Characterization of Ribonuclease A Irradiated with $\gamma$-Rays in the Presence of Cytidylic Acid with Respect to the Interaction of the Enzyme with Folic Acid

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INTRODUCTION

Bovine pancreatic ribonuclease A (RNase A) has a deep cleft which interacts with the substrate, that is the main active site B$_1$-R$_1$-p$_1$ and the sub-site B$_2$-R$_2$ etc. Substrate analog such as cytidine 2' (3')-phosphate (CMP) binds firmly at the B$_1$-R$_1$-p$_1$ site$^{10}$. Folic acid interacts with RNase A$^{8}$ in the manner that the benzene ring of the former binds to the sub-site of the latter and the $\alpha$-carboxylate group to the p$_1$ site$^{9}$. RNase A was chromatographed successfully on an affinity column in which folic acid was introduced in an agarose gel matrix$^{10}$.

Irradiation of RNase A with $\gamma$-rays in the presence of CMP brought about formation of an enzyme derivative which was enzymatically active but differed in the chromato-
graphic behavior. The aim of the present study is to elucidate whether the folic acid-binding site on the enzyme is damaged or modified by \( \gamma \)-irradiation in the presence of CMP.

**MATERIALS AND METHODS**

RNase A was obtained from Boehringer/Mannheim GmbH and used without further purification. CMP (Sigma) was an equimolar mixture of 2'- and 3'-isomers as revealed by ion-exchange chromatography. Other chemicals were of chemical grade. Amounts of the irradiated enzyme were calibrated by amino acid analysis of the acid hydrolysates of the products.

Amberlite IRC-50 chromatography of the enzyme was performed according to Hirs et al.\(^5\). Affinity chromatography on a folate-Sepharose column was described\(^6\). Gel filtration through a Sephadex G-25 column was done after Hummel and Dreyer\(^7\). Polyacrylamide gel electrophoresis was run at pH 4\(^9\). Methods of enzyme assay using RNA\(^9\), C-cyclic-p\(^9\) and CpU\(^11\) as substrates were described elsewhere.

A solution for \( \gamma \)-irradiation was prepared by dissolving RNase A alone (3.6\( \times \)10\(^{-5}\)M) or RNase A and CMP in distilled water at the concentrations of 3.6\( \times \)10\(^{-5}\) M and 1.5\( \times \)10\(^{-8}\) M, respectively, and adjusted pH to be 5.6. The solution (4.0 ml) was irradiated at room temperature with a 3,000 Ci \( ^{60}\)Co source at the dose rates of 14-18 krads/min. The irradiated enzyme was recovered from the solution by gel filtration through a Sephadex G-25 column with 0.1 N acetic acid, followed by lyophilization.

**RESULTS**

When enzyme activity was measured using RNA as substrate, RNase A was completely protected from inactivation by \( \gamma \)-irradiation in the presence of CMP at doses up to at least 500 krads, while the survival of the enzyme in the absence of the nucleotide was less than one percent (Fig. 1). This is in accordance qualitatively with the data presented by Ukita and Waku\(^5\).

The chromatographic pattern on an Amberlite IRC-50 column of the irradiated enzyme in the presence of CMP at the dose of 450 krads (\( \gamma \)-C-RNase) was similar to that reported by Ukita and Waku\(^5\) and the amount of intact enzyme survived in the product was estimated to be about 20 percent (data not shown). The electrophoretic behavior of \( \gamma \)-C-RNase on polyacrylamide gel was similar to that on filter described by the same authors\(^5\) (data not shown).

As evident from the Lineweaver-Burk plots (Fig. 2), the Km-values for RNase A and \( \gamma \)-C-RNase were the same for two different substrates. This is in accordance with the result obtained by Ukita and Waku\(^5\). The \( V_{\text{max}} \) values for the irradiated enzyme were 2.0- and 2.3-times smaller than those for the native enzyme when C-cyclic-p and CpU were used as substrate, respectively (Table 1). This is in contrast to the result reported by Ukita and Waku\(^5\) who obtained the same \( V_{\text{max}} \) value for both the native
and irradiated enzyme*.

Affinity of γ-C-RNase for folic acid was measured by the Hummel and Dreyer gel filtration method\(^7\). The binding ratio of the ligand to the enzyme was calculated to be 0.37 mol/mol when 4 × 10\(^{-8}\) M of the former was used. The same value was obtained for the native enzyme** at the same concentration of the ligand**.

The chromatographic pattern of γ-C-RNase on a folate-Sepharose affinity column was similar to that of the native enzyme\(^5\), though small fractions were detected which were enzymatically active and emerged somewhat faster than the main peak (Fig. 3).

DISCUSSION

Behavior of γ-C-RNase on IRC-50 chromatography and polyacrylamide gel electrophoresis suggests that the product(s) obtained by γ-irradiation of RNase A in the presence of CMP was similar to that described by Ukita and Waku\(^5\). Both preparations were enzymatically active when RNA was used as substrate and when the enzyme was assayed at pH 7.3 (present case) or at pH 7.6 (Ukita and Waku).

Kinetic analysis of the enzyme activity revealed that the Km-values for native RNase A and the irradiated γ-C-RNase were the same and the \(V_{\text{max}}\)-values for the latter were about one-half the values for the former in both cases when C-cyclic-p and CpU were used as substrates. The enzyme was assayed in a 0.01 M acetate buffer at pH 5.0 under the condition which the maximum interaction between the enzyme and the substrate was observed. Ukita and Waku\(^5\), on the other hand, assayed the enzyme in a Na\(_2\)CO\(_3\)-CO\(_2\) buffer at pH 7.6 using C-cyclic-p as substrate and found no difference in the values of both Km and \(V_{\text{max}}\) for the native and irradiated enzymes. The apparent discrepancy between these two cases is supposed to result from the different composition and different values of pH and ionic strength of the buffers used.

The fact that the Km-values for RNase A and γ-C-RNase were the same suggests

*) The \(V_{\text{max}}\)-value were estimated from Fig. 11 in Ref. 5, since no value was reported in literature.

**) The binding ratio was estimated to be 0.37 mol/mol from Table I in Ref. 2.
that the main active site B1-R1-p1 of the enzyme remained intact after γ-irradiation.

Practically little difference was observed between the affinities of RNase A and γ-C-RNase for folic acid as revealed by gel filtration and affinity chromatographic studies. These results suggest that the B2 sub-site that is the folic acid-binding site is insensitive to γ-irradiation.

The decrease in the values of $V_{\text{max}}$ by γ-irradiation suggests any modification to make the reaction rate decrease at some site(s) other than the B1-R1-p1 and B2 sites.
Fig. 3. Chromatographic pattern of \( \gamma \)-C-RNase on a folate-Sepharose column. \( \gamma \)-C-RNase (0.024 \( \mu \)mol) was eluted with a NaCl gradient from a folate-Sepharose column (1 x 26 cm). —, Absorbance at 220 nm, —, enzyme activity using RNA as substrate in arbitrary units.

REFERENCES