A METHOD FOR DEMONSTRATING THE STEPWISE ADDITION OF GLYCINE FROM TRANSFER RNA INTO THE MUREIN PRECURSOR OF STAPHYLOCOCCUS AUREUS[1]

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Received April 22, 1969

Summary: The mechanism of the incorporation of pentaglycine from transfer RNA into a lipid intermediate in Staphylococcus aureus murein biosynthesis was investigated. The average peptide length of the incorporated glycine increased with time, suggesting a stepwise mechanism for glycine incorporation into the lipid intermediate.

The addition of five glycine residues onto the ε-amino group of the lysine of the lipid intermediate[2] is an unresolved part of the biosynthesis of Staphylococcus aureus murein (Matsuhashi et al., 1967). The incorporation of glycine is sensitive to ribonuclease (Chatterjee and Park, 1964), and glycyl-t-RNA[3] serves as the donor of glycine in this reaction sequence (Matsuhashi et al, 1965 and 1967; Park and Chatterjee, 1966). Measurements of incorporated glycine and end-group analysis of incorporated lysine (from UDP muramyl pentapeptide) by Matsuhashi et al. (1967) indicate that, on average, 4-6 glycines are transferred from glycyl-t-RNA to each acceptor lysine in the lipid intermediate. The N-terminal end of the polyglycine chain remains free at this stage.

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[1]Supported by research grant AI 05090 and traning grant no. GM 01136 from the National Institutes of Health.

[2]Lipid-pyrophosphate (N-acetyl glucosaminyl-N-acetyl muramyl-L-alanyl-γ-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine).

[3] t-RNA = transfer ribonucleic acid.

The question explored here is whether the glycines are transferred to the lysine one at a time, or as a chain of 5 residues. We were unable to detect glycine polymers bound to the t-RNA under a variety of conditions. On the other hand, an increase with time in the average length of the glycine peptide on the lipid intermediate was found. This indicates that the glycines were added in a stepwise fashion.

Methods

The incorporation of glycine was studied in a cell-free system using a membrane fraction[4], from S. aureus, as the source of phospholipid and incorporating enzymes.

The method consisted of the following steps:

(1) Preparation of the membranes containing preformed lipid intermediate acceptor: 755 μg. membrane protein[5], 200 mμmoles ATP, 16 mμmoles UDP N-acetyl muramyl pentapeptide, and 188 mμmoles UDP N-acetyl glucosamine were incubated in a final vol. of 100 μl containing 0.05 M tris-HCl, pH 8.0, 0.01 M $MgCl_2$, 0.003 M mercaptoethanol, and 0.1 M NH_4Cl for 21 minutes at 37°C, and centrifuged at 39,000 x g for 10 minutes in order to remove the soluble components.

(2) Preparation of C^{14}-glycyl-t-RNA: 3.46 mμmoles C^{14}-glycine, 100 mμmoles ATP, 10.5 μg activating enzyme[6], and 44.5 μg t-RNA[7] were incubated at 37°C

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[4]Membranes were prepared from S. aureus strain H by the method of Park and Chatterjee (1966).

[5]Protein was determined by the method of Lowry et al. (1951), using a bovine serum albumin solution as the standard.

[6]"activating enzyme" = a partially purified soluble activating enzyme, prepared as described by Park and Chatterjee (1966).

[7]Crude t-RNA prepared by a modification of the method of Zubay (1966), omitting the isopropanol fractionation step.

for 5 minutes in 100 ml containing 0.05 M tris-HCl, pH 8.0, 0.01 M $MgCl_2$, 0.003 M mercaptoethanol, and 0.1 M NH_4Cl, in order to saturate the t-RNA with C^{14}-glycine. Under these conditions, the t-RNA was saturated with 6.7 $\mu\mu$moles glycine and the activating enzyme maintained the glycyl-t-RNA in a saturated condition in the subsequent incubation at 15°C.

(3) Transfer of C^{14}-glycine from t-RNA to lipid acceptor: This mixture was incubated at 15°C with the membranes from step 1 in a final volume of 200 μl containing the same final concentrations of tris-HCl, $MgCl_2$, mercaptoethanol, and NH_4Cl, plus 100 μg/ml chloramphenicol. The reaction was stopped with cold 0.3N perchloric acid, at various times, and the membranes were sedimented at 27,000 x g for 10 minutes and washed with water at 39,000 x g for 10 minutes repeatedly.

(4) Extraction of the lipid intermediate and hydrolysis to obtain the dissaccharide-pentapeptide-polyglycine: The lipid intermediate was quantitatively extracted by the method of Strominger et al. (1966). The butanol layer was washed twice with cold water to remove non-lipid radioactivity. The butanol extract was dried by aeration, resuspended in 0.1 ml absolute ethanol, and boiled for 10 minutes after addition of 0.9 ml of 0.011 N HCl. This treatment solublized at least 98% of the radioactivity and ruptured less than 2% of the glycyl-glycine bonds. The hydrolysate was centrifuged at 39,000 g for 10 minutes and the pellet discarded.

