Purification, characterization and binding interactions of the Chinese-cobra (*Naja naja atra*) serum antitoxic protein CSAP

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The characterization of the single-chain protein in Chinese-cobra (*Naja naja atra*) blood serum, which yields strong specific protection against the venom of the same snake, is reported. The protein, CSAP (cobra serum antitoxic protein), was purified to electrophoretic homogeneity. Over the pH range 5–9 it formed stable complexes with the neuro- and the cardio-toxin of the snake. The molecular size of the CSAP was estimated to be 70.3 ± 0.3 kDa. Tryptic hydrolysis of CSAP yielded several peptides that were able to bind to the toxin. The native CSAP maximally bound 8 ± 1 toxin molecules/molecule. Six tryptic fragments, containing 5–39 residues, were sequenced. The longest

of these displayed sequence similarity to rat serum albumin. The protective effect of the CSAP was demonstrated *in vivo* on mice and *in vitro* by measurement of the rate of haemolysis. Kinetic and thermodynamic parameters of the binding interactions of the neurotoxin and the CSAP were determined from the rates of displacement of ^{125}I -labelled toxin from its complexes with the CSAP by unlabelled toxin by using a DEAE-cellulose filter assay for CSAP–toxin complexes. The toxin molecules rapidly dissociated from one type of site and slowly from a second. The binding capacity and concentration of the CSAP suffice to explain the protective effect of the latter against the toxin.

INTRODUCTION

Many poisonous snakes, probably all, are resistant to their own venom but not to the toxins of other snake families, unless the genetic relationship is very close. The mechanism of this exclusive protection is an interaction between the toxin and an endogenous protein which is secreted into the blood [1]. The chemical nature of this protective substance has so far not been investigated in detail, but Clark and Voris [2] found that the antitoxin in the serum of the Eastern diamondback rattlesnake (*Crotalus adamanteus*) is an albumin-like protein. Also, Omori-Satch et al. [3] purified an antihemorrhagic factor from the serum of the habu snake (*Trimeresurus flavoviridis*) which possessed the electrophoretic properties of a serum albumin or an α_1 -globulin and had a molecular mass of 70 kDa.

Whereas most serum antitoxins of venomous snakes have been discovered by the observation of their protective effect [2–10], CSAP probably was the first to be discovered by electrophoresis. When a mixture of the serum and the venom of the cobra was subjected to electrophoresis, a new band was found which migrated behind that of serum albumin towards the positive electrode. Neither a mixture of human serum and cobra venom nor one of cobra serum and venom of other elapid snakes displayed such a band.

Cobra toxins cause fatal physiological disorders, e.g. neurotoxicity and haemolysis, as well as cardiac and muscular failure, many of which can be explained by the inhibition of synaptic ion channels, by the action of snake phospholipase A_2 and by changes in the membrane structure [11,12]. The toxins may be divided into two distinct classes according to their size: one group of 6.8–7 kDa and the other of ~ 8 kDa. Since the small toxins are the more numerous and since all the toxins interact with the CSAP in a similar fashion, the present investigation was confined to the small toxins. Their size distribution is narrow.

Hence the existence of the large toxins is probably a result of gene diversification by alternative splicing [12a].

The present paper describes the purification by a new method and the characterization of CSAP. This information is likely to be useful in further work on the elucidation of the self-detoxification phenomenon in venomous snakes. Moreover, the resemblance of CSAP to mammalian serum albumins may allow a manipulation of the ligand interactions of the antitoxin with methods developed for the serum albumins of higher species or by injection of native or chemically modified CSAP after cobra bites. Multiple ligand interactions of biopolymers is a complex but physiologically important phenomenon which includes aspects of co-operativity, critical transition and other non-linear relationships [13].

