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Staphylococcal α-toxin: Oligomerization of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles

(toxin–membrane interaction/hydrophilic–amphiphilic protein transition/transmembrane pore/protein–detergent interaction)

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ABSTRACT **Native *staphylococcus aureus* α-toxin is secreted as a hydrophilic polypeptide chain of M_r 34,000. The presence of deoxycholate above the critical micellar concentration induced the toxin monomers to self-associate, forming ring or cylindrical oligomers. The oligomers were amphiphilic and bound detergent. In deoxycholate solution, the protein–detergent complexes exhibited a sedimentation coefficient of 10.4 S. A M_r of 238,700 was determined by ultracentrifugation analyses at sedimentation equilibrium. Because quantitative detergent-binding studies indicated a protein/detergent ratio of approximately 5:1 (wt/wt), the protein moiety in each protein–detergent complex was determined to be approximately M_r 200 000, corresponding to a hexamer of the native molecule. The amphiphilic toxin hexamers were ultrastructurally indistinguishable from the cytolytic, annular toxin complexes that form on and in biological target membranes. They bound lipid and could be incorporated into artificial lecithin lipid vesicles. The transition of toxin protein molecules from a hydrophilic monomer to an amphiphilic oligomer through self-association has thus been shown to be inducible solely through contact of the native protein molecules with an appropriate amphiphilic substrate.**

Staphylococcal α-toxin is secreted as a water-soluble polypeptide chain with a sedimentation coefficient of 3.3 S and a molecular weight of 34,000 (1–4). Early studies by Freer *et al.* suggested that α-toxin damages biological membranes through a tight association with the lipid bilayer (5, 6). Membrane binding was thought to be accompanied by an oligomerization of toxin molecules to form annular, membrane-bound structures. Evidence confirming this proposal was recently obtained in our laboratory. An 11_S protein molecule was isolated from detergent-solubilized, toxin-treated membranes and shown to represent the membrane-bound form of α-toxin. That the toxin oligomer isolated from membranes was amphiphilic was concluded from its tendency to aggregate in aqueous media in the absence of detergents, its ability to bind detergent and lipid, and its low elutability from membranes through ionic manipulations. In the electron microscope, the membrane-derived toxin oligomer exhibited a ring or cylindrical structure with a central pore. Because marker release studies indicated that toxin-damaged biological membranes exhibited functional lesions of discrete pore size, we proposed that α-toxin might damage membranes through oligomerization on and in the membrane to form a ring-structured complex that is partially embedded within the lipid bilayer, generating a transmembrane pore (4). This process would be strikingly analogous to the mechanism of complement-mediated membrane perturbation (7, 8).

The binding of terminal complement components C5b-C9 and the binding of α-toxin to biological target membranes are unique in that primarily hydrophilic polypeptides self-associate to form membrane-penetrating, amphiphilic complexes. The factors responsible for such conformational changes are unknown. In this communication, we report that, simply through contact with deoxycholate detergent micelles, native α-toxin molecules can be induced to self-associate to form amphiphilic, ring-structured hexamers that are indistinguishable from the cytolytic membrane complexes. The presence of an appropriate physicochemical environment therefore appears to be the only condition necessary for inducing the hydrophilic–amphiphilic transition of this protein.

MATERIALS AND METHODS

Unless otherwise stated, all biochemicals were obtained from Serva (Heidelberg) and Merck (Darmstadt). A preparation of purified α-toxin isolated from bacterial culture supernates was generously provided in lyophilized form by the Behringwerke, Marburg. Immediately prior to use, the toxin preparation was dissolved in buffer and chromatographed over a Sephacryl S-300 column (Pharmacia, Uppsala, Sweden; 2.6 × 60 cm) in 10 mM Tris/50 mM NaCl, pH 8.2/15 mM NaN_3 at 4°C. Two protein peaks were separated from each other. The first, accounting for approximately 25% of total protein and eluting in a molecular weight region of 180,000–500,000 was discarded. The second peak, eluting as a sharp, symmetrical peak at a molecular weight region of 30,000–40,000, accounted for residual protein and represented the α-toxin monomer. These fractions were pooled, concentrated by ultrafiltration (BM 10 membranes from Berghoff, Tübingen), and used in further experiments.

