Effects of pH on the Aggregation of Choline-treated E-Amidase of *Streptococcus pneumoniae*

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Pneumococcal E-amidase (36.5 kDa, inactive form of the amidase) incubated with choline aggregates to form a high molecular mass (> 500 kDa), active C-amidase upon the removal of choline by dialysis at neutral pH. When choline was removed at alkaline pH (pH 8-9), aggregation of the enzyme was severely inhibited. The non-aggregated enzyme obtained at alkaline pH easily aggregated in the absence of choline and was active when the pH was shifted to neutral (pH 7). Furthermore, the high molecular mass amidase formed at neutral pH was dissociated at pH 9 to a low molecular mass (about 36.5 kDa). The dissociated amidase also reaggregated and was active when it was simply exposed to neutral pH. Since the original E-amidase itself neither aggregated nor was active by such a pH shift, these amidases obtained after the removal of choline at alkaline pH were suggested to be the intermediate molecules of the choline-mediated conversion of inactive E-amidase to the active form.

Streptococcus pneumoniae produces a powerful autolytic enzyme (N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28))^{1,2)} that participates in several important biological phenomena.^{3,4)} Høltje and Tomasz⁵⁾ speculated that inactive E-amidase is produced as a precursor *in vivo* and upon treatment with lysis-inducing agents, is converted to a catalytic active form (C-amidase) depending on the presence of choline residues in teichoic acid. The inhibitory effect of lipoteichoic acid^{5,6)} and the specific regulation of the enzyme activity by choline residues in the teichoic acid of this bacterium were reported.⁷⁻¹⁰⁾

E-amidase can be converted *in vitro* into C-amidase by incubation with either pneumococcal cell walls containing choline residues (called cell wall-mediated conversion)¹¹⁾ or choline (choline-mediated conversion)^{12,13)} or its structural analogues, aminoalcohols,^{14,15)} in high concentrations. In the choline-mediated conversion, E-amidase aggregates to form active C-amidase which has an apparent molecular mass (> 500 kDa) much higher than

that of the original E-amidase (36.5 kDa) upon subsequent removal of choline by dialysis. Incubation with choline and its subsequent removal are both required for the aggregation, and the enzyme does not aggregate in the presence of choline.¹⁵⁾ It is not known how formation of a high molecular mass amidase requires subsequent removal of choline. It is also unknown whether formation of a high molecular mass amidase is essential for the catalytic activity of amidase since the enzyme activity is inhibited in the presence of choline and its assay is impossible without removal of excess choline.

In this paper, we report factors affecting formation of a high molecular mass material in the choline-mediated conversion of E-amidase to C-amidase. Alkaline pH during the removal of choline drastically inhibited the aggregation of enzyme protein, but not that during the incubation of E-amidase.

Materials and Methods

Bacterial strains and media. Experiments were done with

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noncapsular *Streptococcus pneumoniae* strain R6, a derivative strain of the Rockefeller University strain R36A.¹⁶⁾ An autolysin-defective mutant strain, Cw1,¹⁷⁾ was used for the preparation of cell walls. For preparation of E-amidase, R6 cells were grown at 37°C without aeration in a chemically defined Ced medium¹⁸⁾ supplemented with 40 µg/ml of ethanolamine.⁵⁾ Cw1 cells were grown in C + y medium.¹⁹⁾

Preparation of autolysin. E-amidase was prepared from strain R6 cells grown in ethanolamine-containing medium: cells in exponential-growth phase (2×10^8 cells/ml) were harvested by centrifugation and suspended with 50 mM Tris-HCl (pH 7.0). After disruption of the cells by sonication with a Branson Sonifier Cell Disrupter 200, the sonicated lysate was centrifuged ($12,000 \times g$, at 4°C for 10 min). The supernatant was passed through a Millipore filter (type HA, pore size 0.45 µm) and partially purified through a Bio-gel A-5 m column. Fractions containing the amidase were pooled and stored at -20°C.

Preparation of cell wall. Substrate cell wall was prepared from autolysin-defective mutant (Cw1) cells grown in C + y medium as described previously.¹⁵⁾

Assay for autolysin activity. Amidase activity was measured by the rate of cell wall hydrolysis at 37°C as described elsewhere.¹³⁾

Conversion of E-amidase. Cell wall-mediated conversion was done by incubating E-amidase with cell walls at 0–4°C for 10 min. Enzyme activity was measured by the rate of cell wall hydrolysis in the cell wall-amidase mixture at 37°C. Choline and aminoalcohol-mediated conversion was done using the following aminoalcohols: choline, 2-diethylaminoethanol (DEEA), 2-ethylaminoethanol (EEA), and N-methylaminoethanol (MEA). E-amidase was incubated at 37°C for 30 min with appropriate concentrations of aminoalcohols in 1.0 ml of Tris-HCl (pH 7.0) and then dialyzed against the same buffer at 4°C for 20 hr. The dialyzed materials were used as aminoalcohol-converted amidase.

