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Cold-Labile Hemolysin Produced by Limited Proteolysis of θ-Toxin from *Clostridium perfringens*†

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ABSTRACT: A nicked toxin whose hemolytic activity is temperature dependent was obtained by limited proteolysis of θ-toxin (M_r 54 000) with subtilisin. The nicked toxin (Cθ) is a complex of two fragments: the N-terminal fragment (M_r 15 000) with basic isoelectric point and the C-terminal fragment (M_r 39 000) with the single cysteinyl residue of the toxin whose reduced form is essential for the hemolytic activity. Cθ hemolyzes erythrocytes only at temperatures above 25 °C, whereas the native toxin hemolyzes them even at 10 °C. At temperatures below 25 °C, Cθ does not hemolyze them although it does bind to membrane cholesterol and although no distinct difference was observed between the secondary structure of Cθ and that of native toxin. It was found that Cθ binds to the cells only in a reversible manner at low temperature, while the native one binds irreversibly to the cells within 10 min, which explains the cold lability of Cθ on hemolysis. The structural basis of the cold lability was discussed through comparison of Cθ with another nicked derivative of θ-toxin that was also obtained.

θ-Toxin (perfringolysin O) is an exotoxin produced by *Clostridium perfringens* type A, which belongs to a group of oxygen-labile or thiol-activated hemolysins (Mitsui et al., 1973; Smyth, 1975; Yamakawa et al., 1977). Other hemolysins that belong to the same group such as streptolysin O (Alouf & Raynaud, 1973), cereolysin (Cowell et al., 1976), tetanolysin (Lucain & Piffaretti, 1977; Mitsui et al., 1980), and alveolysin (Geoffroy & Alouf, 1983) were obtained from culture filtrates of *Streptococcus pyogenes*, *Bacillus cereus*, *Clostridium tetani*, and *Bacillus alvei*, respectively. These hemolysins share common properties (Bernheimer, 1976). They are inhibited by a small amount of cholesterol, activated by thiol compounds, and serologically related to each other (Cowell & Bernheimer, 1977).

In recent years the toxins were reported to damage the cell membrane of human fibroblasts (Thelestam & Möllby, 1980), Hela cells (Duncan & Buckingham, 1980), and myocardial cells (Fisher et al., 1981) in addition to the erythrocyte membrane. However, it is not well understood how they damage the cells after adsorption on the membrane cholesterol. Stimulation of ion efflux (Saito, 1983; Blumental & Habig, 1984) has been reported by several workers, but the existence of a causal relation between these phenomena and cell lysis remains to be established. Since the processes leading to hemolysis after adsorption of the toxin on the membrane cholesterol are sequential and rapid, it is difficult to analyze each process biochemically.

One approach to this problem would be studies with modified toxins that bind but do not hemolyze the cells. They would be useful to distinguish essential processes for hemolysis from secondary effects caused by the toxin action. Another approach is to obtain the toxin fragments that have different biological aspects of the processes. For example, colicins E1, E2, and E3 are found to be composed of three functional domains by analysis of the fragments obtained by the limited proteolysis; each fragment is responsible for each distinctive process of adsorption, penetration, and biological activity in the target cells (Ohno-Iwashita & Imahori, 1980, 1982).

In this study we obtained a nickel derivative of θ-toxin by limited proteolysis and found that it binds but does not hemolyze the cells at low temperatures. The nicked toxin and its constituents were isolated, characterized, and compared with another nicked derivative of θ-toxin that we also obtained.

Experimental Procedures

Materials. Trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] was purchased from Worthington. Soybean trypsin inhibitor, subtilisin Carlsberg, and PMSF[1] were from Sigma. Amopholines were from LKB.

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Preparation of θ-Toxin. Culture filtrate (20 L) of *C. perfringens* type A strain PB6K N5 was concentrated and applied to a DEAE-Sephadex column according to Yamakawa et al. (1977). The pass-through fraction, which was used as the starting material, was a gift from Drs. S. Takano and T. Shimizu, Chiba Serum Institute.

