COVALENT ATTACHMENT OF 4-THIOURIDYLIC ACID TO RIBONUCLEASE A BY NEAR-ULTRAVIOLET RADIATION

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SUMMARY : Bovine pancreatic ribonuclease A (EC.2.7.7.16) was irradiated with near-ultraviolet light (334 and 365 nm) in the presence of equimolar amount of a substrate analog 4-thio[^{14}C]uridine 3'-phosphate. Gel-filtration studies revieled that one to two moles of the nucleotide entered into covalent attachment to the enzyme under either aerobic or anaerobic irradiation. Reduction with dithiothreitol of the irradiated protein released about one-third of the attached materials. A model experiment with oxidized glutathione and radioactive 4-thiouridine suggested the formation of aducts between cystinyl residue and the pyrimidine base. The covalent attachment of nucleotide to ribonuclease was independent of inactivation of the enzyme.

4-Thiouridine 2'(3')-phosphate (4-thiouridylic acid) is characteristic for strong absorption near 330 nm and makes a stable equimolar complex with ribonuclease A (1,2). Irradiation of ribonuclease-thiouridylate complex with near-ultraviolet light gave a series of modified proteins of decreased enzymatic activity and no amino acid at the active site was modified however (3-5). The present paper describes some evidences for covalent attachment of the nucleotide by radiation to the enzyme.

MATERIALS AND METHODS

A single component of ribonuclease A was used (3,4). 4-Thio[^{14}C]uridine (0.18 μCi/μmole) was obtained by direct thiation of [U^{14}C]cytidine (Radiochemical Center) by the method of Ueda et al (6). 4-Thio[^{14}C]uridine 3'-phosphate (0.14 μCi/μmole) was synthesized by direct phosphorylation of [U^{14}C]cytidine to obtaine the 2',3'-cyclic phosphate (7), followed by digestion with ribonuclease A and by direct thiation of the digest with H_2S (6). The product was purified on a DEAE-cellulose column (8).

Abbreviation : GSSG, oxidized glutathione.

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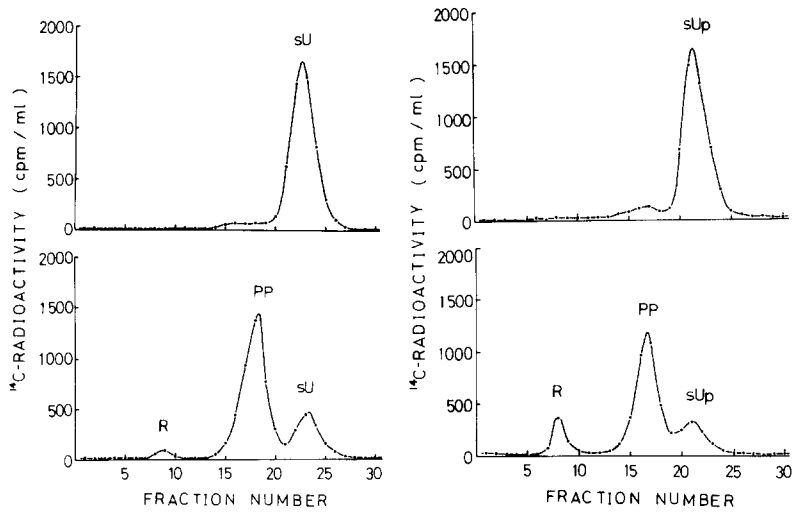

Fig. 1. Sephadex G-25 chromatography of the photoreaction mixture of ribonuclease A and radioactive 4-thiouridine or 4-thiouridylic acid. Column : Sephadex G-25, fine size, 1 x 22 cm. Elution : 1 N acetic acid at 4°C. Collection volume : 3 ml.
Left, with 4-thiouridine; right, with 4-thiouridylic acid. Upper panels, before irradiation; lowere panels, after irradiation. sU, 4-thiouridine; sUp, 4-thiouridylic acid; PP, photoproducts of sU or sUp; R, proteins.

The method of irradiation was as follows. Equimolar mixture of ribonuclease and radioactive 4-thiouridylic acid (0.02 mM each) dissolved in 2 ml of 0.01 M acetate buffer, pH 5.6, was placed in a quarz cell of 10 mm light path, thermostated at 25°C, and was irradiated with light of 334 and 365 nm with a Toshiba SHL 100-UV mercury lamp for 6 hr from 9 cm of the lamp (3,4). The solution was stirred magnetically in the air or agitated by bubbling with nitrogen gas. The irradiated solution was chromatographed on a column of either Sephadex G-25 or G-75 and the protein was recovered from the effluent fractions if necessary.

Reduction of protein with dithiothreitol (4) was carried out under nitrogen barrier. Radioactivity in aqueous solution was measured with a Nuclear Chicago Liquid Scintilation Counter, Model Mark I, at 8 - 9°C with a gel-forming cocktail (9). Enzyme assay was described elsewhere (3).

