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The Thiol-Activated Toxin Streptolysin O Does Not Require a Thiol Group for Cytolytic Activity

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Site-directed mutagenesis of the TGC codon in a cloned streptolysin O (SLO) gene exchanged the single Cys residue in SLO for either Ala or Ser. The parent wild-type SLO (SLO.Cys-530) and the SLO.Ala-530 and SLO.Ser-530 mutant toxins, expressed in *Escherichia coli*, were purified and analyzed. Wild-type SLO.Cys-530 and the SLO.Ala-530 mutant showed no significant differences in their specific hemolytic activities, while the SLO.Ser-530 mutant had a reduced (ca. 25%), but still considerable, specific hemolytic activity as compared with that of wild-type SLO. The parent and mutant toxins extracted from lysed erythrocyte membranes had similar sedimentation profiles on sucrose density gradients, suggesting that the mutations did not affect the ability of SLO to form oligomers in membranes. These results show that the widely held assumption that the in vitro cytolytic activity of SLO requires an essential Cys residue is not true.

Fifteen taxonomically diverse species of gram-positive bacteria, from the genera *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria*, produce a group of immunologically cross-reactive and biologically related membrane-damaging toxins called the "thiol-activated" or "oxygen-sensitive" toxins (for reviews, see references 1, 4, 6, and 24). With one exception (pneumolysin from *Streptococcus pneumoniae*), all are secreted by the producing organisms (1, 12, 24). These toxins damage cholesterol-containing membranes and hence lyse a wide range of eucaryotic cells (1, 3, 10, 24). Their cytolytic activities are irreversibly inhibited by prior incubation of toxin with cholesterol or a number of structurally related sterols (1, 9, 13). Studies on a number of thiol-activated toxins have shown that they bind to cholesterol-containing membranes in a temperature-independent manner and oligomerize in the membrane in a temperature-dependent manner to form large arc- and ring-shaped structures composed of 25 to 100 toxin monomers (1, 6, 7, 10). Once formed, the oligomers appear to be quite stable, and intact oligomers have been isolated by gentle solubilization of lysed erythrocyte membranes (7). Isolated oligomers have been shown not to contain detectable quantities of cholesterol, but they can insert into cholesterol-free liposomes, suggesting that cholesterol may be required only for the initial stages in the cytolytic process (7). Although the role of these toxin oligomers in membrane disruption has not been clearly established, freeze-fracture studies suggest that they span the membrane and, concomitant with their formation, high-molecular-weight cytoplasmic molecules leak from the cell, resulting in cell death (1, 3, 4, 6, 10, 24). Streptolysin O (SLO), from *Streptococcus pyogenes*, is one of the best-characterized thiol-activated toxins and is often considered to be the prototype of this group.

As their name implies, the cytolytic activity of thiol-activated toxins in culture supernatants is rapidly lost upon exposure to air, but completely restored by the addition of reducing agents (4, 24). This has led to the belief that sulfhydryl (—SH) groups play a critical role in toxin activity, a suggestion that is supported by the fact that thiol-blocking agents completely inhibit the cytolytic activity of SLO, pneumolysin, listeriolysin from *Listeria monocytogenes*, and alveolysin from *Bacillus alvei* (1, 2). Recently, the genes for four thiol-activated toxins (SLO, pneumolysin, listeriolysin, and perfringolysin O from *Clostridium perfringens*) have been cloned and sequenced (14, 15, 19, 25–27). A comparison of the deduced amino acid sequences revealed that all four toxins share extensive primary sequence homology (between 42 and 65% of the residues match) and that each toxin contains only one cysteine residue, located at identical positions close to the C-terminal ends of the molecules. This Cys is located within a conserved 12-amino-acid sequence that corresponds to the longest continuous region of identity among the four molecules (Fig. 1), suggesting that it contributes to a functionally important structure (14, 19, 26). Although a single cysteine excludes earlier suggestions that sensitivity to oxidation is due to the formation of intramolecular disulfide bonds, it does not exclude a critical functional role for Cys in toxin activity, as suggested by inhibition with thiol-blocking agents (1, 2). Indeed, the single Cys residue is frequently referred to as the "essential Cys" (1, 6, 26).