Ammonium sulfate was added to the crude toxin solution at a final concentration of 1 M. The solution was then applied to a column of Sepharose 6B on which cholesteryl hemisuccinate was immobilized as a ligand. The column (150 mL) was preequilibrated with buffer A [70 mM sodium phosphate, pH 6.0, containing 78 mM NaCl and 1 M $(NH_4)_2SO_4$]. After the sample was applied, the column was washed with the same buffer. θ-Toxin was eluted with a linear gradient formed from 150 mL each of buffer A and buffer B [70 mM sodium phosphate, pH 6.0, containing 78 mM NaCl, 0.2 M $(NH_4)_2SO_4$, and 40% ethylene glycol], followed by buffer B. θ-Toxin fractions were pooled, dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol), and applied to a Whatman DE-52 column (150 mL) equilibrated with the same buffer. θ-Toxin was eluted with a linear gradient of NaCl concentration (0–0.15 M) in buffer C. Sodium chloride was added to the pooled θ-toxin solution at a final concentration of 0.5 M, and the solution was applied to a phenyl-Sepharose CL-4B column (150 mL) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The column was washed with the same buffer, and θ-toxin was eluted with a linear gradient formed from 750 mL each of buffer D (20 mM sodium phosphate, pH 7.0, containing 0.25 M NaCl) and buffer E (20 mM sodium phosphate, pH 7.0, containing 60% ethylene glycol). Pooled fractions of θ-toxin were dialyzed against 10 mM sodium phosphate buffer, pH 7.0. θ-Toxin and the digested products were stored at 4 °C unless otherwise specified.

The yield of purified θ-toxin was 29 mg from 20 L of cell culture. This represents a 64% overall yield of the toxin. The specific activity of the preparation was about 4.8×10^6 hemolytic units (HU)/mg protein when 1 HU was defined as the amount of the toxin required to cause 50% hemolysis (HD_{50}) of sheep erythrocytes (1 mL of 0.5% suspension) in 30 min at 37 °C.

Determination of Hemolytic Activity of θ-Toxin and Its Derivatives. Hemolytic activity of θ-toxin and its derivatives was determined as described previously (Saito et al., 1982). Toxin solutions were preincubated for activation at 37 °C for 15 min according to Saito et al. (1982). A reaction mixture for the hemolysis assay was composed of 0.75 mL of erythrocyte suspension (0.67%) and 0.25 mL of the activated toxin solution. After incubation for 30 min at 37 °C, the degree of hemolysis was determined by measuring the absorbance at 540 nm of the supernatant. The HD_{50} value was obtained by using the von Krogh equation (Mayer, 1961).

Digestion of θ-Toxin with Proteases. Trypsin or subtilisin Carlsberg was added to a solution of θ-toxin in 50 mM Tris-HCl, pH 8.8 (θ-toxin/trypsin = 20/1 w/w; θ-toxin/subtilisin Carlsberg = 26/1 w/w). The mixture was incubated for 6 h at 25 °C, then, the same amount of the protease was added. The mixture was further incubated overnight at 25 °C. Trypsin was removed from the digest by applying the digest (5 A_{280} units) to a 2-mL benzamidine–Sepharose 6B column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8.0, and 0.5 M NaCl. The pass-through fraction was dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and used as trypsin-free digest of θ-toxin. In some experiments soybean trypsin inhibitor was added to the digest, and the mixture was analyzed without further purification. The digestion with subtilisin Carlsberg was stopped by the addition of 3.3 mM PMSF. The digest (2.5 A_{280} units) was diluted with 4 volumes of distilled water and applied to a 1.5-mL DE-52 column equilibrated with 20 mM Tris-HCl, pH 8.8. The protease passed through, and the digested toxin was eluted with a linear gradient of NaCl concentration (0–0.3 M) in the same buffer. Recoveries of the digested toxins with trypsin and with subtilisin Carlsberg were 65% and 56%, respectively.

Isolation of the Fragments Produced by Digestion with Trypsin and Subtilisin Carlsberg. The protease-free digests of θ-toxin were applied to a TSK gel-TMS 250 column (0.4 × 10 cm; Toyo Soda Manufacturing Co. Ltd.) and developed with a linear gradient of acetonitrile (0–60%) in 0.1% trifluoroacetic acid. The T1, T2, C1, and C2 fragments (see Results) were eluted at the concentrations of 37%, 34%, 36.5%, and 28% acetonitrile, respectively. A solution of each fragment was dried and used for N-terminal analysis and amino acid analysis. The recoveries of the fragments corresponded to 66% and 57% of the applied proteins for the digests with trypsin and with sutilisin Carlsberg, respectively.

N-Terminal Sequence Analysis. N-Terminal sequences of θ-toxin and its fragments were analyzed by using a protein sequencer, Model 470 A (Applied Biosystems).

