SEQUENTIAL ADDITION OF GLYCINE FROM GLYCYL-tRNA TO THE LIPID-LINKED PRECURSORS OF CELL WALL PEPTIDOGLYCAN IN STAPHYLOCOCCUS AUREUS

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SUMMARY

Evidence was provided that in the enzymatic synthesis of the cell wall peptidoglycan in Staphylococcus aureus pentaglycine is introduced by sequential addition of glycine from glycyl-tRNA to the ε-amino terminal of lysine residues in the lipid intermediates, the oligoglycine chain being elongated at the N-terminal.

The cell wall peptidoglycan of Staphylococcus aureus is constructed from N-acetylglucosaminyl-N-acetylmuramyl (-tetrapeptide) glycan cross-linked by pentaglycine bridges between the ε-amino terminal of L-lysine and the carboxy terminal of D-alanine in neighbouring tetrapeptide strands (-L-ala·D-glu·L-lys·D-ala) (Strominger and Ghuysen, 1967). This pentaglycine bridge is a peculiar feature of the peptidoglycan of S. aureus. Previous biosynthetic works provided evidence for the mechanism of introduction of the pentaglycine showing that the glycine donor is glycyl-tRNA, and the acceptor is

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lipid (C_{55}-isoprenol)-PP-N-acetylmuramyl- or disaccharide-pentapeptide (-L-ala·D-glu·L-lys·D-ala·D-ala), pentaglycine being introduced onto the lysine ε-amino group of the latter (Matsuhashi, Dietrich, and Strominger, 1965, 1967). However, it is unknown if mRNA and ribosomes participate in the mechanism and if a definite tRNA is required for synthesis of cell wall peptidoglycan.

We investigated the mechanism of formation of the pentaglycine side chain by Edman degradation of labeled products, prepared enzymatically from lipid-PP-disaccharide-pentapeptide and a system generating radioactive glycyl-tRNA. The results described here clearly indicate that glycine residues are added sequentially on the ε-amino group of L-lysine on the lipid intermediates to form the pentaglycine side chain. Thus this mechanism differs from mRNA coded peptide synthesis on ribosomes.

Lipid-PP-disaccharide-pentapeptide was obtained enzymatically from uridine diphosphate(UDP)-N-acetylmuramyl-pentapeptide and UDP-N-acetylglucosamine by a particulate enzyme preparation from Micrococcus lysodeikticus (Katz, Matsuhashi, Dietrich, and Strominger, 1967). Addition of glycine was achieved with a particulate enzyme (crude ribosome fraction) of S. aureus strain H (Matsuhashi and Strominger, 1967) with homologous tRNA, crude activating enzyme and ATP. Products were routinely isolated and analyzed by paper chromatography and thin layer chromatography on Silica gel G with the solvent isobutyric acid-M-ammonia (1 : 0.6 v/v). For other analytical methods used, see Kato et al., 1968.

Three series of experiments were made to demonstrate the mechanism described above.

1) The direction of growth of the pentaglycine chain was investigated by double labeling experiments as follows. Two sets of reaction system containing ^{14}C-glycine and ^{3}H-glycine respectively, were incu-

bated at 20° for 60 minutes. Just sufficient glycine was added to be used up in this period (main labels). Then, a slight excess of ^{3}H-glycine or ^{14}C-glycine was added reversing the label, and the mixtures were incubated further for 10 minutes (pulse labels). Two kinds of lipid containing double-labeled glycine were thus obtained. One was labeled early and mainly with ^{14}C-glycine and then pulse labeled with ^{3}H-glycine and the other was labeled initially with ^{3}H-glycine and then pulse labeled with ^{14}C-glycine. These lipids were extracted with n-butanol at pH 4 and hydrolyzed with 0.01 N HCl to obtain disaccharide-pentapeptide-oligoglycine which was purified by paper chromatography. By dinitrophenolyzation of the terminal amino group the average chain length was approximately estimated as 3.7 for both products. The purified samples of disaccharide-pentapeptide-oligoglycine were then subjected to four cycles of Edman degradation and the radioactivities of the phenylthiohydantoin(PTH)-glycine released in each cycle were measured. Fig. 1 shows the change of the ^{3}H/^{14}C ratio of PTH-glycine in the successive degradation cycles. The PTH-glycine released in the first cycle corresponds to the N-terminal glycine residue and that released at the second cycle corresponds to the glycine residue next to the N-terminal, and so on. The closed circles are for the product first labeled with ^{14}C-glycine and then pulse labeled with ^{3}H-glycine. The open circles are for the product first labeled with ^{3}H-glycine and then pulse-labeled with ^{14}C-glycine. The results in Fig. 1 clearly show that the late pulse labels were mainly recovered in the first degradation cycle, that is, from the N-terminal residue with less and less recovery in subsequent cycles. Conversely, recovery of early labels increased in successive cycles, that is, the labels were in internal residues of the oligoglycine chain.