To examine directly the role of Cys in the activity of thiol-activated toxins, we have constructed and analyzed a number of mutant SLO derivatives that differ only with respect to the Cys residue. Here we report that a Cys residue is not essential for SLO cytolytic activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* LE392 [F$^-$ *hsdR514 supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ$^-$] and JM103 [Δ(*lac pro*) *thi strA supE endA sbcB hsdR* (F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15)], which were used as the hosts for recombinant plasmids and M13 phages, have been described by Maniatis et al. (18) and Messing et al. (20). Phages M13mp18 and M13mp19 and the vector plasmid pUC18 have been described by Yanisch-Perron et al. (28). The construction of the SLO$^+$ hybrid plasmid, pMK157, has been described previously (15), and the construction of other plasmids is described in the Results. Growth conditions for *E. coli* strains and phages were as described previously (15, 17).

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Toxin	(Residues)	
SLO	(525 - 542)	I M A R E C T G L A W E W W R K V I
PLY	(423 - 440)	V K I R E C T G L A W E W W R T V Y
LLO	(479 - 496)	V Y A K E C T G L A W E W W R T V I
PFO	(453 - 470)	I K A R E C T G L A W E W W R D V I

FIG. 1. Conserved sequences encompassing cysteine in SLO, pneumolysin (PLY), listeriolysin (LLO), and perfringolysin O (PFO). The relationships between the complete sequences of these toxins are described in detail in references 14, 19, and 26.

DNA isolation, cloning, sequencing, and synthesis procedures. Restriction endonucleases, T4 DNA ligase, and DNA polymerase (Klenow) were obtained from CP Laboratories (Bishops Stortford, United Kingdom), NBL Ltd. (Cramlington, Northumberland, United Kingdom), or Amersham International (Amersham, United Kingdom) and were used according to manufacturer instructions. The procedures for plasmid or phage DNA purification, cloning, and sequencing have been described previously (14, 15, 17, 21). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (model 381A), using reagents obtained from Applied Biosystems (Warrington, United Kingdom) and following the manufacturer's instructions. Before use, the purity of oligonucleotides was confirmed by electrophoresis on 20% (wt/vol) polyacrylamide gels, and if necessary the oligonucleotides were purified from the gel (11).

Construction of a stable, high-copy-number SLO$^+$ plasmid, pMK206. The structure of the low-copy-number SLO$^+$ hybrid plasmid pMK157 and the nucleotide sequence of the cloned *slo* gene have been described previously (14, 15). Previous attempts to clone the streptococcal DNA sequences from pMK157 into high-copy-number plasmid vectors failed due to detrimental effects on the host strain that rapidly selected for plasmids where the streptococcal sequences had been deleted (15). Plasmid pMK157 contains 6.2 kilobase pairs of cloned streptococcal DNA, and the *slo* gene was located at one end of these sequences, within 50 base pairs (bp) of the vector-cloned DNA junction (15). In this study, a 2.0-kilobase-pair *Fsp*I-generated fragment from pMK157, containing the entire *slo* gene and 115 bp of adjacent plasmid vector sequences, was cloned into the *Sma*I site of pUC18 (28) to produce a high-copy-number SLO$^+$ hybrid plasmid termed pMK206. The *Fsp*I fragment cloned in pMK206 extends from 124 bp 5′ to the *slo* gene in pMK157 into vector sequences 165 bp beyond the 3′ end of *slo*. *E. coli* containing pMK206 expresses SLO at sufficient levels to facilitate its purification, though this expression is not controlled by the inducible pUC18 *lac* promoter. Cells containing pMK206 are stable, indicating that earlier stability problems were due to streptococcal sequences outside the *slo* gene.

Construction of SLO mutants. The procedure used to exchange the single Cys codon (TGC) in *slo* for other codons is outlined in Fig. 2. Digestion of pMK206 DNA with the restriction endonucleases *Sna*BI and *Bam*HI generates two fragments, which were separated and purified by agarose gel electrophoresis. The single *slo* TGC codon is located within 20 bp of the *Sna*BI-generated end of the smaller (322 bp) of these two fragments. *Hpa*II, which produces 14 fragments on digesting pMK206 DNA, cleaves the small, 322-bp *Sna*BI-*Bam*HI pMK206 fragment once, to generate a 22-bp *Sna*BI-*Hpa*II fragment containing the TGC codon and a 300-bp *Hpa*II-*Bam*HI fragment containing the 3′ end of *slo*. These fragments were separated by gel electrophoresis, and the *Hpa*II-*Bam*HI fragment was recovered from the gel.

