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RENATURATION OF A REDUCED TAIWAN COBRA CARDIOTOXIN

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Summary

Refolding of a denatured protein obtained by reducing cardiotoxin from the Taiwan cobra with mercaptoethanol has been carried out in aqueous and non-aqueous solutions.

Oxidation of the reduced protein in 0.05 M phosphate buffer (pH 7.2) resulted in isolating an active protein which showed, as compared to native cardiotoxin, identical conformation and biological activities such as lethality, antigenicity and muscle contracture inducing activity. On the other hand, the reduced protein was undergoing incorrect SS-pairing and several inactive products were formed in a mixture of 1,2-ethanediol and 1-propanol (1:1; v/v).

Introduction

Snake cardiotoxin is a membrane-active polypeptide which causes irreversible depolarization of cell membranes [1]. In accordance with the results of Lo et al. [2], there are four cardiotoxin isomers in the venom of Taiwan cobra (*Naja naja atra*). Narita and Lee determined the primary structure for one of them [3]. Recently, the amino acid sequences of the four isotoxins in Taiwan cobra venom have been elucidated [4-7]. The major toxin is cardiotoxin analogue III [6] which is identical with cardiotoxin of Narita and Lee. It is known that cardiotoxin is a single basic polypeptide comprising 60 amino acid residues. There are four disulfide linkages in this rather small protein molecule. Based on the procedures proposed by Chou and Fasman [8], Chen et al. reported that this protein is devoid of helical structure but it contains a considerable amount of β -strand and β -turn conformations [9]. This is further confirmed by the optical study [10].

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Another toxin, cobrotoxin, was also isolated from Taiwan cobra [111]. Its structure was studied in detail and refolding of the reduced cobrotoxin was reported by Yang [12]. The results indicated that the integrity of disulfide bonds in cobrotoxin is essential for lethality. The condition for refolding was also applied to the attempted chemical synthesis of cobrotoxin [13].

This paper reports the reoxidation of a reduced cardiotoxin analogue III in aqueous and in non-aqueous solution. This study permits us to evaluate: (a) the physical restriction of disulfide bonds and the activity associated with side chains in maintaining the framework of cardiotoxin. (b) The physical state of cardiotoxin in the membrane structure for its cytolytic activity. (c) The possibility of chemical synthesis of reduced cardiotoxin and folding to the natural state to complete total synthesis.

Materials and Methods

Cobra cardiotoxin was prepared and repeatedly purified on a CM-Sephadex C-50 or C-25 column according to the previous procedure [2]. The homogeneous preparation, testing with acrylamide gel electrophoresis, was used for the study. The solution was further clarified by passing it through Millipore filter of 5 μ m pore size before optical study. The protein concentration was determined spectrophotometrically, using an extinction coefficient $E_{1cm}^{1\%}$ at 280 nm, of 6.82 and 6.05 for native and reduced toxins, respectively [10].

All chemicals used were reagent grade from commercial sources. Water was double distilled.

Thiol groups were assayed spectrophotometrically by Ellman reagent, 5,5'dithiobis-2-nitrobenzoic acid (DTNB). In a solution of DTNB and cystein methyl ester hydrochloride, the molar absorbance of 2-nitromercaptobenzoate was determined as 13 600 M⁻¹ · cm⁻¹ at 412 nm [14].

Disc gel electrophoresis generally followed the method of Davis [15]. It was carried out in 10% acrylamide gel at room temperature, 6 mA/tube, for 40 min in 0.35 M β -alanine acetate (pH 4.5). Proteins were stained with Amido Black.

Antisera against purified cardiotoxin were prepared according to the previous report [16]. Rocket immunoelectrophoresis [17] at room temperature for 3 h at 9 mA/plate was performed on a glass plate (8×8 cm) coated with 1% agarose in 1/15 M phosphate buffer (pH 6.0) containing the antisera. The precipitin lines could be seen directly by eyes or visualized after stained with 0.5% Coomassie Brilliant Blue in a solution of acetic acid/methanol/water (10 : 20 : 70, v/v/v) and destained by the same solvent system.

Cardiotoxin was reduced in accord with the procedure of Anfinsen et al. [18] with some modifications. A tube containing 36 mg of toxin in 2 ml of 0.05 M phosphate buffer in the presence of 6.0 M guanidine hydrochloride (pH 7.6) was flushed with a stream of nitrogen gas for 10 min. 50 μ l of β -mercaptoethanol was added to the solution and the tube was sealed and kept for 5 h at room temperature. Then two drops of glacial acetic acid were added to stop the reaction. The reaction mixture was passed immediately through a Sephadex G-25 column (2 × 30 cm), using 0.1 M acetic acid in the presence of 0.01 M β -mercaptoethanol as an eluant. The unretarded portion containing the

reduced cardiotoxin was collected, lyophilized to remove β -mercaptoethanol and obtain the reduced cardiotoxin (34 mg). One part of the reduced cardiotoxin (17 mg) was dissolved in 21 ml of aqueous solution (0.05 M phosphate buffer, pH 7.2) and stood at room temperature for aerobic oxidation. The condition used was similar to that of cobrotoxin [12] only with more dilute solute concentration. Another part (17 mg) was dissolved in 21 ml of nonaqueous solution (a mixture of 1,2-ethanediol and 1-propanol (1:1 v/v)) and also stood at room temperature for aerobic oxidation. At several time intervals, an aliquot of the reaction mixture was assayed for the thiol groups.

