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Structural requirements of choline derivatives for 'conversion' of pneumococcal amidase

A new single-step procedure for purification of this autolysin

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Tertiary amines appear to be the minimal structure needed to convert in vitro the inactive form (E-form) of pneumococcal amidase to the catalytic active form (C-form). Diethylethanolamine was one of the compounds that converted the E-form, a finding that has been used successfully to develop an affinity chromatography system in DEAE-cellulose for the rapid and efficient purification of lytic enzymes of pneumococcus and its bacteriophages.

Choline; DEAE-cellulose; Amidase; Autolysin; (Pneumococcus)

1. INTRODUCTION

Streptococcus pneumoniae contains a powerful lytic enzyme, an *N*-acetylmuramoyl-L-alanine amidase, that participates in separation of daughter cells at the end of cell division [1] and in the irreversible effects (lysis and bactericidal action) caused by β-lactam antibiotics [2]. The active form of this amidase (C-form) is found only in pneumococci that contain choline in the teichoic acids of the cell wall and this aminoalcohol has been identified as an allosteric ligand necessary for recognition and degradation of cell walls by the enzyme. Replacement of choline by ethanolamine renders the cell wall of *S. pneumoniae* resistant to the action of the amidase, these ethanolamine-grown cells containing an inactive form of pneumococcal amidase (E-form). The E-form can be converted into the C-form in the presence of either the substrate (cell walls containing choline) or 2% choline. Depending on the procedure used to carry out the process of 'conversion', the molecular mass of the resulting C-form of the amidase varies: the cell wall-converted enzyme has a high molecular mass (>500 kDa) whereas the choline-converted form has a molecular mass of 36 kDa, identical to that of the E-form. These results mean that a change in the value of the molecular mass is not required for the conversion, although this change depends upon the substance used for activation [3,4].

The process of enzymatic activation has been recently studied at the molecular level using several deleted and fused amidases and we have demonstrated the importance of the C-terminus in catalytic activation of the wild-type enzyme [5]. To investigate further the biochemical basis of this process, we show here that a large number of small compounds with a chemical structure similar to that of choline are capable of converting the E-form autolysin to the C-form. One of these compounds has been used successfully to develop a procedure for the rapid purification of large amounts of pneumococcal amidase and of other lytic enzymes.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains*

S. pneumoniae R6 is a derivative of Rockefeller strain R36A. *Escherichia coli* RB 791 (pGL100) is a strain that overproduces the pneumococcal amidase [6].

2.2. *Assay of autolytic activity*

Assay conditions for testing compounds used for activation were as follows: 100 μl of extracts obtained by sonicating washed cells of *E. coli* RB791, containing the E-form amidase, in TM buffer (50 mM Tris-maleate, pH 6.9) were incubated with 10 μl of each choline-related compound (from Merck or Fluka) at a final concentration of 0.1 or 0.5 M for 15 min at 0°C ('activation step'). Subsequently, the mixture was diluted with 1000 vols TM buffer to avoid the inhibitory effect of the compounds tested on the enzymatic reaction. 10 μl diluted enzyme were added to a reaction mixture containing 10 μl [^{3}H]choline-labeled cell wall extract and 200 μl TM buffer. The mixture was incubated for 10 min at 37°C, the reaction being stopped and processed as in [3].

3. RESULTS AND DISCUSSION

3.1. *Effect of choline-like molecules on the conversion process*

Extracts obtained from *E. coli* RB791 containing the E-form of the amidase were used to test the capacity of several compounds with a chemical structure similar to that of choline to convert the E-form of the enzyme to the C-form. Table 1 shows that choline (compound A) can mediate the process of conversion of the E-form of the enzyme as recently demonstrated [4]. Dimethylethanolamine (B) was also capable of converting the enzyme whereas monomethylethanolamine (C) and ethanolamine (D) were not. These results are consistent with previous observations that choline and phosphorylcholine block the hydrolytic activity of the pneumococcal autolysin in a noncompetitive fashion and suggest that these compounds can be efficiently bound to the enzyme [7]. In contrast, ethanolamine, phosphorylethanolamine and monomethylethanolamine had no effect on enzyme adsorption to the cell wall or on hydrolytic activity [7]. There are few data available on the effects of dimethylethanolamine on the pneumococcal amidase. It has been shown that this compound, when replacing choline in the culture medium, can be incorporated into teichoic acids of the cell wall [8]. In addition, on infection of these bacteria with bacteriophage Dp-1 up to 70% neutralization of Dp-1, with respect to that observed in pneumococci containing choline, was obtained [9]. These results suggest that only one methyl group can be removed to achieve partial activation of the amidase.