EXPERIMENTAL

Materials

The venom of *N. naja atra* was milked from snakes collected in China. The serum of this snake was bought from the Tiantai Experimental Snake Farm, Zhejiang, China. The cobra venom samples were pooled and centrifuged at 3000 rev./min for 30 min. The supernatant was freeze-dried and stored at -30°C until use. The snake serum was also stored at -30°C .

Human serum

Control samples were supplied by a clinical laboratory of Zhejiang University.

Reagents

Sephadex G-25, G-100, G-150, Blue Dextran and Polybuffer

Abbreviations used: CSAP, cobra serum antitoxic protein; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); PVDF, poly(vinylidene difluoride); RBC, red blood cells; T, free toxin; AT, antitoxin–toxin complex.

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were supplied by Pharmacia. Myoglobin, the urea nitrogen kit, Tos-Phe-CH₂Cl (tosylphenylalanylchloromethane; 'TPCK')-treated trypsin and molecular-mass markers were provided by Sigma, whereas the DEAE ion-exchange paper was a product of Whatman. Poly(vinylidene difluoride) (PVDF) transfer membranes were obtained from Millipore. X-OMAT diagnostic film was a product of Eastman-Kodak. Servalyte Precotes 3–10 were obtained from Serva. All other chemicals were of AR or CP grade and purchased in China or from Merck. The rabbit antiserum against the cobra albumin was a gift from Professor R. Mao, Defence Research Laboratory, Taipei, Taiwan.

Methods

Electrophoresis

The toxin-CSAP complexes were separated by vertical PAGE in discs and slabs. In most cases the gels were stained with Coomassie Brilliant Blue R-250. The albumin was identified by immunoelectrophoresis [12]. A test for glycoproteins in the cobra serum was performed by the staining procedure of Kayser [13]. Isoelectrofocussing was performed in an LKB 2117 Multiphor II apparatus at constant current and 150–1700 V.

Chromatofocusing

The pI of CSAP was also determined on a Pharmacia f.p.l.c. apparatus using a Mono P column and eluting with Polybuffer. The sample was eluted at pH 6.8.

Protein assay

The total protein content was determined by the method of Doumas et al. [14]. A sample (20 µl) of CSAP solution was added to 1 ml of biuret reagent and left for 10 min at 23 °C. The A_{540} was compared with that of standard proteins. The albumin concentration was measured by a modification of the method of Doumas et al. [15]. A sample (10 µl) was mixed with 1 ml of Bromocresol Green reagent at 23 °C. After 1 min, the A_{628} was read. Protein nitrogen was assayed by the method of Berthelot [16] after digestion of the protein. The molar concentration of protein was calculated by dividing (g of nitrogen/litre) × 6.25 by the known molecular mass.

Preparation of a neurotoxin-Sepharose 4B affinity column

The cobra neurotoxin was prepared by the method of Kaneda et al. [17], freeze-dried and stored at –20 °C. The neurotoxin (40 mg) was dissolved in 0.1 M NaHCO₃/0.5 M NaCl, pH 8.5, and suspended with 4 g of preswelled CNBr-Sepharose 4B. The suspension was stirred overnight at 4 °C and then transferred to a 1 M ethanolamine solution at pH 8.0. The affinity gel was washed with 0.1 M acetate/0.5 M NaCl, pH 4.0, followed by 0.1 M Tris, pH 7.4.

Purification of CSAP

An aqueous solution of cobra serum was mixed with the same volume of saturated (NH₄)₂SO₄ solution with continuous stirring. After 12 h at 4 °C, the suspension was centrifuged for 30 min at 10000 *g*. The supernatant was dialysed against several changes of distilled water and then passed through a 1.5 cm × 5 cm column of neurotoxin-Sepharose 4B. The adsorbed proteins were eluted with 10 mM acetate of decreasing pH value. The CSAP was released from the column at pH 4.4–5.8.

