Naturally Occurring Disulfide-bound Dimers of Three-fingered Toxins

A PARADIGM FOR BIOLOGICAL ACTIVITY DIVERSIFICATION

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Disulfide-bound dimers of three-fingered toxins have been discovered in the Naja kaouthia cobra venom; that is, the homodimer of α-cobratoxin (a long-chain α-neurotoxin) and heterodimers formed by α-cobratoxin with different cytotoxins. According to circular dichroism measurements, toxins in dimers retain in general their three-fingered folding. The functionally important disulfide 26–30 in polypeptide loop II of α-cobratoxin moiety remains intact in both types of dimers. Biological activity studies showed that cytotoxins within dimers completely lose their cytotoxicity. However, the dimers retain most of the α-cobratoxin capacity to compete with α-bungarotoxin for binding to Torpedo and α7 nicotinic acetylcholine receptors (nAChRs) as well as to Lymnea stagnalis acetylcholine-binding protein. Electrophysiological experiments on neuronal nAChRs expressed in Xenopus oocytes have shown that α-cobratoxin dimer not only interacts with α7 nAChR but, in contrast to α-cobratoxin monomer, also blocks α3β2 nAChR. In the latter activity it resembles α7-bungarotoxin, a dimer with no disulfides between monomers. These results demonstrate that dimerization is essential for the interaction of three-fingered neurotoxins with heteromeric α3β2 nAChRs.

Three-fingered toxins (TFTs) are the main components of the Elapidae snake venoms. TFTs consist of one polypeptide chain, their spatial structure being characterized by a hydrophobic core stabilized by four disulfide bridges, which confine three polypeptide loops (fingers). In cobra venom TFTs are represented mainly by α-neurotoxins and cytotoxins. So-called short-chain α-neurotoxins (60–62 amino acid residues, 4 intramolecular disulfides) effectively block nicotinic acetylcholine receptors (nAChRs) of muscle-type, and long-chain α-neurotoxins (65–75 residues with an additional disulfide in central loop II) block neuronal homopentameric α7 nAChR as well (1, 2). These toxins are widely used as tools in the nAChR studies. Cytotoxins, structurally related to short-chain α-neurotoxins, manifest another activity; they non-selectively disrupt cell membranes and, thus, kill the cells (3).

Another example of TFT interacting with nAChR are κ-bungarotoxins (κ-Bgts), minor components of the krait (Elapidae) venom (4, 5). All κ-Bgts consist of 66 amino acid residues and, similar to long-chain α-neurotoxins, contain five disulfide bonds. However, in contrast to α-neurotoxins, κ-Bgts practically do not block muscle-type nAChRs and only weakly act on α7 nAChR but with high efficiency interact with α3β2 neuronal receptors (6). Despite the vast array of data on structure-activity relationship for α-neurotoxins and κ-Bgts, it is not yet clear what are the main structural features determining the specificity of a toxin to the particular receptor type. Recently, based on the x-ray structure of α-cobratoxin (α-CT) with acetylcholine-binding protein, Bourne et al. (7) suggested that Lys-29 is the main residue determining the difference in specificity between α-neurotoxins and κ-bungarotoxins. However, A29K mutation in the long type α-neurotoxin substantially decreased the affinity to α7 nAChR but did not induce any affinity to α3β2 receptor (8). It should be mentioned that the unique structural property of κ-Bgts distinguishing them from all other TFTs is their dimeric nature; both in crystal and solution these toxins exist as a non-covalent dimer of two identical molecules (9, 10). Although it was suggested that dimeric nature of κ-Bgt may play a role in its binding to receptors (11), there is no clear understanding of the dimerization importance for the biological activity.