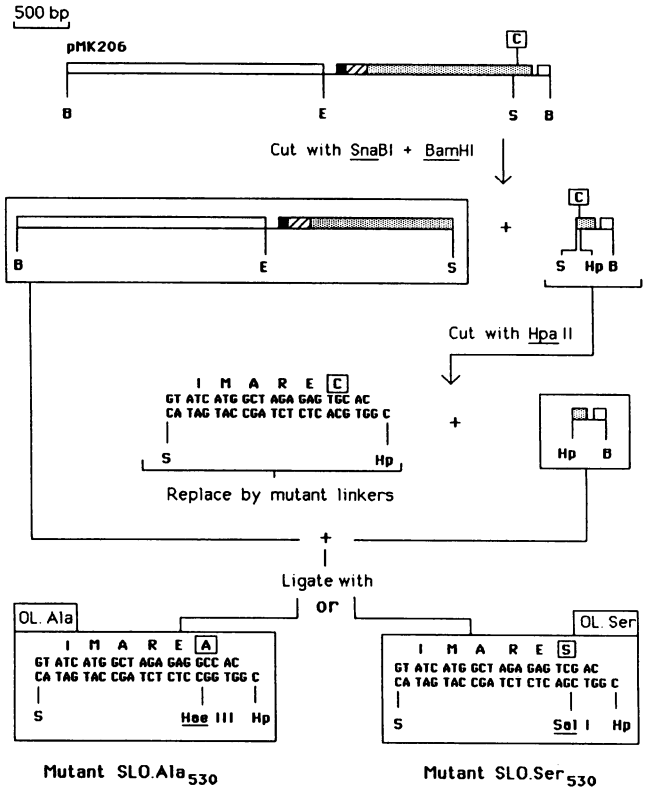

FIG. 2. Construction of SLO.Ala-530 and SLO.Ser-530 mutants. The structure of the SLO$^+$ hybrid plasmid, pMK206, is outlined at the top of the diagram, and the strategy used to construct the site-directed mutations is outlined below. In the line drawings, the blank boxes represent the vector DNA and the shaded boxes represent the *slo* gene. The black box at the left end of the *slo* gene corresponds to the predicted signal peptide, and the adjacent box (hatched) corresponds to the N-terminal sequences in the secreted high-molecular-weight form of SLO that are thought to be removed subsequent to secretion to generate the low-molecular-weight form of the toxin (14). The single Cys codon in *slo* is indicated by the boxed C above the gene. The letters below the gene indicate cleavage sites for the following restriction endonucleases: B, *Bam*HI; E, *Eco*RI; S, *Sna*BI; Hp, *Hpa*II. The scale of the line drawings is indicated in the top left corner of the diagram. OL.Ala and OL.Ser describe the synthetic oligonucleotide linkers used to construct the mutants, and the restriction endonuclease fragments of pMK206 to which they were ligated are enclosed in boxes.

Short double-stranded linkers, designed to replace the 22-bp *Sna*BI-*Hpa*II fragment with sequences where the TGC codon was altered to GCC (Ala) or to TCG (Ser), were constructed by synthesizing and annealing complementary oligonucleotides. These linkers were designed so that the desired codon change would introduce a new restriction endonuclease cleavage site, in order to facilitate subsequent screening for mutants. These new sites are *Hae*III in the case of the GCC (Ala) linker and *Sal*I in the case of the TCG (Ser) linker. The large *Bam*HI-*Sna*BI pMK206 fragment and the 300-bp *Hpa*II-*Bam*HI fragment were ligated with either the GCC (Ala) or the TCG (Ser) linkers to generate the pMK206 mutants pMK206.Ala-530 and pMK206.Ser-530.

Peptide synthesis and antisera. Peptide SLOs[104-123], corresponding to residues 104 to 123 of the deduced SLO sequence (14), was synthesized, and antipeptide antiserum was produced as described previously (21).

