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Protein-Bound Choline Is Released from the Pneumococcal Autolytic Enzyme during Adsorption of the Enzyme to Cell Wall Particles

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The inactive precursor form of the pneumococcal autolytic enzyme cloned in *Escherichia coli* was isolated by affinity chromatography on Sepharose-linked choline. The enzyme was recovered in an electrophoretically pure and activated form by elution from the affinity column with radioactive choline solution. When radioactive choline was used for elutions, the enzyme protein isolated contained protein-bound choline, at approximately 1 mol of choline per mol of enzyme protein, indicating the presence of a single choline recognition site. Radioactive choline remained bound to the enzyme protein during dialysis, precipitation by trichloroacetic acid or ammonium sulfate, and during gel filtration, but not during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Incubation of the choline-labeled autolysin with pneumococcal cell walls at 0°C resulted in the adsorption of the enzyme to the wall particles and a simultaneous release of free choline from the enzyme protein. It is suggested that the choline molecules that became bound to the enzyme protein during the activation of autolysin are expelled from the choline-binding site and replaced by choline residues from the wall teichoic acid as the autolysin molecules adsorb to their insoluble substrate before the onset of enzymatic wall hydrolysis.

The major pneumococcal autolysin, *N*-acetylmuramoyl-L-alanine amidase (amidase) is known to contain specific recognition sites for choline residues which are distinct from the catalytic site of this enzyme (6). Choline is a normal structural component of the pneumococcal wall teichoic acid (3, 7, 11), and earlier studies indicate that prior to the hydrolysis of the muramoyl-L-alanine bonds in the pneumococcal wall, the amidase molecules have to adsorb to the insoluble wall particles by attaching to the choline residues of the wall teichoic acid (5); the functional role of the choline recognition site in the amidase may be in mediating this unique adsorption step (5). Nevertheless, this proposition raises a dilemma. The pneumococcal amidase is known to exist in two forms: an inactive (precursor) form, which can be converted, in vitro or in vivo, to the other form, an enzymatically active C form (12), by exposure of the precursor form to choline-containing cell walls, wall or lipoteichoic acids (2, 6, 12), or free choline (3). During the process of enzyme conversion the converting agent becomes strongly bound by the enzyme (2, 6). Thus, the active form of the amidase already has its choline recognition site occupied, and, therefore, attachment to the wall substrate must involve either a second choline site or replacement of the resident choline-containing agent from the enzyme by the choline moieties of the cell wall.

The purpose of this study was to perform an experimental test of these possibilities.

MATERIALS AND METHODS

Isolation of amidase. Amidase was produced by cultures of *Escherichia coli* CM21 carrying the cloned pneumococcal *lytA* gene on plasmid pGL80 (4). The inactive precursor E form (12) of the amidase is produced under these conditions (4). The cells were harvested by centrifugation (10,000 × *g* for 10 min) and disrupted by ultrasonic treatment for 10 min at 40% output in a sonicator (model W-225; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) in the presence of detergent (1% Brij 35) and 1 M LiCl. Debris were removed by centrifugation, and the supernatant was dialyzed at 4°C for 18 h against 50 mM Tris hydrochloride buffer (pH 7.0) containing 0.1% Brij 35. The supernatant was then concentrated against Aquacide (Calbiochem-Behring, La Jolla, Calif.), loaded on a Sephacryl S-200 (Pharmacia Inc., Piscataway, N.J.) gel filtration column (2.5 by 70 cm), and eluted with the above buffer. Fractions of 5.3 ml were collected and assayed for amidase activity, and the fractions showing peak activity (fractions 3 through 5) were pooled.

Assay of amidase activity. Amidase activity was assayed by mixing 2.5 μl of the enzyme solution with 50 mM Tris hydrochloride buffer (pH 7.0) containing 0.1% Brij 35 (50 μl) and ^{14}C-lysine-labeled walls (200 μg and 1.25 × 10^5 cpm in 50 μl). The mixture was incubated for 60 min at 37°C and centrifuged. Soluble radioactivity in the supernatant was determined. One unit of amidase enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μg of cell wall material under the assay conditions in 10 min.

Assay of protein-bound [^{3}H]choline. The [^{3}H]choline-labeled enzyme (25 μl) was mixed with cold trichloroacetic acid (TCA) (final concentration, 10%) and bovine serum albumin (Cohn fraction V; Sigma Chemical Co., St. Louis, Mo.) (final concentration, 0.1%). After 10 min at 0°C the mixture was filtered onto membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and the radioactivity of the precipitate was determined by scintillation counting.