In this paper we report identification in the Naja kaouthia cobra venom of a new class of naturally occurring TFTs, which represent disulfide-bound heterodimers of α-cobratoxin (α-CT), a long-chain α-neurotoxin, with cytotoxins, as well as a homodimer of α-CT. In these dimers α-CT retains its ability to...
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interact with muscle-type and α7 nAChR, although with decreased affinity, whereas cytotoxins become completely non-cytotoxic. In addition to the ability to interact with the above receptor subtypes, α-CT homodimer acquires the capacity to block α3β2 neuronal nAChR. Such an interaction was never observed for monomeric α-neurotoxins. Dimeric α-CT from cobra venom may be considered as a functional analogue of krait κ-Bgts. This is a first example of post-translational modification resulting in α-neurotoxin capable to block both α7 and α3β2 neuronal nAChRs.

EXPERIMENTAL PROCEDURES

Materials—The *N. kaouthia* Thailand cobra venom was obtained from living adult cobras kept in captivity as described earlier (12). α-CT and cytotoxins (CX) 1, 2, and 3 were purified from this venom according to Kukhtina *et al.* (13). N-Ethylmaleimide (NEM) was from Fluka (Hamburg, Germany), 4-vinylpyridine was from Aldrich, tris(2-carboxyethyl)phosphine (TCEP) was from Sigma, modified porcine trypsin was from Promega (Madison, WI), dithiothreitol was from Bio-Rad, and monoiodinated (3-[125I]iodotyrosyl54)-α-bungarotoxin (~2000 Ci/mmol) was from Amersham Biosciences. Other reagents were of the highest quality commercially available.

Isolation of the Disulfide-bound Dimers—For separation of dried venom, gel filtration of crude cobra venom was carried out using a Sephadex G50 sf (Amersham Biosciences) column (4.5 × 150 cm) in 0.1 M ammonium acetate buffer, pH 6.2, containing 0.01% sodium azide; cation-exchange HPLC of fraction II from gel filtration was performed on a HEMA BIO CM column (10 × 250 mm, Tessek, Czech Republic) in ammonium acetate gradient, pH 7.5, and reverse-phase HPLC was carried out using Phenomenex C18 (4.6 × 250 mm) or Vydac C18 (10 × 250 mm) columns in acetonitrile gradient with 0.1% trifluoroacetic acid as in Osipov *et al.* (14). For separation of liquid venom, 2.5 ml of crude liquid venom collected from 8 adult cobras were applied within 1 h after collection onto a gel-filtration column and separated under the conditions described above for dried venom. Fraction II thus obtained was directly applied onto a Vydac C18 (10 × 250 mm) column. After the column was equilibrated with 15% acetonitrile, 0.1% trifluoroacetic acid for 15 min, a gradient to 45% acetonitrile in 30 min was run at a flow rate 2 ml/min.

Exposure to Denaturing Conditions—Each dimeric protein (20 μg) was dissolved in 130 μl of 6 M guanidine hydrochloride and incubated overnight at room temperature. The mixtures were analyzed by reverse-phase HPLC using a Phenomenex C18 (4.6 × 250 mm) column and then by SDS-PAGE. The proteins incubated without guanidine hydrochloride served as a control.

Total and Partial Reduction and S-Alkylation—Total reduction of the proteins and alkylation with 4-vinylpyridine were performed as in Osipov *et al.* (14). Selective reduction was carried out under nitrogen for 20 min at room temperature by two methods following the recommendations from Martin *et al.* (15) and Gray (16). In the first method, 1:1 and 2:1 of DTT to protein molar ratios were employed in a 0.2 M Tris-HCl buffer, pH 8.5, followed by the addition of excess of 4-vinylpyridine and incubation under the nitrogen for 45 min. In the second method, 1:1, 2:1, and 15:1 TCEP to protein molar ratios were employed in 0.17 M sodium citrate buffer, pH 3.0. Then the excess of NEM suspended in the same buffer was added, and reaction mixture was stirred under nitrogen for 45 min. The resulting products were separated using a Phenomenex C18 (4.6 × 250 mm) column as above. The main product was additionally incubated with TCEP (2:1) in 0.17 M sodium citrate buffer, pH 3.0, in the presence of 6 M guanidine hydrochloride for 45 min and then alkylated by NEM as above.