(i) F.p.l.c. hydrophobic-interaction chromatography. A sample (5 mg) from affinity chromatography was dissolved in buffer A [20 mM imidazole/HCl/0.8 M (NH₄)₂SO₄, pH 7.0] and applied to an f.p.l.c. phenyl-Superose 5/5 column. The proteins were eluted with buffer B (20 mM imidazole/HCl, pH 7.0).

(ii) Determination of the molecular size of the CSAP and its complexes with the toxin. SDS/PAGE [18,19] and gel filtration on a Sephadex G-150 column (10 cm × 120 cm; equilibrated with 0.05 M sodium phosphate buffer, pH 7.0) were used to estimate the molecular size of CSAP, but only gel filtration was used to determine the sizes of the CSAP-toxin complexes. Since albumins, like CSAP, are globular proteins, these methods are expected to yield good estimates of the molecular mass.

¹²⁵Iodination

The iodination method of Flanagan et al. [20] was used. Excess free [¹²⁵I]iodide was separated from the labelled neurotoxin in a column of Dowex 1 × 8 ion-exchange resin containing 0.01 % NaN₃. The radioactivity was measured in a γ-radiation counter (BF 5300). The radioactivity on the DE-81 filters was determined by the method of Schmidt and Raftery [21]. A sample was incubated with an excess of ¹²⁵I-neurotoxin in 10 mM sodium phosphate buffer, pH 7.4. After 30 min at 25 °C, an aliquot (30 µl) was transferred to a disc (2.3 cm diameter) of DEAE-cellulose paper (DE-81). The disc was washed with 10 mM sodium phosphate, pH 7.4. After three washing cycles over a period of 90 min the disc was dried and the radioactivity was measured in a γ-radiation counter.

Digestion of CSAP with Tos-Phe-CH₂Cl-treated trypsin

A solution of Tos-Phe-CH₂Cl-treated trypsin (12.5 mg) in 1.0 ml of 10 mM Tris/HCl buffer, pH 8.0, was added to a solution of CSAP (1.0 g in 40 ml of this buffer). The digestion was terminated after 48–72 h at 25 °C by adjustment of the pH to 5.0. The solution was concentrated to 10 ml by freeze-drying. Aliquots (200 µl) were applied to a Superose 12 f.p.l.c. column. The fractions were purified on either an f.p.l.c. phenyl-Superose HR 5/5 hydrophobic-interaction column or an f.p.l.c. MonoQ HR 5/5 ion-exchange column.

Electrophoresis of the tryptic peptides

Two-dimensional PAGE was performed by the method of Das and Baese [19]. In the first dimension the proteins were separated by isoelectric focusing (pH 3–10) in a round gel (1 mm diameter) containing 6 M urea and in the second dimension by SDS/PAGE. The pI values for the fragments were determined by isoelectric focusing on a plate gel, whereas their molecular sizes were ascertained by SDS/PAGE using a 7.5–15 % gradient or 10 %-non-gradient polyacrylamide gel.

Protein blot and autoradiography

The tryptic peptides of the CSAP were separated by SDS/PAGE on a 10 % gel. The peptides were then transferred to a PVDF membrane at 25 °C by a semi-dry blot procedure (Phase G.m.b.H.) for 60 min at a constant current of 0.8 mA/cm² in 30 mM Tris in aq. 20 % (v/v) methanol [22]. The membrane was saturated with 3.0 % gelatine in Tris-buffered saline, pH 7.4, for 2 h at room temperature and then transferred to ¹²⁵I-neurotoxin solution containing the same buffer, in which it was kept at 4 °C overnight. The membrane was washed four times with Tris-buffered saline, dried at 60 °C, and Kodak HX-OMAT film was

exposed to it for 48 h at -70°C . The radioactivity was elevated with a Desaga CD60 densitometer.

The protective effect of CSAP on mice injected with cobra venom

The LD_{50} value was determined by the method of Meier and Theakson [23]. Groups of 8–10 male NIH (National Institutes of Health) mice, each weighing about 20 g, were used. Each mouse received a dose of $\sim 100\ \mu\text{g}$ of CSAP in 0.2 ml of saline intraperitoneally. After 10 min the mouse was given an injection of cobra venom.