Purification of SLO and its mutant derivatives. Wild-type

SLO and its mutant derivatives, expressed from cloned genes in *E. coli*, were purified using a procedure adapted from that of Bhakdi et al. (5). Preliminary experiments showed that both the parent and mutant toxins can be released from *E. coli* by an osmotic shock procedure designed to release periplasmic proteins (22), suggesting that they are located predominantly in the periplasm (data not shown), although this procedure was not efficient when scaled up for large culture volumes. For large-scale preparations, SLO was released from cells by a lysozyme-EDTA treatment that also resulted in a limited degree of cell lysis.

E. coli LE392, containing pMK206 or the mutant plasmids, was grown at 37°C, at 200 rpm to an A_{600} of 1.0 in 7.5 liters of L broth supplemented with 100 μg of ampicillin per ml, and the cells were harvested by centrifugation at ca. 10,000 × *g* for 5 min. All subsequent steps were carried out at 4°C. The cells were suspended in 300 ml of buffer A, which consisted of 25 mM Tris hydrochloride (pH 8.0) containing 50 mM NaCl, 1 mM EDTA, 5% (vol/vol) glycerol, 2 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. Lysozyme was added from a 1-mg/ml (in buffer A) stock solution to give a final concentration of 60 μg/ml. After 30 min, 30 ml of 250 mM EDTA (in buffer A) solution was added and the suspension was incubated for a further 15 min, followed by the removal of cells and cell debris by centrifugation at 16,000 × *g* for 20 min. The supernatant was rapidly passed over a column (18 by 2.5 cm) of DEAE-Sephacel which had previously been equilibrated with buffer A. The column was washed with the same buffer, and SLO or its mutant derivatives, which did not bind to the column, were recovered in the wash eluent. After hemolytically active fractions were pooled, 25 g of solid polyethylene glycol 8000 per 100 ml was added and stirred slowly into solution. After a further 30 min of stirring, the resulting precipitate was harvested by centrifugation at 18,000 × *g* for 1 h. The pellets were carefully drained and suspended in 75 ml of buffer B (10 mM Tris hydrochloride [pH 9.0], 3 mM sodium azide, 1 mM dithiothreitol) containing 10 mM NaCl. This material was then loaded onto a DEAE-Sephacel column (12 by 2.5 cm) that had been preequilibrated with buffer B containing 10 mM NaCl. The column was washed with 80 ml of buffer B + 10 mM NaCl and then with 20 ml of buffer B + 50 mM NaCl, before elution with a linear salt gradient formed from 200 ml of buffer B + 50 mM NaCl and 200 ml of buffer B + 200 mM NaCl. Fractions containing hemolytic activity were identified and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). Active fractions were combined according to their purity as judged by SDS-PAGE, dialyzed against buffer A, and then concentrated by ultrafiltration using a Filtron Technology Co. (Clinton, Mass.) ultrafiltration apparatus with a UF-polyethersulfone membrane, designed to retain globular proteins which have a molecular weight in excess of 50,000. A similar degree of purity was achieved with SLO and the mutant derivatives (Fig. 3). The purified toxins were stored at −80°C for up to 6 months without noticeable loss of activity or any effect on the stabilities of the high- and low-molecular-weight forms, as judged by SDS-PAGE. However, repeated freeze-thawing was found to be detrimental to the hemolytic activity and therefore the purified toxins were stored in small volumes.

Hemolytic titration. The cytolytic activity of SLO and its mutant derivatives were measured by assaying hemolytic activity, essentially as described by Bhakdi et al. (5). Human erythrocytes were washed three times with phosphate-buffered saline (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]) and resuspended to a concentration of 2.5×10^8 erythrocytes per ml in phosphate-buffered saline. Titrations were performed in phosphate-buffered saline containing 0.5% (wt/vol) bovine serum albumin. A 50-μl volume of the erythrocyte suspension was added to 50 μl of serially diluted toxin (twofold dilutions) in 96-well microtiter plates, and after the plates were incubated for 30 min at 37°C, the wells corresponding to ca. 50% hemolysis were determined visually. The hemolytic titer was defined as the reciprocal of the highest dilution of toxin that produced >50% hemolysis. When required, cholesterol was added to the toxin diluent (phosphate-buffered saline) to a concentration of 5 μg/ml. Protein concentration was determined by the Bradford assay (8), and specific activity was defined as the number of hemolytic units per milligram of protein.

