*Proc. Nat. Acad. Sci. USA*
Vol. 68, No. 11, pp. 2627-2630, November 1971

# Abnormal Autolytic Enzyme in a Pneumococcus with Altered Teichoic Acid Composition

**(cell wall/lysis/enzyme activation)**

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*Communicated by George Palade, July 26, 1971*

**ABSTRACT** **Pneumococci in which the choline component of the cell wall teichoic acid was replaced by ethanolamine contain an abnormal autolytic enzyme that has a low molecular weight and low activity in contrast to the enzyme typical of choline-containing bacteria that has a high molecular weight and high activity. The abnormal autolysin can be converted to the normal (choline-type) enzyme by incubation *in vitro* with choline-containing cell walls.**

The cell walls of *Diplococcus pneumoniae* can be fractionated into two major macromolecular components that are covalently linked: a peptidoglycan of conventional type and a polysaccharide (1) or a complex "teichoic acid" that contains choline as a structural component (2). All the macromolecular form of choline appears to be present in the teichoic acid, which seems to be essential for some cellular functions, since the bacteria require choline for growth (3). Furthermore, pneumococci in which the cell-wall choline was biosynthetically replaced by structural analogs, such as ethanolamine, develop a number of physiological defects, such as abnormal cell division, resistance to lysis by detergents (4), and tolerance of extremely high doses of bacteriolytic antibiotics (5); ethanolamine-containing cell walls cannot be solubilized by the pneumococcal autolytic enzyme (1).

In the present communication, we report a further and quite surprising physiological change: cells with the anomalous aminoalcohol in their teichoic acid contain an abnormal autolytic enzyme with low molecular weight and catalytic activity. This altered enzyme can be converted to the "normal" enzyme (typical of choline-grown cells, see *Methods*) by incubation *in vitro* with choline-containing cell walls.

## MATERIALS AND METHODS

Pneumococci (strain R 36 A) were grown in a chemically defined medium (6) containing either choline ("choline-grown" cells) or ethanolamine ("ethanolamine-grown" cells). The terms "C-form" and "E-form" autolysin will be used throughout the paper to indicate autolytic enzymes typical of choline-grown and ethanolamine-grown bacteria, respectively. Cell walls of choline-grown or ethanolamine-grown bacteria labeled with radioactive [methyl-$^3$H]choline, [1,2-$^{14}$C]ethanolamine, or [U-$^3$H]lysine were prepared from mid- or late-logarithmic phase cultures (2). The soluble radioactive products of autolysin action were characterized by chromatography on gel filters (Sephadex and Biogel) and DEAE-Sephadex (2).

The activity of the autolytic enzyme was determined by measurement of the release of nonsedimentable radioactivity from choline- or lysine-labeled cell walls (2). Before centrifugation of the samples (International Microcapillary centrifuge, model MB, 14,000 $\times$ *g*, 5 min), autolysin action was terminated by the addition of formaldehyde (1 drop of 38% $CH_2O$ to 0.3-ml samples). The unit of autolysin activity was arbitrarily defined as the amount of enzyme capable of releasing, in nonsedimentable form, 1200 dpm from choline-labeled cell walls (specific radioactivity 4–5 $\times$ $10^7$ dpm/mg) in 30 min at 37°C. All chemicals used were commercially available (analytical grade) reagents.

## RESULTS

Autolysins prepared from both choline-grown and ethanolamine-grown cells degrade lysis-sensitive pneumococcal cell walls with equal rates, at an optimum pH of 6.5, to identical kinds of soluble products, namely, relatively small peptide fragments and choline-containing macromolecules of heterogeneous, but fairly reproducible, size distribution and ionic properties (see Fig. 2 of ref. 2). The findings indicate that the autolysins prepared from both choline-grown and ethanolamine-grown pneumococci have the same substrate specificity (L-alanine-muraminidase, ref. 2).

However, at pH 7.6, (somewhat above their pH optimum), some important differences were detected between the two forms of the enzyme. While both forms could completely solubilize cell walls at this pH to yield products identical to those obtained by autolysis at the optimum pH of 6.5, their rates of hydrolysis were quite different. Fig. 1 demonstrates the peculiar (accelerating) kinetics of the action of E-form autolysin.