The protective effect of CSAP against a haemolysis caused by cobra cardiotoxin or venom

The method of Kihara [24] was used to measure the sensitivity of rabbit or guinea-pig erythrocytes to cobra toxins. Blood collected by heart puncture was protected against coagulation by citrate. The red blood cells (RBC) were sedimented, washed with saline, centrifuged [$3 \times 10\ \text{min}$ at $4000\ \text{rev./min}$ ($\sim 1500\ g$)] and suspended in saline. Samples were shaken with toluene, centrifuged and diluted to an A_{540} about 1 for haemoglobin assay. A sample ($250\ \mu\text{g}$) of toxin or venom in 0.1 ml of saline was added to 2 ml of the RBC suspension and the mixture was incubated at 30°C for 30 min. Then the suspension was centrifuged and the A_{540} was read. Amounts of 50–500 μg of CSAP in 0.05 ml saline were added to a series of test tubes, each containing 250 μg of toxin in 0.05 ml of saline. The tubes were incubated for 10 min at 30°C . Then 2 ml of RBC suspension was added and the tubes were incubated for further 30 min. The haemolytic effect was measured after a final centrifugation.

Amino acid sequencing

The tryptic peptides were dissolved in 0.1% trifluoroacetic acid and purified by h.p.l.c. on a C_{18} column (pump 2248; Pharmacia-LKB) using an acetonitrile gradient. They were sequenced by Dr. F. Buck, Department of Cell Biochemistry, University of Hamburg, Hamburg, Germany, using automatic Edman degradation and reversed-phase h.p.l.c. with photometric detection of amino acid phenylthiohydantoin derivatives (Applied Biosystems model 470A sequencer).

Kinetic measurements

The binding assay, in which the free antitoxin and the toxin-antitoxin complexes (AT), owing to their acidity, are bound to the DEAE-paper was carried out as described by Klett et al. [25]. [The free toxin (T) passes through the paper.] The filter was washed twice with PBS and counted for ^{125}I -T in the AT complexes. The specific activity of the KI used was 13 mCi of $^{125}\text{I}/\mu\text{g}$ and that of the toxin was $3.6 \times 10^9\ \text{c.p.m./mol}$. The antitoxin (0.5 ml) was incubated with ^{125}I -labelled toxin ($10\ \mu\text{l}$) in 10 mM sodium PBS at pH 7.4 and 25°C overnight ($\sim 16\ \text{h}$) with shaking. Then 400 μl of a solution of unlabelled toxin was added to displace the labelled toxin molecules. Final concentrations were: CSAP, 1.18 nM; ^{125}I -toxin: 47 nM; unlabelled toxin, 93.8 μM . The total volume was 1 ml, and the background radioactivity was 45 c.p.m.

RESULTS

The binding of the cobra venom to CSAP could be demonstrated by electrophoresis on starch or polyacrylamide. When the duration of such experiments was varied and the patterns were

compared, then the migration of distinct AT complexes of different degrees of saturation of the CSAP with toxin was observed (see Figure 1). Under the conditions of the experiment, three such complexes could be discerned which moved with constant velocity toward the positive electrode (see Figure 2).

Both affinity and f.p.l.c hydrophobic-interaction chromatography were effective methods of purification of the CSAP (see Table 1). Whereas peaks 1–3 in Figure 3 show a single SDS/PAGE band (at 70 kDa), and hence solely consist of CSAP, presumably in different states of oligomerization, peak 4 also contained other serum proteins. Inclusion of 2-mercaptoethanol

Figure 1 Time dependence of the electrophoretic pattern of ^{125}I -toxin in AT complexes