According to the method of Ginsborg and Warriner [19], the chicken biventer cervicis muscle was used to test the contracture activity of cardiotoxin. The muscle preparation in 20 ml Krebs solution bubbled with 95% O₂ and 5% CO₂ was stimulated indirectly with supramaximal rectangular pulses of 0.5 ms at a frequency of 0.2 Hz. The isometric contractions were recorded with a Grass FT 03 force displacement transducer attached to a Grass 7D polygraph. The experiment was performed at 37 ± 0.5°C with 1.5 μ M cardiotoxin.

Toxicity was assayed with albino mice weighing 15-20 g by intraperitoneal injection. Eight mice were used at each dosage. The LD_{50} was determined according to the method of Litchfield and Wilcoxon [20].

Circular dichroism (CD) was measured with a Jasco J-20 spectropolarimeter under constant nitrogen flush. Fused silica cylindrical cells of various path length were used so that the absorbance of the solution was kept below two. The data were expressed in terms of mean residue ellipticity, $[\theta]$, with a mean residue weight of 112.3.

A Hitachi model 524 UV-Visible spectrophotometer was used for measuring light absorbance.

Results

Reduction and reoxidation of cardiotoxin

The reduced cardiotoxin contains eight sulfhydryl groups. Fig. 1 illustrates the disulfide bond formation when oxidized aerobically the reduced proteins. In aqueous solution the oxidation was rapid at the beginning but became slow after third day. On the basis of the free thiol groups remaining, 90% of the reduced protein could be oxidized at the end of the fifth day. The rate of SSpairing of the reduced protein in non-aqueous solution was slower than that in aqueous solution. Even at the end of the ninth day, there remained 3.2 sulfhydryl groups per protein molecule in the solution.

The product obtained by the oxidation in aqueous solution for five days or in organic solution for nine days is hereafter referred to as aqueous oxidized protein or non-aqueous oxidized protein, respectively. There was a slight turbidity in the aqueous oxidized protein solution but it was not precipitated. Both preparations could be resolved into two fractions by a Sephadex G-50 column using 0.05 M ammonium acetate (pH 6.8) as an eluant. The results are shown in Fig. 2. The aqueous oxidized protein (fraction I) (fractions 20-33, 7 mg) and the non-aqueous oxidized protein (fraction I) (fractions 20-30, 5 mg) were the aggregated products formed by means of intermolecular disulfide linkages, because they were eluted out earlier than native toxin. The aqueous oxi-



Fig. 1. The content of sulfhydryl groups during oxidation of the reduced cardiotoxin. SH groups were titrated with DTNB as described in Materials and Methods. (\bullet), in non-aqueous solution; (\circ), in aqueous solution.

Fig. 2. Chromatography of the reoxidized products on Sephadex G-50 (column dimension, 2.5×60 cm). (-----), aqueous oxidized products; (----), non-aqueous oxidized products; (----), native cardiotoxin.

dized protein (fraction II) (fractions 35-45, 9.5 mg) and non-aqueous oxidized protein (fraction II) (fractions 33-45, 11 mg) showed the same molecular size but not identical biochemical properties (see below).

Characterizations

The electromobility and homogeneity of the oxidized products were determined with disc gel electrophoresis. The results are shown in Fig. 3. The aqueous oxidized protein (fraction II) gave a single band with the same mobility as cardiotoxin. Several smear bands moving slower than native toxin were found in the non-aqueous oxidized protein (fraction II). The aggregated proteins of both aqueous oxidized protein and non-aqueous oxidized protein were hardly moving in the gel.

The conformation of the oxidized proteins were investigated by their CD spectra. As shown in Fig. 4, the CD spectrum of aqueous oxidized protein (fraction II) contained at least five small positive peaks in the region of 250-300 nm and two peaks and two troughs below 250 nm. The intensities of the peak at 195 nm and the trough at 214 nm were 10 900 and $-1500 \text{ deg} \cdot \text{cm}^2$. dmol⁻¹, respectively. The CD spectrum agrees well with that reported for cardiotoxin [10]. The non-aqueous oxidized protein (fraction II) or the aggregated proteins (aqueous oxidized protein (fraction I) and non-aqueous oxidized protein (fraction I)) gave completely different CD spectra when compared with that of cardiotoxin. Unlike the positive CD bands of cardiotoxin in the region of 250-300 nm, all the ellipticities of non-aqueous oxidized protein (fraction II) and the aggregated proteins in the spectral region were negative. A small trough at 202 nm was found in the CD spectra of the aggregated proteins and a broad negative band appeared in the spectrum of non-aqueous oxidized protein (fraction II). In comparison with the CD spectra of unordered forms of polypeptides or proteins [21-23], the characteristics of both spectra of nonaqueous oxidized protein (fraction II) and the aggregated proteins do not show the completely unordered forms.