Gel Chromatography and Detergent-Binding Studies. Sephacryl S-300 columns (1 × 60 cm) were used in the chromatography buffer given above. Deoxycholate was present at 1.25 or 5 mM. [^{3}H]Deoxycholic acid (4.0 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels; New England Nuclear) was added to the chromatography buffer to give 50–200 × 10^3 cpm/ml. Columns were eluted at 3.2 ml/hr, and 1.6-ml fractions were collected. All detergent-binding studies were performed at 22°C. The amount of detergent bound to protein was calculated as described in ref. 9.

Ultracentrifugal Analyses. These were performed as detailed (4) with the exception that equilibrium sedimentation analyses were performed at a rotor velocity of 5600 rpm for determination of molecular weight.

Preparation of Lipid Vesicles. Artificial lipid vesicles of α-lecithin reconstituted with α-toxin were prepared by detergent-

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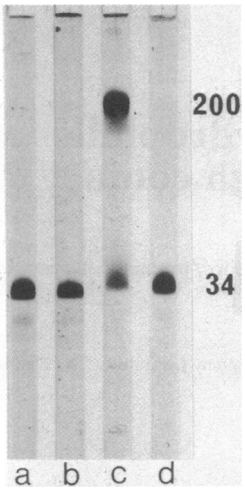

FIG. 1 $NaDodSO_4$/polyacrylamide gel electrophoresis of native, 3.3S α-toxin (lane a), $NaDodSO_4$-treated and boiled protein sample recovered as peak b in Fig. 2*C*, (lane b), and $NaDodSO_4$-treated but unheated protein sample (peak b), (lane c), and $NaDodSO_4$-treated, unheated protein sample from peak a in Fig. 2 (lane d). Direction of electrophoresis, top to bottom; stain, Coomassie brilliant blue. Sizes shown in kilodaltons.

dialysis as described (4). The initial protein/lipid ratio used in the reconstitution experiments was 1:20 (wt/wt).

Other Methods. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed in 5-mm gel rods according to Fairbanks *et al.* (10), with 4.5% gels. Estimation of sedimentation coefficient by sucrose density gradient ultracentrifugation was performed as described (4). Electron microscopy was performed as described (11). Protein was determined by the Lowry procedure (12), with 0.4% $NaDodSO_4$ present in the samples. Initial calibration curves were obtained by amino acid analyses that were performed in parallel.

RESULTS

The properties of native α-toxin have been detailed previously and will only be summarized here. The purity of the α-toxin preparation was >95% as judged by analytical ultracentrifugation and $NaDodSO_4$/polyacrylamide gel electrophoresis. The protein banding at 34,000 daltons (Fig. 1 lane a) in $NaDodSO_4$ gels accounted for >95% of Coomassie-stainable material. A sedimentation coefficient of 3.3 S and a mean molecular weight of 34,000 ± 3900 were found by analytical ultracentrifugation. The protein reacted with antibodies to α-toxin in a toxin neutralization test. The amino acid composition of the protein agreed well with that published by Six and Harshman (13, 14).

