FORMATION OF ACETYLGLYCYLPUROMYCIN BY THE INCUBATION OF HEN'S OVIDUCT MINCES WITH PUROMYCIN

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A hypothesis that the N-terminal acetylamino acid residue functions as the initiator of polypeptide synthesis has been presented (Narita, 1962a, 1962b). Before thorough examinations of the hypothesis are made, a number of evidences (Adams and Capecchi, 1966; Webster et al., 1966; Clark and Marcker, 1966) for the initiation of polypeptide synthesis in microorganisms have been reported since Marcker and Sanger (1964) found formylmethionyl-t-RNA in the cell-free system prepared from E. coli. However, several efforts to elucidate the initiation mechanism of protein synthesis in the cytoplasmic system of higher organisms such as mammals and plants have been unsuccessful (Lengyel, 1967). Since the reported acetylated proteins at their N-terminal ends were those from vertebrates and plants, there still exists a possibility that the N-terminal acetylamino acid functions as the initiator of polypeptide synthesis in higher organisms (Narita et al., 1968). This possibility has been further supported by the finding that acetylphenylalanyl-t-RNA functions as the initiator of polyphenylalanine synthesis directed by polyuridylic acid in E. coli system (Haenni and Chapeville, 1966; Lucas-Lenard and Lipmann, 1967; Economon and Nakamoto, 1968).

We have attempted to study the initiation mechanism of ovalbumin synthesis in hen's oviduct minces, because the protein is known to be ended with the N-acetyl-Gly-Ser- sequence (Narita, 1961; Narita and Ishii, 1962; Baba et al., 1965). Our previous report (Narita et al., 1968) showed that C^{14}-acetate was incorporated into the N-terminal peptide of ovalbumin synthesized by hen's oviduct minces and that this incorporation was strongly inhibited by puromycin.

These findings may support our aforementioned hypothesis. However, another possibility cannot be excluded that C^{14}-acetate is incorporated into the N-terminal glycine during the elongation of the polypeptide. The possibility may be ruled out if labeled acetylglycylpuromycin (abbreviated as Ac-Gly-Puro) would be isolated in the incubation mixture in which hen's oviduct minces are incubated with labeled acetate and glycine in the presence of puromycin, since the formation of Ac-Gly-Puro implies that acetylglycyl-t-RNA, like N-formyl-methionyl-t-RNA_F in E. coli ribosomes (Bretscher, 1966), is presented in the peptidyl site of oviduct ribosomes. This communication describes the formation of Ac-Gly-Puro by the incubation of the oviduct minces with puromycin.

Experimentals

Synthesis of Standard Ac-Gly-Puro. As usual, radioactive acetylglycine was synthesized from glycine-2-H^3 (145 mc/mmole) and acetic-1-C^{14} anhydride (10 mc/mmole) in such a way that the radioactivity ratio of C^{14} to H^3 was approximately 1 to 10. Acetylglycine was separated from the unblocked starting amino acid by passage of the reaction mixture through 1 x 10 cm column of Dowex 50-X16 (H^+ form) and the column was thoroughly washed with water. The effluent and the washings were combined and lyophylized. The radioactivity ratio of C^{14} to H^3 in the synthesized acetylglycine was 1 : 12.5 (C^{14}, 5.0 μc/μmole; H^3, 62.4 μc/μmole).

One equivalent of acetylglycine (1.10 mg) was dissolved in 0.5 ml of tetrahydrofuran (not completely soluble) and 1 equivalent of dicyclohexylcarbodiimide was added. The mixture was shaken for 30 minutes and the precipitate was removed by centrifugation. To a suspension of puromycin·2 HCl (1.2 equivalents) in 0.5 ml of tetrahydrofuran was added over 3 equivalents of triethylamine, and triethylamine·HCl was precipitated soon after the mixture became clear. To the clear solution was added the activated acetylglycine solution described above, and the mixture was evaporated after it was allowed to stand for 2 hours at room temperature. Radioactive Ac-Gly-Puro in the resi-