Non-denaturing PAGE was performed at 25 W. The solvent was 10 mM PBS, pH 6.8 at room temperature. The reaction mixture comprised 0.5 ml of 1.18 nM antitoxin, $10\ \mu\text{l}$ of ^{125}I -labelled toxin (1.29 mg/ml) and 0.5 ml of unlabelled toxin (1.29 mg/ml). Symbols: $\square$, gel α , 45 min, $25\ \mu\text{l}$; $\triangle$, gel β , 90 min, $25\ \mu\text{l}$; $\circ$, gel γ , 135 min, $50\ \mu\text{l}$. The gels were run in parallel and in triplicate. Vertical bars represent standard deviation. Coomassie Blue-stained bands of free antitoxin (A) and AT complexes (AT) are marked by vertical arrows. The ordinate axis shows the fraction of total radioactivity on the gel present in a given segment.

Figure 2 Rate of movement towards the positive electrode of three distinctive protein spots on the electrophoresis gel

$\blacksquare$, $\blacktriangle$ and $\bullet$ represent three AT complexes, namely AT, AT_2 and AT_3 .

Table 1 Purification of CSAP and its tryptic peptides

Step	Protein	Protein yield of step (%)	Total yield (%)
(NH ₄) ₂ SO ₄ precipitation	CSAP	30	30
Neurotoxin–Sephrose 4B affinity chromatography	CSAP	50	15
F.p.l.c. hydrophobic-interaction chromatography	CSAP	40	6
F.p.l.c. Superose 12	Tryptic peptides I–IV	40	40
F.p.l.c. hydrophobic-interaction chromatography	Tryptic peptides II and III	40	16
Neurotoxin–Sephrose 6B affinity chromatography	Tryptic peptide IV	50	8

Figure 3 Elution diagram of a sample (5 mg) of cobra serum albumin from the peak fraction of the affinity column in 0.2 ml of buffer A

The f.p.l.c. column was phenyl-Superose 5/5 at 4 °C. Buffer A was 20 mM imidazole/HCl/0.8 M (NH₄)₂SO₄, pH 7.0; buffer B was 20 mM imidazole/HCl, pH 7.0. Protein was detected by its A₂₈₀. The straight diagonal line represents the buffer gradient.

did not influence this band. The test for glycosides on CSAP using the periodic acid–Schiff reagent was negative, but the CSAP band could be stained with Coomassie Brilliant Blue R-250. Its homogeneity was confirmed by isoelectric focusing.

Physical properties

The molecular mass of CSAP was estimated, both by SDS/PAGE in the presence of a reducing agent and by Sephadex G-150 gel filtration in a non-denaturing solvent, as $70\,300 \pm 300$ Da. Hence the CSAP is a single polypeptide. Its concentration in blood plasma is 22.8 g/l. On analytical gel filtration the CSAP saturated with the neurotoxin produced a single symmetrical peak with a V_e corresponding to a mass of 125 kDa (95% confidence: 115–129 kDa). Similarly, the complex of the CSAP with the cardiotoxin yielded a molecular mass of 124 kDa (95% confidence: 117–132 kDa). Since the molecular mass of the toxins is 6.8–7.0 kDa, the CSAP maximally binds 8 ± 1 mol of toxin/mol of CSAP.

Limited tryptic hydrolysis

The native CSAP was digested with Tos-Phe-CH₂Cl-treated trypsin. Many small acidic peptides were released, which were retained by the neurotoxin affinity column. They are probably

Table 2 Time course of the binding capacity of CSAP for the neurotoxin

Duration of digestion (h)	Binding capacity* (%)
0	100
1	353
2	180
6	140
12	140
24	133
36	153
48	100
72	100

* ¹²⁵I-toxin retained on the DEAE filter in complex with CSAP.