MALDI Mass Spectrometry—MALDI-TOF MS and MS/MS analysis was performed on Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonik) equipped with an Nd laser. The MH+ molecular ions were measured in reflector mode; the accuracy of mass peak measurement was 0.01%. Aliquots (0.5 μl) of the sample were mixed on a steel target with an equal volume of 2,5-dihydroxybenzoic acid (Aldrich) solution (10 mg ml⁻¹ in 30% acetonitrile, 0.5% trifluoroacetic acid), and the droplets were left to dry at room temperature. Every mass spectrum was obtained as a sum of minimum 500 laser shots. Fragment ion spectra were generated by laser-induced dissociation slightly accelerated by low energy collision-induced dissociation using helium as a collision gas. Correspondence of the found masses to the toxin peptides and to MS/MS peptide fragments was manually interpreted with the help of GPMAW 4.04 software (Lighthouse Data) using 0.01–0.02% precision as a criterion. For partial cleavage of disulfide bonds, the energy of laser shots was increased from 15 to 25% maximal value. Tryptsinolysis and recording of MALDI mass spectra of tryptic fragments as well as their MS/MS analysis were performed as in (17).

Edman Degradation—The proteins were subjected to Edman degradation using a protein sequencer 477A (Applied Biosystems, Foster City, CA).

Electrophoresis in Polyacrylamide Gels—SDS-PAGE was carried out according to Smith (18) under non-reducing and reducing conditions in 16.5% gel. Gel thickness was 1.5 mm.

Receptor Binding Studies—For competition binding assays, suspensions of nAChR-rich membranes from *T. californica* electric organ (1.2 nM α-bungarotoxin binding sites suspended in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mg/ml bovine serum albumin), human α7 nAChR-transfected GH4C1 cells (1.2 nM α-bungarotoxin binding sites prepared in PBS with 0.1% Tween 20 (PBS-T) buffer), or solutions of expressed AChBP from *L. stagnalis* (6.4 nM in PBS-T buffer) were incubated for 90 min with various amounts of the toxins followed by an additional 90-min incubation with 0.2 nM [125I]α-bungarotoxin. Non-specific binding was determined by preliminary incubation of the preparations with 1 μM α-CT. The membrane and cell suspensions were applied to glass GF/F filters (Whatman, Kent, England) presoaked in 0.25% polyethyleneimine, and the unbound radioactivity was removed from the filter by washing (2 × 3 ml) with 20 mM Tris-HCl buffer, pH 8.0, con-
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RESULTS

Isolation of the Proteins—Gel filtration of dried crude N. kaouthia venom on Sephadex G-50 resulted in four main fractions (I-IV, supplemental Fig. S1A). Fraction II containing proteins with molecular masses in the range from 9 to 30 kDa was further separated by cation-exchange chromatography (supplemental Fig. S1B). The fractions obtained were analyzed by MALDI MS, and those containing proteins with molecular masses of 15.64 kDa (fraction II-6) and 14.52–14.60 kDa (fractions II-9, II-11, and II-12) were finally purified by reverse-phase HPLC (Fig. 1). New proteins isolated from these fractions were named (after subsequent elucidation of their structure) αCT-αCT (15,640 Da), αCT-CX1 (14,515 Da), αCT-CX3 (14,528 Da), and αCT-CX2 (14,556 Da) (Table 1). The yield of αCT-αCT was 0.04% of dry venom weight, αCT-CX3 was ~0.02% of dry venom weight, and the yield of the two other proteins (αCT-CX1 and αCT-CX2) was less than 0.02% of dry venom weight.

It can be seen from Fig. 1A that there are small peaks eluting before and after αCT-αCT peak. All these small peaks correspond to products with molecular mass equal to that of αCT-αCT and apparently are isomers that can be separated from the main protein. There were similar isomers of αCT-CX, which were also separated by reverse-phase chromatography (Fig. 1, B–D).