Isolation of SLO oligomers. SLO oligomers were isolated from lysed human erythrocyte membranes by gentle solubilization with 250 mM sodium deoxycholate and purified by density gradient centrifugation, as described by Bhakdi et al. (7). After protein was separated from the gradient fractions on SDS-PAGE gels, SLO was identified by immunoblotting with rabbit anti-SLO peptide SLOs[104-123] as the primary antibody and with peroxidase-conjugated sheep anti-rabbit immunoglobulin (Sera-lab. Ltd., Sussex, United Kingdom) as the second antibody, as described previously (17).

RESULTS

SLO mutants. The cloning of the *slo* gene from *S. pyogenes* Richards, to produce a low-copy-number SLO^+ hybrid plasmid (pMK157), and the nucleotide sequence of the cloned *slo* gene have been described previously (14, 15). Plasmid pMK157 expresses low levels of SLO in *E. coli*, making purification of significant quantities of recombinant SLO a very difficult task. To overcome this problem, the *slo* gene from pMK157 was cloned into a high-copy-number vector (Materials and Methods) to produce the SLO^+ hybrid plasmid pMK206. The structure of pMK206 and the position of the single Cys codon in the cloned *slo* gene are described in Fig. 2. This Cys codon (TGC) was exchanged for either an Ala (GCC) or a Ser (TCG) codon by replacing a 22-bp restriction endonuclease-generated fragment of pMK206 with synthetic oligonucleotide linkers incorporating the desired mutant codon (Fig. 2). In designing the oligonucleotide linkers, new restriction endonuclease cleavage sites (*Hae*III in the case of the Cys→Ala mutant and *Sal*I in the case of the Cys→Ser mutant; see Fig. 2) were included to facilitate subsequent screening for mutants. When the resulting mutant plasmids, pMK206.Ala-530 (Cys-530→Ala-530) and pMK206.Ser-530 (Cys-530→Ser-530), were transformed into *E. coli* LE392 and transformants were selected on blood agar plates containing ampicillin, surprisingly, the majority of the transformants were hemolytic. Restriction endonuclease screening of plasmids isolated from hemolytic transformants showed that these plasmids produced the fragment patterns predicted for mutants pMK206.Ala-530 and pMK206.Ser-530. Over 200 bases spanning the putative TGC→GCC and TGC→TCG mutations in pMK206.Ala-530 and pMK 206.Ser-530, respectively, were sequenced, and the predicted structures of these plasmids were confirmed. This indicated that altering the single Cys residue in SLO to either Ala or Ser did not abolish its cytolytic activity.

Purification and specific cytolytic activities of the mutant toxins. To compare the cytolytic activities of the SLO.Ala-530 and SLO.Ser-530 mutant toxins with the activity of the

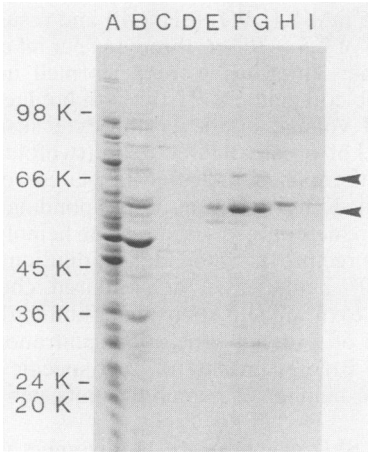

FIG. 3. Purification of SLO expressed in *E. coli*. Coomassie blue-stained, 12.5% (wt/vol) SDS-PAGE gel of protein from hemolytically active fractions at different stages in the purification procedure described in Materials and Methods. Tracks: A, protein released from cells by lysozyme-EDTA treatment; B, polyethylene glycol-precipitated protein from the first (pH 8.0) DEAE-Sephacel column; C through I, protein in representative active fractions from the second (pH 9.0) DEAE-Sephacel column. The positions of the high- and low-molecular-weight forms of SLO are indicated by the arrows to the right of track I. The mobilities of molecular size standards (Sigma Chemical Co., Poole, Dorset, United Kingdom) are indicated to the left of track A. Track F represents the material used for the experiments described in this paper. The SLO.Ala-530 and SLO.Ser-530 mutant toxins behaved in an identical manner to the wild-type SLO during this purification procedure, and material corresponding in purity to SLO in track F was used for further experiments.

