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CONFORMATIONAL ALTERATION IN ALPHA-TOXIN FROM STAPHYLOCOCCUS AUREUS CONCOMITANT WITH THE TRANSFORMATION OF THE WATER-SOLUBLE MONOMER TO THE MEMBRANE OLIGOMER

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SUMMARY: The membrane-damaging alpha-toxin aggregate of Staphylococcus aureus was characterized physicochemically. The aggregate weight of the toxin formed by various methods appeared to be 6 times higher than the molecular weight of the monomer as determined by the laser light scattering technique, suggesting the presence of a hexamer in the membrane. The aggregates fluoresced 20 to 50 % more than the monomer at 336 nm. Circular dichroism measurements revealed that both the monomer and the oligomer showed essentially β-sheet structure with the maximum ellipticity about -8,400 deg.cm^2.dmol^{-1} at 215 nm. Circular dichroism spectrum of the oligomers showed ellipticity difference of -6,600, -44 and +84 deg.cm^2.dmol^{-1}, at 200, 250 and 280 nm, respectively, compared with the monomer. All these results suggest that the conformational change in the toxin molecule occurs concomitant with the transformation of the water-soluble monomer to the membrane-embedded hexamer.

Alpha-toxin, a cytolytic exo-protein produced by Staphylococcus aureus has been accounted to be a major cause of the pathogenecity of this organism (1,2). The mechanism of eliciting the cytolytic activity of this toxin is the formation of the transmembrane hollow or pore in the plasma membrane of susceptible cell (3-5), through which intracellular materials leak out (4-7). The transmembrane pore is formed by an aggregate of the toxin molecules, although the toxin monomer is an water-soluble protein. This cytolytic activity can be easily measured in vitro by the hemolysis (8, 9). In the process of α-toxin action, the water-soluble toxin molecules must be triggered to elicit a conformational change to gain an ability to penetrate into the hydrophobic domain of the target membrane.

We have tested this assumption by measuring the spectroscopic properties of the toxin molecule. The result suggested that the oligomerization accompanied a conformational change(s) in protein molecules.

Abbrevations used: RBC, red blood cell; SDS, sodium dodecylsulfate; DOC, sodium deoxycholate; $C_{12}E_8$, polyoxyethyleneglycol dodecylether; Buffer A, 10 mM Tris-HCl-50 mM NaCl-3 mM NaN_3, pH 8.2; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CD, circular dichroism; LS, light scattering; RI, refractive index.

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MATERIALS and METHODS

Purification of α-toxin. Alpha-toxin was purified from the culture supernatant of S. aureus Wood 46 in Tryptic soy broth. In a typical experiment, a 4 liters of the culture supernatant was fractionated by 70 % saturated $(NH_4)_2SO_4$ at 4 °C. The insoluble materials were dissolved in 10 ml of 50 mM Tris-HCl, pH 8.5 and were passed through a Sephadex G-100 column (2.5 x 100 cm) equilibrated with 50 mM Tris-HCl, pH 8.5. The fractions containing a major hemolytic activity were applied onto two sequentially connected columns (a CM-Sepharose CL-6B and a DEAE-Sephacel, of 0.5 x 20 cm each) equilibrated with 50 mM Tris-HCl, pH 8.5, and the columns were eluted with the same buffer. The toxin preparation in a flow-through fraction appeared to be a homogeneous protein band with an apparent M_r 33,000 as examined by polyacrylamide gel electrophoresis in SDS. Protein was quantified by the Lowry's method (10) or by the absorption at 280 nm. Absorption coefficient at 280 nm at 1 cm light path of 1 mg/ml of the toxin was determined to be 1.97.

Oligomerization of α-toxin. (i) The oligomers induced by DOC (DOC-oligomers) were the procedure described by Bhakdi et al (11). The toxin (1 mg/0.2 ml in Buffer A) was mixed with 20 μl of 68.7 mM DOC in Buffer A, and the mixture was incubated at 25 °C for 30 min. (ii) The oligomers formed on the membrane of phosphatidylcholine liposome (PC-oligomer). The liposome membrane prepared by the procedure described earlier (12) (7.5 mg/0.6 ml in Buffer A) was mixed with 3.45 mg of α-toxin in 0.46 ml of Buffer A and the mixture was kept at 25 °C for 2 h. (iii) The oligomers formed on the membrane of rabbit RBC (RBC-oligomer). The RBC membranes prepared according to the procedure of Dodge et al (13) (2.6 mg protein/ml in Buffer A) were mixed with 3 mg/0.4 ml of purified α-toxin and the mixture was kept at 25 °C for 2 h.