We repeated the fractionation using freshly collected liquid venom, pH 6.0, within 1 h after collection. Fraction II from a Sephadex G-50 column, pH 6.2, was directly applied on a reverse-phase column. MALDI MS analysis of the fractions obtained revealed the presence of all four proteins in the peaks eluting at the expected times (see Fig. 1). Thus, the new proteins are not artifacts generated during drying and/or storage of the venom.

Structural Characterization—Determination of the N-terminal amino acid sequence of αCT-CX3 by Edman degradation up to the 21st residue revealed two sequences, one corresponding to α-CT and another to N. kaouthia cytotoxin 3 (supplemental Scheme SI).

When analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) under non-reducing conditions, αCT-αCT showed one band with an apparent molecular mass of about 19 kDa, whereas αCT-CX1, αCT-CX2, and αCT-CX3 showed apparent molecular masses of about 16–17 kDa (Fig. 2A). At electrophoresis under reducing conditions all those bands disappeared, and new bands with lower molecular masses were observed. αCT-αCT shows one new band of 7.8 kDa, corresponding to the mass of α-CT. αCT-CX1, αCT-CX2, and αCT-CX3 gave rise to two bands with masses corresponding to those of α-CT (7.8 kDa) and cytotoxin (about 7 kDa) (Fig. 2B). At the same time incubation of each protein in 6 M guanidinium chloride for 24 h did not induce changes either in electrophoretic pattern under non-reducing conditions or in the retention times in reverse-phase HPLC. These results suggest the presence of a disulfide bond(s) between two polypeptide chains constituting each isolated protein. The dis-
crepancy in molecular masses of dimers determined by MALDI MS (Table 1) and by SDS-PAGE (Fig. 2A) can be explained by unusual electrophoretic behavior of dimers due to their uncommon molecular size or detergent binding ability. Such deviations from mass/mobility relationship were observed for several polypeptides (e.g. for heat-stable enterotoxin (23)).

MALDI mass spectra of αCT-CX1, αCT-CX3, and αCT-CX2 have been taken at low energy of laser shots (15% of maximal value) and at increased energy (25%). For αCT-CX1 an increase in the energy of laser shots (supplemental Fig. S2) results in the appearance of new m/z signals corresponding to molecular masses of the toxins forming heterodimers; that is, α-CT (m/z 7821) and cytotoxin 1 from the same venom (m/z 6693). A similar behavior was observed for other heterodimers (Table 1). Because a disulfide bond within a protein is broken most easily under conditions of MALDI MS (24), these data support our suggestion about the nature of the bond(s) between the polypeptide chains in the dimers.

For further structural characterization the new proteins were completely reduced and alkylated with 4-vinylpyridine. Purification of alkylated αCT-αCT by reverse-phase HPLC resulted in a single peak of polypeptide with a molecular mass of 8874 Da. This modified polypeptide was digested with trypsin, and the peptides obtained were analyzed by MALDI MS (supplemental Table S1). Some tryptic peptides were sequenced using the collision-induced dissociation method (25). The MS data showed unequivocally that polypeptide of 8874 Da was α-CT (Swiss Prot #P01391) with all 10 cysteines pyridylethylated. Thus, protein αCT-αCT consists of two equal polypeptide chains of α-CT.

Alkylation of reduced αCT-CX1, αCT-CX2, and αCT-CX3 with 4-vinylpyridine followed by reverse-phase HPLC gave rise to two peaks of approximately the same intensity for each protein. Analysis of these products by MALDI MS indicated that one component had molecular mass of 8875 Da and another, ~7600 Da (Table 1). The alkylated products were digested with trypsin and analyzed as indicated above for αCT-αCT. The data obtained showed that product of 8875 Da corresponded to α-CT, whereas the second components are highly (more than 85% covering by tryptic fragments) homologous to cytotoxins from N. kaouthia venom (supplemental Table S1). Thus, αCT-CX1 contains a polypeptide with an amino acid sequence highly homologous to cytotoxin 1 (Swiss Prot #P60305), αCT-CX2 highly homologous to cytotoxin 2 (Swiss Prot #P01445), and αCT-CX3 highly homologous to cytotoxin 3 (Swiss Prot #P01446).

