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# Streptolysin O: the C-terminal, tryptophan-rich domain carries functional sites for both membrane binding and self-interaction but not for stable oligomerization

Silvia Weis, Michael Palmer *

*Institute of Medical Microbiology, University of Mainz, Augustusplatz, 55101 Mainz, Germany*

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**Abstract**

Streptolysin O belongs to the class of thiol-activated toxins, which are single chain, four-domain proteins that bind to membranes containing cholesterol and then assemble to form large oligomeric pores. Membrane binding involves a conserved tryptophan-rich sequence motif located within the C-terminally located domain 4. In contrast, sites involved in oligomerization and pore formation have been assigned to domains 1 and 3, respectively. We here examined the functional properties of domain 4, which was recombinantly expressed with an N-terminal histidine tag for purification and an additional cysteine residue for covalent labeling. The fluorescently labeled fragment readily bound to membranes, but it did not form oligomers nor lyse cell membranes. Moreover, the labeled fragment did not detectably become incorporated into hybrid oligomers when combined with lytically active full-length toxin. However, when present in large excess over the active toxin, the domain 4 fragment effected reduction of hemolytic activity and of functional pore size, which indicates interference with oligomerization of the lytically active species. Our findings support the notion that domain 4 of the streptolysin O molecule may fold autonomously, is essential for membrane binding and is capable not of irreversible but of reversible association with the entire toxin molecule. 

*Keywords:* Thiol-activated toxin; Pore-forming toxin; Protein oligomerization; Membrane binding

## 1. Introduction

Streptolysin O (SLO) is secreted by the Gram-positive bacterium *Streptococcus pyogenes* and belongs to the highly homologous family of thiol-activated cytolysins, which group of toxins is remarkable for its strict specificity for membranes containing cholesterol [1]. Binding to the sterol occurs at the level of the monomer, whereas pore formation coincides with the formation of large, ring-shaped, membrane-inserted oligomers of approx. 30 nm diameter [2]. Apart from these rings, arc-shaped oligomerization intermediates have been characterized that constitute pores of reduced functional size and are lined on the facing side by a free edge of the target membrane [2,3]. While the X-ray structure of the monomer has been determined for one of the toxins (Fig. 1) [4], crystallographic analysis of the pore oligomers has been precluded by their extraordinarily large

* Corresponding author. Present address: Department of Medical Biochemistry and Genetics, Texas A and M University, 440 Reynolds Medical Building, TAMU 1114, College Station, TX 77843-1114, USA;
E-mail: mpalmer@medicine.tamu.edu

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size and their heterogeneity. Nevertheless, it has become clear that, among the four domains of the monomeric molecule, the C-terminal domain 4 has an essential role in membrane binding [5], while domains 1 and 3 participate in oligomerization and concomitant membrane permeabilization [6,7]. However, it has not been unequivocally determined whether these functions can exclusively be assigned to the respective domains in question. In the present study, we posed the following questions. (1) Is domain 4 capable of membrane binding independently of the rest of the toxin molecule? (2) Is domain 4 involved in the subsequent steps of oligomerization and pore formation? The answers provided contribute to the understanding of structure-to-function relationships of the thiol-activated toxins.