As a model for the enzyme-nucleotide system, mixture of 0.5 mM GSSG and 5 mM

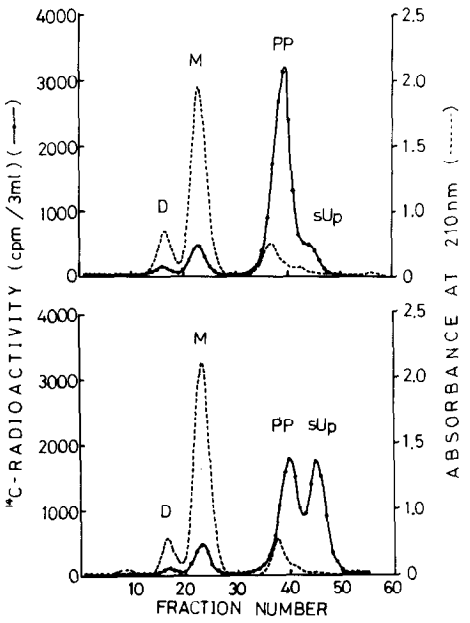

Fig. 2. Sephadex G-75 chromatography of the photoreaction mixtures of ribonuclease A and radioactive 4-thiouridylic acid. Column : Sephadex G-75, 1,5 x 90 cm. Elution : M/15 sodium phosphate buffer, pH 7.4 at 4°C. Collection volume : 3.5 ml.
Upper panel, irradiated in the air; lower panel, irradiated in an atmosphere of nitrogen. M, ribonuclease monomers; D, ribonuclease dimers.

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radioactive 4-thiouridine dissolved in distilled water was irradiated for 2 to 4 hr in the air. The product was chromatographed on an Avicel SF thin-layer plate with a solvent system of n-butanol - pyridine - acetic acid - water (15:10:3:12, by vol) and was detected by ninhydrin and radioautography.

RESULTS

When ribonuclease was irradiated in the air in the presence of 4-thio[^{14}C]uridine or 4-thio[^{14}C]uridylic acid, some radioactivity was detected in the protein fraction (sign R in Fig. 1) by chromatography on a Sephadex G-25 column, while no radioactivity was observed in the fraction without irradiation. The amount of the attached radioactive materials to the protein was only 2 per cent for the thionucleoside (3 % inactivation) and 10 to 14 per cent for the thionucleotide (18 - 20 % inactivation), indicating that specific interaction between the enzyme and the nucleotide is essential for the covalent attachment.

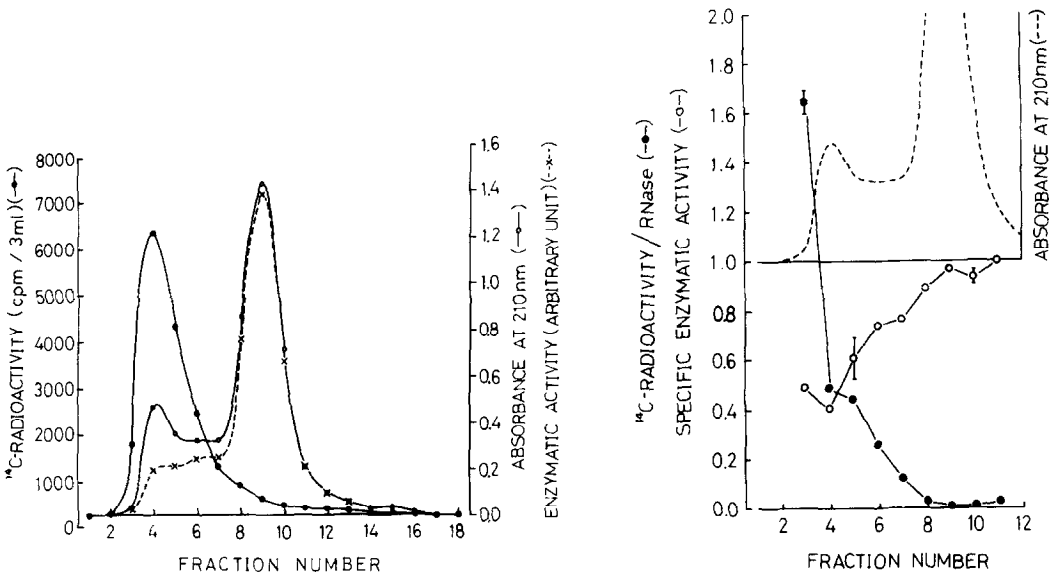

Fig. 3. IRC-50 chromatography of the irradiated ribonuclease. Sample : protein fractions recovered from the reaction mixture of ribonuclease A and radioactive 4-thiouridylic acid irradiated in the air. Column : Amberlite CG-50 (IRC-50), 0.9 x 29 cm. Elution : 0.2 M sodium phosphate buffer, pH 6.2 at 4 C. Collection volume : 3.5 ml.
Left, absorbance at 210 nm (—•—), radioactivity (—○—) and enzymatic activity (-×-); right, amount of bound nucleotide (—•—) and specific enzymatic activity (—○—).