Determination of molecular and aggregate weights. The molecular or aggregate weight was determined by the laser LS technique combined with the high performance gel permeation chromatography and refractometry (for details, see ref. 14-16).

Other methods. CD was measured by using a JASCO spectropolarimeter J-500 equipped with a DP-501 data system. Fluorescence was measured by using a Shimadzu fluorophotometer RF-450 equipped with a recorder DR-3.

RESULTS and DISCUSSION

State of the toxin aggregates. Since the oligomeric aggregates of α-toxin molecules can be formed on plasma membrane, liposome membrane and in DOC micelle, it must be confirmed that the aggregates formed by these techniques are the same in physicochemical criteria. Fig. 1 depicted an SDS-polyacrylamide gel electropherogram of the purified oligomers as well as their heat-dissociated monomers. The result showed that the oligomers were electrophoretically homogeneous.

The oligomers were separated from the monomers by the gel filtration through a Sephacryl S-300 column in the presence of $C_{12}E_8$ and their low-angle LS, UV-absorption and RI were measured in the presence of 2 mM $C_{12}E_8$. $C_{12}E_8$ does not cause neither oligomerization of the monomer nor dissociation of the oligomer. A typical elution profile was shown in Fig. 2. Molecular- and aggregate weights of the protein moiety in the surfactant was calculated according to the procedure described earlier (14-16). Thus, the molecular weight of native α-toxin molecule appeared to be 32,400 (Table 1). The aggregate weights of the oligomers formed in DOC, PC-membrane and RBC-membrane were calculated to be 203,000, 186,000 and 189,000, respectively (Table 1), indicating the presence of hexamers as suggested previously (3-5, 17-19).

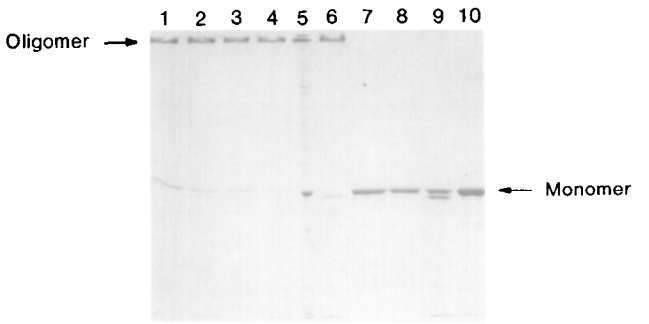

Fig. 1: Polyacrylamide gel electrophoresis of the oligomers formed by various methods. The toxin oligomers were formed in DOC, PC-membrane and RBC-membrane as described above. A part of the sample was treated with 100 μg/ml of trypsin at room temperature to confirm the trypsin resistance (5). They are dissolved in the sample buffer and run for electrophoresis in the buffer system described by Laemmli (22) with or without heating at 95 °C for 5 min. Lanes 1, 2 and 7, DOC-oligomer native, trypsin-treated and heated; lanes 3, 4 and 8, PC-oligomer native, trypsin-treated and heated; lanes 5, 6 and 9, RBC-oligomer native, trypsin-treated and trypsin-treated-heated; lane 10, α-toxin monomer heated in the sample buffer.

This is the first report, to our knowledge, that the aggregate weight and the subunit number of the membrane-assembled α-toxin were determined by the technique based on the firm physical rule.