Purification of amidase and conversion to the active form by affinity chromatography on choline-Sepharose and labeling of the enzyme with radioactive choline. The crude amidase was loaded on a choline-Sepharose column (2.5 by 12 cm) (4). In

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some experiments, DEAE-Sepharose was used instead of choline-Sepharose (9). The column was washed with 1 bed volume of buffer (Tris hydrochloride, pH 7.0), then with 0.5 M NaCl in the same buffer, and finally with buffer alone. The enzyme bound to the column was eluted with a 2% solution (15 ml) of radioactive choline (specific activity, 73 μCi/mmol). Fractions were monitored for radioactivity, and the pooled radioactive fractions were extensively dialyzed in 20-mm MWCO tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with cutoff value of 2,000 against a total of 8 liters of 50 mM Tris hydrochloride buffer (pH 7.0) with 0.1% Brij 35 at 2°C. The dialyzed material was then concentrated to 400 μl against Aquacide and loaded on a gel filtration column (model P6; 0.9 by 29 cm; Bio-Rad Laboratories, Richmond, Calif.). Elution was performed with the above buffer, and fractions (1.5 ml) were assayed for amidase activity, soluble and TCA-precipitable radioactivity, and protein with BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.). Fractions showing amidase activity were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Amidase preparations were run on 10% polyacrylamide gels in which bisacrylamide was replaced by *N,N'*-diallylytartardiamide (10). The protein bands were stained with Coomassie brilliant blue (1), cut out, and dissolved in 50 mM Tris hydrochloride for the determination of radioactivity. Molecular weight standards were the LMW SDS-PAGE kit from Pharmacia (4). The M_r 14,400 standard runs with the front.

RESULTS

Properties of the amidase labeled with radioactive choline. The amidase preparation eluted from the affinity column by the radioactive choline solution was passed through a P6 column and analyzed by SDS-PAGE. The results indicated the presence of pure amidase: only the protein band doublet characteristic of the enzyme preparations (4, 9) was detectable in the first peak. No protein was detectable in the later fractions. All the radioactive choline counts in peak 1 were precipitable with cold TCA, along with the protein, whereas peak 2 contained residual free choline that was not precipitable with TCA and whose quantity varied somewhat from preparation to preparation. The amidase recovered in the P6 fractions was in the activated form, as evidenced by the usual criteria (12). The specific radioactivity of [^{3}H]choline used in the conversion assay was 50 μCi/mmol. The specific radioactivities of the choline-labeled amidase of three separate preparations recovered in the P6 fraction were 77, 223, and 206 dpm/μg of pure amidase protein, and the corresponding numbers of choline molecules bound per enzyme molecule of 35 kilodaltons were 0.4, 1.1, and 1.03, i.e., remarkably close to 1.0. A value less than 1 may indicate the presence of inactive enzyme molecules (e.g., molecules that had nonfunctional choline sites). The radioactive choline label was bound to the amidase protein by the following criteria. (i) The radioactive label was retained during extensive dialysis. (ii) The radioactive label was quantitatively precipitated along with the protein by cold 20% TCA and by 50% ammonium sulfate. There was no detectable radioactive choline coprecipitating with albumin in appropriate control experiments. (iii) Radioactivity, protein, and enzyme activity eluted together from the P6 column (Fig. 1). (iv) Choline was not bound to the enzyme by covalent bonds, since no radioactivity could be detected in the two protein bands isolated from the SDS-PAGE gel (Fig. 1, insert, and data not shown). (v) Although the precursor form of amidase produced by the *E. coli* system could be quantitatively adsorbed to the choline-Sepharose, the radioactive choline-labeled enzyme was no longer retained by the affinity column, indicating that the choline recognition site of the enzyme, required for the affinity chromatography, was occupied. In this experiment, choline-labeled amidase (10.4 μg of protein; 3,900 cpm) was loaded onto the affinity column. Elution with 50 mM Tris buffer (pH 7.0), containing 0.1% Brij 35 removed all radioactivity as well as amidase activity. All amidase activity as well as 85% of the radioactivity eluted in peak 1, and all radioactivity in this peak was precipitable with cold TCA. A smaller second peak contained 15% of the radioactivity, which was TCA soluble. No detectable amidase activity was removed from the affinity column by the same eluent, if the material adsorbed was the precursor (E-form) enzyme. (vi) The association between radioactive choline and enzyme was not perfectly stable: an enzyme preparation (5.2 μg of protein containing 1,200 cpm of TCA-precipitable radioactive choline) gradually lost protein-bound label during storage at 0°C (5% was lost in 2 weeks and about 18% was lost in 5 weeks).