Deoxycholate-Induced Oligomerization of α-Toxin. α-Toxin monomer (1.5 mg in 1 ml buffer) was treated with 1.25 mM deoxycholate for 20 min at 22°C and chromatographed over a Sephacryl S-300 column equilibrated with 1.25 mM deoxycholate. This concentration is well below the critical micellar concentration of the detergent (15). The protein eluted in a sharp, symmetrical peak (Fig. 2*A*) at K_{av} 0.55, corresponding to a molecular weight region of 30,000–40,000. No binding of detergent was discerned. A second toxin sample was pretreated similarly with 3.75 mM deoxycholate and supplemented with radioactive detergent to give an equivalent ratio of radioactive/unlabeled detergent as in the chromatography buffer. When chromatographed over the same column, the protein now eluted in two peaks. That the new peak at K_{av} 0.25 (designated "b") bound detergent was apparent from the appearance of a detergent peak that coeluted with the protein (Fig. 2*B*). A second peak, identical to that of native 3.3S toxin monomer, bound no detergent. When the toxin sample was pretreated with 6.25 mM deoxycholate similarly supplemented with radioactive detergent, all of the protein was converted to the new protein peak "b" of high molecular weight (Fig. 2*C*). The coeluting detergent peak was well separated from the second detergent peak corresponding to free deoxycholate detergent micelles. The third radioactive peak corresponded to deoxycholate monomers that were retarded by the Sephacryl gel and appeared after the total (bed) volume of the column.

From these experiments, the amount of deoxycholate remaining bound to the high molecular weight form of α-toxin after chromatography under the given conditions was determined to be approximately 17% (wt/wt, detergent/protein). Another binding study was performed with 5 mM DOC present in the chromatography buffer. In this experiment, 10 ml of native α-toxin (0.5 mg/ml) was made 2.5 mM in unlabeled deoxycholate. The sample was concentrated by ultrafiltration to 1 ml, during which the concentration of deoxycholate increased to above the critical micellar concentration. This procedure was found to be optimal for obtaining monodispersed high molecular weight toxin complexes in sufficient concentration for the detergent-binding studies and for ultracentrifugal analyses. Upon chromatography over the Sephacryl column, the toxin again eluted symmetrically at K_{av} 0.25, and the amount of detergent bound to the protein was determined to be approximately 20% (wt/wt) in these experiments (Fig. 3).

Molecular Weight of Oligomeric α-Toxin. The protein peak eluted from the column shown in Fig. 3 was subjected to ultracentrifugation analysis. A symmetrical 10.4S peak accounting for >95% of sedimenting material was found by velocity sedimentation. At sedimentation equilibrium, a mean molecular weight of 238,700 ± 23,000 was determined. The partial specific volume of α-toxin was 0.72 as determined from its amino acid composition, and the partial specific volume of the detergent–protein complex was 0.73 [a value of 0.778 used for deoxycholate (16)]. From these values, with correction for the weight of bound detergent (16), the molecular weight of the oligomeric form of α-toxin was determined to be approximately 200,000. Thus, the oligomers most probably represent hexameric complexes of the native toxin molecules.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Lanes b and c in Fig. 1 depict $NaDodSO_4$ gel electrophoresis of the toxin hexamer recovered from the Sephacryl column in Fig. 2*C* (peak b). Electrophoresis of $NaDodSO_4$-treated but unheated samples led to the appearance of a major high molecular weight (200,000) protein band and a weakly stained band of low molecular weight (lane c). When the same sample was boiled in $NaDodSO_4$ prior to electrophoresis, the high molecular weight band was converted to the native (molecular weight 34,000) band (lane b). Unheated, $NaDodSO_4$ treated toxin samples recovered from the low molecular weight peak (peak a, Fig. 2) gave rise to the native band (lane d). These results confirm the temperature-dependent dissociation of the toxin hexamer to the monomer form in the presence of $NaDodSO_4$ as reported previously (5, 17).