## 2. Materials and methods

### 2.1. Construction and purification of the SLO domain 4 fragment (r-d4)

The SLO gene region corresponding to domain 4 (amino acids 459–571) of the SLO gene was amplified by PCR. The 3′-PCR primer was designed to hybridize downstream from the coding region, while the 5′-primer (GAA ACA ACA GGT ACC GAG TGC ACT AGT GG) corresponded to the boundary of domains 3 and 4; the first two underlined nucleotides served to introduce an unique *Kpn1* site for use in cloning, whereas the third one served to change tyrosine 460 to cysteine in order to facilitate covalent labeling of the recombinant protein. The unique wild type cysteine residue of SLO at position 530 was replaced by alanine, which mutation was derived from plasmid pMK206 [8] that served as the template in PCR. The PCR product was inserted into the expression vector pQE-30 (Qiagen) to provide for an N-terminal purification tag of six histidine residues. Liquid cultures of *Escherichia coli* strain TG1 transformed with the recombinant plasmid were grown in LB medium and supplemented with IPTG (0.5 mM) to induce protein expression. The cells were harvested by centrifugation, resuspended with 20 mM Tris-HCl, 0.5 M NaCl (pH 8.0) and lysed by ultrasonication (Branson probe sonifier 250). The inclusion bodies released by this procedure were recovered by centrifugation (20 min, $30\,000 \times g$) and washed three times by resuspension with 20 mM Tris-HCl, 0.3 M NaCl, 2% Triton X-100 and centrifugation. They were then solubilized with 8 M urea, 20 mM Tris-HCl, 0.3 M NaCl (pH 8.0) and applied to a chromatography column filled with Chelating Sepharose FF (Amersham Pharmacia) which had been presaturated with $NiCl_2$. The column was washed with 8 M urea, 0.5 M NaCl, 0.1 M sodium phosphate, 10 mM Tris, pH 8.0, and then with the former buffer but adjusted to pH 6.3. Finally, the domain 4 fragment (hereafter referred to as r-d4) was eluted with 8 M urea, 0.5 M NaCl, 50 mM MES, pH 5.5. The eluate was recovered in fractions that were analyzed for purity by SDS-PAGE.

The purification of several point mutants of streptolysin O (C530A, N402C, and S101C) fused to maltose-binding protein of *E. coli* has been described previously [9].

### 2.2. Cysteine-specific covalent labeling or chemical cleavage

For covalent labeling with fluorescein maleimide or tetramethylrhodamine maleimide (both from Molecular Probes, Eugene, OR), the r-d4 protein (1–2 mg) was first transferred by gel filtration into 8 M urea, 50 mM sodium phosphate, pH 7.5 using a PD10 column (Amersham Pharmacia) and then supplemented with the respective labeling reagent (dissolved in DMSO) to 0.5 mM. After incubation at room temperature for 15 min, excess reagent was removed by gel filtration as above. Labeling yield was estimated spectrophotometrically to $\geq 90\%$ with fluorescein and to $\geq 75\%$ with tetramethylrhodamine, respectively, whereby the $A_{280}$ of r-d4 was determined from its amino acid sequence according to [10]. The molar extinction coefficients of the labels (as stated by the supplier) were 83 000 $M^{-1}$ $cm^{-1}$ for fluorescein and 91 000 $M^{-1}$ $cm^{-1}$ for tetramethylrhodamine, respectively.

Cysteine-specific chemical cleavage was carried out according to [11] with minor modifications. Labeling with nitrothiocyanobenzoate (NTCB, 5 mM; Sigma) was carried out for 60 min in 8 M urea, 50 mM Tris-HCl, 1 mM EDTA, pH 7.5. The protein was then immediately transferred by gel filtration into 6 M guanidine chloride, 0.1 M Tris-HCl, 1 mM EDTA,

pH 9.0 and incubated at room temperature for 24 h. The sample was then passed over Chelating Sepharose FF to remove the cleaved histidine tag as well as any uncleaved protein. The cleaved r-d4 protein devoid of the histidine tag was collected with the flow-through, concentrated by ethanol precipitation and redissolved with 8 M urea/50 mM sodium phosphate, pH 7.5. Homogeneity of the cleaved protein was confirmed by Tris/Tricine-SDS-PAGE according to [12]. (Note that in all of the experiments described below involving labeled or unlabeled r-d4, the histidine tag was retained unless stated otherwise.)

### 2.3. Renaturation of r-d4

The unlabeled, labeled, or cleaved r-d4 protein, dissolved in 8 M urea/50 mM sodium phosphate (pH 7.5), was diluted into 50 vols. of 5 mM Tris-HCl, 125 mM NaCl, 0.1% BSA, pH 9.0 and incubated at room temperature for 10 min. These diluted solutions were employed in all of the below experiments relating to the biological activity of r-d4.