Gel filtration. Sephadex G-200 (Pharmacia, superfine) columns were equilibrated with 50 mM Tris-HCl at various pH and the same buffer was used as eluent.

Results

Inhibition of the aggregation of choline-treated E-amidase by the removal of choline at alkaline pH

To substantiate the manner of aggregation of E-amidase in choline-mediated conversion, we examined factors affecting the formation of

Fig. 1. Effects of pH during the Removal of Choline on the Aggregation of Choline-treated E-amidase.

E-amidase (500 units) was incubated at 37°C for 30 min with 50 mg/ml of choline in 1.0 ml of 50 mM Tris-HCl (pH 7). The samples were then dialyzed at 4°C for 20 hr against 50 mM Tris-HCl at pH 7 (A), pH 8 (B), and pH 9 (C). The dialyzed sample was put on a Sephadex G-200 column (2.6×35 cm), and eluted with the same buffer as used for dialysis. Fractions of 2.5 ml were collected and the pH was adjusted to neutral. Each fraction was divided into two and the first was assayed for amidase activity (○), and the second was assayed for amidase activity after cell wall-mediated conversion to detect the remaining E-amidase, if any (●).

The arrow indicates the elution position of blue dextran and the bar that of E-amidase.

a high molecular mass amidase. Figure 1 shows the effect of pH during the removal of choline. E-amidase was incubated at pH 7 with a high concentration of choline and the sample was dialyzed against Tris buffer at various pH. When choline was removed at neutral pH (pH 7), the enzyme aggregated and all the enzyme activity was eluted at a position of a high molecular mass on a Sephadex G-200 gel chromatography (Fig. 1A). However, when choline was removed at alkaline pH, aggregation of the enzyme protein was drastically inhibited; at pH 8, the enzyme appeared at the intermediate molecular size (Fig. 1B) and at

pH 9, the enzyme was eluted at a position quite similar to that of the original E-amidase (36.5 kDa) (Fig. 1C). Addition of several detergents and chaotropic ions during the removal of choline did not cause inhibition of the aggregation (data not shown).

Completion of the aggregation upon pH shift

Since the non-aggregated amidase obtained by the removal of choline at pH 9 was similar in size to that of the original E-amidase, we were interested in examining the nature of such a non-aggregated amidase in the absence of choline.

Though the catalytic activity of the original E-amidase becomes detectable only after it was converted by cell walls or choline,^{6,12,13} the non-aggregated amidase obtained at pH 9 was active without any conversion treatment (Fig. 1C). Judging from the enzyme activity of the peak in Fig. 1, the non-aggregated amidase had nearly the same activity as that of the amidase that had completed the aggregation by dialysis at pH 7. Moreover, the non-aggregated amidase could easily form into a high molecular mass when the amidase was exposed to neutral pH (Fig. 2). Similar aggregation was observed when the intermediate molecular mass-material obtained at pH 8 was exposed to neutral pH

Fig. 2. Completion of the Aggregation of the Non-aggregated Amidase upon pH Shift.

The non-aggregated amidase at pH 9 (fractions 40–46 in Fig. 1C) was pooled and dialyzed against pH 7–Tris buffer. The dialyzed sample was put on a Sephadex G-200 column equilibrated with 50 mM Tris-HCl (pH 7) and eluted with the same buffer. Two and a half ml of fractions were collected, and the amidase activity was assayed as described in the legend to Fig. 1.

The arrow indicates the elution position of blue dextran and the bar that of E-amidase.

(data not shown). The same pH shift did not cause the aggregation of the original E-amidase. These results suggest that the non-aggregated amidase obtained by the removal of choline at alkaline pH has acquired a tendency to aggregate and is the intermediate molecule in choline-mediated conversion.

Effects of pH during the incubation of E-amidase with choline

Figure 3 shows the effects of pH during the incubation of E-amidase with choline; E-amidase was treated at various pH with a high concentration of choline and then all the samples were dialyzed at neutral pH (pH 7) to remove excess choline. The samples were put

Fig. 3. Effects of pH during the Incubation of E-amidase with Choline on the Subsequent Aggregation.