Electron Microscopy and Membrane Reconstitution. When aliquots of the 3S toxin peak corresponding to the toxin monomer (peak a, Fig. 2) from Sephacryl columns were examined in the electron microscope, only a finely granular material was seen (Fig. 4*A*). By contrast, the 10.4S toxin hexamer (peak b in Fig. 2*C* and peak fraction in Fig. 3) from the columns contained myriads of ring structures (Fig. 4*B*) 8.5 nm in diameter, and containing central pores approximately 2.5–3 nm in diameter. These ring structures were identical to those formed on and

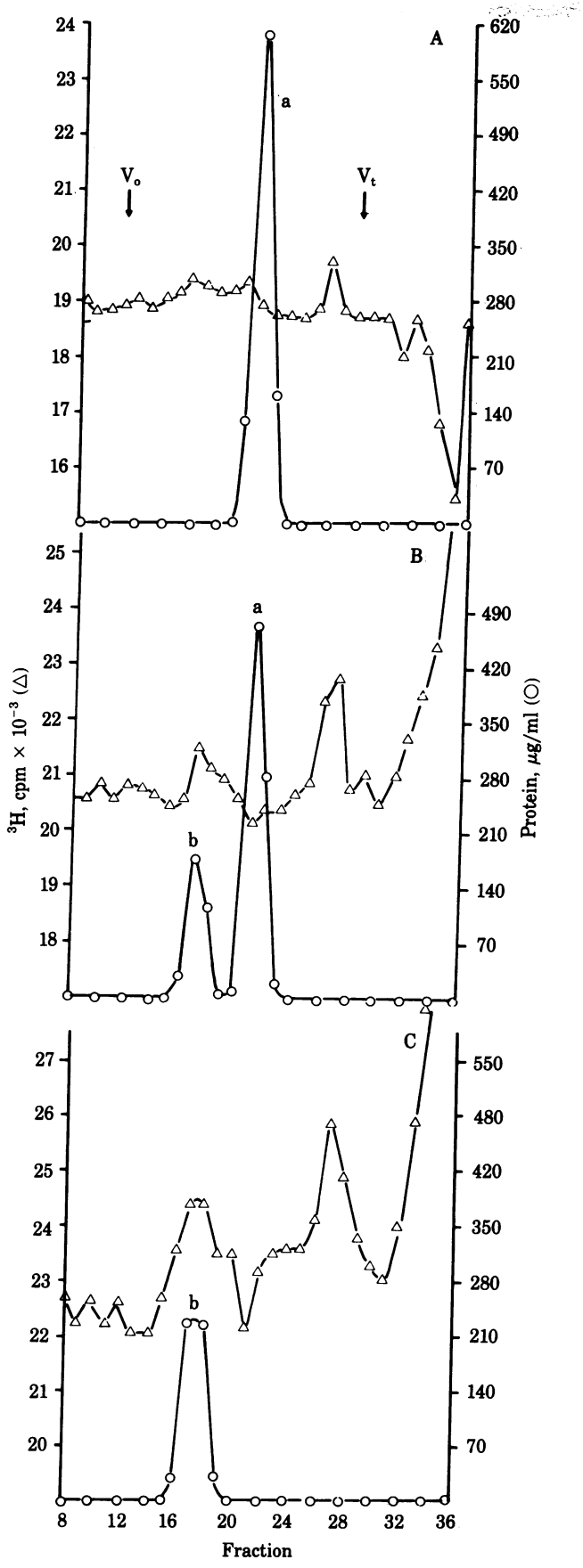

FIG. 2. Elution of α-toxin upon gel chromatography over Sephacryl S-300 in the presence of 1.25 mM deoxycholate. A sample of α-toxin was treated with 1.25 mM (*A*), 3.75 mM (*B*), or 6.25 mM (*C*) deoxycholate and chromatographed. Concentrations of deoxycholate around and above the critical micellar concentration (*B* and *C*) induced oligomerization of the toxin molecules to form higher molecular weight complexes, apparent from the shift in elution position of the toxin. Peak a, 3.3S toxin monomer; peak b, 10.4S toxin hexamer. V_o and V_t, column void volume and the total (bed) volume, respectively. [^{3}H]Deoxycholate was present in the chromatography buffer. Note the binding of detergent by the high molecular weight form of the α-toxin, and the absence of binding by the toxin monomer.