due was separated from unreacted acetylglycine and by-products (mainly acetylglycyldicyclohexyl urea) and purified successively by paper chromatography with n-butanol-acetic acid-water (4 : 1 : 1, v/v; Rf 0.78) and by paper electrophoresis at pH 3.5 (pyridine-acetic acid-water, 1 : 10 : 289, v/v and 50 V/cm for 1.5 hours). It was shown that Rm, the ratio of travelled distance from glycine to Ac-Gly-Puro to that from glycine to lysine was 0.19. The ultraviolet absorption spectrum of Ac-Gly-Puro in 50 % ethanol was identical with that of puromycin.

Incubation Mixture. The incubation mixture consisted of 1 g of hen's oviduct minces in 10 ml of Krebs-Ringer-Tris buffer of pH 7.4 (Umbreit et al., 1949) containing 1 mM C^{12}-amino acid mixture (minus glycine), 10 mM glucose, 1 mM dithiothreitol, 2 mM Ac-Gly-Ser-OH and 4 mg puromycin. After preincubation of the mixture for 10 min., 50 μc of C^{14}-acetate (31 mc/mmole) and 500 μc of H^3-glycine (1940 mc/mmole) were added. The reaction mixture was further incubated at 37°C for 30 min., and then it was subjected to freezing-thawing for three times to disrupt oviduct cells. The mixture was homogenized with 5 ml of Krebs-Ringer-Tris buffer and centrifuged at 12,000 r.p.m. for 20 min. Trichloroacetic acid crystals was added to the supernatant in a final concentration of 5 %. The resulting precipitate was removed by centrifugation at 3,000 r.p.m. for 15 min. and the precipitate was washed with 5 % trichloroacetic acid. The supernatant after the trichloroacetic acid treatment and the washings were combined. The clear solution thus obtained was shaken three times with an equal volume of ether to remove lipids which interfere fractionation of Ac-Gly-Puro. The pH of the aqueous phase was adjusted to 7 with N/10 NH_4OH and Ac-Gly-Puro formed was extracted three times with an equal volume of sec.-butanol. The organic phase was evaporated and the identification of Ac-Gly-Puro was made by successive use of paper chromatography (n-butanol-acetic acid-water) and paper electrophoresis at pH 3.5. The distribution of radioactivity on paper was determined with a radiochromatogram scanner and C^{14} and H^3 radioactivities of the extract of the pieces of the paper with water or 50 % ethanol were de-

termined with a Beckman LS 150 liquid scintillation counter after each of extracts was dried and dissolved in 0.2 ml of hyamine.

Results and Discussion

Partition coefficients of chemically synthesized Ac-Gly-Puro between water and ethylacetate, n-butanol, sec.-butanol and ethylene chloride were measured to find out the best solvent for extraction of Ac-Gly-Puro from the reaction mixture. The most suitable solvent was sec.-butanol for this purpose; partition coefficient was 0.83. The evaporated sec.-butanol extract of the incubation mixture with puromycin was subjected to paper chromatography (n-butanol-acetic acid-water). A part of the paper chromatogram corresponding to the position of synthetic Ac-Gly-Puro was cut and the material was extracted with 50 % ethanol, though no prominent radioactive peak could be found in this region. The extract was electrophoresed at pH 3.5 as mentioned above and a radioactive peak was observed, which shifted slightly from that of the synthetic Ac-Gly-Puro (see upper and middle pannels of Fig. 1). The material extracted from the radioactive peak, however, behaved identically with the authentic sample on a paper chromatogram prepared with tert.-amyl alcohol-phthalate buffer at pH 6 (Rf 0.77). Furthermore, re-electrophoresis of the radioactive material was carried out with the synthetic Ac-Gly-Puro on the same paper sheet and radioautogram was prepared. As shown in Fig. 1 (lower pannel), the extracted radioactive material from the incubation mixture behaved identically with the synthetic Ac-Gly-Puro. It must be added that such radioactive material was not formed when puromycin was removed from the incubation mixture. Hydrazinolysate of the radioactive material purified by the re-electrophoresis as described above revealed the presence of C^{14}-acetylhydrazide (Rf 0.58) and H^{3}-glycine hydrazide (Rf 0.30) on the paper chromatogram (pyridine-aniline-water, 9 : 1 : 4, v/v) (Narita, 1961). The same chromatographic pattern was obtained with the hydrazinolysate of the synthetic Ac-Gly-Puro, suggesting probable identity of the radioactive material with the synthetic Ac-Gly-Puro.