Amino Acid Analysis. Proteins were hydrolyzed in vacuo at 110 °C with 6 N HCl for 20 h, and the hydrolysates were analyzed on a Hitachi 835-50 amino acid analyzer.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-500A spectropolarimeter. On the basis of amino acid analyses, the mean residue weight and the extinction coefficient ($E_{280}^{0.1\%}$) of θ-toxin were estimated to be 110.8 and 1.6, respectively, and these values were used for calculation of the mean residue ellipticity ($[\theta]$ in deg cm^2 dmol^{-1}) of θ-toxin and its digested derivatives.

Electrophoresis. PAGE without SDS was performed in 7.5% gel at pH 8.3, according to Ornstein (1964) and Davis (1964). Analytical SDS gels (12%) were prepared and run according to Laemmli (1970). The molecular weights of θ-toxin and the fragments were estimated by SDS–PAGE using Bio-Rad low molecular weight protein standards. Two-dimensional gel electrophoresis (nonequilibrium pH gradient electrophoresis) was performed according to O'Farrell et al. (1977). Where specified, urea was omitted from the gel.

Results

Digestion of θ-Toxin with Proteases. θ-Toxin (M_r 54 000) was digested with subtilisin Carlsberg or trypsin under the conditions described under Experimental Procedures, and the digests were analyzed by SDS–PAGE (Figure 1). In each digestion, θ-toxin was cleaved into two fragments, C1 (M_r 39 000) and C2 (M_r 15 000) by the digesion with subtilisin Carlsberg (Figure 1, lane 2) and T1 (M_r 29 000) and T2 (M_r 26 000) by trypsinolysis (lane 3). The fragments were further analyzed by two-dimensional gel electrophoresis with isoelectric focusing in the presence or absence of urea in the first dimension and then by SDS–PAGE in the second dimension (Figure 2). C1 and C2 fragments comigrated with each other in the absence of urea in the first-dimensional gel (Figure 2A), even though they have quite different p*I*s, as shown in the gel with urea (Figure 2B). These results indicate that the toxin digested with subtilisin Carlsberg (referred to as Cθ) exists as a complex of C1 and C2 in the absence of urea. When the digest was run on polyacrylamide gel at pH 8.3 without SDS and the hemolytic activity of proteins eluted from the gel slices was assayed, the activity was accompanied with one protein band composed of C1 and C2 (data not shown), showing that

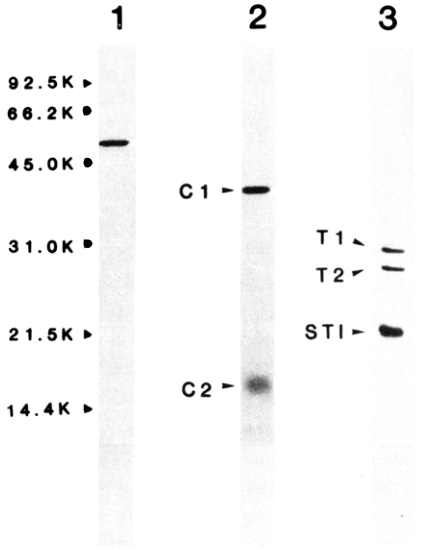


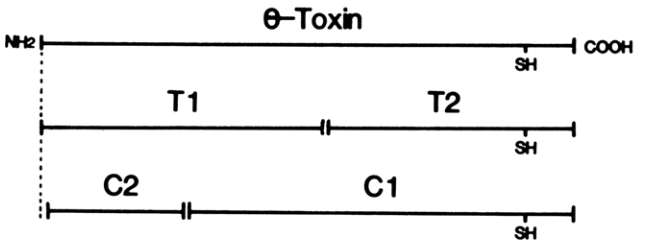
FIGURE 1: SDS–PAGE of digests of θ-toxin with proteases. Purified θ-toxin (lane 1) and digests of θ-toxin with subtilisin Carlsberg (lane 2) and with trypsin (lane 3) were run on a gel, and protein bands were stained with silver nitrate. The migration positions of molecular weight standards are indicated by arrows. STI represents soybean trypsin inhibitor.

Cθ has hemolytic activity. Similar experiments showed that the toxin digested with trypsin (referred to as Tθ) is a complex of T1 and T2 (Figure 2C,D) and has hemolytic activity.

Characterization of Toxin Fragments. The amino acid compositions of isolated fragments are listed in Table I. The sum of the amino acid compositions of C1 and C2 or T1 and T2 is very similar to that of θ-toxin, indicating that the proteolysis occurred at quite limited point(s) under our conditions.

