in Fig. 2. Other experiments showed that the annealing did not affect the degree of inactivation.

The concentration of SH groups titrated with pCMB was about ten times as large as that of the radicals in irradiated RNase; 7.78\(\times\)10\(^{-4}\)M and 18.2\(\times\)10\(^{-4}\)M of SH groups were formed when 4.97\(\times\)10\(^{-5}\)M and 12.6\(\times\)10\(^{-5}\)M of radicals were formed respectively. It is known that SH groups are produced by rupture of disulfide bonds of RNase and the amount of SH groups and the inactivation are in linear relationship\(^9\).

**DISCUSSION**

By comparing ESR spectra of RNase and amino acids, it was found that the spectrum of RNase is similar to a superposition of those of Tyr, Phe and cystine, so we concluded that RNase
come to have free radicals in its chromophores by 254 m\(\mu\) light irradiation. Energy transfer, through which radicals were formed in nonabsorbing residues, was not occurred.

Considering the observation in \(\gamma\)-ray irradiation\(^{10}\), the effects of annealing and of oxygen on the ESR spectra of Tyr and Phe might be explained in terms of the formation of delocalized free radicals in the aromatic ring which was once formed by the absorption of UV-light at 77°K, and in terms of subsequent oxidation of the ring and localization of the spins to oxygen atoms after annealing in air. The fact that the irradiated GSSG and GSH have similar ESR spectra (Fig. 1) suggests the formation of the same radicals in both molecules. Studies\(^{11-13}\) on irradiation of single crystal of L-cystine revealed that a stable free radical, \(R\cdot\text{-CH}_2\text{-S}^-\), was formed by the irradiation and that the \(g\)-value of the sulfur radical was highly anisotropic. For example, Akasaka et al.\(^{13}\) showed that three principal components of the \(g\)-value of stable sulfur radical were 2.052, 2.025 and 2.002. The largest value (2.052) is close to the values observed in GSSG (2.059), GSH (2.059) and RNase (2.061). Hence it is quite probable that sulfur radicals were formed in GSSG, GSH and RNase.

The peak at \(g\)-value of 2.061 in RNase, did not change in intensity when the protein was warmed at 195°K, even though Henriksen et al.\(^{14,15}\) observed the change in intensity of a spectrum of sulfur radicals when \(\gamma\)-irradiated protein was warmed. They interpreted that certain radicals, mainly sulfur radicals, were formed through the process of electron migration. Further investigations are required to explain the discrepancy.

The linear relationship between spin concentration and degree of inactivation seems to show that the radical formation by UV-light is one of the important electronic states, which bring about inactivation of RNase. However, it was also found that about two molecules were inactivated along with the formation of one spin (Fig. 2), and that the amount of SH groups formed by UV irradiation was ten times larger than that of radicals. These results suggest that inactivation and also disulfide break of RNase are brought about by more than one mechanisms without passing through radical formation.

REFERENCE