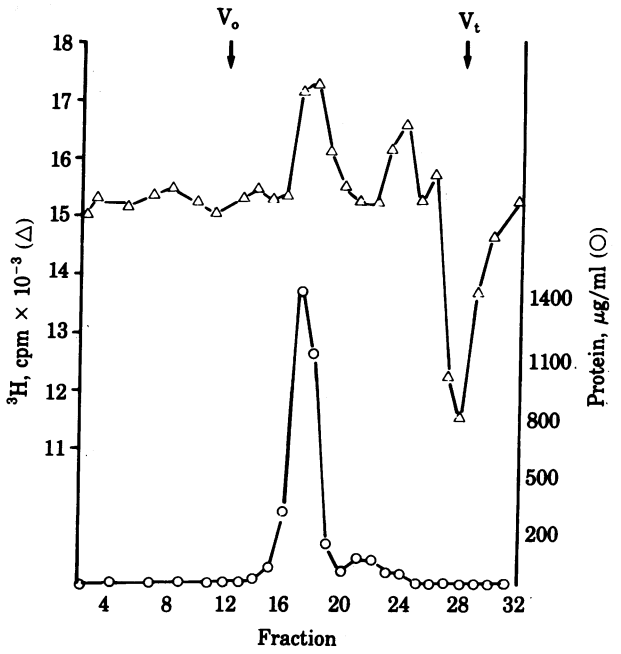

FIG. 3. Measurement of deoxycholate-binding to oligomerized α-toxin at a background detergent concentration of 5 mM. The toxin oligomer bound approximately 20% detergent (wt/wt, detergent/protein).

isolated from toxin-treated, biological target membranes (Fig. 4*C*; ref. 4).

Toxin hexamers from the Sephacryl column were mixed with a solution of deoxycholate/lecithin. After dialysis to remove detergent, the lipid vesicles that formed were isolated by flotation through sucrose as described (4). Approximately 20% of the toxin rings were regularly incorporated into the lecithin bilayers (Fig. 4*D*) and were again morphologically identical to the 11S ring structures previously isolated from biological membranes and similarly reincorporated into liposomes. The toxin molecules were not elutable from the liposomes by treatment with salt buffers of varying ionic strength, but could be released as 11S hexamers by detergent solubilization of the liposomes (results not shown).

Specificity of Detergent-Toxin Interaction. Similar experiments were conducted with Triton X-100, the synthetic lysolecithin analogues 1-dodecylpropanediol-3-phosphorylcholine and 1-lauroylpropanediol-3-phosphorylcholine (kindly supplied by U. Weltzien, Freiburg), and $NaDodSO_4$. None of these detergents induced oligomerization of α-toxin at concentrations above the critical micellar concentration. Moreover, oligomerization did not occur when toxin was first treated with Triton X-100 (final concentration) 6 mM and then deoxycholate (6.25 mM) was subsequently added. The presence of pure deoxycholate micelles thus appears to be necessary for inducing oligomerization of α-toxin in solution, and mixed Triton/deoxycholate detergent micelles do not exhibit this property.

Temperature dependence of deoxycholate-induced oligomerization of α-toxin was tested by treating a sample of native

