DISSOCIATION OF AN AUTOLYTIC ENZYME-CELL WALL COMPLEX BY TREATMENT WITH UNUSUALLY HIGH CONCENTRATIONS OF SALT[1]

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SUMMARY. Definitive proof that the autolytic enzyme of Streptococcus faecalis ATCC 9790 is not covalently linked to the cell wall was obtained. The autolysin appears to be bound to the wall by an electrostatic interaction which requires unusually high salt concentrations (e.g., 4 to 10 M LiCl) for disruption. The active form is even more firmly bound to the wall than the latent (proteinase-activatable) form. This unusually strong electrostatic interaction may play some role in controlling the cellular localization as well as action of this potentially dangerous enzyme on its extraprotoplasmic substrate.

Bacterial autolytic enzymes are most frequently found to be "fixed" to the cell wall (1). Until very recently such enzymes have been regarded by many investigators to be an integral part of this extraprotoplasmic structure (1,2,3). For example, Brown et al. (3) have suggested that a portion of the wall teichoic acid and the autolytic amidase of Bacillus subtilis are covalently bound. These same investigators (3) have also pointed out the difficulties in purifying and characterizing such enzymes because of their close association with wall polysaccharides. In mechanically broken cells the autolytic enzyme (a β N-acetylmuramide glycanhydrolase) of Streptococcus faecalis 9790 is found virtually exclusively in the cell wall fraction from which it is not released until wall dissolution is virtually complete (2,5). The active form of the enzyme was found associated primarily with recently synthesized wall, while a latent (proteinase-activatable)

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form was randomly located on the wall surface (5). The strength of the enzyme-wall association, the fact that the enzyme occurs and works on a substrate located outside of the cellular permeability barrier, and the association of the active form with "new wall", all suggested that the enzyme might be "built into" the wall during wall biosynthesis and was perhaps even covalently linked to the wall.

Some indirect evidence against a covalent linkage was found and includes 1) the demonstration that isolated autolysin rapidly, and apparently irreversibly, binds to walls even at 0° (4) and 2) competition studies, which showed that about 85% of the latent autolysin is not wall-bound in whole cells but, because of its unusually high affinity for walls, becomes bound during cell disruption (6). We now find that the autolysin can be removed from isolated walls by very high concentrations (e.g., 5 M) of salts, such as CsCl, LiCl and NH_4Cl, but not by 8 M urea, suggesting that the enzyme is bound to the wall by an electrostatic interaction which can be disrupted only by unusually high salt concentrations.

Previous experiments showed that 8 M urea or 1 M ammonium acetate failed to either remove or inactivate wall-bound autolysin (4). It was considered possible that such reagents might cause autolysin removal, but that upon subsequent dilution, enzyme was rebound to the walls. Therefore, ^{14}C (L-lysine) labeled, sodium decylsulfate inactivated walls (SDS-walls) (2) were mixed with unlabeled walls (1:2.2 by weight) which contained autolysin (LOG walls), exposed to either 8 M urea or 5 M CsCl at 0° for 17 hr, then diluted ten-fold with water, harvested by centrifugation, washed twice with water, and assayed for active and latent autolysin (2,6). Release of ^{14}C from the added SDS-walls during the initial 40% drop in wall turbidity was used as an indicator of the ability of the ^{14}C-walls to compete for the binding of released autolysin. Active enzyme remained bound to the LOG walls after exposure to both CsCl (Fig. 1A) and urea. After exposure to CsCl, complete equilibration of latent autolysin between labeled and unlabeled walls was indicated by an almost parallel ^{14}C release and drop in turbidity (Fig. 1B). Absence of release of ^{14}C during the initial 50% drop in turbidity following treatment with 8 M