It is known that the disulfide bond Cys-26—Cys-30 in the central loop (loop II, Fig. 3) of α-CT can be

| Fig. 1 panels | Protein   | Molecular mass | Observed m/z value for double charged ion | Mass of additional components observed at the elevated laser energy | Masses observed after complete reduction and alkylation by 4-vinylpyridine |
|---------------|-----------|----------------|------------------------------------------|------------------------------------------------------------------|--------------------------------------------------------------------------|
| A             | αCT-αCT   | 15,640 ± 2     | 7822                                     | 6693, 7821                                                       | 8874                                                                     |
| B             | αCT-CX1   | 14,515 ± 2     | 7254 (7255)*                             | 6709, 7821                                                       | ND*                                                                      |
| C             | αCT-CX3   | 14,528 ± 2     | 7263 (7264)*                             | 6737, 7821                                                       | 7554, 8875                                                              |
| D             | αCT-CX2   | 14,556 ± 2     | 7277 (7279)*                             | 7581, 8875                                                       |                                                                          |

* In parentheses m/z values determined at elevated laser energy are shown. Slight shifts in m/z values for double-charged ions observed in some spectra can be explained by decreased precision of mass determination at elevated laser energy, as compared with standard conditions.

* Exact mass was not determined due to a broadness of the signal.
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reduced and alkylated αCT-αCT and αCT-CX3. This means that at least in one α-CT molecule the disulfide at the tip of the central loop in αCT-αCT and in a single αCT in the αCT-CX3 dimer is not involved in the formation of covalent dimer and is as easily available for reduction as in monomeric α-CT. It is also more susceptible to reduction under mildly alkaline conditions than disulfide bonds connecting polypeptide chains in dimers. The described experiment also showed the absence in dimers of free sulfhydryl groups, i.e. polypeptide chains should be connected at least by two disulfide bonds. A 2-fold increase in the amount of the reducing agent (DTT) did not result in any additional protein modification. This indicates that in dimers no other cystines, including Cys-26—Cys-30 in the second α-CT chain of αCT-αCT, are easily reducible.

A similar result was obtained when αCT-αCT was reduced by TCEP under acidic conditions and alkylated by NEM to avoid disulfide isomerization. When 2 mol of TCEP/mol of αCT-αCT were applied followed by NEM alklyation, the increase in mass of the protein was 500 Da, indicating incorporation of 4 NEM into the molecule. The MALDI MS of the tryptic digest of the modified protein in general looked similar to that of αCT-αCT reduced with DTT. One new peak could be with confidence assigned to a fragment of αCT, namely, to fragment 50–68 bearing 1 NEM group at Cys-57 (confirmed by MS/MS data). At the same time, m/z values of the both unmodified fragments 24–33 and 50–68 were present in the mass spectra, indicating that they were each alkylated in one of the chains.

Further modifying αCT-αCT with 4 groups of incorporated NEM (after reducing it with 2 mol of TCEP per mole of protein), we obtained the product αCT-αCT+6 NEM (of 16,400 Da), which could be split into two chains under the action of DTT, αCT+2NEM and αCT+4NEM. Analysis of these fragments gave hints to localization of the dimer-forming disulfides (see supplemental Table SII). However, the reliable conclusions cannot be done due to limitations of methods used.

Circular dichroism (CD) spectra of isolated dimers are shown in Fig. 4 together with the spectra of the toxins forming these proteins. The spectrum of αCT-αCT (Fig. 4A) in general resembles that of α-CT. However, there are some differences in the 190–225-nm range, indicating only small changes in the conformation of polypeptide backbone caused by dimerization. The CD spectra of αCT-CX1, αCT-CX2, and αCT-CX3 (Fig. 4, B–D) are more different from the superposition of the spectra of α-CT and corresponding cytotoxin than in the case of αCT-αCT and α-CT. This difference is especially evident in the spectral range from 190 to 220 nm corresponding to the dichroism of peptide bonds. The smallest changes were observed for αCT-CX2, where only band intensity changed, whereas in the spectrum of αCT-CX3 the band extrema were shifted by 5–10 nm as compared with the “averaged” spectrum of α-CT and cytotoxin. The observed spectral differences may be related to the changes both in the length and in the twisting of β-sheet structures (26). However, the general patterns of heterodimer spectra are characteristic for TFT. This means that TFT fold in dimers should not be substantially changed. Fluorescence spectra (not shown) of α-CT and αCT-αCT registered at excitation
wavelengths of 280 and 292 nm were practically identical, indicating the equivalent tryptophan microenvironment in both proteins.