SLO.Cys-530 parent, these toxins were purified from *E. coli* LE392 containing pMK206.Ala-530, pMK206.Ser-530, or pMK206, respectively. The specific hemolytic activity reported for the most highly purified preparations of SLO from *S. pyogenes* culture supernatants is 800,000 hemolytic units per mg of protein (1, 5). On SDS-PAGE, SLO purified from *S. pyogenes* culture supernatants resolves into a high-molecular-weight minor band and a lower-molecular-weight major band, and both forms have been shown to have identical specific hemolytic activities (5). Two similarly sized SLO antigens (M_r 68,000 and M_r 61,000) have been detected previously, by immunoblotting, in *E. coli* expressing the cloned gene (15). In this study, the recombinant wild-type SLO expressed in *E. coli* was purified to a specific hemolytic activity of 850,000 to 1,200,000 hemolytic units per mg of protein, which is equivalent to the best preparations obtained from *S. pyogenes* culture supernatants (differences of up to twofold in the measured hemolytic activity were considered to be within the bounds of experimental error, due to the nature of the assay). The purification of SLO from *E. coli* is summarized in Fig. 3. The purified toxin used in subsequent assays (track F) contains both the high- and low-molecular-weight forms of SLO (identified by immunoblotting; data not shown) as well as three minor contaminating bands that could not be separated by further purification without substantial loss of activity. The activity of the purified toxin was not affected by the addition of reducing agents, which is consistent with previous observations on highly purified SLO from *S. pyogenes* culture supernatants (1). The SLO.Ala-530 and SLO.Ser-530 mutant toxins were purified from *E. coli* to the same extent as the wild-type SLO, as judged by SDS-PAGE.

TABLE 1. Specific hemolytic activities of SLO and the mutant toxins

Purified toxin	Specific hemolytic activity[a] (HU/mg)	
	−Cholesterol	+Cholesterol[b]
SLO.Cys-530 (wild type)	850,000	3,000 (0.35)
SLO.Ala-530	750,000	3,000 (0.4)
SLO.Ser-530	200,000	700 (0.35)

[a] A difference of up to twofold in the measured hemolytic activity is within the bounds of experimental error. HU, Hemolytic units.

[b] The figures in parentheses show the percentages of activity remaining in the presence of 5 μg of cholesterol per ml.

The specific hemolytic activities of the purified wild-type SLO.Cys-530 and mutants SLO.Ala-530 and SLO.Ser-530 are described in Table 1. There were no significant differences in the specific hemolytic activities of wild-type SLO and the SLO.Ala-530 mutant, while the SLO.Ser-530 mutant had a reduced, but still considerable activity. Moreover, the cytolytic activities of all three toxins were equally inhibited by cholesterol (Table 1) or by neutralizing anti-SLO sera (data not shown). No differences were observed, during prolonged (6 months) storage, between the wild-type and mutant toxins with respect to the stabilities of their specific hemolytic activities or the integrity of their high- and low-molecular-weight forms, as judged by SDS-PAGE.

Oligomer formation in membranes. To determine the abilities of the mutant toxins to form oligomers in membranes, human erythrocyte membranes from cells that had been lysed with SLO or the mutant toxins were extracted gently with deoxycholate and fractionated on sucrose density gradients, as described by Bhakdi et al. (7). Membranes from osmotically lysed erythrocytes and purified toxins treated with deoxycholate in the gradient loading buffer (no membranes) were included as controls. Gradient fractions were analyzed by SDS-PAGE and immunoblotting with a monospecific anti-SLO peptide (SLO.s[104-123]) antiserum (Fig. 4). In the absence of membranes, the wild-type SLO.Cys-530 and both the SLO.Ala-530 and SLO.Ser-530 mutant toxins were detected in low-density fractions, whereas, in each case, toxin extracted from lysed cell membranes sedimented into high-density fractions. This result is identical to that previously obtained using SLO purified from *S. pyogenes* culture supernatants by Bhakdi et al. (7), who demonstrated that SLO in the high-density fractions was in the form of oligomers corresponding to the typical arc- and ring-shaped structures observed in SLO-treated membranes by electron microscopy. This suggests that the Cys residue in SLO is not essential for oligomer formation.