A closer examination of the two forms of the enzyme revealed that they also differed in physical properties: the bulk of autolysin prepared from choline-grown cells appeared to have a considerably higher molecular weight (excluded by Sephadex G-200 and BioGel P-300, but retained by Agarose-A-5m) than the autolysin extracted from ethanolamine-grown bacteria (retained by Sephadex G-75). Consistent with these observations is the finding that the two forms of autolysin could be separated by $(NH_4)_2SO_4$ precipitation: most of the recovered C-form activity was precipitated with 1 M salt, while most of the E-form activity precipitated with 2 M salt (Table 1). Purification of the enzymes by fractional precipitation with $(NH_4)_2SO_4$ (10-fold for the C-form and about 50-fold for the E-form), or maintenance of the enzyme extracts at their pH optimum of 6.5 throughout

the preparation did not change their kinetic properties or their molecular weight, as detected by gel filtration (E-form: 2–5 × $10^4$, C-form: 1–3 × $10^6$). The molecular weights of the autolysins were also uninfluenced by the method used to extract the enzymes from the bacteria: sonic disruption or mechanical breakage (methods *A* or *B* in legend to Fig. 1) both yielded enzymes of the same characteristic molecular weight. After incubation of mixtures of C- and E-form enzyme extracts at 30°C for 60 min, followed by chromatography on agarose, both activities could be recovered at their characteristic positions in the elution profile.

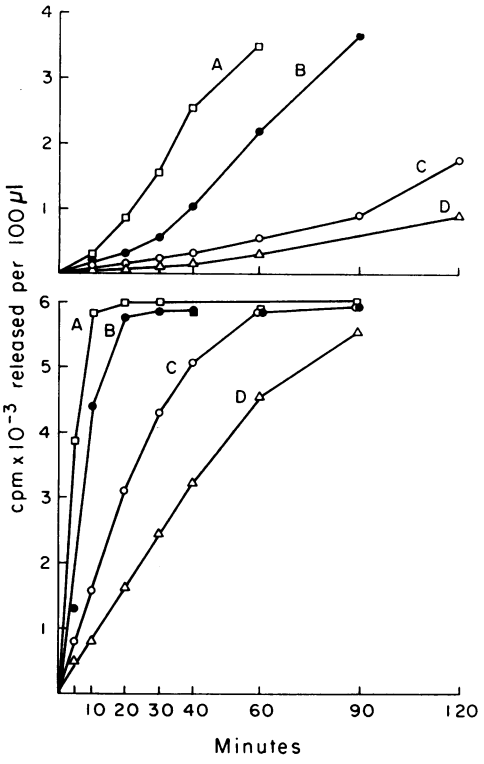


FIG. 1. The kinetics of cell-wall degradation by the E-form and C-form of autolysin at pH 8. E-form (*upper figure*) or C-form (*lower figure*) autolysin at different enzyme concentrations was used to solubilize [$^3$H]choline-labeled cell walls (total radioactivity 6000 cpm per 100 µl). Curves *A*, *B*, *C*, and *D* represent the kinetics of wall hydrolysis by 100, 25, 10, and 5 µl of enzyme extract, respectively; each was added to a total of 1 ml of cell-wall suspension in 0.15 M NaCl–0.01 M $K_2HPO_4$–3 mM 2-mercaptoethanol solution (SPSH). The protein concentration of the enzyme extracts was 6 mg/ml. *Autolysin* was prepared from choline-grown ("C-form" autolysin) or ethanolamine-grown pneumococci ("E-form" autolysin) by the following methods: (*Method A*) a suspension of about $10^{10}$ cells in 4 ml of SPSH was sonicated at 0°C for 2–5 min (Raytheon sonicator; maximum energy output), then centrifuged (20,000 × *g*, 10 min at 0°C); the supernatant solutions were used as crude "autolysin". Extracts of this kind had specific autolysin activities per mg of protein of 10–15 units, in the case of choline-grown cells and 0.5–1 unit, in the case of ethanolamine-grown bacteria, if activity was measured by mixing autolysin and cell-wall substrate at 37°C (in order to minimize "conversion" of autolysin from the E- to the C-form); the same extracts showed 10–16 units/mg of protein autolysin activity, if the incubation of cell-wall substrate and enzyme at 37°C was preceded by a brief exposure of the autolysin to choline-containing cell walls, at 0°C (i.e., under conditions of conversion). (*Method B*): a suspension of bacteria ($10^{10}$/ml) in acetate buffer [0.1 M (pH 4)] was shaken in a Mickle disintegrator at 4°C for 15 min. During rupture of pneumococci at this low pH, 70–90% of the autolysin activity remained attached to the sedimentable cell-wall fraction. Most of the enzyme activity could be eluted from the walls, however, by brief (5–15 min) incubation of the washed cell-wall suspension in SPSH, containing 0.1 M potassium phosphate buffer, pH 8 at 0°C. After centrifugation, the supernatants contained most of the autolytic activity. Method B yielded a 4-fold purified autolysin.

