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Exopenicillinase Synthesis in *Staphylococcus aureus*

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In *Staphylococcus aureus*, penicillinase remaining cell-bound (60 to 75% of original total) after treatment with citrate does not become exopenicillinase. Exopenicillinase in these cells appears only under conditions permitting de novo penicillinase synthesis. By use of ^{14}C-labeled cells, it was shown that exopenicillinase consists of newly synthesized molecules which have not equilibrated with preformed membrane-bound enzyme.

Little information is available on the synthesis and secretion of exopenicillinase in bacteria. In *Bacillus licheniformis*, about half of the penicillinase of cultures in exponential growth is cell-bound and about half is free in the medium. The cell-bound enzyme, which is firmly attached to the cell membrane (5), has been shown from kinetic data to be an intermediate in the natural formation of the exoenzyme. Collins (4) has also shown that during induction the most newly synthesized enzyme is cell-bound. The penicillinase on the cell membrane may be released as free enzyme by treatment with trypsin and some other proteolytic enzymes, and it has been postulated that in this species a similar enzymatic process is involved in the natural formation of exopenicillinase (5, 6).

S. aureus strain 8325 $\alpha i^- p^+$ excretes up to 40% of its total penicillinase as exoenzyme. Under certain conditions of growth (2), the cells retain this potential exopenicillinase; however, it can be liberated from its cell-bound state by incubating the cells in 0.15 M sodium citrate at 37 C for short periods (3). This latter process has been shown to involve neither de novo protein synthesis nor a requirement for energy, and thus represents a liberation of "cell-bound exopenicillinase." A study of the conditions required for the replenishment of this fraction of penicillinase and the nature of this newly replenished enzyme was undertaken to gain information on the synthesis and secretion of exopenicillinase in *S. aureus* without the drawbacks of enzyme dilution and contamination by medium constituents.

The results reported here relating to the synthesis of cell-bound exopenicillinase in *S. aureus* were presented in part to the 12th Annual Meeting of the Australian Biochemical Society, 1968.

MATERIALS AND METHODS

S. aureus strain 8325 $\alpha i^+ p^+$, inducible for penicillinase, and the magnoconstitutive mutant 8325 $\alpha i^- p^+$ derived therefrom were kindly provided by R. P. Novick. The cells were grown in 60 ml of Tris-CY medium (2) in 250-ml Erlenmeyer flasks for 16 hr at 37 C with shaking. To obtain cells with a high penicillinase content, the cells were collected by centrifugation, resuspended in 60 ml of fresh Tris-CY medium, and incubated at 37 C with shaking for 2 hr. Replenishment of external sites with penicillinase was carried out by washing citrate-treated cells once with distilled water and incubating them in Tris-CY medium with shaking at 37 C for the times indicated. Penicillinase was induced in the inducible strain by the addition of 0.25 μg of cloxacillin per ml to the Tris-CY medium.

Cell-bound exopenicillinase was liberated from cells of *S. aureus* by incubating them at 37 C in 0.15 M sodium citrate for 30 min as described by Coles and Gross (3). After incubation, the cells were removed by centrifugation. Where purification of the liberated penicillinase was necessary, the supernatant fluid was treated with Amberlite CG-50 resin (Na^+ form, 300 mg/10^4 units of penicillinase) for 15 min at 4 C. The resin containing the penicillinase was poured into a column and washed with 0.1 M sodium citrate (*p*H 7.6) until the absorbancy of the eluate was zero at both 260 and 280 nm. The penicillinase was then eluted with 0.3 M sodium citrate. Fractions (1.0 ml) were collected and immediately assayed for penicillinase. Under these conditions, the bulk of the penicillinase recovered was eluted in the first three to five fractions. Penicillinase purified in this manner uniformly had a specific activity of 19 to 22 units/μg of protein.

Reconstituted ^{14}C-protein hydrolysate containing 13 radioactive L-amino acids was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y., and puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Cloxacillin was a commercial product of Commonwealth Serum Laboratories, Victoria, Australia. Other materials and methods used were as previously described (2, 3).

RESULTS

After liberation of penicillinase from sites external to the permeability barrier of the cell membrane by treatment with 0.15 M sodium

citrate for 30 min at 37 C, *S. aureus*, constitutive for the production of penicillinase, retains 60 to 75% of its original penicillinase bound to the cell. Conditions were sought whereby these external sites might again be occupied by penicillinase. It was found that replenishment of depleted sites with penicillinase occurred only under conditions which permitted growth. On reincubation in fresh Tris-CY medium, cells depleted of exopenicillinase synthesized an amount of penicillinase approximately equivalent to that released, and external sites were again occupied by penicillinase. This process was essentially complete after 2 hr of incubation, there being very little further increase in penicillinase content after 2.5 hr. It appeared, therefore, that before replenishment of external penicillinase could take place, an equivalent amount of enzyme had to be synthesized by the cells.