The N-terminal amino acid sequence of T1 fragment was determined as Lys-Lys-Ile-Thr-X-Ile-Asn-Gln, which coincides well with that of θ-toxin, determined as Lys-Lys-Ile-Thr-X-Ile-Asn-Gln-X-Ile-Asp-X-Gly-Ile-X-X-Leu. On the other hand, the N-terminal sequence of T2 fragment was determined as Asn-X-Gln-Gln-Tyr-X-Asp-Ile. Thus, T1 contains the N-terminus of θ-toxin, while T2 was assigned to the C-terminal portion. Since the trypsin digestion of C1 produced T2, C1 was also assigned to the C-terminal region. The N-terminal sequence of C2 was found to be Asn-Gln-Ser-Ile-Asp-Ser-Gly-Ile-Ser-Ser-Leu. It shows that C2 was derived from the N-terminal portion of θ-toxin though the N-terminal hexapeptide of the native toxin was cut off by the subtilisin digestion.

Scheme I



θ-Toxin has only one cysteinyl residue, chemical modification of which with *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid) inactivates the toxin. Table I shows that the cysteinyl residue resides in the C-terminal (C1 and T2) fragments. The p*I* values of θ-toxin, C1, C2, T1, and T2 were estimated as approximately 6.9, 5.9, 9.6, 9.7, and 5.2, respectively. Thus, each digested toxin is composed of the N-terminal basic fragment (C2 or T1) and the C-terminal weakly acidic fragment (C1 or T2) with the unique cysteinyl residue (Scheme I).

Hemolytic Activities of Digested Toxins. Protease-free samples of digested toxins (Cθ and Tθ) were prepared as described under Experimental Procedures. Hemolytic activities of Cθ and Tθ were compared with that of θ-toxin at 37 °C (Figure 3). From the linear plots shown in Figure 3B, HD_{50} values of θ-toxin, Cθ, and Tθ were determined to be 0.34, 0.86, and 1.27 ng, respectively, showing that the relative hemolytic

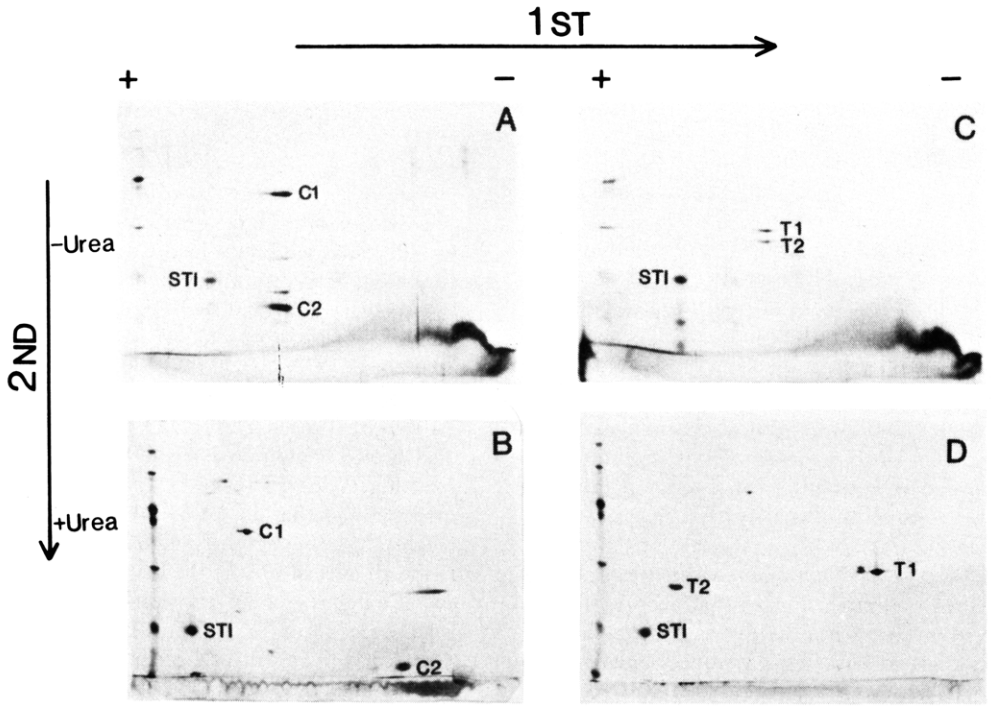


FIGURE 2: Migration of fragments in two-dimensional gels. Nonequilibrium pH gradient electrophoresis was performed for the first dimension, and SDS–PAGE (12%), for the second dimension. The digests of θ-toxin with subtilisin Carlsberg (A and B) and with trypsin (C and D) were applied on the first-dimensional gel with pH 3.5–10 ampholines in the presence (B and D) or absence (A and C) of 9 M urea and run toward the cathode for 5 h at 400 V.