### 2.4. Assay of toxin binding to erythrocytes

100 μl of fluorescein-labeled, renatured r-d4 were added to 1.9 ml of 125 mM NaCl, 0,1% BSA, buffered to pH 6.0 (with 50 mM MES), pH 7.0 (with 50 mM sodium phosphate), or pH 8.0 or 9.0 (with 50 mM Tris-HCl), respectively; the final concentration of r-d4 was 1–2 μg/ml. One half of each of these samples were used to resuspend washed, pelleted human or rabbit erythrocytes to 5% final concentration. Following incubation on ice for 10 min, the erythrocytes were pelleted again. The supernatants were withdrawn, mixed with an equal volume of 0.2 M sodium borate pH 9.2, and assayed for fluorescein fluorescence, whereby the spare halves of the r-d4 dilutions that had not been incubated with erythrocytes served as references. For quantitating the effect of r-d4 upon binding of the entire streptolysin O molecule, the non-lytic fluorescein derivative of the point mutant N402C [13] was incubated with 5% erythrocytes at 2.5 μg/ml both in the absence and in the presence of unlabeled r-d4 (2 μg/ml final concentration, corresponding to a fivefold molar excess over N402C). The extent of binding was quantitated fluorimetrically as before.

### 2.5. Hemolysis and hemolysis inhibition assays

The r-d4 protein (unlabeled, labeled, or NTCB-cleaved) was renatured and then admixed with an equal volume of isotonic buffer varying in pH (see above). From these solutions (which contained 10 μg/ml of the respective derivative of r-d4), twofold serial dilutions were prepared in a round bottom microtiter plate, followed by addition to each well of an equal volume of 2.5% rabbit or human erythrocytes suspended in 5 mM sodium phosphate/125 mM NaCl, pH 7.0. Hemolysis or hemagglutination titers were read visually after incubation at room temperature for 30 min.

For hemolysis inhibition assays, twofold serial dilutions were prepared of the lytically active SLO mutant C530A, starting with 8 μg/ml. To each well of one series, the renatured domain 4 fragment was added to 10 μg/ml, whereas a parallel series was supplemented with buffer only. Finally, rabbit or human erythrocytes were added to 1%. Hemolysis was evaluated after 30 min using a microplate filter photometer set to an extinction wavelength of 620 nm.

### 2.6. Release of calcein and of tetramethylrhodamine-dextran from resealed erythrocyte ghosts

Human red cells were lysed osmotically in the cold, laden with calcein and tetramethylrhodamine-dextran and resealed as described [3]. Two parallel dilution series were prepared of the lytically active SLO mutant C530A at pH 6.0. To each well of one of these series, the renatured, unlabeled domain 4 fragment was added to 10 μg/ml. Then, a constant amount of the labeled ghosts was added. The samples were incubated at room temperature for 15 min and then centrifuged for 3 min in a benchtop centrifuge. The supernatants were withdrawn and assayed for calcein and rhodamine-dextran fluorescence as described [3].

### 2.7. Fluorescence energy transfer assay of oligomer formation

Samples (5 μg each) of the domain 4 fragment labeled with fluorescein and with tetramethylrhodamine, respectively, were mixed and then added to osmotically lysed, washed human erythrocyte

membranes (ghosts) and incubated for 15 min at 37°C. The fluorescence excitation spectrum was obtained between 460 and 560 nm in a SPEX Fluorimax spectrofluorimeter in the ratio mode (emission wavelength: 577 nm; band passes: 2.1 nm). Reference samples were prepared from either labeled species alone plus ghosts. All spectra were subtracted with a scattering blank containing ghosts only without any labeled protein.

To assess incorporation of the domain 4 fragment into hybrid oligomers with the entire toxin molecule, the lytically active cysteine mutant S101C, labeled with fluorescein, was admixed with the tetramethylrhodamine-labeled domain 4 fragment at fivefold molar excess prior to incubation with ghosts. Reference samples were prepared as above.

### 2.8. Analysis of oligomerization by density gradient centrifugation

Human erythrocyte ghosts (100 μl packed volume) were suspended at pH 6.0 and incubated (37°C, 15 min) with various combinations of fluorescein-labeled and unlabeled intact toxin species (see Section 3). They were then recovered by centrifugation, solubilized by addition of sodium deoxycholate to 5% final concentration, and subjected to density gradient centrifugation as described [14]. The gradients were split into eight fractions of equal volumes, which were diluted with 5 vols. sodium borate (50 mM, pH 9.0)/0.25% SDS, incubated at 70°C for 5 min and assayed for fluorescein fluorescence (excitation wavelength: 488 nm; emission wavelength: 520 nm).