Figure 4 Separation of a tryptic digest of CSAP on FPLC Superose 12

Reaction conditions were as follows: molar ratio CSAP/trypsin, 80; pH, 8.0; temperature, 25 °C; duration, 16 h. A sample (5.0 mg) was eluted with 0.3 M urea in 10 mM acetate buffer, pH 5.0.

parts of the binding site(s) for the basic toxins, since they were capable of interacting with the ¹²⁵I-neurotoxin. This is indicated in Table 2, but was confirmed in separate experiments (results not shown). The principal product was, after 48 h, the fragment T-60, and after 72 h the principal products were T-40, T-30 and T-18. The numbers refer to the molecular masses ($\pm 10\%$) as determined by non-reducing PAGE. The full binding activity of the intact CSAP for the ¹²⁵I-neurotoxin was retained after 3 days of tryptic digestion (Table 2). This observation indicated that many of the fragments still possessed the original activity of the native CSAP or an even higher activity. The preincubation of the CSAP with the neurotoxin with cardiotoxin or with octanoic acid did not influence the digestion pattern but retarded the hydrolysis.

Isolation of CSAP tryptic peptides

The digests were fractionated by gel filtration on f.p.l.c. Superose 12 (Figure 4). Peak I (T-60) was purified by f.p.l.c. hydrophobic-

Figure 5 Purification of the tryptic fragments T-60 and T-40 on f.p.l.c. phenyl-Superose HR 5/5

A sample (5.0 mg) from peak 1 in Figure 4 was dissolved in buffer A and eluted as described below. The larger of the shaded peaks contains T-60, the other T-40. Buffer A was 20 mM imidazole/HCl, pH 7.0; buffer B was 0.70 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A. The thicker line represents the percentage transmission at 280 nm. The thinner largely diagonal line is buffer composition gradient.

interaction chromatography, peak 2 mainly contained undigested CSAP, peak 3 consisted of T-30 and peak 4 consisted of T-40 and T-18. We can explain the presence of intact CSAP in peak 2 only by assuming adherence between this protein and its fragments. The inference is supported by the observation of a single SDS-bound electrophoretic band in peaks 1–3 in Figure 3. The peaks 2 and 3 were further fractionated by f.p.l.c. Mono QHR 5/5 ion-exchange chromatography, and T-18 was isolated by a subsequent affinity-chromatography step on neurotoxin–Sepharose CL-6B. The final purification of T-40 and T-60 was achieved by hydrophobic interaction chromatography on an f.p.l.c. phenyl-Superose HR 5/5 column (Figure 5).

Properties of tryptic CSAP fragments

When prior to electrophoresis the purified T-30 was reduced by β -mercaptoethanol, two bands appeared on the gel in addition to a trace of unreduced T-30 (Figure 6, lane 5), i.e. this fragment (and CSAP) probably contains at least one disulphide linkage. However, besides the major band of about 15 kDa, a weak band of a small peptide appeared which may arise from an entrapped impurity or an additional gene product. The measured pI values of the fragments were all below 5. Those of the larger ones had pI values above that of CSAP (4.05 ± 0.05), whereas the small fragments were more acidic than CSAP. T-60 had a higher affinity for the toxin than did CSAP, whereas T-40, T-30 and T-18 possessed 10–40% of the binding capacity of CSAP.

Amino acid sequencing of tryptic peptides

Six tryptic fragments have been sequenced from the N-terminus

Figure 6 SDS/PAGE of purified tryptic fragments of CSAP after reduction with β -mercaptoethanol

Lanes 1 and 7, standard proteins (molecular masses, M , in parentheses): BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), pancreatic trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.2 kDa). Lanes 2–6, CSAP, T-60, T-40, T-30 and T-18 respectively. T-18, -30, -40 and -60 represents tryptic peptides of sizes ~ 18 , 30, 40 and 60 kDa respectively.

(Table 3). Only the first (and longest) displays similarity to mammalian serum albumins. A comparison with the section (residue nos.) 132–170 of the rat albumin with 1–39 of the CSAP fragment no. 1 showed 35% identity in an overlap of 31 amino acids (Table 3, Cobra 9–39 versus rat 140–170).