Table I: Amino Acid Compositions of θ-Toxin and Its Fragments

amino acid	number of residues[a]							
	C1	C2	C1 + C2	T1	T2	T1 + T2	θ-toxin	θ-toxin[b]
Asp	53.6	21.5	75.1	40.3	36.7	77.0	75.4	76.2
Thr	27.2	6.2	33.4	15.7	21.3	37.0	35.4	33.2
Ser	32.1	13.8	45.9	27.9	19.2	47.1	46.8	42.9
Glu	32.0	11.5	43.5	21.8	22.6	44.4	42.8	38.2
Pro	12.1	8.3	20.4	12.1	9.8	21.9	19.1	21.6
Gly	13.3	9.0	22.3	11.8	12.3	24.1	23.1	25.3
Ala	27.8	5.1	32.9	16.0	17.6	33.6	32.3	32.2
Cys	0.6		0.6		0.9	0.9	1.0[c]	0.7[c]
Val	28.4	10.3	38.7	21.9	16.8	38.7	38.7	38.3
Met	3.8	1.2	5.0	3.8	0.4	4.2	4.5	4.5
Ile	17.9	11.5	29.4	16.8	13.8	30.6	31.3	31.1
Leu	21.8	9.2	31.0	19.2	10.1	29.3	30.8	25.7
Tyr	20.6	4.0	24.6	10.3	15.9	26.2	24.8	21.8
Phe	11.4	3.2	14.6	7.8	6.6	14.4	13.7	13.1
Lys	29.6	14.4	44.0	26.5	17.7	44.2	43.6	42.6
His	6.3	0.1	6.4	1.6	5.8	7.4	6.5	5.5
Arg	10.3	6.9	17.2	9.1	6.0	15.1	17.1	11.4
total	348.8	136.2	485.0	262.6	233.5	496.1	486.9	464.3

[a] The values are the numbers of residues in one molecule calculated on the basis of the molecular weights given in the text. [b] From Yamakawa et al. (1977). [c] After oxidation to cysteic acid by performic acid.

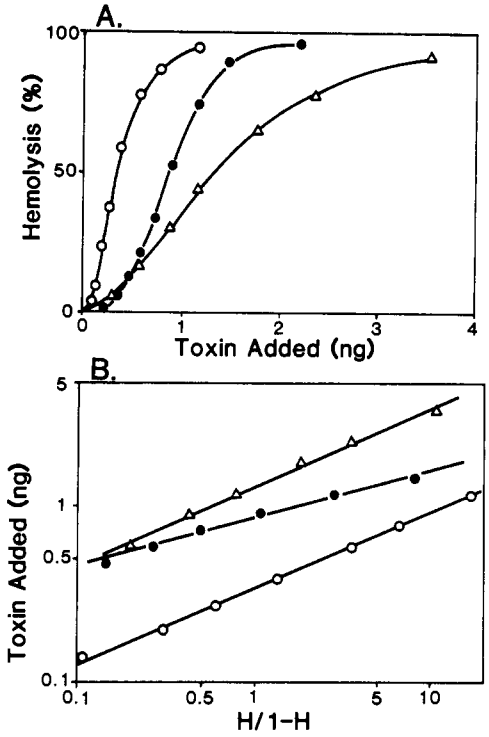


FIGURE 3: Titration of hemolytic activities of θ-toxin and its derivatives at 37 °C: (A) hemolytic activities of θ-toxin (○), Cθ (●) and Tθ (△) measured as described under Experimental Procedures; (B) dose–hemolysis curves in (A) replotted to give linear plots according to the von Krogh equation (Mayer, 1961). H represents the degree of hemolysis.

activities of Cθ and Tθ at 37 °C are 40% and 27% of that of the native toxin, respectively.

The dose–hemolysis plot of Tθ gave a straight line with the same slope as that of native toxin. On the other hand, a shallower slope was obtained in the case of Cθ (Figure 3B), suggesting that Cθ hemolyzes the cells in a manner different from that of the native toxin. Moreover, the hemolytic activity of Cθ depends strongly on incubation temperature (Figure 4). The activity of Cθ decreased markedly with decreasing temperature. At temperatures below 25 °C almost no hemolysis was observed even after the addition of a large excess of Cθ, while both θ-toxin and Tθ lysed more than 90% of the cells under the same conditions.

