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THE SULFHYDRYL GROUPS OF THE THIOL-DEPENDENT CYTOLYTIC TOXIN FROM *Bacillus alvei* EVIDENCE FOR ONE ESSENTIAL SULFHYDRYL GROUP

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SUMMARY

Alveolysin, an extracellular protein toxin ($M_r \simeq 63,000$) excreted by *Bacillus alvei* and purified to homogeneity was shown to contain four cysteine residues. All thiol groups of the hemolytically active toxin preparation were free as found by direct titration by 5,5'-dithiobis (2-nitrobenzoic acid) and confirmed by the absence of disulfide bond. Toxin alkylation with tosyl lysine chloromethyl ketone resulted in the complete loss of hemolytic activity and the disappearance of only one thiol group with no modification of histidine residues. These results support the conclusion that one essential thiol group is implicated in the membrane-disrupting activity of alveolysin.

INTRODUCTION

Alveolysin, a 63,000-dalton, cytolytic and lethal extracellular protein excreted by *Bacillus alvei* (1,2) has been purified to apparent homogeneity (2). This protein toxin is a member of the group of the sulfhydryl-dependent (or oxygen-labile) cytolysins elaborated by gram-positive bacteria of the species *Streptococcus, Clostridium, Bacillus and Listeria* (3→6). These cytolysins share a number of common properties : (i) they are antigenically related, (ii) they are inactivated by cholesterol and related sterols, (iii) their lytic, lethal

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Abbreviations : TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone ; DTT, dithiothreitol ; HU, hemolytic unit ; BAEE, α-N-benzyl-L-arginine ethyl ester ; PBS, phosphate-buffered saline ; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid).

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and other biological effects are lost by oxidation or treatment with thiol-blocking agents and restored by thiol compounds and other reducing agents (3,6). Only reduced toxin binds specifically to its receptor (cholesterol) on eukaryotic cell membrane (3→6).

Our knowledge on the -SH group(s) of the sulfhydryl-dependent cytolysins is still very limited and sequence data are lacking. It has been assumed that these toxins contain at least one disulfide bond in the oxidized (inactive) form which is reduced to free -SH groupsin the activated state (5). Cowell *et al.* (7) detected two half-cystine residues per molecule of cereolysin, thereby providing the first chemical evidence for substantiation of the sulfhydryl mechanism of activation. Two cysteine residues were found in pneumolysin (Mary K. Johnson, personal communication) and one in perfringolysin O (clostridium theta-toxin) (8). The molecular mechanism by which the sulfhydryl group(s) of toxin molecules contribute to the lytic process remains to be elucidated.

As a step toward a better understanding of this mechanism, it appeared interesting to undertake a detailed study of sulfhydryl groups of alveolysin purified by an improved procedure. Four sulfhydryl groups are found in the native toxin molecule. Evidence is provided by selective alkylation with tosyl lysine chloromethyl ketone (TLCK) that one sulfhydryl group is implicated in the cytolytic (hemolytic) activity of the toxin.

MATERIALS AND METHODS

Toxin purification and assay. Alveolysin was purified from the supernatant fluid of 16-liter culture of *Bacillus alvei* strain ATCC 6344 cultivated for 18 hrs at 37°C on a medium containing per liter : Trypticase (Bio-Mérieux, Paris) 30 g ; yeast extract (Difco) 20 g ; Na_2HPO_4. 12 H_2O, 8 g ; NaH_2PO_4. 1 H_2O, 0.7 g ; glucose 5 g ; pH 7.5. The supernatant fluid was concentrated ($\simeq$ 50 fold) by ultrafiltration on Amicon Hollow Fiber H 1 X 50 (DC2 apparatus) (Amicon, Lexington, Mass.). Toxin purification involved four steps (Geoffroy and Alouf, manuscript in preparation). The crude concentrated fluid was chromatographed on a thiopropyl-Sepharose 6B gel (Pharmacia, Uppsala, Sweden) column and the bound material was eluted with dithiothreitol (DTT, Sigma) in phosphate-buffered saline (sodium phosphate 70 mM, NaCl 70 mM) (PBS), pH 7.4. The hemolytic pool from the eluted material was then further purified by gel filtration chromatography on a Sephacryl S-200 gel column (Pharmacia). The active fraction was further chromatographed on Bio-Gel P-100 (Bio-Rad, Richmond, Calif.) column. The hemolytic material constituted the purified toxin which was shown to be homogeneous by immunochemical analysis. A single band was revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reduced conditions. The mol. wt. was 63,000. The hemolytic activity of the toxin was assayed on sheep erythrocytes suspension as reported previously (2). One hemolytic unit (HU) was defined as the amount of test material needed to release the hemoglobin from 50 % of

the erythrocytes. No increase of the hemolytic activity of the purified toxin preparation was observed upon adding cysteine or DTT indicating optimal active state of the toxin. The specific activity of alveolysin preparation was 10^6 HU/mg protein (one HU is equivalent to 1 ng protein, *i.e.* 1.6×10^{-5} nmoles).