## 3. Results

### 3.1. Refolding of the domain 4 fragment and its binding to erythrocytes

The amino acid residues 461–571, corresponding to the C-terminal domain 4 of the folded structure

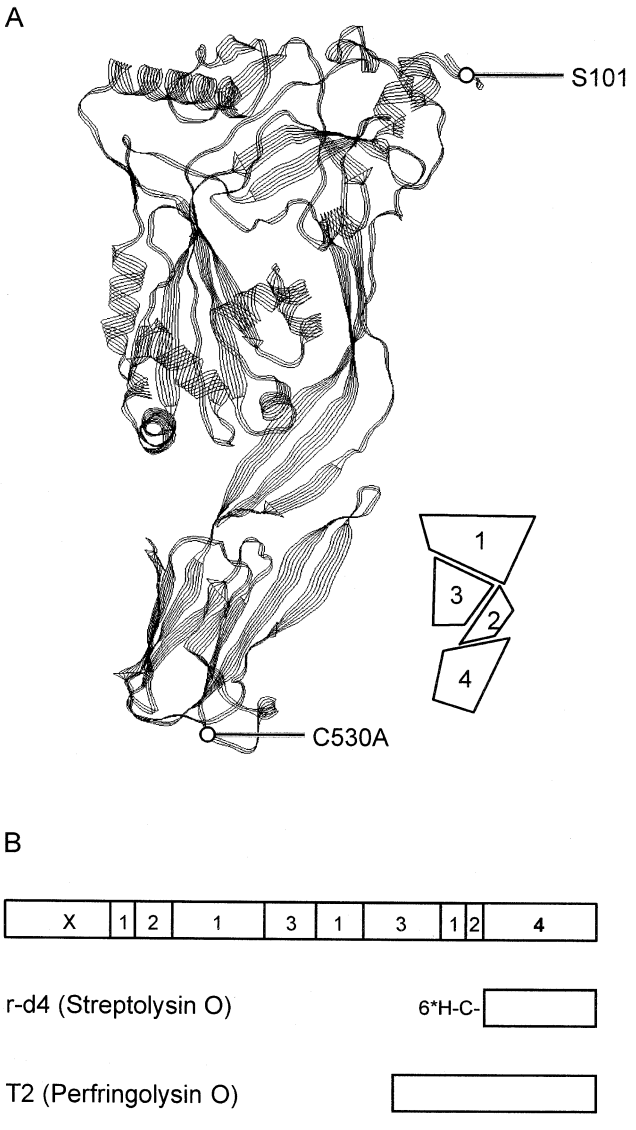


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Fig. 1. Structure of the streptolysin O monomer in solution (based on the crystal structure of the homologous toxin perfringolysin O [4]). (A) Folded structure. The four domains are identified in the accompanying sketch. The first amino acid residue covered by this structure corresponds to residue S101 of streptolysin O. Residue C530 is the only cysteine of wild type streptolysin O; with all the mutants employed in this study, it was replaced with alanine which substitution does not affect lytic activity [8]. (B) Top: assignment of tertiary to primary structure. Domain 4 comprises a contiguous stretch at the C-terminus of the sequence. In contrast, domains 1–3 each consist of two or more distantly located segments. X: the 100 N-terminal amino acids of streptolysin O (which are not required for lytic activity [9]) are not covered by the crystal structure. Middle: the recombinant fragment r-d4 corresponds to domain 4 of streptolysin O, N-terminally extended by six histidines and a unique cysteine residue. Bottom: the tryptic fragment T2 of perfringolysin O (see Section 4) comprises domain 4 as well of the adjacent parts of domains 1–3.

of SLO (Fig. 1A), were recombinantly expressed with a purification tag comprising six histidine residues and an extra cysteine attached to its N-terminus (Fig. 1B); this protein is hereafter referred to as r-d4. When r-d4, thiol-specifically labeled with fluorescein, was diluted from 8 M urea into buffers varying in pH, incubated at room temperature, and centrifuged, most of the fluorescence was found to precipitate at pH $\leq 7$. In contrast, the fluorescence was quantitatively recovered in the supernatant at pH 9.0, indicating that under this condition the protein had escaped aggregation. The r-d4 fragment was therefore initially renatured at pH 9 in all subsequent experiments.