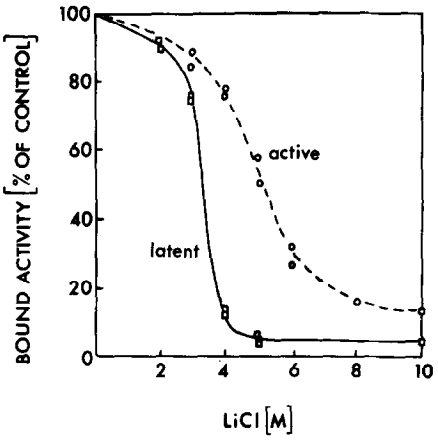

Fig. 1. Loss of turbidity (dashed lines) and release of ^{14}C label (solid lines) from mixtures of LOG and SDS-^{14}C-lysine labeled walls (2.2:1 by weight) after treatment at 0° for 17 hr with either 5 M CsCl (circles), 8 M urea (triangles) or untreated control (squares). After treatment, wall suspensions were diluted ten-fold with water, harvested by centrifugation, washed 3 times with water and assayed for active (A) and latent (B) autolysin (1). In part A, the drop in turbidity and the loss of ^{14}C of the 8 M urea treated sample was very similar to that of the 5 M CsCl treated sample and is not shown. In part B, the release of ^{14}C and the drop in turbidity of the control and urea treated samples were very similar. Therefore, only the ^{14}C release of the urea treated sample and the turbidity decrease of the control are shown. Release of soluble radioactivity from the walls was measured as previously described (5,6).

urea indicated that this reagent failed to cause latent autolysin release from LOG walls.

Significant inactivation of latent autolysin by 5 M CsCl treatment was not observed since the rate of wall dissolution after exposure to 5 M CsCl was virtually the same as the control (Fig. 1B). High concentrations (over 5 M) of CsCl, NH_4Cl, and LiCl were all found to release autolysin from walls. Because of its high solubility and low density (which facilitates separation of walls from the salt solution by centrifugation) LiCl was used for further studies. Little autolysin was removed from the walls by LiCl treatment unless a concentration of 4 M or higher was used (Fig. 2). At 4 M only about 15% of the latent form, but about 75% of the active form, remained wall-bound. For maximum reduction of the amount of the active form left on the walls, higher

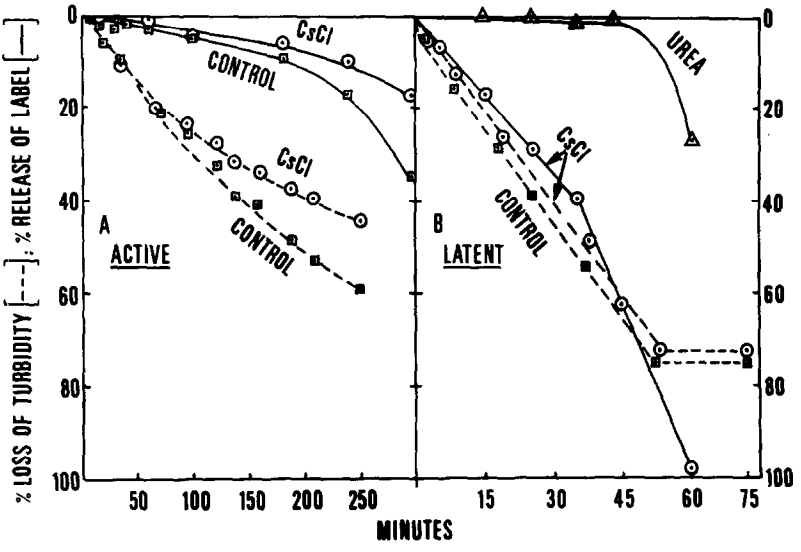

Fig. 2. The percent of the initial active and latent forms of the autolysin which remain bound to walls after treatment of 4 mg wall samples with 6 ml of LiCl of the molarity indicated at 0° for 17 hr. Following treatment, wall suspensions were harvested, washed twice, and assayed for active and latent autolysin.

concentrations (e.g., over 8 M LiCl) were required. About half of the latent activity no longer present on the walls after exposure to 4.5 M LiCl could be recovered from the supernate after dilution to 0.45 M and binding of the activity to SDS-walls by exposure for 10 min at 0° followed by 5 min at 37°.