That replenishment is dependent on de novo protein synthesis is shown by the sensitivity of the process to inhibition by chloramphenicol and puromycin. Cells, containing 880 units of cell-bound penicillinase/ml after treatment with citrate, contained only 12 units of citrate-releasable penicillinase/ml after incubation for 2 hr in the presence of either 60 μg of chloramphenicol/ml or 20 μg of puromycin/ml; in the absence of these inhibitors, 280 units of penicillinase/ml could be released by treatment with citrate. Very little transfer of cell-bound penicillinase to external sites took place when either of these two inhibitors of protein synthesis was present in the incubation medium. These inhibitors can, of course, be expected to inhibit the synthesis of all proteins, including penicillinase, in the constitutive mutant. To determine whether protein synthesis in general or penicillinase synthesis in particular is required for replenishment of external sites, the inducible strain 8325 $\alpha i^+ p^+$ was used. This strain, when fully induced, behaves like the constitutive mutant with respect to the liberation properties described (2, 3). However, when it was grown in the absence of cloxacillin after treatment with 0.15 M sodium citrate, conditions under which synthesis of all proteins other than penicillinase would be expected to be normal, there was almost no transfer of internally located penicillinase, and the external sites remain nearly devoid of this enzyme (Table 1).

The above data do not indicate whether during replenishment an equivalent amount of preformed penicillinase is transferred across or from the cell membrane, or whether it is newly synthesized enzyme that becomes located on these external sites. An attempt to answer this question was made by growing the constitutive mutant 8325$\alpha i^- p^+$ in radioactive medium, liberating the penicillinase from external sites, allowing replenishment in nonradioactive medium, and determining the specific radioactivity of the externally located penicillinase. Results of a typical experiment are given in Table 2. Under the conditions used to obtain uniform labeling, the proteins eluted from the CG-50 column had a specific radioactivity of about 6,500 counts per min per

TABLE 1. *Requirement for penicillinase synthesis for replenishment of external sites by S. aureus strain 8325 $\alpha i^+ p^+$*

Replenishment time	Penicillinase (units/ml of culture)	
	Total	External
min		
0	360	—
120	1,280	370
120[a]	370	28

[a] No inducer.

TABLE 2. *Radioactivity of penicillinase located on external sites of S. aureus strain 8325$\alpha i^- p^+$ after replenishment in ^{12}C-medium compared with that of penicillinase originally present*[a]

Growth medium	Radioactivity (counts/min)				Purified penicillinase (units/ml)				Specific radioactivity of penicillinase (counts per min per unit)			
	1[b]	2	3	Total	1[b]	2	3	Total	1[b]	2	3	Mean
^{14}C-medium.....	317	258	74	649	1,000	675	250	1,925	0.317	0.382	0.296	0.337
^{12}C-medium.....	27	19	13	59	625	360	310	1,295	0.043	0.053	0.042	0.046

[a] The growth medium contained 0.6 μc of ^{14}C-reconstituted protein hydrolysate, and replenishment was carried out in ^{12}C-medium for 60 min, during which time the total amount of penicillinase almost doubled. The cells were again treated with citrate, and both lots of liberated penicillinase were purified as described in Materials and Methods. Portions (0.5 ml) of fractions containing high penicillinase activity were precipitated with trichloroacetic acid and assayed for radioactivity as described previously (3). The measurements of radioactivity are corrected for background (12 counts/min), and at least 6,000 counts were recorded for each determination.

[b] CG-50 column fraction number.

mg, and the purified penicillinase, 0.337 counts per min per unit of penicillinase. Variations in the results with individual fractions selected to arrive at this latter mean did not exceed 12%. After reincubation in ^{12}C-containing medium, and following the same procedures, a mean value for the specific radioactivity of penicillinase of only 0.046 counts per min per unit was obtained, with a maximum variation from this in individual fractions of 15%.

DISCUSSION

From the results presented, it can be seen that penicillinase remaining cell-bound after treatment of cells of *S. aureus* with citrate does not become exopenicillinase. The requirement for specific de novo synthesis of penicillinase for replenishment of external sites can be explained by the fact that it is the newly formed enzyme that becomes exopenicillinase. During replenishment (Table 2), a total of only 59 counts/min, representing a *maximum* of 177 units of old penicillinase (if *all* radioactive protein in the second sample of purified penicillinase were penicillinase) in a total of 1,295 units, was found on replenished sites. Because such a low level of radioactivity was found in the penicillinase fractions, the final purity of the fraction is not crucial to the argument.

If external sites had been preferentially replenished by preformed penicillinase, a specific radioactivity similar to that of penicillinase from fully labeled cells would be expected. If all of the penicillinase produced by the cell entered some common pool where it became freely miscible, a specific radioactivity about 50% of that furnsihed by fully labeled cells would be expected, since during reincubation in ^{12}C-medium the total penicillinase content almost doubled. The low specific radioactivity actually observed indicates that external sites are predominantly replenished by newly synthesized penicillinase, and that which remains cell-bound after removal of exopenicillinase does not materially contribute to repopulation of external sites. This situation in *S. aureus* contrasts with the conclusion of Collins (4) that in *B. licheniformis* the cell-bound enzyme consists mainly of the most recently synthesized molecules.

Our results with *S. aureus* have several features in common with those presented by Chesbro and Lampen (1), who studied penicillinase secretion in growing cultures of *B. licheniformis*. For example, these authors noted a close coupling of the rates of penicillinase synthesis and secretion and concluded that a long-lived membrane-bound intermediate is not mandatory in the secretion of penicillinase. In *S. aureus*, the great bulk of the cell-bound enzyme, which is firmly attached to the cell membrane (7; Coles and Gross, *unpublished data*), cannot be regarded as an intermediate in the formation of the exoenzyme. This means that some type of compartmentation of penicillinase within the cell membrane of *S. aureus* must exist, in addition to the type of compartmentation suggested by the experiments of Richmond (7).

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