**Biological Activity of Dimers**—α-CT is a specific blocker of muscle and neuronal α7 nAChR types (27). Therefore, we have tested the activity of dimers in competition experiments using the membranes from electric organ of electric ray *T. californica* as a source of muscle-type nAChRs and transfected cell line GH4C1 as a source of human α7 nAChR (28). The soluble AChBP from *L. stagnalis* mollusk (which potently binds long-chain α-neurotoxins (29, 30)), was also used in the competition experiments. Radioactive 125I-labeled α-Bgt was used as a ligand. Dimer formation reduces the activity of α-CT (Fig. 5) depending on the nature both of the dimer and the receptor; αCT-αCT was more active on Torpedo nAChR and AChBP than αCT-CX2 and αCT-CX3, whereas αCT-αCT was the least active on α7 nAChR. αCT-CX2 and αCT-CX3 were almost equipotent on Torpedo nAChR, whereas on α7 and AChBP their activities differed 2–2.4-fold. In general, α-CT incorporated into a dimer retained to a certain extent its ability to bind nAChRs and AChBP.

Electrophysiological experiments have shown that αCT-αCT blocks acetylcholine-induced current in recombinant human α7 and α3β2 nAChRs expressed in *Xenopus* oocytes (Fig. 6). For α7 nAChR, the IC50 of dimer is 1 order of magnitude higher than that of α-CT, which is in accord with the binding data. In the case of α3β2 nAChR, the IC50 of dimer is about 0.15 μM. This value is very close to that (0.1–0.12 μM) determined for α-Bgt in the assay on chick ciliary ganglion (31). We show for the first time that α-Bgt blocks human α3β2 nAChR much more potently (IC50 3 nM), and a similar value (2.3 nM) for rat α3β2 nAChR has been reported earlier (11). No interaction between α3β2 nAChR and α-Bungarotoxin was observed at concentration up to 10 μM (31, 32). We show also that monomeric α-CT has practically no effects on α3β2 nAChR (IC50 > 300 nM). Thus, upon dimerization α-CT acquires the ability to block α3β2 nAChR like α-neurotoxins.

It is interesting that α-Bgt is also active against human α7 nAChR (IC50 10 nM). It should be noted that similar activity of α-Bgt was earlier observed on chimeric α7-5HT3 receptor (27).

Cytotoxicity of αCT-CX2 and αCT-CX3 was determined on the cell line PC12 that is very susceptible to cytotoxic components from snake venom (21). Thus, CX3 from *N. kaouthia* induced the death of more than half of the cells at concentration of 20 μM, whereas both αCT-CX2 and αCT-αCX3 produced no changes in PC12 cells at concentration up to 10 μM. It indicates that cytotoxic activity of cytotoxin moieties in the dimers is greatly diminished or probably completely destroyed.
DISCUSSION

Cobra venoms are mixtures of proteins having diverse functional and structural properties. More than 100 proteins were identified in *Naja atra* cobra venom by a proteomic approach (33). However, all these proteins can be classified only into about 10 structural types. Their vast majority, both by number and by content in the venom, belongs to the family of TFTs. Studying the venom of cobra *N. kaouthia* in the well established family of TFTs, we have found for the first time a glycosylated member (34). In the present work we describe some other minor components from *N. kaouthia* venom, principally new types of TFTs, namely an α-CT homodimer and α-CT het-

erodimers with cytotoxins, all covalently bound by disulfide bridges.