Binding of r-d4 to both human and rabbit erythrocytes was maximal at pH $\leq 7$ and decreased toward higher pH values (Fig. 2A). When erythrocytes were laden with r-d4 at pH 6, washed, and then resuspended and incubated at pH 9, the major fraction of r-d4 was recovered in the supernatant, indicating that binding was reversible (data not shown). At the modest concentrations of toxin employed in these experiments, r-d4 did not interfere with membrane binding of the full-size streptolysin O molecule (Fig. 2B).

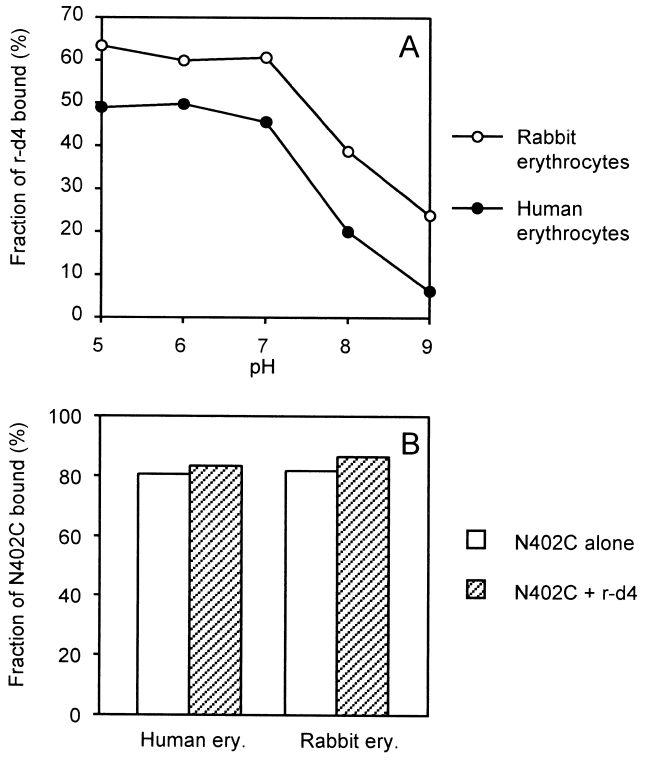


Fig. 2. Binding of recombinant domain 4 (r-d4) to erythrocytes. (A) Fluorescein-labeled r-d4 was renatured by dilution at pH 9.0 and then incubated with rabbit or human erythrocytes at various pH values. Binding was then quantitated fluorimetrically by its depletion from the supernatant. (B) Unlabeled r-d4 was renatured, admixed at fivefold molar excess with the fluorescein-labeled, non-lytic point mutant N402C, and incubated with erythrocytes. Binding of N402C was quantitated as above. (All values represent means of triplicates.)

The r-d4 molecule was entirely devoid of hemolytic activity, but it effected agglutination of both human and rabbit erythrocytes. Hemagglutination was abolished upon removal of the histidine tag by cysteine-specific chemical cleavage (cf. Fig. 1B). Since this cleavage method results in blocking of the cysteine thiol group [11], the cleaved r-d4 could not easily be labeled for the quantitation of binding. However, the cleaved fragment shared with the unlabeled one the ability to inhibit hemolysis (see below), which suggests that it also retained the capability of binding to membranes.

### 3.2. *The domain 4 fragment inhibits hemolysis by intact streptolysin O*

We next examined the functional interaction between r-d4 and lytically active SLO molecules. To this end, the hemolytic capability of the active SLO mutant C530A was quantitated in the presence of various amounts of r-d4. Fig. 3A shows that the fragment inhibited the hemolytic activity of C530A in a dose-dependent manner, suggesting interference of r-d4 with the oligomerization of C530A. The pH dependence of this inhibitory action paralleled that of membrane binding (data not shown), which suggests that inhibition of hemolysis requires prior membrane binding of r-d4. To further characterize the influence of r-d4 upon oligomerization, r-d4 and C530A were then also applied to resealed erythrocyte ghosts that had been laden with two soluble markers of different size (calcein, $M_r$ 622, and tetramethylrhodamine-dextran, $M_r$ 64 000). When C530A was applied alone, the effusion of dextran ceased more readily with increasing dilution of the toxin than the release of calcein (Fig. 3B). This was consistent with previous results and with the existence of incomplete, arc-shaped toxin oligomers creating pores of reduced functional diameter and preferably