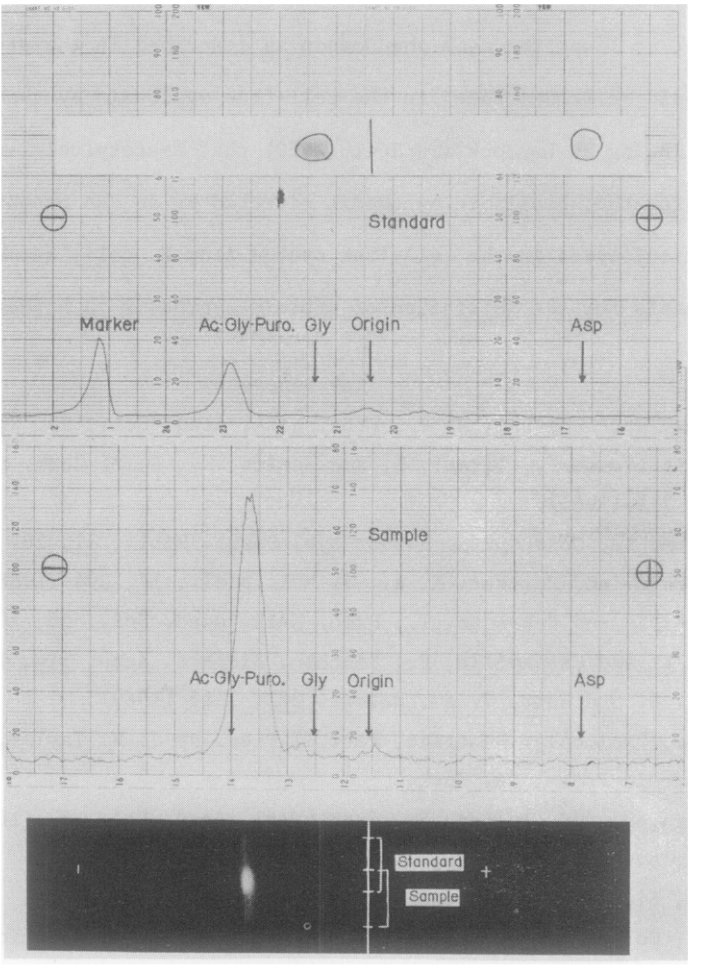

Fig. 1. Electrophoretic pattern of Ac-Gly-Puro at pH 3.5. In upper and middle pannels are shown charts by a radiochromatogram scanner of the chemically synthesized Ac-Gly-Puro and the formed one by the incubation of hen's oviduct minces with C^{14}-acetate, H^3-glycine and puromycin, respectively. Lower pannel shows radioautogram of co-electrophoretogram of the synthetic (standard) and the formed Ac-Gly-Puro.

When C^{14}-acetate and H^3-puromycin were used, the same results as those described above were obtained.

These findings support our hypothesis of the initiation of ovalbumin synthesis by considering the fact that formylmethionylpuromycin was released in the E. coli cell-free system incubated with formylmethionyl-t-RNA_F and

puromycin (Bretscher, 1966). In order to obtain conclusive evidence for our hypothesis, at least the same phenomenon as described in the present communication should be demonstrated in the cell-free ovalbumin synthesizing system. A recent finding by Laycock and Hunt (1969) that N-acetylvalyl-t-RNA seemed essential for the hemoglobin synthesis directed by an RNA preparation from rabbit reticulocytes in the cell-free system from E. coli, seems to support our hypothesis for the initiation of protein synthesis in higher organisms.

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