Fig. 3. Disc gel electrophoregrams of native cardiotoxin and the reoxidized products. 1, native cardiotoxin; 2, aqueous oxidized protein (fraction II); 3, aqueous oxidized protein (fraction I) 4, non-aqueous oxidized protein (fraction II).

Fig. 4. CD spectra of native cardiotoxin, reduced cardiotoxin and the reoxidized products. ———, native cardiotoxin; — · - · -, aqueous oxidized protein (fraction II); • — — •, reduced cardiotoxin in 0.1 M acetate/ 10^{-2} M β -mercaptoethanol; — – , reduced cardiotoxin in phosphate buffer at the beginning of oxidation; — · - , aqueous oxidized protein (fraction I) · · · · · , non-aqueous oxidized protein (fraction II).



Fig. 5. Contracture inducing activities of native and renatured cardiotoxins. A, Native cordiotoxin; B, renatured cardiotoxin aqueous oxidized protein (fraction II).

Fig. 6. Rocket immunoelectrophoresis of native cardiotoxin and the reoxidized products. 1, Native cardiotoxin (1 mg/ml); 2, aqueous oxidized protein (fraction II) (1 mg/ml); 3, aqueous oxidized protein (fraction I) (4 mg/ml); 4, non-aqueous oxidized protein (fraction II) (4 mg/ml); 5, reduced cardiotoxin (8 mg/ml).

Bioassays

The non-aqueous oxidized protein (fraction II) or the aggregated proteins showed no lethality at the dose of 6 μ g per gram of body weight of albino mice. Aqueous oxidized protein (fraction II) was toxic. Its LD₅₀ was 2.5 μ g per gram of body weight, which was comparable with the toxicity of native cardiotoxin (LD₅₀ : 2.4 μ g per gram of body weight). The aqueous oxidized protein (fraction II) could induce contructure in the chicken skeletal muscle as potent as cardiotoxin (Fig. 5).

The result of rocket immunoelectrophoresis is shown in Fig. 6. The reduced protein, non-aqueous oxidized protein (fraction II) or the aggregated proteins showed no binding with the anti-cardiotoxin sera. On the other hand, aqueous oxidized protein (fraction I) could bind with anti-cardiotoxin serum as strong as cardiotoxin. We have performed the oxidation by dissolving non-aqueous oxidized protein (fraction II) in 0.05 M phosphate/ 10^{-4} M β -mercaptoethanol (pH 7.2) for around four days, the products showed the same antigenicity as native cardiotoxin. Seemingly, the disulfide bonds exchanged and rearranged to correct pairing.

Discussion

This study shows that the reduced cardiotoxin can be renatured as cobrotoxin in aqueous but not in non-aqueous solution. It is well known that solvent plays an important role for keeping the conformation of a protein molecule. In aqueous solution, the arrangement of amino acid residues in the reduced toxin is sterically suitable for the correct SS-pairing. While in organic solution, the environments around the side chains of the reduced toxin can not result in a steric structure favoring the right disulfide bond formation. Hung and Chen indicated that the native conformation of cobra cardiotoxin in aqueous solution can be retained in the solvent less polar than water [10]. In a mixed solvent of 1,2-ethanediol and 1-propanol, the non-polar amino acid residues could not transfer from the core of protein to the interface between the toxin molecule and the solvent. However, when the toxin is reduced and dissolved in organic medium, the activities associated with the side groups for the "native format" no longer exist and enable the non-polar amino acid residues to transfer again to the protein core. The failure of refolding the reduced toxin in organic solvent reflects that the integrity of disulfide linkages in the natural state is to maintain the framework of cardiotoxin and prevent the penetration of solvent into the protein molecule. Moreover, "that the conformation of correct SS-bond arrangement in a protein in aqueous solution must be the thermodynamically stable one" [24] is evident from the fact that the inactive scramble products of non-aqueous oxidized protein (fraction II) can be reoxidized in aqueous solution to an active protein.

Snake cardiotoxin can impair cell membranes of different origins. They lose the activity when they are unfolded by breaking the disulfide bonds. Having incubated a mixture of cobra cardiotoxin and liposome of phospholipid, we found that the toxin destablizes the structure of lipid bilayer but the other snake toxins like neurotoxins show no such activity (Chen, Y.H., unpublished). This discovery accompanying the results of Vincent et al. [25] supports that the rupture of cell membranes by cardiotoxin arises mainly from their actions on the phospholipid. The present study shows that the reduced cardiotoxin is soluble in organic solvent but it can not be renatured in non-aqueous solution. It seems that the reduced cardiotoxin could be compatible with the hydrocarbon of lipid bilayer but it could not restore to an active protein in the membrane structure. This may be the main reason why cardiotoxin looses its biological activity when its disulfide bonds are broken. Apparently, maintaining the active conformation of cardiotoxin in the membrane structure is crucial for its cytolytic effect.

Like cobrotoxin, reduced cardiotoxin refolded successfully in phosphate buffer and the desired product can be isolated with about 60% yield. The same condition will be used for the total synthesis of cardiotoxin and other analogues.

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