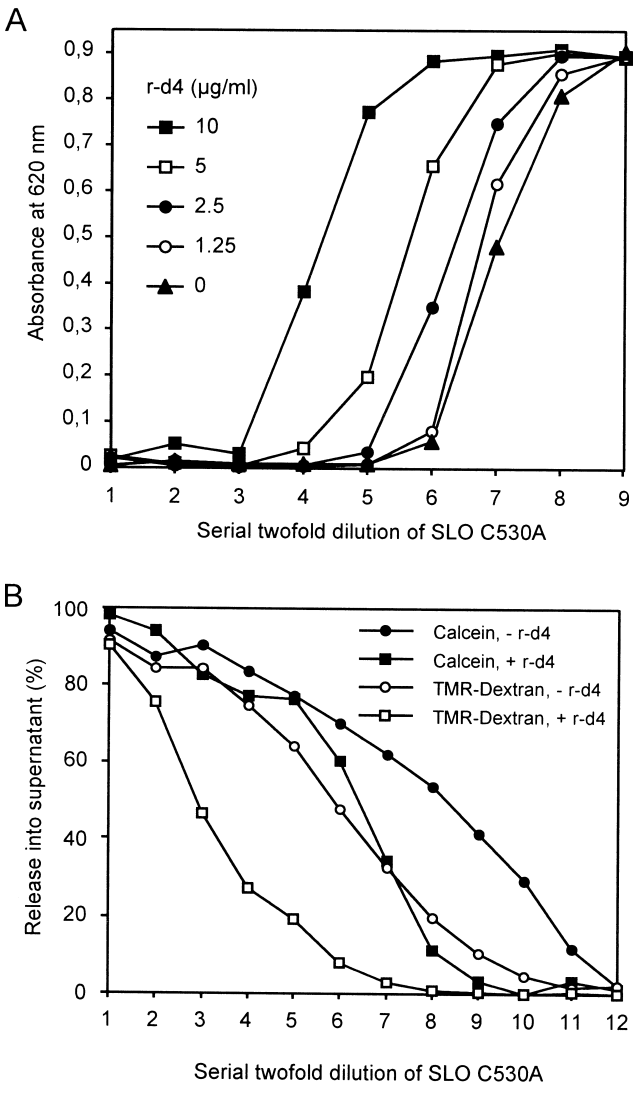


Fig. 3. Effect of r-d4 upon membrane permeabilization by the lytically active mutant C530A. (A) Inhibition of hemolysis. Serial twofold dilutions of C530A were supplemented with r-d4 at the amounts indicated. Rabbit erythrocytes were added, and the amount of cells remaining unlysed after incubation for 30 min was quantitated by absorbance at 620 nm. (B) Release of fluorescent markers of different size. Calcein ($M_r$ 620) and tetramethylrhodamine-dextran ($M_r$ 64 000) were simultaneously included within resealed erythrocyte ghosts, which were exposed to serial dilutions of C530A, either with or without supplementation by r-d4 (10 μg/ml). The release of either marker was quantitated fluorimetrically.

being detected at limiting concentrations of toxin. While the release of either marker was readily reduced by the inclusion of r-d4 (10 μg/ml), this inhibitory effect commenced at higher concentrations of C530A with the dextran than with calcein. The enhanced discrimination in the release of the two markers suggests that r-d4 effected a gradual reduction in size of the pores and, by inference, of the oligomers formed by C530A. These findings therefore constitute additional evidence that r-d4 interferes with oligomerization.