LOG walls were exposed to 4.5 M LiCl for intervals of 0 to 60 min at 0°, filtered (3), washed twice and assayed for latent autolysin which remained bound. Only about 15% of the latent activity remained wall-bound whether the exposure time was zero min or 60 min (filtration time - 1 to 2 min). This suggests that enzyme release was extremely rapid if not instantaneous. At high wall concentrations, less autolytic activity was removed by 4.5 M LiCl than at lower concentrations (Table I). Latent autolysin released from LOG walls by 4.5 M LiCl after dilution to 0.18 M LiCl was stable at 0° for 17 hr, but 95% of the activity was lost at 52° in 6 min.

Discussion - Dissociation of the autolysin wall complex by salt, but

Table I. Percent of autolysin remaining in the LOG walls after treatment with 4.5 M LiCl at 0° for 10 min. Following treatment, cell wall suspensions were harvested, washed twice with water, and assayed for total autolysin (2)

Cell wall concentration (mg/ml)	11	5.5	2.75	1.4	0.7	0.35	0.17
% autolysin remaining on the walls	39	26	19	18	17	14	12

not with urea, makes it clear that the enzyme is not covalently linked to the wall. In view of the extremely rapid, if not instantaneous, release, the interaction appears to be electrostatic. Further, the dependence of enzyme release on cell wall concentration (Table I) suggests a physical equilibrium between wall-bound and unbound autolysin.

The requirement for salt concentration above 4 M to release most of the wall-bound autolysin suggests an unusually strong (probably multiple), electrostatic interaction. This situation is not entirely unique since other systems, particularly those involving very large molecules or particulate structures, require high salt concentrations for dissociation. For example, 1) high salt concentrations (e.g., 5 M CsCl or 2 M LiCl) are required to remove many proteins from ribosomes (7); and 2) a 2 M LiCl treatment removed hen egg-white lysozyme but not ATPase from membrane ghosts of S. faecalis (8). Also, RNA polymerase strongly binds to DNA, but this association is sensitive to an increase in ionic strength so that complex formation can no longer be detected at NaCl or KCl concentrations greater than 0.35 M (9). It would not be surprising if other wall or membrane associated enzymes of Gram-positive bacteria were released upon treatment with unusually high concentrations of salt.

Higher salt concentrations are required to remove (or cause movement of) the active form than latent autolytic activity. This suggests that the

active form has an ever higher affinity for the wall than does latent enzyme. It seems possible that this increased affinity could be related to removal of amino acid residues from the latent enzyme by proteinase activation. A few differences in properties of the latent and active forms have already been detected. These include: 1) In whole cells the active form occurs in the wall-bound state, while about 85% of the latent form binds to the wall during cell disruption (6). 2) Wall-bound latent autolysin appears to be somewhat more heat-stable than the active form (4). Excellent recovery of latent autolysin was indicated by comparable rates of wall lysis before and after exposure to 5 M CsCl in the mixing experiment shown in Figure 1. However, only about one half of autolysin released after treatment with 4.5 M LiCl was recovered from the supernate. This decreased recovery could be due to the known lability of highly purified soluble autolysin (4) or to less than optimal conditions for the rebinding of the enzyme.

Previous experiments showed that a partially purified autolysin preparation (which still contained considerable amounts of wall polysaccharides) could bind efficiently to trichloroacetic acid extracted or periodate treated walls (4). This makes it less likely that the phosphate-containing, non-peptidoglycan wall polymers (teichoic acids?) play an essential role in the electrostatic interaction between autolysin and walls.

LOG walls can bind at least three times more autolysin than they normally contain when isolated, suggesting the presence of a large number of binding sites. Evidence has been presented indicating that, in exponentially growing cells, the active form is localized at the site of wall extension (6,10) and suggesting that latent enzyme is bound to the walls before activation (6). Increased affinity after activation makes it even less likely that the active enzyme can be displaced from these sites.

It is tempting to speculate that the unusually strong electrostatic interaction of autolysin and cell wall plays some role in controlling its cellular localization and activity.

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