Spectroscopic properties of the α-toxin monomer and oligomer. In the process of the oligomer formation, the water-soluble α-toxin molecule turns to be the water-insoluble membrane protein or the surfactant-solubilized protein oligomer. This drastic change of the protein environments may elicit a certain conformational change in the toxin molecule. We have examined fluorescence emissions of tryptophan residues in the monomers and the oligomers in the presence of 2 mM $C_{12}E_8$. The oligomers formed in DOC, PC-membrane and the RBC-membrane were extracted by 1 % of $C_{12}E_8$ and purified by passing through a Sephacryl S-300 column. The monomers were dissolved in 1 % of $C_{12}E_8$ in an identical absorption at 280 nm to the respective oligomers. Emission spectra of the protein showed the following; (i) The oligomers fluoresced more intense than the monomers under the identical conditions. (ii) Extent of fluorescence increment was not always the same, but was reproducible among the oligomers formed by the different methods. Increments of fluorescence intensity in the oligomers were 33, 48 and 10 % for DOC-, PC- and RBC-oligomer, respectively. Reason for this difference is not clear at present. (iii) The emission maximum of the oligomers was shifted from 332 to 336 nm. These results suggested that the protein conformation or the environment around the tryptophan residues changed, concomitant with the oligomer formation (Fig. 3).