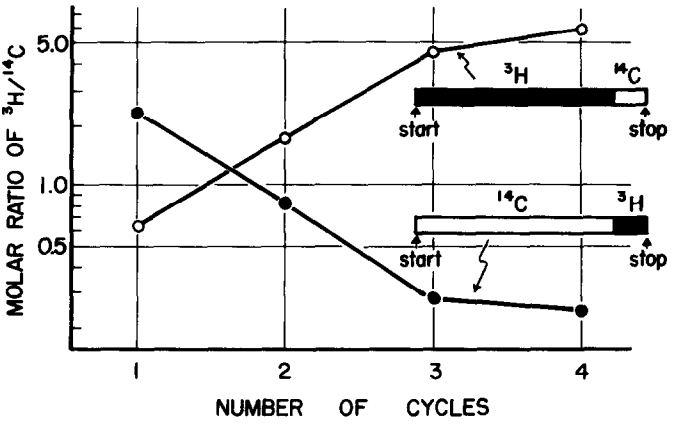

Fig. 1. $^3H/^{14}C$ ratio of PTH-glycine released in each cycle of Edman degradation. Two sets of reaction mixtures containing in a final volume of 0.2 ml: Tris HCl, pH 8.0, 38 μmoles; $MgCl_2$, 1.9 μmoles; KCl, 19 μmoles; sucrose, 31 mg; ATP, 0.7 μmole; L-alanine, 0.1 μmole; 2-mercaptoethanol, 0.9 μmole; Na-deoxycholate, 90 μg; crude tRNA from S. aureus, 200 μg; lipid-PP-disaccharide-pentapeptide (containing some lipid-PP-N-acetylmuramyl-pentapeptide), ca. 3.0 mμmoles; and crude ribosome fraction of S. aureus strain H (100,000 x g precipitate in the presence of 10 mM $MgCl_2$), 470 μg as protein were incubated at 20° for 60 minutes with 5.4 mμmoles of (tube 1) ^{14}C-glycine (25.1 mC per mmole) or (tube 2) ^3H-glycine (203 mC per mmole). Then 18 mμmoles of ^3H-glycine (203 mC per mmole) or ^{14}C-glycine (63.3 mC per mmole) were added, reversing the labels, and the incubation was continued for a further 10 minutes. Double-labeled samples of lipid-PP-disaccharide-pentapeptide-oligoglycine were extracted from the final reaction mixtures with n-butanol in the presence of pyridine-acetic acid (pH 4). After removal of solvents in vaccuo the samples were subjected to hydrolysis with 0.01 N HCl at 100° for 20 minutes. Aliquots of the hydrolysates were subjected to N-terminal analysis by dinitrophenolyzation. Other aliquots containing either 1.05 mμmoles (tube 1) or 0.94 mμmole (tube 2) glycine were subjected to four cycles of Edman degradation. Released PTH-glycine extracted with ethyl acetate, giving a single radioactive spot on thin layer chromatography, was counted in toluene-PPO-POPOP in a liquid scintillation spectrometer. In successive cycles 348, 297, 114, and 63 μμmoles glycine (by radioactivity ^{14}C plus ^3H) were recovered from tube 1 and 351, 318, 180, and 69 μμmoles from tube 2. The ordinate represents the molar $^3H/^{14}C$ ratio of PTH-glycine released in each cycle from tube 1 (●——●) and from tube 2 (o——o).

From these results it can be concluded that the oligoglycine chain is elongated at the N-terminal. However, it is uncertain if the oligoglycine chain is directly formed on the lipid-PP-disaccharide-pentapeptide or if it is first formed on some other pentaglycine

carrier, for example, an appropriate tRNA, and then transfered to the lipid compound.

2) The next experiment showed that the formation of the glycine chain of the lipid occurs sequentially. Lipid-PP-disaccharide-pentapeptide was incubated with S. aureus enzymes, ^{14}C-glycine, tRNA and ATP. The incorporation of ^{14}C-glycine reached a plateau after 60 minutes incubation. Two sampled of labeled lipid compounds were isolated after 10 minutes (early product) and 180 minutes (late product) incubation. They were treated as in the preceeding experiment to obtain disaccharide-pentapeptide-oligoglycine. Analysis of N-terminal glycine by dinitrophenolyzation showed that the average chain lengths were 3.0 and 5.2 respectively. Samples were then subjected to six cycles of Edman degradation. The actual amount of the molecule with the chain length n was calculated as the difference between the PTH-glycine released at the (n)th cycle of the degradation from that at the (n+1)th cycle. Fig. 2 shows calculated values for the distribution of oligoglycine chain lengths in the early (left) and late (right) products. The early product consisted mainly of lipid-PP-disaccharide-pentapeptide containing triglycine and some diglycine, tetraglycine and monoglycine. On the other hand, in the late product it contained mainly pentaglycine and tetraglycine. There were also small amounts of triglycine and hexaglycine. From these results it is quite clear, that the pentaglycine chain on the lipid compounds is formed through a shorter chain. The chain length increases by addition to the amino terminal, as shown in the first experiment, so the pentaglycine chain must be formed by sequential addition of single glycine molecules to the amino terminal of the oligoglycine chain on the lipid-PP-disaccharide-pentapeptide.