(5) Measurement of the average peptide length of the C^{14}-glycine in the dissaccharide-pentapeptide-polyglycine: The butanol extract was incubated with 1580 μmoles DNFB, 1150 μmoles triethylamine, 2.5 μmoles cold pentaglycine internal standard and 5 ml absolute ethanol for 1 hour at room temperature[8] to dinitrophenylate the N-terminal glycine. The mixture was dried, re-

[8]This procedure is based on a modification of the method of MacFarlane (1962).

suspended in 3 ml of 5.7 N HCl, and exhaustively extracted with two volumes ethyl ether.

The resultant acid layer was hydrolyzed at 104°C for 4 hours in the dark in a sealed tube. The absorption of each sample, nearly all of which was due to the standard DNP pentaglycine, was read at 420 mμ before and after hydrolysis. A 10-15% loss of the absorption at 420 mμ occurred on hydrolysis, although 100% of the radioactivity was recovered. It was assumed that this represented destruction of DNP glycine bonds during hydrolysis. The N-terminal glycine (i.e. DNP glycine) was corrected for destruction by dividing the observed amount by the absorption yield of the individual sample. The hydrolysate was extracted three times with two volumes ethyl ether. This step quantitatively extracted DNP glycine, leaving the free glycine in the water layer. The ether layer was taken to dryness and counted by liquid scintillation in 20 ml of a solution composed of 2000 ml toluene, 1000 ml ethylene glycol monoethyl ether, 12 g 2,5 - diphenyloxazole and 0.3 g p-bis-2 (5-phenyloxazolyl) -1- benzene (Hall and Cocking, 1965). The water layer was dried and aliquots taken for determination of radioactivity and of free amino groups, using the ninhydrin method of Cocking and Yemm (1955). Corrections were made for slight quenching caused by the yellow dinitrophenylated material. The material was exposed to the light as little as possible in order to minimize photodestruction (3% occuring in 70 minutes at room temperature in 5.7 N HCl).

The total glycine divided by the corrected DNP glycine gave the average peptide length (APL) for the glycine incorporated into the lipid intermediate. By this procedure, it was shown that the DNP pentaglycine standard was converted to one DNP glycine and four free glycines. The absorption at 420 mμ and the ninhydrin values were used for determining the amounts of DNP glycine and of free glycine, respectively. (97% of the recovered absorption was ether-

soluble, 95% of which was due to pure DNP glycine).

RESULTS AND DISCUSSION

In Figure 1, the rate of C^{14}-glycine incorporation into the lipid intermediate and the corrected average peptide length (APL) of the C^{14}-glycine are presented. These APL values were about 12% lower than the values which were uncorrected for destruction of the DNP glycine bond. From repeated analyses of pentaglycine internal standards, it appears as if the corrected APL values were more reliable than the uncorrected ones. The results indicate that there is a gradual increase in APL from 1.5 to 3.0 glycines. Other experiments using different membrane preparations and relatively long incubation periods have shown increases in APL with time to values as high as 5.0.

Matsuhashi et al reported that 4-6 glycines were attached to one lysine

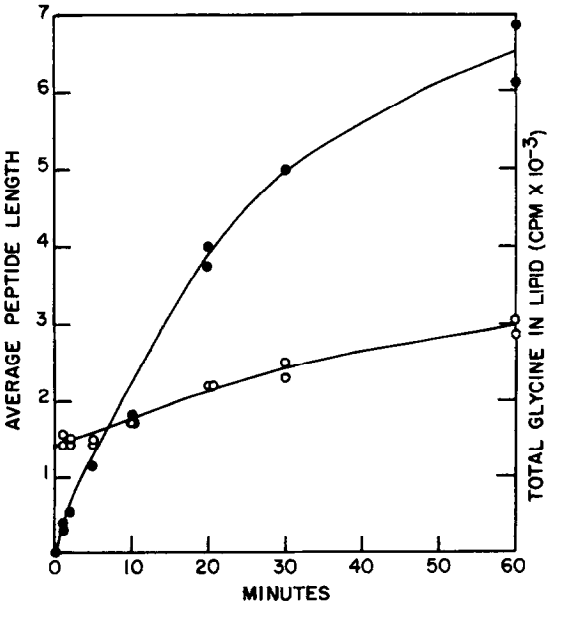

Fig. 1 Increase in the average peptide length of the C^{14}-polyglycine in the lipid intermediate (o——o) and the total glycine incorporated (●——●) with time at 15°C. CPM = counts per minute.

on the lipid (1967). This observation depended on accurate measurement of the ϵ-amino groups of radioactive lysine that remained free. However, it was difficult to adapt this method to the brief incubation periods where short polyglycine chains might be detected, since over 90% of the lysines had not yet reacted with glycine. The N-terminal analysis of glycine, rather than lysine, proved to be a more precise tool for studying incorporation at early times.

Our results suggest that glycine is added to the lipid intermediate from t-RNA in a stepwise fashion. With this method one can investigate whether addition of the first glycine to the epsilon amino group of lysine involves a different mechanism than the subsequent addition of glycine to N-terminal glycine.

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