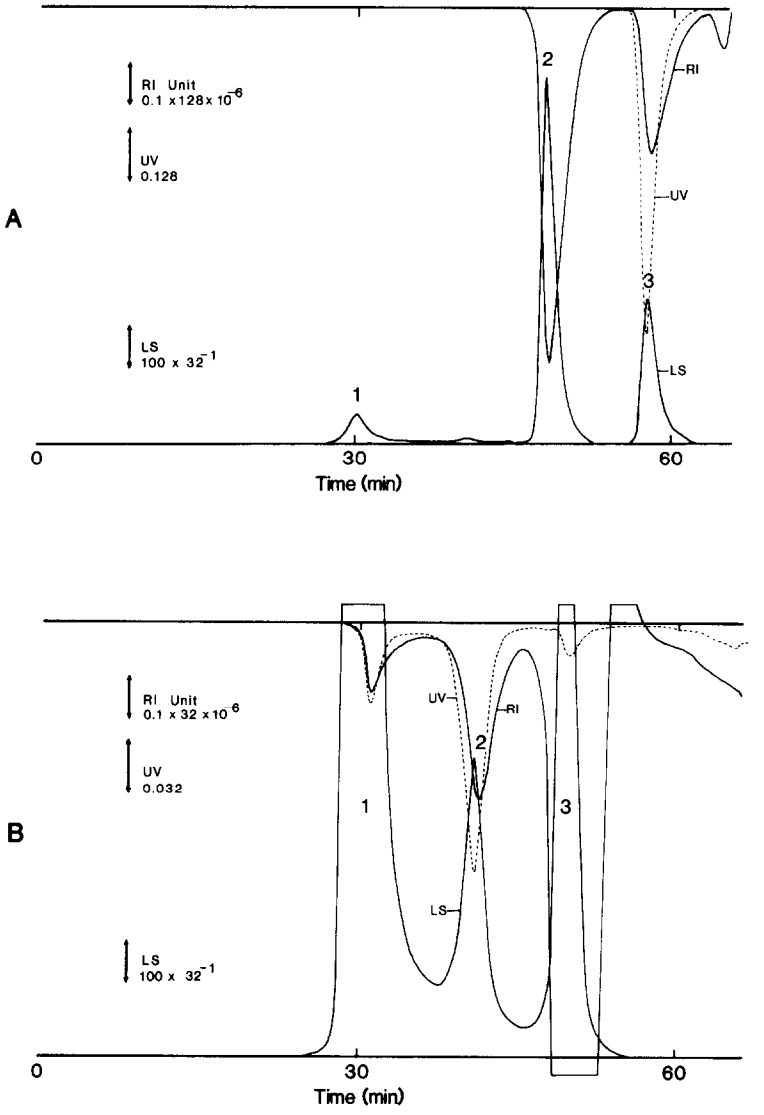

Fig. 2: Typical tracings of the detector response of a LS photometer, a UV-detector and a RI meter. A TSKgel G3000SW column (0.75 x 60 cm) was connected to a high performance liquid chromatography system in the following sequence; a double plunger pump - a column - a differential UV-flow detector - a laser LS photometer - a differential RI meter and the signals from the detectors were recorded. A, α-toxin monomers were dissolved in a solution containing 2 mM $C_{12}E_8$-10 mM Hepes-100 mM NaCl-3 mM NaN_3, pH 7 (0.3 mg/ml) and a 0.1 ml portion was injected into the column equilibrated with the above solution. The column was eluted with the same buffer in the rate of 0.4 ml/min at 15 kg/cm^2. Peaks 1, 2 and 3 correspond to an unidentified material (probably a spontaneously formed higher aggregate of the toxin), $C_{12}E_8$-micelle and the toxin monomer, respectively. B, The purified RBC-oligomers were dissolved at 1.5 mg/ml in a solution described above. A portion of this material (0.2 mg/0.13 ml) was injected into a column and the column was eluted as above. Peaks 1, 2 and 3 are unidentified material (see above), the toxin oligomer and the micelle, respectively. Molecular (aggregate) weight of protein or protein moiety of protein surfactant complex was calculated from a calibration line on the basis of LS/RI·1/(dn*/dc) of the reference proteins of known molecular weight, where LS, RI and (dn*/dc) are the LS intensity, the relative RI and the specific RI increment of the protein (or protein-surfactant complex). (dn*/dc) was calculated as $RI/(A_{280\,nm}/\kappa)$, where $A_{280\,nm}$ and κ are absorption at 280 nm and an absorption coefficient of the individual protein, respectively (for a detail, see ref. 14-16). Reference proteins used were ribonuclease, chymotrypsinogen A, alcohol dehydrogenase and bovine serum albumin.

Table 1. Aggregate weight and subunit number of alpha-toxin oligomer determined by the method of the laser LS technique in the presence of $C_{12}E_8$

Alpha-toxin	Aggregate weight	Subunit[b]
monomer	32,400[a]	
DOC-oligomer	203,000	6.2
PC-oligomer	186,300	5.7
RBC-oligomer	189,400	5.9

[a] Molecular weight
[b] Aggregate weight of oligomer/molecular weight of monomer

Since the fluorescence increment was recognized in the oligomers, we have examined the conformational states of the toxin monomer and the oligomer by recording CD spectra. Fig. 4A showed a typical trace of the recordings. The CD profiles between the monomer and the oligomer from 190 to 205 nm appeared to be significantly different but that from 215 to 240 nm were indistinguishable. The maximum molar ellipticity of both the monomer and the oligomer appeared to be about -8,400 deg.cm^2.$dmol^{-1}$ at 215 nm. Mathemetical treatment of the data according to the procedure described by Chen et al (20) showed that the monomers contained α-helix, β-sheet and random coil, 11, 61 and 28 %, respectively and those in the oligomers were 12, 58 and 30

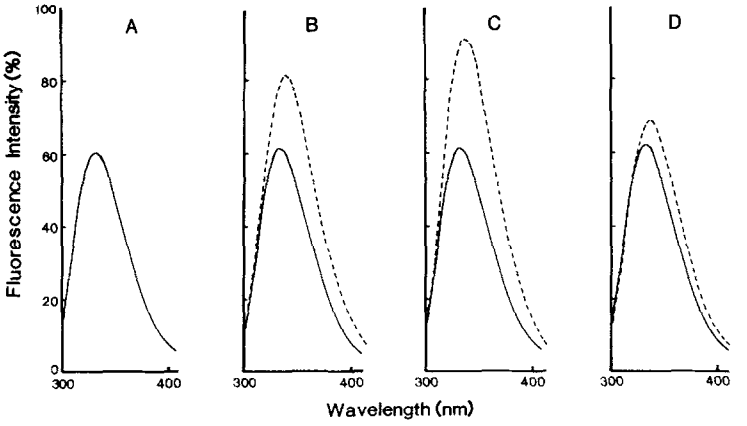

Fig. 3: Fluorescence spectra of α-toxin. The toxin monomers and the oligomers were dissolved in an appropriate solution to $A_{280\ nm}$ = 0.30 (0.152 mg/ml). The fluorescence spectra were recorded from 300 to 400 nm with an excitation wavelength at 280 nm at room temperature. Slit widths for an excitation and an emission were 5 and 10 nm, respectively. A, the monomer in Buffer A; B, the monomer and DOC-oligomer in Buffer A containing 1.25 mM DOC; C, the monomer and PC-oligomer in Buffer A containing 2 mM $C_{12}E_8$; D, the monomer and RBC-oligomer in 2 mM $C_{12}E_8$-10 mM Hepes-100 mM NaCl-3 mM NaN_3, pH 7. Solid and dotted lines show the emission spectra from the monomer and the oligomer, respectively.