It is uncertain whether the hexaglycine shown in Fig. 2 was formed by a broad substrate specificity of the glycine transfer

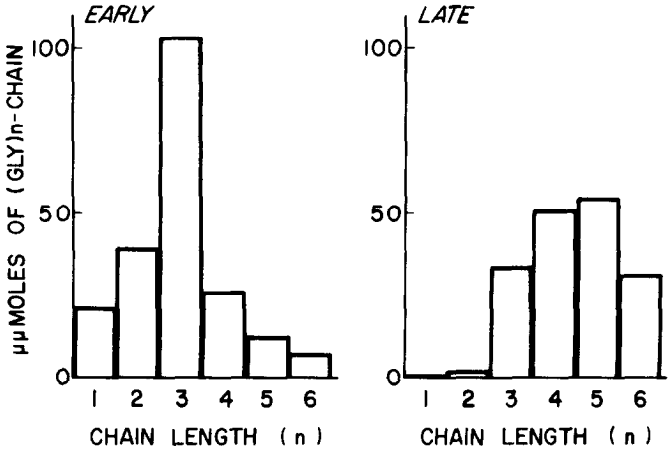

Fig. 2. Chain length distribution in the early (left) and late (right) products. Ordinates represent the amount of oligoglycine chain with the chain length n (abscissa) calculated as described in the text. Experimental conditions were as for Fig. 1. except that only ^{14}C-glycine was used for labeling.

TABLE I

Disappearance of ε-amino terminal of lysine on addition of glycine.

Incubation time min	ε-DNP-lysine cpm	Free lysine cpm
0	2020	30
10	300	3670

Lipid-PP-disaccharide pentapeptide labeled with L-lysine-^{14}C (about 30,000 cpm per mμmole) was used as subtrate. Other experimental conditions were as for Fig. 1.

enzyme or whether it was simply the result of incomplete Edman degradation. However, its appearance does not affect the main conclusion drawn from this experiment.

3) The last experiments showed that the ε-amino terminal of L-lysine

was the site to which glycine is sequentially added. Incorporation of glycine into the lipid-PP-disaccharide-pentapeptide resulted in rapid disappearance of the ε-amino group of L-lysine in the pentapeptide. As shown in Table 1, almost all the ε-amino group of lysine disappeared after 10 minutes incubation, at which time the mean chain length of oligoglycine was 3. Repeated Edman degradation of the products resulted in the appearance of the ε-amino terminal of lysine and this was determined as phenylthiocarbamyl(PTC)-lysine after acid hydrolysis. The number of degradation cycles required for exposure of lysine, expressed as a percentage of the lysine exposed at the (n)th cycle, was in good agreement with the distribution of chain lengths of oligoglycine shown in Fig. 3. Thus oligoglycine chain is definitely formed sequentially on the ε-amino terminal of L-lysine in these lipids.

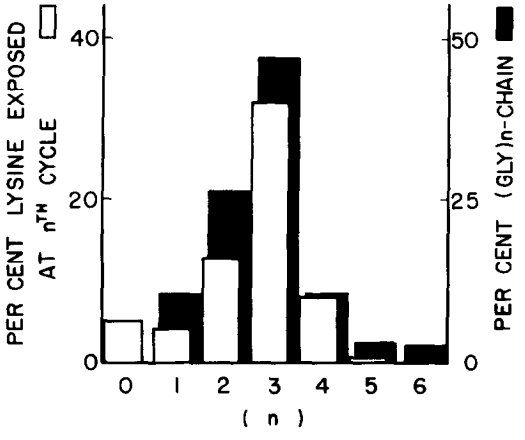

Fig. 3. Coincidence of the amount of lysine (labeled with ^{14}C) exposed at the (n)th cycle of Edman degradation with the distribution of the chain lengths of oligoglycine (labeled with ^{3}H). The products obtained after 10 min incubation shown in Table I were used.

REFERENCES

Kato, K., Hirata, T., Murayama, Y., Suginaka, H., and Kotani, S., Biken J. 11, 1 (1968).

Katz, W., Matsuhashi, M., Dietrich, C. P., and Strominger, J. L., J. Biol. Chem. 242, 3207 (1967).
Matsuhashi, M., Dietrich, C. P., and Strominger, J. L., Proc. Natl. Acad. Sci. U. S. 54, 587 (1965).
Matsuhashi, M., Dietrich, C. P., and Strominger, J. L., J. Biol. Chem. 242, 3191 (1967).
Matsuhashi, M., and Strominger, J. L., Abstracts from the VII-th Internat. Congress of Biochem. in Tokyo, 1967.
Strominger, J. L., and Ghuysen, J.-M., Science, 156, 213 (1967).