The effect of oxygen was examined by comparing the chromatograms on Sephadex G-75 of the products irradiated in the air and in an atmosphere of nitrogen (Fig. 2). The protein fractions were well separated into monomer and dimer fractions (4) and the distribution patterns of radioactivity revealed that oxygen little affects on the covalent attachment of nucleotide to the enzyme as well as on the formation of the dimer of protein. The monomer fraction foranaerobic irradiation had full enzymatic activity (102 %) in spite of attchment of appreciable amount of nucleotide (11 per cent). This finding leads to the conclusion that the attachment of nucleotide is not responsible for the inactivation of enzyme.

The protein fractions recovered from the photoreaction mixtures were treated with dithiothreitol. Under either aerobic or anaerobic irradiation , about one-third (35 and 34 per cent , respectively) of the radioactivity originally bound to protein was transfered by reduction to low-molecular weight fractions and the rest remained boud (Fig. 4). This is

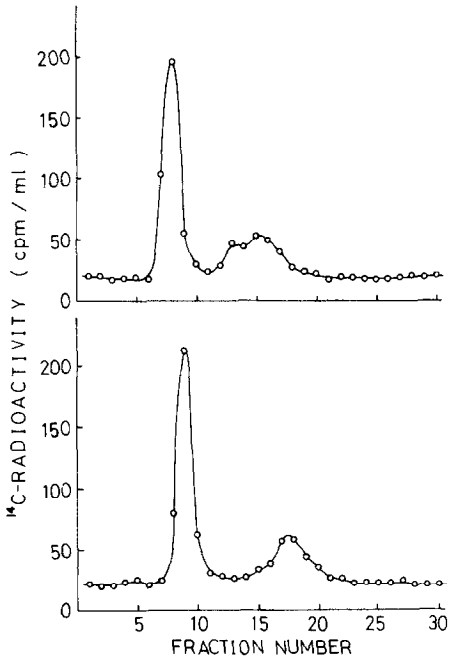

Fig. 4. Sephadex G-25 chromatography of the irradiated ribonuclease after treatment with dithiothreitol. Sample : protein fraction recovered from the photoreaction mixture of ribonuclease A and radioactive 4-thiouridylic acid, followed by treatment with dithiothreitol. Chromatography : see Fig. 1.

Upper panel, irradiated in the air; lowere panel, irradiated in an atmosphere of nitrogen.

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a direct evidence for presence of two types of bonding that has been deduced from the experiments without radioactive tracer (4).

Irradiation of GSSG in the presence of radioactive 4-thiouridine gave two radioactive and ninhydrin-positive spots on a thin-layer plate. Treatment of the photoproducts with dithiothreitol brought about disappearance of one spot and decrease in the amount of radioactivity to about one-half, suggesting that half of the bonding will be disulfide bond.

DISCUSSION

The most probable target for the photo-labeling of ribonuclease A is a cystinyl residue which does not occupy the active site of the enzyme. This is compatible with the finding that the covalent attachment by radiation is independent of inactivation of the enzyme, for modification of amino acids other than at the active site causes no inactivation of

enzyme in most cases. The reducible type of the bonding for attachment will be a mixed disulfide formed by disulfide exchange between -S-S- linkage of a cystinyl residue in the enzyme and 4-thio-group of the pyrimidine base in the nucleotide.

Covalent attachment of 4-thiopyrimidine nucleotides to proteins by near-ultraviolet radiation has been demonstrated in another system too (10). The present system provides an excellent model to elucidate the mechanism of photo-affinity labeling (11), since ribonuclease A is an almost unique example of protein whose tertiary structure is well known among the subjects studied hitherot by this method.

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REFERENCES

1. Sawada,F. and Ishii,F. (1968) J. Biochem. (Tokyo) 64, 161 - 165.
2. Samejima,T., Kita,M., Saneyoshi,M. and Sawada,F. (1969) Biochim. Biophys. Acta 179, 1 - 9.
3. Sawada,F. (1969) J. Biochem. (Tokyo) 65, 767 - 776.
4. Sawada,F. and Kanbayashi,N. (1973) J. Biochem. (Tokyo) 74, 459 - 471.
5. Sawada,F. (1974) Photochem. Photobiol. 20, 523 - 526.
6. Ueda,T., Imazawa,M., Miura,K., Iwata,R. and Odajima,K. (1971) Tetrahedron Letters 27, 2507 - 2510.
7. Ueda,T. and Kawai,I. (1970) Chem. Pharm. Bull. (Tokyo) 18, 1303 -2308.
8. Saneyoshi,M. and Sawada,F. (1969) Chem. Pharm. Bull. (Tokyo) 17, 181 - 190.
9. Anderson,L.E. and McClure,W.O. (1973) Anal. Biochem. 51, 173 - 179.
10. Frischauf,A.F. and Scheit,K.T. (1974) Biochem. Biophys. Res. Commun. 53, 1227 - 1233.
11. Ruoho,A.E., Kiefer,H., Roeder,P.E. and Singer,S.J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2567 - 2571.