### *In vitro* conversion of the E-form autolysin to the C-form

The accelerating rate of cell-wall hydrolysis by the E-form autolysin suggested that the enzyme might be undergoing a change in its physical properties while catalyzing cell-wall hydrolysis *in vitro*. This was indeed found to be the case: by the end of the solubilization of the cell-wall substrate, the molecular weight of the reisolated E-form autolysin became indistinguishable from that of the C-form. Further experiments showed that this increase in size, accompanied by dramatically improved catalytic properties, can be achieved by a brief exposure of the E-form autolysin to *choline-containing cell walls* at low (0°C) temperature. After such a brief "preexposure" of E-form enzyme to (nonradioactive) choline-containing cell walls, followed by high-speed centrifugation in the cold (20,000 × *g*, 10 min) C-form autolysin of characteristic molecular weight could be recovered from the supernatant solution. Such supernatants could hydrolyze added radioactive cell walls with greatly increased rates and "normal" (ie., nonaccelerating) kinetics. The increase in

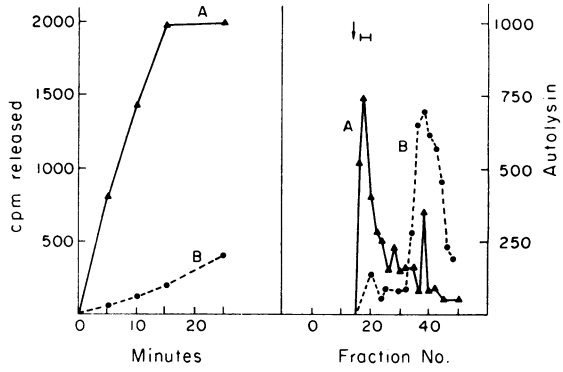


FIG. 2. *In vitro* conversion of the E-form to the C-form autolysin. The *left side* of the figure shows the kinetics of cell-wall hydrolysis by the same autolysin prepared from ethanolamine-grown bacteria, *before* (curve *B*) and *after* (curve *A*) conversion of the autolysin to the C-form. In curve *B*, the enzyme extract (100 µl) and substrate wall suspension (labeled with [$^3$H]choline) were mixed and incubated at 37°C. In curve *A* the same enzyme and cell-wall suspension were mixed at 0°C, and—5 min later—incubated at 37°C. The *right side* of the figure shows the elution of autolysin (prepared from ethanolamine-grown cells and purified about 50-fold by $(NH_4)_2SO_4$ precipitation) from agarose-A *before* (*dashed lines*) and *after* (*solid lines*) exposure to a suspension of nonradioactive, choline-containing cell walls, at 0°C for 10 min. Autolysin prepared from choline-grown cells (crude or 15-fold purified by $(NH_4)_3SO_4$ precipitation) was eluted from the agarose column in the same fractions as the "converted" E-form enzyme.

The horizontal bar indicates the elution of blue dextran (molecular weight = 2 × $10^6$) from the column. The void volume (*arrow*) was determined by passing a suspension of *Bacillus subtilis* phage SP 82 through the column.

TABLE 1. *Some properties of the C- and E-forms of pneumococcal autolysin*

| Source of autolysin | Form | Molecular weight | Concentration of precipitating $(NH_4)_2SO_4$(M) | Effect on autolysin activity of: (rate with / rate without) prior treatment or addition: Prior treatment with cell wall at 0°C | Addition with substrate $PO_4^{-3}$ | Addition with substrate $Mg^{+2}$ | Addition with substrate EDTA | pH 6.5 |
|---|---|---|---|---|---|---|---|---|
| Ethanolamine-grown pneumococci | E | $2–5 \times 10^4$ | 2 | 10–30 | 12 | 14 | 3–4 | 13 |
| Ethanolamine-grown pneumococci | C (converted from E) | $1–3 \times 10^6$ | – | 1 | 1.5 | 1.4 | 1 | 2 |
| Choline-grown pneumococci | C | $1–3 \times 10^6$ | 1 | 1 | 1.4–1.8 | 1.7 | 0.7–1.2 | 2 |

Approximate molecular weights of the autolysins were determined by gel filtration; the E-form autolysin was excluded by Sephadex G-50 but retained by Sephadex G-75; C-form autolysin was excluded by Sephadex G-200 and BioGel 300 and eluted from Agarose-A-5M with blue dextran. Autolysin activities were determined from the initial rates of cell-wall hydrolysis. The concentrations of the stimulatory ions were: 0.1 M ($PO_4^{-3}$), 4 mM ($Mg^{+2}$), and 1.5 mM (EDTA).