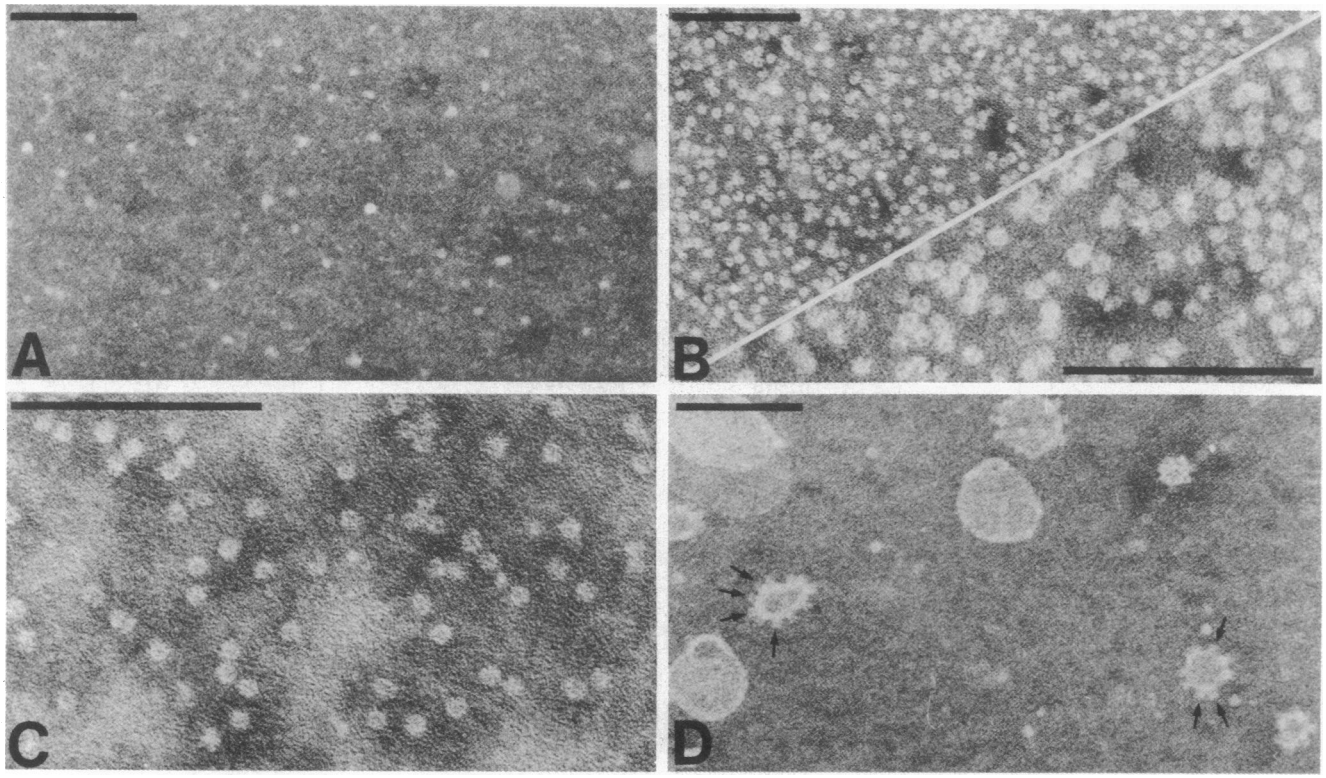

FIG. 4. (*A*) Native 3.3S α-toxin, showing the presence only of finely granular material. (*B*) Aliquot of peak b (Fig. 2*C*), showing the presence of ring-shaped molecules (8.5 nm in outer diameter) with pores of 2.5–3.0 nm in diameter. These molecules are indistinguishable from toxin oligomers isolated from toxin-treated erythrocytes, shown for comparison in *C*. (*D*) Lecithin liposomes carrying the deoxycholate-induced toxin hexamers (arrows). (Stained with sodium silicotungstate; scale bars indicate 100 nm.)

toxin (2 mg/ml) with 6.25 mM deoxycholate at 0°C or 22°C for different time periods and then sedimenting the toxin in sucrose density gradients. At 22°C, the oligomerization of α-toxin was virtually instantaneous, all the protein immediately sedimenting as an 11S peak. At 0°C, oligomerization was markedly retarded but did occur, and total conversion to the 11S form was complete after 60 min (data not shown).

DISCUSSION

The unusual observation made in this study is that oligomerization of primarily hydrophilic α-toxin molecules to form amphiphilic, ring-structured hexamers can be induced in a two-component system solely through contact of the protein molecules with deoxycholate detergent micelles. A particular physicochemical microenvironment alone thus suffices to trigger the conformational changes in the toxin molecules that lead to self-association and exposure of apolar surfaces. If an analogous mechanism can be taken to operate at the membrane level, the hydrophilic–amphiphilic transition of α-toxin molecules in a target membrane also might be expected to depend solely on the presence of an appropriate environment, possibly represented by the lipid bilayer itself (18).