Titration of disulfide groups by electrolytic reduction. Total disulfide content of alveolysin was determined by the method of Weitzman (9) based on electrolytic cleavage of disulfide bonds at mercury pool cathode in acidic buffer containing guanidine/HCl, followed by estimation of the liberated thiol with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (10).

Titration of thiol groups with 5,5'-dithiobis (2-nitrobenzoic acid). Free -SH groups of alveolysin were determined on aliquots of native and denatured (see preceding paragraph) toxin preparation. In all instances, thiol estimations were calculated on a basis of a molar absorbance of 13,600 for 5-carboxy-4-nitrophenol at 412 nm. The A_{412} was read against a toxin-free blank treated in parallel.

Inhibition by N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK). Toxin reaction with TLCK (Sigma) was carried out on 1 mg of alveolysin in 1 ml of PBS buffer pH 6.8 by incubation with 100 µl of 10^{-2} M TLCK in distilled water for 30 min at 37°C. The mixture was further dialyzed against PBS and lyophilized.

Reduction and carboxymethylation. The toxin was substituted as described by Waxdal *et al.* (11). It was denatured in 0.5 M Tris/HCl buffer pH 8.1 containing 6M guanidine/HCl and 0.002 M EDTA. To this solution, DTT (50 moles/mole of sulfhydryl groups in the protein) was added and the tube was flushed (30 s) with nitrogen, then maintained at 50°C for 3-4 hrs. The tube was then cooled at room temperature and a solution of recrystallized iodoacetic acid (2-fold molar ratio to the DTT added) in 1N NaOH was added. After alkylation for 20 min in the dark, reaction mixture was dialyzed against 0.01 M ammonium bicarbonate and lyophilized.

Amino acid analysis. Amino acid analysis of alveolysin was performed on a Beckman Multichrom B with a ICAP 10 integrator calculator, using a single column procedure (12). Toxin samples (1 mg) were hydrolyzed *in vacuo* in 6N HCl for 20 hrs. Half-cystine was estimated as cysteic acid after performic oxidation (13) or as S-carboxymethyl cysteine.

Testing esterase and proteolytic activities. Esterase activity was tested according to Mitchell and Harrington (14) on 50 µg (10^4 HU) of alveolysin. Toxin sample was incubated with α-N-benzoyl-L-arginine ethyl ester (BAEE) (Sigma) using 2 ml aliquots of buffer (2.5 mM DTT, 50 mM Tris/HCl, pH 7.4) containing 7.5×10^{-4} M BAEE. A positive control was run with a sample of the thiol protease, clostripain (EC 3.3.22.8) as described by Gilles *et al.* (15). Proteolytic activity was assayed by the Azocoll substrate (Calbiochem, Los Angeles, Ca) method described by Jackson and Matsueda (16).

RESULTS

Sulfhydryl groups content of alveolysin. The determination of the thiol groups of the toxin under various conditions is reported in Table 1. Amino acid analysis showed four cysteine residues on the basis of a molecular weight of 63,000. In the native toxin preparation four thiol groups were directly accessible to 5,5'-dithiobis (2-nitrobenzoic acid) reagent. Titration of the denatured preparation by the same reagent did not disclose

TABLE 1

EFFECT OF TLCK ON SULFHYDRYL GROUPS OF ALVEOLYSIN

	Apparent -SH groups	Histidine residues[e]
Native toxin	3.8[a]	9.7
	≃ 4.0[b]	
Denatured toxin (guanidine/HCl)	≃ 4.0[b,c]	
TLCK-treated toxin	≃ 2.7[d]	9.7

a) Determined by amino acid analysis as cysteic acid after performic oxidation
b) Titration by 5,5'-dithiobis (2-nitrobenzoic acid)
c) Electrolytic cleavage by the method of Weitzman (9)
d) Determined by amino acid analysis as S-carboxymethyl-cysteine as described in Methods
e) Determined by amino acid analysis.

additional thiol groups. Moreover no disulfide bond was found as shown by the electrolytic cleavage method. These results indicate that four free thiol groups are present on the hemolytically active toxin molecule as obtained by the thiol-Sepharose chromatographic procedure described.

Effect of TLCK and other sulfhydryl reagents on the hemolytic activity of alveolysin. The hemolytic activity of alveolysin tested on sheep erythrocytes (5 HU toxin, equivalent to 5 ng incubated with $3x10^8$ erythrocytes) in the presence of increasing amounts of TLCK (Table 2) at 37°C was completely inhibited by 1 mM of this reagent. Inhibition was temperature-dependent as no inactivation of the lytic activity was observed

TABLE 2

INHIBITION OF THE HEMOLYTIC ACTIVITY OF ALVEOLYSIN BY TLCK

Residual activity %	TLCK mM
100	none
80	0.01
70	0.05
60	0.10
44	0.50
0	1

Alveolysin was incubated at 37°C with TLCK in PBS pH 6.8 for 15 min and assayed as described in the text.

at 22°C. In contrast, inhibition over a temperature range of 5-37°C was observed with a variety of SH-blocking agents such as p-chloromercuribenzoate, N-ethylmaleimide, DTNB, 4,4'-dithiopyridine and $HgCl_2$ under similar concentrations of toxin and reagent (unpublished data). Inhibition by TLCK and N-ethylmaleimide was not reversed by adding thiols in contrast to the other reagents mentioned.