TABLE 2. *Conversion of the E-form to the C-form autolysin*

| Prior treatment of E-form enzyme (0°C for 10 min, unless specified otherwise) | Effect on autolysin activity (rate with / rate without prior treatment) | Form of enzyme after prior treatment (filtration on Agarose) |
|---|---|---|
| None | 1 | E-form |
| Choline-containing cell wall (μg/ml) | | |
| 50 | 11.6 | C-form |
| 20 | 7.7 | |
| 10 | 6.7 | |
| 5 | 4.0 | |
| 1 | 1.2 | |
| 20 (60 min) | 8.6 | |
| 25 (heated at 100°C for 10 min) | 12 | |
| "highly purified" | 12 | |
| Enzymatic hydrolysate of choline-containing cell wall (25 μg/ml) | 2 | |
| Choline-containing cell wall residue after removal of teichoic acid | 1 | |
| Purified teichoic acid extracts (prepared by $KIO_4$, formamide, and $Cl_3CCOOH$) | 1* | |
| Choline-containing cell wall, in presence of LiCl (4 M) | 1 | |
| Choline, phosphorylcholine, CDP-choline (each at 10 μg/ml) | 1 | |
| Ethanolamine-containing cell wall (30 μg/ml) | 1 | E-form |
| 0.1 M phosphate buffer (pH 6.5); for 30 min | 1 | E-form |
| $MgSO_4$, 4 mM; for 30 min | 1 | E-form |
| EDTA 1.5 mM; for 30 min | 1 | |

Choline-containing cell walls were "stripped" of their teichoic acid component by three separate methods: (*a*) treatment with $10^{-2}$ M $KIO_4$ at 0°C in the dark for 60 min; (*b*) extraction with formamide at 175°C for 60 min; (*c*) extraction with 5% trichloroacetic acid at 90°C for 60 min. The insoluble (peptidoglycan) residue of cell walls was isolated by centrifugation; the supernatants, containing the teichoic acid fragments, were passed through columns of Sephadex G-25 (in water) and concentrated by lyophilization. An increase in the rate of autolysin activity more than twice that without prior treatment (see below) was taken as an indication of conversion of the E-form to the C-form enzyme. In some cases, the molecular weight of the enzyme was also determined by gel filtration before and after treatment by elution from Agarose-A-5M (*last column*). The following extra purification steps [i.e., in addition to the steps routinely used (2)] were applied for the preparation of "highly purified" cell walls. Treatments (at room temperature) for 30–60 min with (*i*) 8 M LiCl; (*ii*) 6 M urea in Tris–EDTA–borate buffer (pH 8.9); (*iii*) 0.1 M EDTA: (*iv*) acetone, and (*v*) extraction with chloroform–methanol 1:1 at 70–80°C for 10 mins, then, (*vi*) freezing and thawing, and (*vii*) treatment with 500 μg/ml of deoxycholate at 37°C for 10 min.

* In one experiment out of five, the addition of a hot-formamide extract of teichoic acid increased the activity of E-form enzyme about five times.

specific enzyme activity occasionally approached 30- to 50-fold (Fig. 2). These findings clearly indicate that the mechanism of "conversion" to the larger and catalyti ally more effective enzyme was not simply due to a physical attachment of autolysin at the low temperature to the cell-wall substrate. Besides the increased size and activity the E-form enzyme that was converted to the C-form (by previous incubation with choline-containing cell walls in the cold) also became similar to the autolysin found in choline-grown cells in a number of other properties, such as a greatly decreased sensitivity to stimulation by several ionic conditions (Table 1).