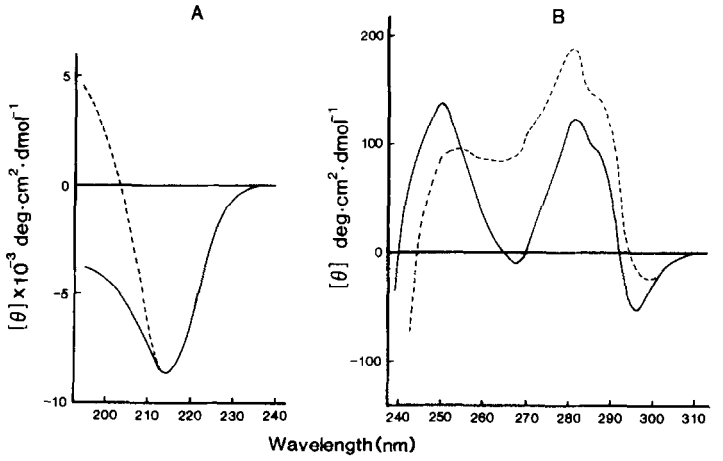

Fig. 4: CD spectra of α-toxin monomer and the oligomer. The monomers and the purified RBC—oligomers were dissolved at $A_{280\ nm}$ = 1.04 (0.529 mg/ml) in 2 mM $C_{12}E_8$-10 mM Hepes-100 mM NaCl-3 mM NaN_3, pH 7 and the CD spectra were recorded. A, record from 190 to 240 nm at wavelength expansion 5 nm/min, a time constant 0.25 sec, a scan speed 50 nm/min and a light path 0.01 cm at room temperature. The mean residue weight was calculated from the amino acid composition. The curve presented was the average of 16 repeated scans. B, record from 240 to 310 nm. Conditions are similar to above except that a light path was 1 cm. The scan was repeated 4 times. Solid and dotted lines show the monomer and oligomer, respectively.

%, respectively. As long as these calculations are concerned, the oligomerization showed no significant conformational change in the backbone structure of the protein molecule. However, a significant change in CD spectra between the monomer and the oligomer was seen in the region from 190 to 205 nm. The difference of ellipticity between them at 200 nm appeared to be 6,600 $deg.cm^2.dmol^{-1}$. This significant difference in the ellipticity must be meaningful and, therefore, we believe that the conformational change in the protein molecule occurred concomitant with the monomer to the oligomer transformation. Since, there is no model study in such change at 190 to 215 nm region, to our knowledge, we are unable to characterize the nature of the alteration. However, it must be stressed that the change of CD spectrum at this region was reproducibly observed (16 repeated experiments) and the change was significantly large. One reason why no model study could be made in such change at this region might be that a thermodynamically-unstable model compound (like the monomer) could not be synthesized, or even if it was made the compound readily transformed into a stable form (like the oligomer). In fact, α-toxin tends to form aggreagate spontaneously at relatively high concentration (about 1 mg/ml) by a light mechanical force like a vortexing. A similar change of CD profile upon spontaneous insertion of a water-soluble protein into membrane was reported in δ-toxin from S. aureus (21).

As shown in Fig. 4B, the changes of CD spectrum at 250 and around 280 nm, corresponding to tyrosine and tryptophan residues, respectively, were observed, concomitant with the monomer to the oligomer conversion. The data confirmed the result of fluorescence change. The ellipticity changes at 250 and 280 nm by the oligomerization of the toxin molecule were -44 and +84 $deg.cm^{2}.dmol^{-1}$, respectively. The conformational change of the toxin molecules was observed consistently regardless of the method of the oligomer formation.

The results reported in this paper revealed a major conformational transition of the toxin molecule upon the oligomer formation as detected in the change of CD spectrum and of the fluorescence intensity. The studies on the conformational change of the protein molecules concomitant with the transformation of the water-soluble to the membrane protein are essential to elucidate a mechanism of the transmembrane protein assembly. The studies also contribute in understanding of the pathogenecity of the staphylococcal infection.

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