The hydrophilic–amphiphilic oligomerization of α-toxin in solution is dependent on the presence of deoxycholate micelles. Oligomerization does not occur at detergent concentrations below the critical micellar concentration, or in the presence of other tested detergents. A charge effect of the detergent alone cannot be responsible because mixed micelles of Triton and deoxycholate also fail to induce oligomerization. Possibly, other soluble substrates will eventually be found that can mimic the action of deoxycholate. The reported appearance of ring structures in preparations of native α-toxin may be due to the presence of such contaminating substances in the preparations. The reported presence of lytically inactive high molecular weight toxin forms in bacterial culture supernates (18) may also be due to spontaneous oligomerization induced in a similar manner.

Once formed, the toxin hexamers are entirely stable and do not fully dissociate unless boiled in $NaDodSO_4$. The hydrodynamic data indicate the presence of a homogeneous population of toxin oligomers of molecular weight 200,000, corresponding to hexamers of the native toxin molecules. Their amphiphilic nature is apparent from their ability to bind large amounts of detergent and their lipid-binding capacity. Furthermore, removal of deoxycholate from the toxin hexamers by dialysis was found to cause their aggregation (data not shown). The same properties are shared by 11S toxin complexes formed on and isolated from biological target membranes (4). Ultrastructurally, the membrane-derived complexes are also indistinguishable from the deoxycholate-induced hexamers. All the evidence therefore points to molecular identity between the hexamers formed in solution with the toxin rings formed on membranes. Future studies into the mechanism through which protein oligomerization causes exposure of apolar molecular surfaces will be aided by the possibility of studying this process in solution under controlled conditions such as have been described here.

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1. Arbuthnott, J. P. (1970) *Microbial Toxins*, eds. Montie, T. C. Kadis, S. & Ajil, S. A. (Academic, New York), Vol. 3, pp. 189–236.
2. Bernheimer, A. W. (1974) *Biochim. Biophys. Acta* **344**, 27–50.
3. Rogolski, M. (1979) *Microbiol. Rev.* **43**, 320–360.
4. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. & Wellensiek, H. J. (1981) *J. Cell Biol.*, in press.
5. Freer, J. H., Arbuthnott, J. P. & Bernheimer, A. W. (1968) *J. Bacteriol.* **95**, 1153–1168.
6. Freer, J. H., Arbuthnott, J. P. & Billcliffe, B. (1973) *J. Gen. Microbiol.* **75**, 321–332.
7. Mayer, M. M. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2954–2958.
8. Bhakdi, S. & Tranum-Jensen, J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5655–5659.
9. Helenius, A. & Simons, K. (1972) *J. Biol. Chem.* **247**, 3656–3661.
10. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
11. Tranum-Jensen, J., Bhakdi, S., Bhakdi-Lehnen, B., Bjerrum, O. & Speth, V. (1978) *Scand. J. Immunol.* **7**, 45–56.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. C. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Six, H. R. & Harshman, S. (1973) *Biochemistry* **12**, 2672–2677.
14. Six, H. R. & Harshman, S. (1973) *Biochemistry* **12**, 2677–2683.
15. Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415**, 29–79.
16. Tanford, C. & Reynolds, J. A. (1976) *Biochim. Biophys. Acta* **457**, 133–170.
17. Cassidy, P. & Harshman, S. (1979) *Biochemistry* **18**, 232–236.
18. Arbuthnott, J. P., Freer, J. H. & Billcliffe, B. (1973) *J. Gen. Microbiol.* **75**, 309–319.
19. Arbuthnott, J. P., Freer, J. H. & Bernheimer, A. W. (1976) *J. Bacteriol.* **94**, 1170–1177.