Among proteins of non-venomous origin, the examples containing several copies of TFT-like folds can be found in literature; e.g. a polypeptide chain of γ-type inhibitors of phospholipases A2 from sera of different snakes contains two repeats with TFT-like fold (35), and urokinase-type plasminogen activator receptor contains three repeats. However, in these proteins the disulfides are only within TFT-like domains but not between them (36).

Dimerization of venom proteins is a well known phenomenon. For example, heterodimeric toxins (not of TFT nature) were isolated from venoms of ants (37), spiders (38), and scorpions (39). Recently, a new class of disulfide-bound pseudo-homodimeric neurotoxins was found in the venom of marine cone snails (40). In snake venoms, dimeric phospholipases A2 and disintegrins, including disulfide-bound homodimeric dis-integrin (41), and disulfide-bound heterodimeric C-type lectin-like protein (42) were described.

As far as TFTs are concerned, their non-covalent association is well known. There are some data that, to exert their cytolytic action on membranes, the TFT cytotoxins should form dimers (43) or trimers (44). α-Bgt, which blocks αβ2 nAChR, is very close to α-CT structurally but exists as a dimer both in solution and crystal (9, 10). However, in this case, dimerization does not involve the rearrangement of disulfide bonds and formation of disulfides between monomers.

Earlier three disulfide-bound dimeric toxin-like proteins were isolated from mamba venom (45). These proteins were called “synergistic” toxins as they were not toxic themselves but did increase toxicity of TFTs when injected together with them. Their amino acid sequences slightly resemble TFT’s, with the position of one cysteine being changed. Neither disulfide pairing and spatial structures for these proteins nor intrinsic activity of the dimers and constituting monomers was established.

Therefore, α-CT-αCT and αCT-CX dimers are the first examples of naturally occurring TFTs in which toxin monomers are bound by disulfide bridges. In contrast to the synergistic toxins, these dimers possess their own activities, although dimerization affects the biological activity of constituting monomers. To test the activity of α-CT within the dimers, we used three different proteins which the native toxin binds efficiently. These were nAChR from the electric organ of *T. californica*, neuronal α7 nAChR, and AChBP from *L. stagnalis*. The results obtained (Fig. 5) indicate that α-CT moiety within the dimers preserves its capacity to interact with all proteins tested. However, the potency was diminished in all cases, depending on the nature both of the dimer and of the binding protein. The lowest decrease in affinity was observed for the binding of homodimer to *Torpedo* nAChR, and the highest was for the binding of αCT-CX3 to AChBP. It is well documented that the central loop of α-neurotoxins is important for their binding to neuronal nAChR (e.g. see Ref. 27). The capacity to bind to nAChRs shows that the central loop in dimers (at least one loop in homodimer) is accessible for interaction with the receptor.

Dimerization with α-CT proved fatal for the cytotoxic activity of cytotoxins. Probably, α-CT within a heterodimer interferes with the formation of membrane-active non-covalent...
The most interesting is that dimeric \( \alpha\)-CT acquires the ability to block both \( \alpha/7 \) and \( \alpha/3/3 \) \( \alpha\)-nAChRs (Fig. 6) similarly to \( \kappa\) Bgt. This means that all amino acid residues essential for the interaction with \( \alpha/7 \) \( \alpha\)-nAChR are present in the \( \alpha\)-CT, and toxin dimerization is the necessary condition for the realization of this interaction. Because of its interaction with \( \alpha/7 \) \( \alpha\)-nAChR, dimeric \( \alpha\)-CT from cobra venom may be considered as a functional analogue of \( \kappa\) Bgt found so far only in krait venoms.

One natural disulfide bond, Cys-26—Cys-30, in the tip of the central loop II of the dimers (Fig. 3) is preserved as follows from its selective reduction and alkylation characteristic for the monomeric \( \alpha\)-CT. Interestingly, unmodified peptide 24–33 arising apparently from that portion of \( \alpha\)-CT containing 6 NEMs.