### 3.3. *The domain 4 fragment does not oligomerize on membranes*

Despite its lack of hemolytic activity, the domain 4 fragment might still form oligomers on membranes, as exemplified by non-lytic mutants of other pore-forming toxins [15]. In order to examine whether r-d4 would form such non-lytic oligomers, the fragment was thiol-specifically labeled with fluorescein and with tetramethylrhodamine, respectively. When located close to one another, these two labels form an efficient couple for fluorescence energy transfer. Mixing of the two labeled species prior to incubation with the membranes should result in the formation of mixed oligomers and, therefore, in the enhancement of tetramethylrhodamine fluorescence upon excitation of fluorescein due to energy transfer. This,

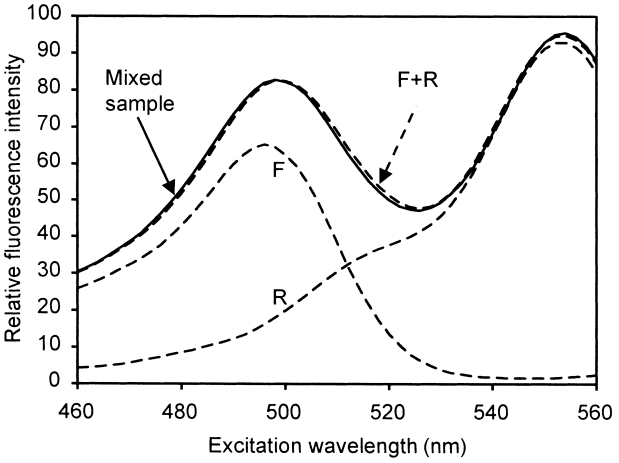


Fig. 4. Analysis of oligomerization of r-d4 by fluorescence energy transfer. The r-d4 protein, labeled with fluorescein (F) or tetramethylrhodamine (R), was incubated with washed erythrocyte membranes, and fluorescence excitation spectra were obtained (emission wavelength: 577 nm). The two labeled species were admixed and then incubated with membranes. The ensuing spectrum (Mixed sample) is congruent with F+R, the numerical sum of he individual samples F and R, indicating lack of energy transfer.

however, was not observed: the excitation spectrum of fluorescence detected at 577 nm (representing the emission maximum of the rhodamine-labeled species) obtained with such a mixed sample corresponded exactly to the sum of the two spectra recorded with the individual components (Fig. 4). This result indicates that r-d4 lacks oligomerizing capability.

In a second experimental approach, the membranes were incubated with fluorescein-labeled r-d4, solubilized with the detergent deoxycholate, and the oligomeric state of the protein examined by density gradient centrifugation. Fig. 5 shows that r-d4 was recovered with the top fractions of the gradients, indicative of a state of low molecular weight. In contrast, the lytically active point mutant S101C was recovered from the bottom fractions, reflecting its conversion into large oligomers upon contact with membranes. Oligomerization of S101C persisted in the presence of unlabeled r-d4, i.e. the fragment had no detectable inhibitory effect at the high toxin concentrations employed in this experiment. Importantly, when fluorescently labeled r-d4 was combined with the unlabeled, lytically active SLO point mutant C530A, no fluorescence was detected in the bottom fractions, indicating that the labeled fragment had failed to enter into hybrid oligomers with the active species. The latter conclusion was then confirmed by the above fluorescence energy transfer approach, which failed to detect any energy transfer from the fluorescein-labeled active mutant S101C to rhodamine-labeled r-d4 upon co-incubation with membranes (data not shown). In sum, the experiments did not yield any evidence of stable association of the r-d4 fragment, neither with itself nor with full-length SLO molecules.