Immunological reactivity

A single band (not shown) was found on immunoelectrophoresis of a mixture of CSAP and anti-(cobra albumin) antiserum. No cross reaction between this antiserum and human or bovine serum albumin occurred.

Protective effect of CSAP against cobra toxins

Mice that had received an injection of CSAP prior to the injection of cobra venom had an LD_{50} value which was 3.5 ± 0.3 -fold higher than that of unprotected mice (Figure 7). A similar dose–response relationship was found when guinea-pig erythrocytes were treated with CSAP prior to their exposure to the cardiotoxin (Figure 8).

Measurement of the rate of displacement of toxin molecules from AT complexes

The antitoxin was equilibrated for 24 h at 4 °C with a 40-fold molar excess of ^{125}I -labelled neurotoxin. Then the system was perturbed by the addition of a 2000-fold excess of unlabelled toxin. The time course of the release of radioactivity from the complex is shown in Figure 9. It may be simulated by the sum of two exponential terms. The segment representing the late phase is shown in Figure 10(a). The linear portion of this plot was used to evaluate the time constant λ_2 of this phase. After subtraction of the contribution of the corresponding exponential term from the total radioactivity, the procedure was repeated to determine the other time constant λ_1 (Fig. 10b). The ordinate intercepts of the two plots yielded the pre-exponential factors C_1 and C_2 in the simulation function:

$$y = C_0 + C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t)$$

In (b), symbols are defined as follows: $\square$, identity; $\cdot$, conservative replacements; $\cdot\cdot$, distant replacement

No.	Sequence by Edman degradation
1	<div> <div>1</div> <div>10</div> <div>20</div> <div>30</div> </div> TSSTGTISPFVHPNAEEAXQAFQNDRD (S) VLAQYIFXL (S) R
3	<div> <div>1</div> <div>10</div> <div>20</div> <div>30</div> </div> LASQISATDFGAILTLTQVTPXATLEDL (K)
3	<div> <div>1</div> <div>10</div> </div> HVDDQHSTIR
4	<div> <div>1</div> </div> NHPELSK
5	<div> <div>1</div> </div> FIETHEK
6	<div> <div>1</div> </div> FVNAK

Species	Partial sequence
Rat (132-170)*	KDDNPNLPPFQRPEAEAMCTSFQENPTSTFLGHYLLHEVPA : : : : : : : : : : : : : : : : : :
Cobra (no. 1†, 1-39)*	TSSGTGISFPFVHPNAEEAXQAFQNDNRDSVLAAQYIFXLS 1 10 20 30

† Fragment number.

The kinetic experiments showed that two mutually independent classes of binding sites for the neurotoxin exist on the cobra serum albumin. The affinity is almost the same at all the sites, but

Figure 7 Protective effect of CSAP towards the venom

(a) Dose-response relation for freeze-dried cobra venom dissolved in saline and injected intraperitoneally into mice (20 g, male). The regression line is given by the equation $D = 1.60 + 0.528 D/T$; $r = 0.96$. The LD_{50} was 1.60 ± 0.12 mg/kg. D is the dose (mg/kg) and T the survival time (h). The vertical bars give the S.D. (b) The same plot as in (a), but for mice which had received ~ 100 μ g of partially purified CSAP/mouse intraperitoneally 10 min before being injected with the venom. The regression line is given by the equation $D = 5.60 + 0.370 D/T$; $r = 0.89$. The LD_{50} was 5.60 ± 0.25 mg/kg.

Figure 8 Protective effect of CSAP against haemolysis caused by cobra cardiotoxin

Cardiotoxin (250 μ g in 0.1 ml of 0.9% NaCl) was mixed with different amounts of CSAP in 0.1 ml of 0.9% NaCl. After incubation for 10 min, 2 ml of guinea-pig RBC suspension were added. The mixture was incubated for 30 min, then centrifuged; the A_{540} of the supernatant was read.