Table 2 summarizes experiments aimed at learning which component of the choline-containing cell walls was responsible for the "conversion" phenomenon. While highly purified walls were active converting agents, cell walls stripped of their teichoic acid component were inactive. The soluble teichoic acid preparations that were extracted (and unavoidably degraded to various degrees) by the "stripping" procedures were also inactive in causing conversion. None of these treatments had any effect on the activity of C-form autolysin; attempts to convert the C-form to the E-form by incubation of the enzyme with ethanolamine-containing cell

walls at various pH values and temperatures, and for various periods of time, were all unsuccessful. Table 2 also shows that previous exposure of the E-form enzyme (in the absence of cell walls) to ionic conditions that stimulate enzyme activity when added *together* with the substrate did not result in the conversion to C-form autolysin; the E-form enzyme retained its original molecular weight and low specific activity. On the other hand, mixing the E-form enzyme with the substrate in the presence of $PO_4^{-3}$ and $Mg^{+2}$ ions caused rapid degradation of cell walls, accompanied by conversion of the enzyme to the C-form. These observations suggest that the dramatic (10–15 fold) increase in the activity of E-form autolysin observed in the presence of $PO_4^{-3}$ and $Mg^{+2}$ ions or at low pH (see Table 1) is the composite of two effects: a modest, 1.5- to 2-fold stimulation of the rate of autolysin action (also observed with C-form enzyme) and a rapid conversion of the E-form enzyme to the catalytically more effective C-form. At pH values above 6.5 and in the absence of the stimulating ions, the conversion of enzyme forms occurs much faster at 0°C than at 37°C; the reasons for this requirement for low temperature are not known.

## DISCUSSION

A connection between teichoic acids and lysis sensitivity has been noted in several bacterial species. Thus, sensitivity to streptolysins was correlated with the presence of alanine-containing teichoic acids in streptococci (7). The specific autolytic activity of a staphylococcal mutant that is defective in teichoic acid was much lower than that of the wild type (8); sensitivity of *Streptococcus faecalis* cell walls to the homologous autolytic muramidase was somewhat decreased after the removal of teichoic acid (9). Pneumococcal cell walls "stripped" of their teichoic acid component lose sensitivity to homologous autolysin; cell walls in which the choline component of the teichoic acid was biosynthetically replaced by ethanolamine also become resistant to autolysin (2).

In all these cases, the absence or compositional change of teichoic acids modifies the sensitivity of a bacterial structural component to some lytic agent.

In the present communication, we describe a different type of alteration of the lytic system of a bacterium: in this case, the introduction of abnormal amino alcohol into the cell wall structure resulted in the replacement of the normal, high molecular weight autolysin with an inactive, low molecular weight enzyme.

The "small" autolysin molecules that one can isolate from ethanolamine-grown bacteria cannot degrade ethanolamine-containing cell walls, and they appear to have little if any lytic effect on the normal choline-containing cell walls. The simplest interpretation of our findings is that the catalytic activity (and, in fact, even the detection) of the E-form autolysin is due to the aggregation of this enzyme to the active, high molecular weight C-form under the assay conditions. While the rate of appearance of this active enzyme varies from practically zero (in the presence of 4 M LiCl) through rather slow (at pH 8) to fast (at pH 6.5, in the presence of $Mg^{+2}$ or $PO_4^{-3}$ ions), nevertheless the agent responsible for the conversion of inactive to active enzyme is invariably the choline-containing cell wall itself. Since this conversion can occur at 0°C and depends on the concentration of the cell wall, it is likely that the small "E-form" autolysin molecules aggregate to the active form on the surface of the cell wall, while interacting, in some way, with the choline-containing teichoic acids. The data indicate that this interaction need not involve a firm physical attachment to the cell wall. Whether or not the entrapment of soluble polysaccharide (cell wall) material contributes to the aggregation process is presently being investigated.

Whether or not the increase in molecular size in itself is a sufficient condition for the appearance of autolysin activity is not clear. Acquisition of biological activity *via* assembly of smaller protein subunits, however, is not uncommon in phenomena involving cell surfaces. One specific example would be the assembly of subunits that precedes the appearance of lytic activity during complement fixation (10).

Activity of the pneumococcal autolytic enzyme(s) have been implicated in several physiologically important phenomena, such as cell separation and antibiotic sensitivity. Clearly, the activity of this enzyme must be carefully regulated *in vivo*, and experiments are in progress to test the possible physiological (regulatory) significance of the enzyme aggregation phenomenon. Some evidence for the existence of inactive autolysin precursors has been reported in streptococci. The fact that the molecules of the pneumococcal E-form enzyme promptly aggregate to form large, active autolysin upon encountering their "normal" substrate suggests that even within choline-grown bacteria, there may exist a similar, postribosomal event that converts inactive or low-activity autolysin precursors to active enzyme by interaction with teichoic acid chains at the cell surface.

This investigation was supported by a grant from the National Institutes of Health, U.S. Public Health Service.

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