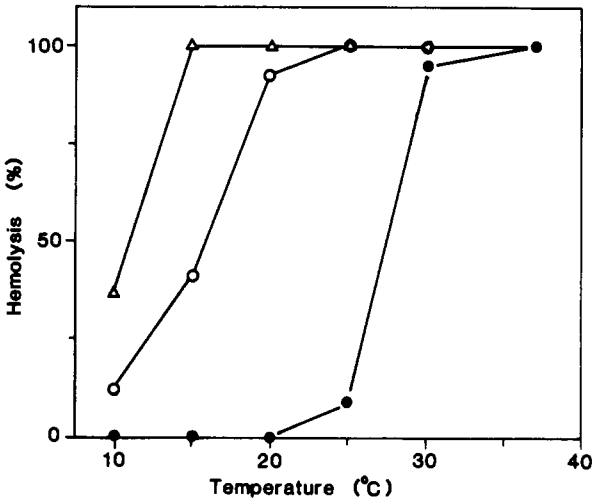


FIGURE 4: Temperature dependency of hemolytic activities of θ-toxin and its derivatives. Sheep erythrocytes were incubated with 40 HU of θ-toxin (○), Cθ (●), or Tθ (△) for 30 min at the indicated temperatures, and the degree of hemolysis was determined.

The binding activity of Cθ was examined with erythrocytes at the temperature where Cθ does not hemolyze the cells. The cells were incubated with Cθ for the indicated times at 20 °C, and the amount of bound toxin was determined by measuring hemolysis at 37 °C as described in the legend of Figure 5. The binding activity of Cθ was almost the same as that of θ-toxin (Figure 5, solid lines). The amounts of Cθ bound to the cells within 5 min at 20 °C were sufficient to cause 100% hemolysis when the cells treated with Cθ were incubated at 37 °C (Figure 5, dotted lines). Thus, Cθ binds to the cells but does not hemolyze them below 25 °C.

Since membrane cholesterol has been reported to be the primary receptor for thiol-activated hemolysins on the cell surface (Bernheimer, 1974), we examined whether Cθ binds specifically to cholesterol on the cell surface even at low temperatures. The affinity of Cθ for cholesterol was investigated (Table II). Cholesterol at 10^{-7} M reduced the amounts of bound toxins to 32–48% of the control values. Ten times higher concentration of cholesterol (10^{-6} M) completely inhibited the binding of Cθ as well as θ-toxin, at both 20 and 37 °C. Thus, Cθ, as nearly equal as θ-toxin, binds specifically to the membrane cholesterol even at low temperatures.

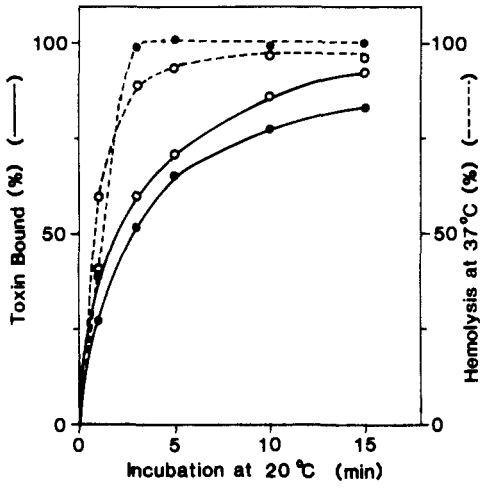


FIGURE 5: Binding of θ-toxin and Cθ to erythrocytes at 20 °C. θ-Toxin (○) or Cθ (●) (5 HU) was incubated with sheep erythrocytes (1 mL of 0.5% suspension) for the indicated times at 20 °C, and then the cells were pelleted by a short spin (10 s) in an Eppendorf centrifuge at room temperature. Fresh sheep erythrocytes (0.5 mL of 1% suspension) were added to the supernatant, and the mixture was incubated for 30 min at 37 °C. The amounts of the toxins in the supernatant were determined by using the standard dose–hemolysis plots of the toxins. The amounts of bound toxins (solid lines) were obtained by subtracting those of unbound toxins from 100%. In the same experiment, the degree of hemolysis by the bound toxins was determined by resuspending the centrifuge pellet mentioned above in 1 mL of the same buffer and incubating the suspension for 30 min at 37 °C (dotted lines). The degree of hemolysis by θ-toxin was only 3% after 15 min at 20 °C without subsequent incubation at 37 °C.