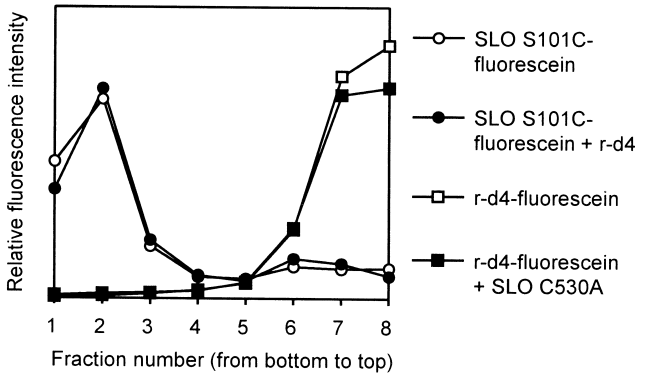


Fig. 5. Analysis of oligomerization of r-d4 by density gradient centrifugation. Fluorescein-labeled streptolysin O mutants were incubated with erythrocyte membranes. The samples were solubilized with sodium deoxycholate, placed on top of sucrose density gradients, and subjected to ultracentrifugation. The active mutant S101C is recovered from the bottom fractions, indicating its transformation into oligomers upon contact with membranes. In contrast, fluorescein-labeled r-d4 is recovered from the bottom fractions, which indicates lack of oligomerization. The labeled r-d4 also failed to be incorporated into hybrids with the unlabeled active mutant C530A.

## 4. Discussion

The elongated, four-domain molecular structure of the thiol-activated toxins was initially characterized by electron microscopy and hydrodynamic analysis [16], but only from the crystal structure [4] it has become clear that the membrane-binding domain is formed by a contiguous C-terminal part of the protein sequence. In contrast, the polypeptide chain runs back and forth through the residual three domains, each of which therefore comprises several shorter segments scattered along the sequence (cf. Fig. 1B). Thus, if the molecule is proteolytically cleaved in the middle and the two ensuing fragments are separated, only domain 4 will remain intact, whereas domains 1–3 will be split apart. Accordingly, in two earlier studies, an N-terminal tryptic fragment of perfringolysin (named T1) was found to be devoid of any detectable activity, whereas the C-terminal T2 fragment retained the activity of binding to membranes and of interfering with the oligomerization of functionally active, intact toxin [17,18]. The question now arose whether or not both of these partial activities of the T2 fragment could be ascribed to its domain 4 section. The findings of the present study indicate that this is indeed the case, although the interference with oligomerization of wild type toxin appears to be less pronounced with the streptolysin O r-d4 fragment. Reportedly, the hemolytic activity of the corresponding wild type perfringolysin molecule was entirely abrogated by T2 when the latter was present in sufficient excess [18]. In contrast, with r-d4, hemolysis by intact streptolysin O was diminished but not entirely abolished. The apparently lower inhibitory activity of r-d4 as compared to T2 might conceivably arise by its lack of the elements derived from

domains 1–3. Alternatively, the difference in inhibitory efficacy might be due to intrinsic differences of the two homologous but still distinct toxins. In any event, T2 and r-d4 apparently share the same mode of action. Both of them fail to form stable oligomers on their own. While the possibility of stable integration into hybrid oligomers with intact toxin was not definitely addressed with T2 [18], we here found that no such stable interaction occurred with r-d4. Nevertheless, both fragments clearly interfere with oligomerization of the wild type molecules, which was shown by a reduced oligomer average size in the case of T2 and, more indirectly, by the reduction of pore size due to r-d4. In the absence of irreversible association of r-d4 with the intact toxin molecules, the most likely mode of this inhibitory effect appears to consist in its reversible interaction with monomers or growing oligomers of intact streptolysin O.

An interesting fortuitous finding consists in the agglutination of erythrocytes depending on the N-terminal histidine tag of r-d4. This non-specific effect may be expected also to arise in the case of other membrane-binding proteins when recombinantly expressed as histidine-tagged fusions. Even in the absence of specific binding, the histidine tag may confer a non-specific interaction with erythrocyte membranes upon proteins intended to remain soluble. To effect agglutination, the histidine tag will have to protrude from the specifically bound membrane, which in the case of streptolysin O means that the N-terminal part of domain 4 does neither insert nor closely approach the target lipid bilayer. From the inhibition of intact streptolysin O by the membrane-bound fragment, it would then appear that the intact molecule attains a similar orientation with respect to the membrane, so that we hypothesize that also within the membrane-bound intact toxin molecule the N-terminal part of domain 4 remains located remotely from the lipid bilayer.

In sum, our study shows that domain 4 of streptolysin O is an autonomously folding unit, that it binds to membranes independently of the rest of the molecule, and that it associates with lytically active toxin molecules in an abortive, reversible fashion that nevertheless points to a role of this domain in the formation of the pore oligomer. These findings signify a more stringent assignment of function to parts of the structure than hitherto derived with proteolytic fragments and point mutants.

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