E-amidase (500 units) was incubated at 37°C for 30 min with 50 mg/ml of choline in 1.0 ml of 50 mM Tris-HCl at pH 7 (A), pH 8 (B), and pH 9 (C). The samples were then dialyzed against 50 mM Tris buffer at pH 7 at 4°C for 20 hr. The dialyzed samples were put on Sephadex G-200 columns equilibrated with 50 mM Tris buffer (pH 7) and eluted with the same buffer. Fractions of 2.5 ml were collected and assayed for enzyme activity with (●) or without (○) cell wall-mediated conversion before assay as described in the legend to Fig. 1.

The arrow indicates the elution position of blue dextran and the bar that of E-amidase.

on Sephadex G-200 columns and eluted at neutral pH. The aggregation of E-amidase did occur in all cases (Fig. 3B, C), indicating that alkaline pH during incubation with choline did not affect the formation of a high molecular mass amidase.

Dissociation and reaggregation of the high molecular mass, C-amidase

To examine the effects of alkaline pH on the

high molecular mass amidase, C-amidase obtained by choline-mediated conversion (at pH 7) was exposed to alkaline pH by dialysis and fractionated on a Sephadex G-200 column. As seen in Fig. 4B, the high molecular mass amidase was dissociated and all the enzyme activity was eluted at a position similar to that of the original E-amidase. The same treatment at pH 7 did not cause the dissociation (Fig. 4A).

The dissociated amidase at pH 9 could also form into a high molecular mass when the amidase was simply exposed to neutral pH; the dissociated amidase (fractions 39–43 in Fig. 4B) was dialyzed against pH 7 and then refractionated on a Sephadex G-200 column. The enzyme activity was detected at a similar position to that of the high molecular mass C-amidase (Fig. 4C). These results indicate that aggregation and dissociation of choline-treated E-amidase is reversible and controlled *via* pH shift in the absence of choline.

Similar dissociation at pH 9 was observed when the high molecular mass amidase obtained by other aminoalcohol-mediated conversion at pH 7, such as *N*-methylaminoethanol (MEA), 2-ethylaminoethanol (EEA) and 2-diethylaminoethanol (DEEA), was exposed to pH 9 (data not shown).

Fig. 4. Dissociation and Reaggregation of the High Molecular Mass, C-amidase under Alkaline Conditions.

E-amidase (500 units) was incubated with choline and dialyzed against Tris buffer (pH 7) as described in the legend to Fig. 1. The dialyzed sample was passed through a Sephadex G-200 column equilibrated with the same buffer. Fractions containing the high molecular mass amidase (see Fig. 1A) were pooled and served as choline-converted amidase. The choline-converted amidase was then dialyzed against 50 mM Tris-HCl at pH 7 (A) and pH 9 (B). These dialyzed samples were put on a Sephadex G-200 column equilibrated with the same pH-buffer as used for dialysis, respectively. Two and a half ml of fractions were collected and amidase activity was measured as described in the legend to Fig. 1.

The dissociated amidase at pH 9 (fractions 39–43 in panel B) was pooled and dialyzed against 50 mM Tris-HCl buffer at pH 7. The dialyzed sample was refractionated (panel C). The arrow indicates the elution position of blue dextran and the bar that of E-amidase.

Discussion

Pneumococcal inactive E-amidase can be converted *in vitro* into the active form by incubation with choline and its subsequent removal.^{12,13} The aggregation of E-amidase was observed upon the removal of excess choline.¹⁵ The results described in this paper showed that the step of removal of the excess choline was sensitive to alkaline pH; when choline was removed at pH 9, the aggregation of the amidase was severely inhibited. Due to these findings, we were able to produce non-aggregated amidase in the absence of choline, which seemed to be the intermediate molecule in the choline-mediated conversion. It is interesting to note that the non-aggregated amidase readily aggregates upon exposure to

neutral pH. Our observations indicate the following reversible aggregation and dissociation of choline-treated amidase *via* pH shift in the absence of choline.

It became clear that E-amidase had acquired a tendency to aggregate during the incubation with choline. What is the effect of choline on E-amidase? It is possible that choline molecules induce some structural or conformational change in E-amidase that is essential for the aggregation under the proper conditions. This is consistent with the recent observation from circular dichroism study of the amidase that the conformational change of C-terminal regions of the amidase and the pneumococcal phage lysin occurred in the presence of choline.^{20,21)}

It still remained obscure whether or not the aggregation of E-amidase is necessary for the appearance of its catalytic activity, since choline-treated amidase readily aggregates at neutral pH, at which the enzyme activity is assayed, and aggregation may occur before the enzyme acts on the substrate. Further investigation will be necessary to elucidate the possible role of choline in the formation of a high molecular mass, active amidase during conversion of inactive E-amidase.

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