DISCUSSION

The experiments reported in this paper provide clear evidence that the widely held assumption that the cytolytic activity of SLO requires an essential Cys residue is not true. Replacing the single Cys residue in SLO with Ala had no significant effect on the specific hemolytic activity of the toxin, at least in in vitro assays. This surprising finding is not confined to SLO. In an accompanying paper, Saunders et al. (23), using a similar approach, provide clear evidence that Cys is not an essential residue for the cytolytic activity of a second thiol-activated toxin, pneumolysin from *S. pneumoniae*. Thus, we must now question the assumption of an "essential Cys" in the case of other members of this family of thiol-activated toxins, particularly since those that have

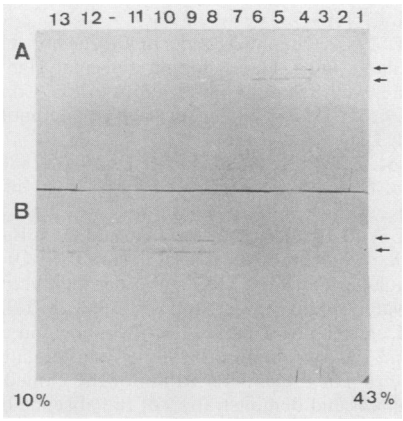

FIG. 4. Toxin oligomer formation in erythrocyte membranes. The figure shows immunoblots from an experiment performed with the SLO.Ala-530 mutant. Wild-type SLO.Cys-530 and the SLO.Ser-530 mutant produced identical results. The tracks correspond to fractions from the bottom (track 1, 43% [wt/vol]) to the top (track 13, 10% [wt/vol]) of sucrose density gradients, separated on mini-SDS-PAGE gels and immunoblotted with an anti-SLOs[104-123] serum. (A) Fractions from a gradient loaded with deoxycholate-solubilized, toxin-lysed erythrocyte membranes; (B) fractions from a gradient loaded with deoxycholate-treated toxin (no membranes). The high- and low-molecular-weight forms of SLO are indicated by the arrows to the right of the panels. No antigen was detected in fractions from control gradients loaded with osmotically lysed erythrocytes (no toxin).

been cloned and sequenced have been found to share extensive structural homologies with SLO and pneumolysin (14, 19, 26).

The designation of SLO and related toxins as thiol-activated toxins was based originally on the reversible sensitivity of these toxins to oxidation when culture supernatants (or crude lysates, in the case of the nonsecreted pneumolysin) are exposed to air, and this provided the initial impetus for the assumption that these toxins must contain one or more essential Cys residues. Although this designation accurately describes the behavior of these toxins in crude culture supernatants or lysates, it does not necessarily imply that an —SH group is essential for cytolytic activity. The observation that sensitivity to oxidation by air decreases during toxin purification (1) strongly suggests that in crude preparations toxin is reversibly inactivated by the formation of disulfide links with other molecules, such as cosecreted —SH-containing proteins or —SH-reactive smaller molecules. Rather than blocking an essential —SH group, cross-linking to such molecules could inhibit activity simply by sequestering the toxin or inducing a gross conformational change. However, purified preparations of a number of thiol-activated toxins, including SLO and pneumolysin, were shown to be reversibly inactivated by treatment with oxidizing agents such as $HgCl_2$ or *p*-chloromercuribenzoate and to be irreversibly inactivated by a number of —SH-blocking agents, such as iodoacetamide, iodoacetate, or ethylmaleimide (2). The ability of such relatively small groups, cross-linked to Cys, to inhibit cytolytic activity provided stronger evidence to support the assumption that the —SH group played a critical role in toxin function. We now know that this assumption is not correct and that an alternative explanation must be provided for the inhibitory effects of oxidizing or —SH-blocking agents. The simplest explanation is that Cys is located at a functionally important site in the toxin molecule and that while the —SH group per se is not essential for the function of this site, it can react with external oxidizing or —SH-blocking agents to covalently bind chemical groups that distort the critical function of adjacent residues in the molecule. This is supported by the fact that in the four sequenced thiol-activated toxins the single Cys (discounting the second Cys in the signal peptide of perfringolysin O) is located within a conserved 12-amino-acid sequence that corresponds to the longest continuous region of identity between these molecules, suggesting that this may be a functionally important site. In addition, although replacing Cys with Ala did not significantly affect the specific hemolytic activity of SLO, exchanging the Cys for Ser reduced activity to ca. 25%. Exchanging Cys (R = $—CH_2—SH$) for Ser (R = $—CH_2—OH$) is unlikely to have induced any gross alteration in conformation, particularly since the mutant retained significant, albeit reduced, hemolytic activity. This suggests that the reduction in activity is more likely to be due to a localized distortion of a functionally important site. This site would include conserved residues adjacent to the Cys in the primary sequence, but may also include more distant residues in the primary sequence that are brought into juxtaposition with the Cys region in the folded molecule.