The experiments with TCEP revealed another disulfide in \( \alpha\)-CT which can be reduced without resulting in splitting the dimer, namely Cys-57—Cys-62. This conclusion is based on detecting the fragment 50–68 with the NEM group attached to Cys-57 and supported by finding the fragment 50–68 with three attached NEM in the products of tryptic hydrolysis of modified \( \alpha\)-CT-CX3 containing 6 NEMs.
The presented results on the reduction and alkylation of disulfide bonds demonstrate that αCT-αCT and αCT-CX are individual molecules rather than mixtures of disulfide isomers. Such a conclusion could be already made for αCT-αCT because Fig. 1A shows its separation from isomers. For αCT-CX3, indirect evidence of its “isomeric homogeneity” could be its HPLC peak, which was as sharp as the peak of individual αCT-αCT. A high similarity of the CD curves of αCT-αCT and monomeric αCT also made unlikely the presence of many shuffled disulfides.

At present we cannot identify the intermolecular disulfides. Because both disulfide bonds Cys-26—Cys-30 in the central loop II of αCT-αCT (Fig. 3) are preserved in the dimer, the disulfides of hydrophobic core (where all the disulfide bonds of cytotoxins and four of five of α-CT are located) should be involved in the formation of dimers. Therefore, the two polypeptide chains are connected most probably in the region of toxin hydrophobic cores. Such a connection would leave three α-CT loops at least partially available for receptor binding, which is in accord with the registered activity of dimers against nAChRs and AChBP.

Interestingly, systematic removal of disulfide bonds (one at a time) in κ-Bgt by site-specific mutagenesis revealed a different role for each bond (11). Removal of either Cys-46—Cys-58 or Cys-59—Cys-64, belonging to the C-terminal loops, interfered with the ability of the toxin to fold. In contrast, removal of either Cys-3—Cys-21 or Cys-14—Cys-42 did not interfere with the toxin folding and yielded molecules with full biological activity. In view of these data, we suppose that some of the latter cysteines might take part in dimer formation.

Noteworthy, identification of disulfide-bound dimeric TFTs became possible only due to isolation of intact proteins from the venom. Sequencing the genes, cDNAs or completely reduced and Cys-alkylated proteins can give only the primary structures of monomeric chains. In our case these are the well preserved N. kaouthia cobra venom was investigated by a combination of two-dimensional gel electrophoresis, liquid chromatography, and tandem mass spectrometry. Although the authors have seen some difference between electrophoretic patterns of crude venom under reducing and non-reducing conditions, no dimeric TFT was revealed among 61 proteins found. Because the dimers make only 0.01—0.04% of the dried venom weight, combination of several types of chromatography was essential for detecting these true minor components.

It appears that dimerization is one of the ways to induce a new type of activity and/or temporarily change or block another activity. Indeed, it is dimerization that endowed α-CT with a potency to block α3β2 nAChR, at the same time reducing its activity toward other nAChR subtypes. However, dimerization with α-CT completely blocks the cytotoxic activity of cytotoxins. Interestingly, a substantial decrease of cytotoxicity was found earlier for another minor component of cobra venom, namely for a glycosylated cytotoxin. Because the activity could be totally restored upon deglycosylation, it was suggested that glycosylation might be a temporary mechanism of diminishing the toxicity (34).

In summary, naturally occurring TFTs are shown to form disulfide-bound dimeric proteins with a new architecture but in general maintaining the three-finger fold. Functionally, dimerization brings about some decrease in the affinity of a snake α-neurotoxin to its well known targets, distinct nAChRs and AChBP, and results in complete loss of cytotoxic activity of the cytotoxin part. More interesting, the dimeric α-CT acquires the capacity to interact with neuronal α3β2 nAChR, the property that has not been observed so far for any snake α-neurotoxin. This is a good example of biological activity diversification, when the decrease in one type of activity (interaction with α7 nAChR) is compensated by acquisition of new type of activity (interaction with α3β2 nAChR).

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