Replacement of a single methyl group of choline by a bulky benzyl group gives rise to a compound with a potentially steric impediment to use in activation. However, *N*-benzyl-*N,N*-dimethylethanolamine (E) was capable of converting the enzyme, indicating that the enzyme could accept at least a moderate increase in volume of the substituent in this part of the molecule. Furthermore, benzoylcholine (F) was shown to be a good activator of the enzyme, which is not surprising in view of the fact that the in vivo activator of the enzyme is the teichoic acid containing ribitol phosphorylcholine. Consequently, esterification of the alcohol group of choline should not represent a problem for the role of the compound as an activator. To investigate further the importance of the hydroxyl group, we substituted the ethanol group of dimethylethanolamine with ethanothiol (G) or hydroxypropyl (H) groups and found that both compounds were capable of converting the enzyme, indicating that the presence of the hydroxyl group was not relevant, since substitution by a thiol group does not cause any blockage in the activation process. This conclusion is best illustrated by the fact that tetramethylammonium (K), representing substitution of the ethanol group of choline by one of methyl, is also capable of converting the enzyme.

To ascertain whether the presence of two methyl groups was a specific requirement for conversion we substituted both methyl groups of dimethylethanolamine by two ethyl groups and observed that diethylethanolamine (I) was also a good activator, a finding that has been used successfully for purification purposes as discussed below. In addition, it is interesting to note that triethanolamine (J) was incapable of converting the enzyme when used at 0.1 M, although an activation effect was observed at 0.5 M, suggesting that the presence of polar groups might interfere with the conversion process.

The observation that the ethanol group did not represent a specific requirement for the conversion process prompted us to investigate the effect of several alkylamines. Triethylamine (N) and trimethylamine (L) turned out to be good ac-

Table 1

Analogs of choline as activators of the pneumococcal amidase

	Compound $R_2-N^+(R_1)(R_4)-R_3$				Conversion (concentration)	
	R_1	R_2	R_3	R_4	0.1 M	0.5 M
(A)	$-CH_2CH_2OH$	$-CH_3$	$-CH_3$	$-CH_3$	+	+
(B)	$-CH_2CH_2OH$	$-CH_3$	$-CH_3$	$-H$	+	+
(C)	$-CH_2CH_2OH$	$-CH_3$	-H	-H	−	−
(D)	$-CH_2CH_2OH$	-H	-H	-H	−	−
(E)	$-CH_2CH_2OH$	$-CH_2Ph$[a]	$-CH_3$	$-CH_3$	+	+
(F)	$-CH_2CH_2OCOPh$	$-CH_3$	$-CH_3$	$-CH_3$	+	+
(G)	$-CH_2CH_2SH$	$-CH_3$	$-CH_3$	-H	+	+
(H)	$-CH_2CH_2CH_2OH$	$-CH_3$	$-CH_3$	-H	+	+
(I)	$-CH_2CH_2OH$	$-CH_2CH_3$	$-CH_2CH_3$	-H	+	+
(J)	$-CH_2CH_2OH$	$-CH_2CH_2OH$	$-CH_2CH_2OH$	-H	−	+
(K)	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	+	+
(L)	$-CH_3$	$-CH_3$	$-CH_3$	-H	−	+
(M)	$-CH_3$	$-CH_3$	-H	-H	−	−
(N)	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	-H	+	+
(O)	$-CH_2CH_3$	$-CH_2CH_3$	-H	-H	−	−
(P)	$-CH_2CH_2CH_3$	-H	-H	-H	−	−

[a] Ph, phenyl

tivators, although the latter was active only when used at 0.5 M. In contrast, dimethyl- (M), diethyl- (O) and propyl- (P) amines were not capable of activating the amidase even at 0.5 M, indicating that only tertiary amines were able to activate the enzyme.

From the above results we might suggest that tertiary alkylamines are the minimal structures required for in vitro catalytic activation of the E-form of the pneumococcal amidase. On the other hand, we have recently found that the carboxyl-terminal domains of pneumococcal amidase and of the muramidase coded by pneumococcal bacteriophage Cp-1 show considerable homology and appear to be involved in specific recognition of choline-containing cell walls [10]. This domain shows a set of repeated sequences, each of about 20 amino acid residues in length, where a group of four hydrophobic amino acids (a tryptophan, two tyrosines and a phenylalanine or leucine) are always present in the middle of the six repeated sequences. Taking together these findings and the observation that the presence of polar groups blocks the process of activation as shown above, it is tempting to speculate on the importance of a hydrophobic interaction between choline derivatives and these hydrophobic domains of the E-form amidase for catalytic activation of the enzyme.

3.2. *Rapid purification of pneumococcal amidase on DEAE-cellulose columns*

The interaction between diethylaminoethanol (DEAE) and pneumococcal amidase suggested the possibility that the use of DEAE-cellulose would lead to the development of an affinity chromatography system for rapid purification of the enzyme. When crude extracts obtained by sonication of *E. coli* RB791 (pGL 100) were applied onto a DEAE-cellulose column, washed with 3 vols TM buffer containing 1.5 M NaCl, the enzyme was adsorbed to the column due to the affinity of the amidase for DEAE and was later eluted with TM buffer plus 1.5 M NaCl containing 2% choline. A prominent band of molecular mass 36 kDa corresponding to that of the E-form amidase was found when the active fractions eluting from the column were analyzed for