Figure 9 Time course of the release of radioactivity (125 I-toxin) from AT complexes bound to DEAE-cellulose after displacement by a large excess of unlabelled toxin

Initial concentrations were as follows: antitoxin, 1.18 nM; 125 I-toxin, 45 nM. The exchange of toxin was initiated by the addition of 93.8 μ M unlabelled toxin. The solvent was 10 mM buffered saline, pH 4 at 24 $^{\circ}$ C. The curve is a theoretical one computed from the data in Table 4. The right ordinate axis is the molar ratio of exchangeable labelled toxin to total antitoxin (T_{ex}/A_0). The left ordinate axis shows total radioactivity (γ in the text) bound in AT complexes retained by the filter.

Figure 10 Resolution of two dissociation reactions of the neurotoxin (T) from the AT complexes

(a) The slow step; (b) the fast step. The vertical bars represent the S.D.

the rate constants for the two types of site differ by orders of magnitude. This may indicate that their accessibility, rather than the nature of the interacting surfaces, differ and that conformational changes in the CSAP, like the known ligand-induced

Table 4 (a) Observed kinetic parameters pertaining to eqn. (3) and (b) kinetic and thermodynamic parameters derived from eqn. (3)

(a)

Step	No.	(s ⁻¹)	exp I_y^*
Fast	1	$(6.5 \pm 0.6) \times 10^{-4}$	0.80 ± 0.04
Slow	2	$(2.8 \pm 0.4) \times 10^{-5}$	0.52 ± 0.02

(b)

Step	k_{+1} [(mol · s) ⁻¹]	k_{-1} (s ⁻¹)	K_{ass} (l · mol ⁻¹)	ΔG_0 (25 °C) (kJ/mol)
Fast	3080 ± 400	$(5.2 \pm 0.6) \times 10^{-4}$	$(6.0 \pm 1.1) \times 10^6$	38.1 ± 0.4
Slow	162 ± 30	$(2.0 \pm 0.3) \times 10^{-5}$	$(8.1 \pm 1.9) \times 10^6$	38.9 ± 0.8

*exp $I_y = \frac{k_{+1}}{\lambda}$; I_y = ordinate intercept in the plot of $\ln([AT] - [AT]_{\infty})/[AT]_0$ versus t (see the text).

rearrangements in mammalian albumins [26], accompany the exchange reactions. The enhanced toxin-binding capacity of the tryptic fragment T-60 lends some support to this view.

Apparently the cobra survives accidental poisoning by its own seeping venom gland with the aid of its serum albumin. This protein is more acidic than other albumins and would therefore be expected to bind the cobra neurotoxin, which is very basic (pI 10.5) by ionic linkages, although also other bond types, e.g. hydrogen bonds and hydrophobic interactions, may play an important part. In addition, the latter may provide the specificity of the interaction that the ionic bonds are incapable of providing. If the free neurotoxin were present in the blood, it would associate with synaptic proteins with a binding constant in the nanomolar range and block the synaptic transmission if the concentration of the toxin were sufficient [27]. However, the antitoxin effectively competes with the synapses for the binding of the neurotoxin, because the association constant is in the micromolar range, the concentration of the CSAP is high and the binding is multiple (up to 8 ± 1 molecules of toxin/antitoxin molecule). Hence the concentration of the free toxin is kept at a very low level and the snake is spared discomfort. The toxin must be bound to the CSAP in such an orientation that the hydrophobic groups, which are known to interact with the synapsis,

are masked. The affinity of the CSAP for the neurotoxin corresponds to that of HSA for unconjugated bilirubin [28].

This system may be regarded as a model of many binding processes in snake blood, since many toxins exhibit similar binding properties. Besides, it is an interesting example of complex multiple protein-protein interactions. Such processes are widespread in biological systems and are involved in many important physiological mechanisms, including the folding of nascent protein chains and refolding aided by chaperones after passage through a membrane in the extended-chain conformation [29].

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