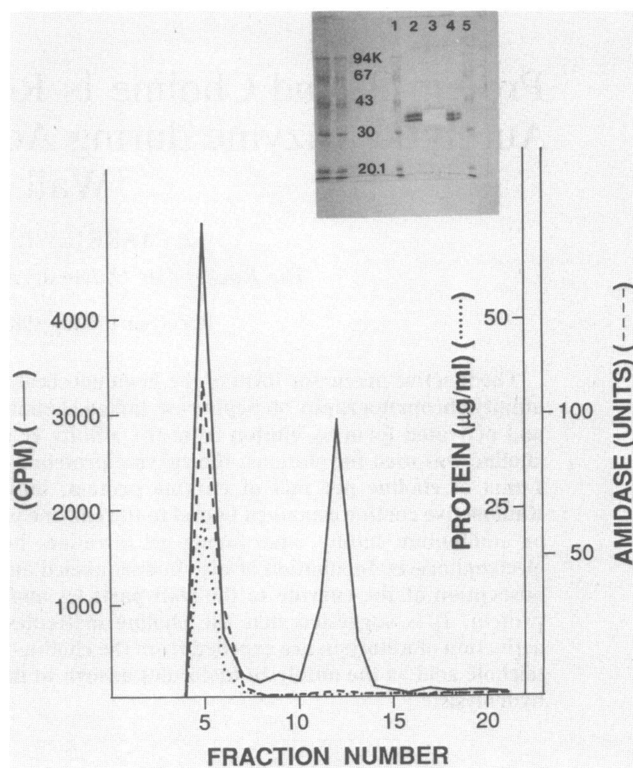

FIG. 1. Gel filtration of [^{3}H]choline-labeled amidase on a P6 column. Amidase isolated, purified, and labeled with [^{3}H]choline, as described in the text, was passed through a P6 column and eluted with 50 mM Tris hydrochloride buffer (pH 7.0) containing 0.1% Brij 35. Fractions (1.5 ml) were analyzed for protein (·····), radioactivity (——), and amidase activity (– – –). The insert shows the results of an SDS-PAGE analysis of the fractions. Lanes: 1 and 5, molecular weight standards (in thousands); 2 through 4, 13, 13, and 6.5 μg of protein from fraction 5, respectively. Material from lane 3 was cut out, eluted, and used for the determination of counts per minute.

Loss of protein-bound choline during adsorption of the choline-labeled amidase to cell walls. Various amounts (100 to 1,800 μg) of purified pneumococcal cell walls (with choline-containing wall teichoic acids) were incubated in small

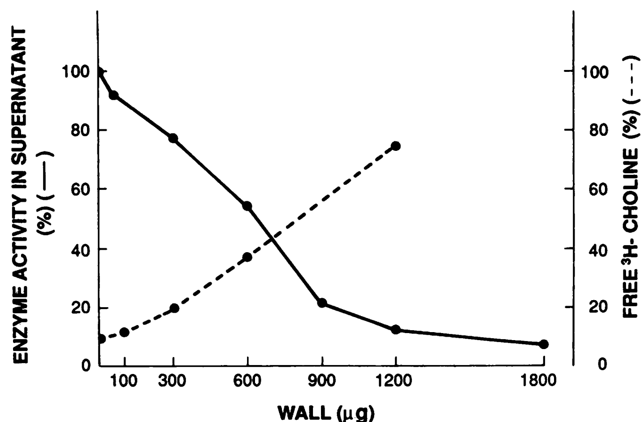

FIG. 2. Release of protein-bound [^{3}H]choline from the amidase during adsorption of the enzyme to choline-containing pneumococcal cell walls. [^{3}H]choline-labeled amidase was incubated with various amounts of cell walls at 0°C. After a 10-min incubation, the suspensions were centrifuged and the supernatants were analyzed for amidase activity and radioactivity not precipitable with cold TCA.

centrifuge tubes, each with 5.2 μg of radioactively labeled amidase (specific radioactivity, 8.03×10^9 cpm of [^{3}H]choline per mmol of enzyme) in 50 mM Tris hydrochloride buffer (pH 7.0) containing 0.2% Brij 35 in a total of 155 μl at 0°C. After 10 min the mixtures of wall and enzyme were centrifuged (10,000 × *g* for 10 min), and the supernatants were assayed for amidase activity and for TCA-precipitable and TCA-soluble radioactivity.