Table II: Inhibition of Binding of the Toxins to Erythrocytes by Cholesterol[a]

toxin	temp (°C)	toxin bound to cells (HU) at cholesterol (M) 10^{-6}	10^{-7}	10^{-8}	0
θ-toxin	37	0	0.37	0.89	0.92
	20	0	0.38	0.92	1.20
Cθ	37	0	0.56	1.07	1.18
	20	0	0.55	1.33	1.39

[a] θ-Toxin or Cθ (1.5 HU) was added to the assay mixture (1 mL) containing 0.5% sheep erythrocytes and various amounts of cholesterol and incubated for 10 min at 20 °C or for 2 min at 37 °C. The cells were pelleted by a short spin. The degree of hemolysis by the bound toxins was determined as described in the legend of Figure 5 and was used for the calculation of the amount of bound toxins.

However, further investigation revealed that the difference between Cθ and θ-toxin resides in the reversibility of binding (Table III). Once θ-toxin was incubated with erythrocytes for 10 min at 20 °C, the bound θ-toxin could not hemolyze newly added cells, indicating that it binds to the cells irreversibly within 10 min. In contrast, even after incubation for 80 min at 20 °C, Cθ redistributed among the previously and newly added cells, resulting in hemolysis of the latter cells. These results strongly suggest that Cθ binds to the cells only in a reversible manner at that temperature. Progression of Cθ to irreversible binding either might not occur or might be very slow on the cell membrane.

Secondary Structure of Digested Toxins. The secondary structures of Cθ and Tθ were compared with that of native toxin by means of circular dichroism measurements (Figure 6). The spectrum of θ-toxin shows a pattern typical of proteins that are rich in β structure such as β-lactoglobulin (Townend et al., 1967) and acetoacetate decarboxylase (Lederer, 1968). The toxin contains 42% β structure, 54% random coil, and only 4% α helix, on the basis of a computer analysis according to Greenfield and Fasman (1969).

Table III: Reversibility of Binding of Cθ[a]

toxin added	incubn at 20 °C (min)	A_{540} 2nd addn of erythrocytes −	+	hemolysis of secondary added cells
θ-toxin	10	0.338	0.351	0.013
Cθ	10	0.351	0.772	0.421
	20	0.368	0.838	0.470
	40	0.376	0.867	0.491
	80	0.349	0.818	0.469
100% hemolysis		0.375	0.938	0.563

[a] Ten hemolysis units of θ-toxin or Cθ was incubated with sheep erythrocytes (1 mL of 0.25% suspension) for the indicated times at 20 °C, and bound toxins were spun down with the cells. Each pellet was resuspended in 1 mL of the same buffer and divided into two equal portions. One portion (0.5 mL) was added to 0.5 mL of 0.375% erythrocyte suspension. The other portion was added to 0.5 mL of phosphate-buffered saline (Saito et al., 1982) as a control. Then, both mixtures were incubated for 30 min at 37 °C to determine the degree of hemolysis. The additional incubation for 10 min at 20 °C prior to the incubation at 37 °C did not change the results. The value of 100% hemolysis was determined by the addition of distilled water.

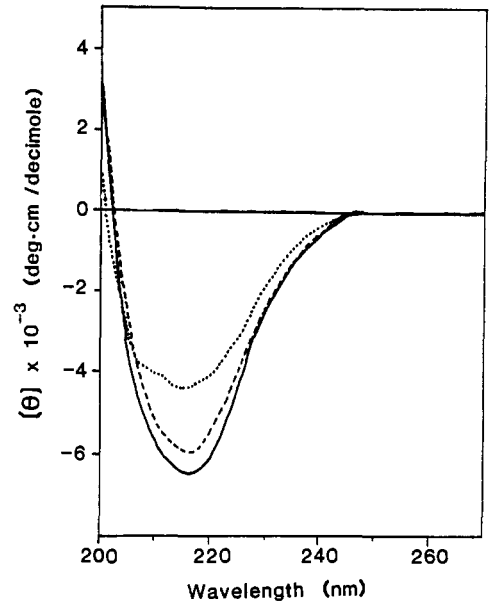


FIGURE 6: Circular dichroism spectra of θ-toxin and its derivatives. Spectra were measured in 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl at 22 °C. θ-Toxin (—); Cθ (- - -); Tθ (···).