homogeneity by SDS-PAGE (fig.1). The presence of a slight band of lower molecular mass in the cloned protein seems to represent a degradation product of the enzyme, since this band increases upon storage. It is important to emphasize that, as reported [4], the choline-eluted fractions showed C-form activity, although E-amidase had been applied. This purification system presents remarkable advantages over affinity chromatography on choline-Sepharose. Thus, DEAE-cellulose is a cheaper product and very easy to handle. Its use avoids the expensive and time-consuming manipulation needed to carry out the coupling reactions of choline with Sepharose. Furthermore, DEAE-cellulose columns can be easily regenerated by conventional chemical treatments with NaOH and HCl. This procedure avoids the presence of residual amounts of enzymes that have been previously purified on the columns, a difficult task when using choline-Sepharose matrixes. Under our experimental conditions up to 1 mg highly purified amidase can be obtained from one cycle of purification, using a DEAE-cellulose column of 5 ml. Finally, DEAE-cellulose has also been used successfully to purify a pneumococcal phage-coded muramidase and a new lytic enzyme (a glycosidase) present in *S. pneumoniae* (not shown).

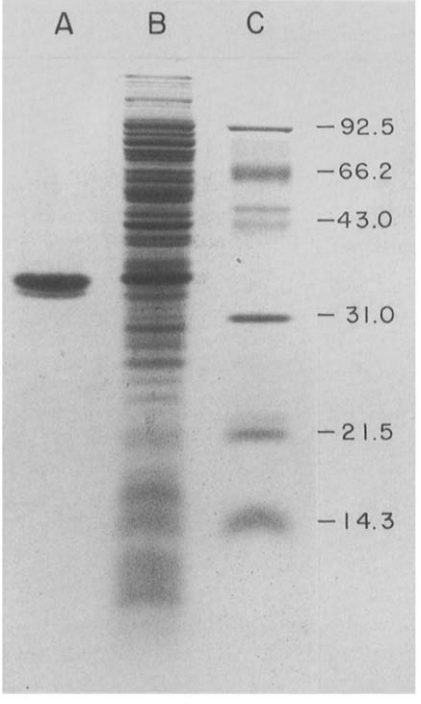

Fig.1. Purification of pneumococcal amidase on DEAE-cellulose. (A) Purified pneumococcal amidase obtained by affinity chromatography on DEAE-cellulose. (B) Total extract obtained from *E. coli* RB791 (pGL100). (C) Protein standards (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor; lysozyme). Molecular masses are indicated (in kDa).

3.3. *Biological effect of DEAE on* S. pneumoniae *cultures*

We were interested in establishing the effect of DEAE on the amidase activity in vivo. *S. pneumoniae* grown in C-medium [4] in the presence of 0.1 M DEAE shows inhibition of cell separation and therefore of chain formation (fig.2) without affecting the growth rate. The morphology of these long chains was somehow different from that previously found when pneumococcus was grown in the presence of 2% choline, ethanolamine or lipoteichoic acid [4], suggesting that DEAE might be incorporated as a component of teichoic acids of the cell wall, a possibility that is currently under investigation.

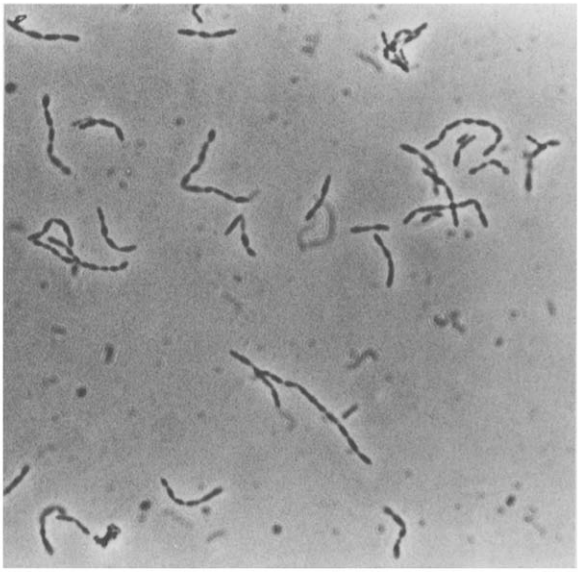

Fig.2. Effect of DEAE on *S. pneumoniae* cell morphology. Pneumococci were grown in CpH 8 medium [3] containing 2% DEAE and a sample was withdrawn at the mid-log phase of growth and photographed (× 940).

Furthermore, no lysis in the stationary phase of growth was observed and a penicillin-tolerance response was found when these cultures were incubated in the presence of penicillin (0.1 μg/ml). Addition of 2% DEAE to the growing medium also prevented the detergent-induced lysis typical of cultures of pneumococcus (not shown). On withdrawal of DEAE from the medium and growing of cells in fresh C-medium, *S. pneumoniae* recovers its normal biological properties. These experiments demonstrate that the presence of a high concentration of DEAE leads to phenotypical characteristics of pneumococci in which the endogenous activity of the autolytic amidase is inhibited in a reversible way.

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