DISCUSSION

The purpose of these studies was to examine in somewhat more detail a peculiar feature of the pneumococcal autolytic amidase, namely, its requirement for choline residues in its cell wall substrate (7). The existence of a choline recognition-binding site within the enzyme protein has been quite evident for some time, both from biochemical (2, 5, 6) and from genetic studies (8). In the studies described in this communication we chose a direct approach to this problem by attempting to tag this choline-binding site in the autolysin with radioactive choline. The data described indicate that we have succeeded in preparing choline-labeled autolysin in sufficiently stable form to test the fate of the protein-bound choline residues during attachment of the enzyme to its cell wall substrate.

Figure 2 shows the gradual binding of the choline-labeled amidase to the cell wall substrate during coincubation of wall and enzyme at low temperature. An earlier study has shown that the disappearance of free enzyme from the supernatant under these incubation conditions was caused by the adsorption of enzyme molecules to the cell wall particles, or, more specifically, to choline residues in the wall teichoic acid polymers (5). In the experiment illustrated in Fig. 2, the gradual disappearance of free (unadsorbed) enzyme was accompanied by a parallel accumulation of free (TCA-soluble) choline in the supernatants. Addition of 40 mM ethanolamine to the incubation mixtures did not prevent the accumulation of free choline or the adsorption of enzyme to the wall (4), and there was no adsorption of enzyme and no release of free choline if the pneumococcal walls were replaced by heterologous (group A streptococcal) walls. A closer examination of the two curves in Fig. 2 shows that the

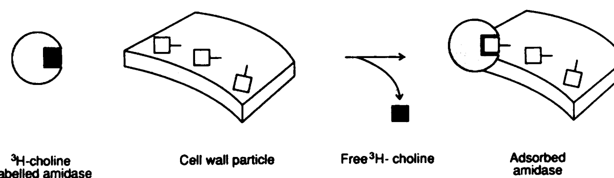

FIG. 3. Model for the expulsion of protein-bound [^{3}H]choline from the choline recognition site of amidase by choline residues in the wall teichoic acid, during adsorption of the enzyme to the pneumococcal wall particles. Circle with indentation represents the amidase and its choline recognition site, which contains a radioactive choline molecule (■) that has become bound by the enzyme in the process of conversion of the inactive to the active form. This radioactive choline is expelled during attachment of the enzyme to the choline residues (□) of cell wall particles.

appearance of free (unbound) choline almost quantitatively parallels the loss of free (unadsorbed) enzyme; i.e., the two curves in Fig. 2 intersect remarkably close to the 50% point. These observations strongly suggest that the release of free choline from the protein is a direct consequence of the enzyme adsorption to the cell wall particles, a process known to be mediated by the interaction between a site on the enzyme protein and the choline moieties of wall teichoic acid (5). The data in Fig. 2 suggest that during the first stage of attack on the substrate particles (i.e., during enzyme adsorption), the choline residues in the wall teichoic acid compete for the same choline recognition site that has become occupied by the radioactive choline molecule used in the preparative procedure, i.e., during conversion of the precursor (E form) of the enzyme produced by *E. coli* to the active C form (Fig. 3). Genetic studies suggest that a choline recognition site resides close to the carboxy terminus of the autolysin molecule (8).

Although it is quite clear that the *lytA* gene codes for the precursor (E form) of the amidase, the nature of the choline-containing material that performs the role of enzyme activation (conversion) in vivo is not yet known. The use of the radioactively labeled free choline in the model experiments described was appropriate, since it has allowed the recognition of the near stoichiometry between choline release and the process of enzyme adsorption; with the polyvalent converting agents, such as wall teichoic acids (5), this would not have been possible. If continued occupancy of the choline recognition site in the amidase is essential for activity, choline moieties residing in this site (e.g., the choline residues built into the wall substrate) may undergo a constant turnover during hydrolysis of the cell wall, and such a process may even allow the translocation of an enzyme molecule within the same wall particle to move from one choline residue of a teichoic acid chain to another.

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