No significant difference was observed between the circular dichroism spectrum of Cθ and that of native toxin at 22 °C (Figure 6) in spite of the drastic difference in their hemolytic activity. The cleavage of θ-toxin with trypsin caused more change in the spectrum, though Tθ has hemolytic activity even at low temperatures. Thus, the difference in the hemolytic activity among θ-toxin, Cθ, and Tθ at low temperatures is not reflected on their secondary structure described here.

Discussion

We have obtained two nicked derivatives of θ-toxin, Cθ and Tθ, by limited proteolysis with subtilisin Carlsberg and trypsin, respectively. Both nicked toxins exist as complexes of N-terminal basic fragments (C2 and T1) and C-terminal weakly acidic fragments (C1 and T2). Since the sum of the amino acid compositions of C1 and C2, or T1 and T2, is very similar to that of θ-toxin, subtilisin Carlsberg or trypsin cleaved the toxin at very limited point(s) without releasing an appreciable number of amino acids. The single cysteinyl residue, which is essential for hemolysis, was assigned to the C-terminal

fragment in each case. The C-terminal fragments, but not the N-terminal fragments, cross-reacted with the antiserum against another thiol-activated hemolysin, tetanolysin (M. Iwamoto and Y. Ohno-Iwashita, unpublished results), suggesting the possibility that thiol-activated hemolysins might share a common structure in their C-terminal portions around the cysteinyl residue.

It is interesting that cleavage at different sites of the toxin molecule yields products with different characters. The cleavage with subtilisin Carlsberg at a point(s) 15 kDa from the N-terminus produced a nicked toxin (Cθ) having 40% of the hemolytic activity of native one at 37 °C. However, the hemolytic activity of Cθ is drastically dependent on the incubation temperature. It causes almost no hemolysis at temperatures below 25 °C, though it does bind to the cells in this temperature range. It is unlikely that at low temperatures Cθ binds nonspecifically to some component other than cholesterol on the cell surface, because θ-toxin and Cθ have comparable affinities for cholesterol at both 20 and 37 °C (Table II). On the other hand, the cleavage with trypsin at some point(s) in the central region of the toxin molecule does not produce such a cold-labile toxin, although the secondary structure was changed more by the digestion than by that with subtilisin. The product, Tθ, has rather higher hemolytic activity than θ-toxin at low temperatures.

From Arrhenius plots, Bernheimer calculated the activation energy of the hemolytic process for θ-toxin, pneumolysin, tetanolysin, and streptolysin O (1947). He reported that a break in the Arrhenius plots between 15 and 20 °C was observed for tetanolysin and streptolysin O, but no break point was observed in the case of θ-toxin or pneumolysin. It would be interesting to investigate what causes the temperature-dependent change in the former two toxins and to compare their behavior with the change in θ-toxin induced by proteolysis.

It was reported that there are at least two steps in the hemolysis by thiol-activated hemolysins (Kanbayashi et al., 1972; Alouf, 1976). The initial step is reversible binding of the toxins to the cells and occurrs even at 4 °C. The subsequent step(s) are temperature-dependent and irreversible, which is required for hemolysis. The binding of θ-toxin and Tθ must be irreversible at temperatures above 10 °C, since hemolysis was caused by the toxins in this temperature range (Figure 4). In fact, the binding of θ-toxin became irreversible within 10 min at 20 °C (Table III). In contrast, the binding of Cθ remains reversible even after 80 min at 20 °C. Presumably, conversion of binding from reversible to irreversible does not proceed well when Cθ is incubated with the cells at low temperatures. It remains to be determined whether the conversion of reversible to irrevesible binding was achieved by conformational change of the toxin or some factor(s) in addition to membrane cholesterol is required for irreversible binding of the toxin. On this point, it is unlikely that the N-terminal hexapeptide lost in Cθ might have some role on the conversion of reversible to irreversible binding, since a derivative of θ-toxin lacking of the N-terminal heptapeptide was also obtained during the purification and it has the same hemolytic activity as the native toxin does (Y. Ohno-Iwashita, unpublished results).

A change of the higher order structure was observed by circular dichroism measurement, when θ-toxin was incubated with cholesterol–lecithin liposomes, but not with lecithin liposomes (Y. Ohno-Iwashita, unpublished results). The possibility that the conformational change just mentioned above may have some role on the irreversible binding of the toxin is now under investigation.

Thus, the cold-labile nicked toxin obtained here should be a useful tool to analyze the mechanism of the toxin action following the binding processes. In addition, the structural data of θ-toxin reported here should be quite helpful to elucidate general structural features of thiol-activated cytolysins, since they share many common properties.

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