Carboxymethylation of TLCK-treated alveolysin. Toxin preparation (1 mg) treated with an excess of TLCK was modified by iodoacetic acid under denaturing and reducing conditions (see Methods). As shown in Table 1, only 2.7 residues of S-carboxymethylcysteine were found. No substitution of histidine residues was observed by amino acid analysis.

Testing for a possible proteolytic or esterolytic activity of alveolysin. No evidence for such an activity was found by using Azocoll reagent treated with 10 µg (10^4 HU) of toxin under the conditions described by Jackson and Matsueda (16). On the other hand, no hydrolysis of BAEE was observed under the conditions stated in Methods.

Effect of protease inhibitors on the hemolytic activity of alveolysin. No inhibition of the hemolytic activity of a toxin preparation (20 HU) with either diisopropylfluorophosphate (10^{-3} M) or benzamidine (10^{-2} M) was observed.

DISCUSSION

Among the fifteen antigenically, chemically and functionally related sulfhydryl-dependent bacterial cytolysins, only streptolysin O, pneumolysin, listeriolysin, perfringolysin O, cereolysin and alveolysin have been purified to homogeneity (see 5,6,17 for a review). These toxins are constituted by a single-chain polypeptide of molecular weight ranging from 51,000 (perfringolysin O) to 68,000 (streptolysin O).

As stated by Smyth and Duncan (5), a fuller understanding of the mode of action of these toxins at the molecular level can only ultimately be obtained through better knowledge of structure-activity relationship. To this end the characterization and study of the -SH group(s) of the purified toxins are obviously of great interest. The cysteine content of streptolysin O and listeriolysin is still not known. The first report on cysteine residues was that of Cowell *et al.* (7) who found two half-cystine residues in cereolysin produced by *B. cereus*. By polyacrylamide gel electrophoresis, two forms of the toxin separable on charge basis were observed : oxidized (inactive) and reduced (active). The former disappeared upon mild reduction of toxin prepa-

ration with DTT. This finding was consistent with the idea that the -SH groups are in a disulfide bond in the oxidized form. Pneumolysin also contained two cysteine residues (see Introduction). In contrast, one cysteine residue was found in perfringolysin O by Yamakawa *et al.* (8). In this case, an intramolecular disulfide bridge in the oxidized form of the toxin is not conceivable. These investigators considered that the oxidized toxin has been formed through intermolecular disulfide bridge between the free -SH group and thioglycolic acid present throughout the purification procedure. Previously, Mitsui *et al.* (18) did not find cysteine residues in a preparation of perfringolysin of lower specific activity and amino acid composition different from Yamakawa's preparation. Moreover, Mitsui *et al.* (19) did not use performic acid-oxidized protein in their analysis and if the toxin contains one cysteine residue, conventional analysis may not detect it (7). Recently, these investigators (19) suggested that the active form of the toxin had free -SH groups which are changed to disulfide bonds with loss of activity by exchange reactions with disulfide reagents.

The work reported here showed that alveolysin in the optimal active (hemolytic) state contained four cysteine residues. All thiol groups were free, as found by titration of these groups, on both native and denatured toxin. This conclusion was confirmed by the absence of disulfide bonds. The presence of four cysteine residues instead of one or two as found for the three toxins mentioned above is not surprising. It is well established that SH-dependent cytolysins show dissimilarities in amino acid composition, isoelectric point and molecular weights (5).

A new insight into structure-activity relationship resulted from alkylation of alveolysin by TLCK used here for the first time as a probe of a sulfhydryl-dependent toxin. TLCK was first used as an alkylating agent designed as an active site-directed inhibitor of trypsin in which case it stoichiometrically reacted with a single histidine residue (20). It was further shown that TLCK was capable to alkylate one essential thiol-group at the active site of three thiol-proteinases, papain (21), ficin (22) and clostripain (15, 22). In all cases, enzymatic activity was lost. For the latter enzyme histidine residues were not modified (22). TLCK interaction with alveolysin gave similar results. Approximately one cysteine residue disappeared after reaction with the reagent and no modification of histidine residues was found. Hemolytic activity was completely lost in parallel. TLCK appears therefore as an -SH reagent which selectively modifies one thiol

group of alveolysin. Other related ketones will probably act similarly. This thiol group is obviously the most reactive of the four thiol groups of the molecule. This property leads to infer that the TLCK-sensitive cysteine residue is essential for toxin binding (the oxidized toxin does not bind) on target cells as the primary step in membrane disruption. It should be noted that alveolysin and very likely related toxins are neither proteolytic nor esterolytic proteins as shown here and in other studies (5).

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