The hemolytic activities of wild-type SLO.Cys-530 and the SLO.Ala-530 and SLO.Ser-530 mutants were equally inhibitable by cholesterol, and all three toxins appear to be equally capable of forming oligomers in erythrocyte membranes. Previous models for the cytolytic mechanism of SLO suggested that the 3′ β-hydroxyl group of membrane cholesterol might interact with the —SH group in SLO and thereby induce a conformational change that allows SLO to insert into the membrane, where lateral diffusion leads to SLO oligomerization and the formation of hydrophilic pores (1). Our results show that this model is incorrect in that an —SH group is not required for interaction with cholesterol or for oligomer formation, but they do not refute the main features of the model. A more detailed analysis of the kinetics of cytolysis and of the structures of the oligomers formed, particularly by the SLO.Ser-530 mutant, which has a reduced activity, will be required to determine whether there are subtle differences in the mechanisms of cytolysis induced by the wild-type and mutant toxins.

The fact that Cys is not essential for the cytolytic activities of SLO (this study) and pneumolysin (23), or for the sublytic effects of pneumolysin on polymorphs (23), raises the question of why this residue has been so strongly conserved in thiol-activated toxins produced by taxonomically diverse species of bacteria. In the four sequenced toxins the Cys residue is located in a similar, very strongly conserved, and probably functionally important region (14, 19, 26; above), and by inference from their common biological properties it seems likely that this may also be the case for other members of this family of toxins. Although highly speculative, the following and not necessarily mutually exclusive possibilities should now be considered. There may be other biologically and structurally related toxins that contain a residue such as Ala in place of Cys, but these would not have been recognized as members of this group as they would lack the key characteristic of thiol activation. While there is no evidence in the literature to support this suggestion, it should be remembered that the majority of membrane-damaging toxins have been very poorly characterized. Another possibility that should be considered is that the cytolytic activity of thiol-activated toxins is fortuitous and that the primary function of these molecules for the producing organisms requires an essential Cys residue, but that this primary

function has not yet been recognized. In this respect it is worth noting that many of the species that produce thiol-activated toxins are not obligate parasites, but spend much of their existence in environments such as soil. Alternatively, the current belief that the primary function of these molecules is to act as virulence factors may be correct, and Cys, while not being required for cytolytic activity in vitro, may play a critical role in vivo. This could be in an unrecognized biological activity that is not reflected by the assays used to characterize the SLO and pneumolysin mutants, or it could be related to toxin stability in vivo. Perhaps thiol-activated toxins need to be stabilized in vivo by forming disulfide bonds with other molecules and are transported to target membranes in this stabilized form, where glutathionelike reduction systems release active toxin. It will be interesting to determine whether there are any differences in the physiological effects of the wild-type SLO.Cys-530 and the SLO.Ala-530 and SLO.Ser-530 mutant toxins when administered to laboratory animals.

The work described here may lead some readers to ask if the term "thiol-activated toxins" is still an appropriate designation for this family of membrane-damaging toxins. Since this is a well-established term and, more importantly, accurately describes the behavior of these toxins in crude culture supernatants of cell lysates, we believe that it should be retained.

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