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Acetylcholine receptor–α-bungarotoxin interactions: Determination of the region-to-region contacts by peptide–peptide interactions and molecular modeling of the receptor cavity

(α-neurotoxin/binding site/three-dimensional structure/synthetic peptides)

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ABSTRACT **In previous studies from this laboratory, the binding regions of α-neurotoxins on human and *Torpedo* acetylcholine (AcCho) receptors (AcChoRs) and the binding regions for the receptor on the toxin were characterized with synthetic peptides of the respective molecules. In the present work, peptides representing the active regions of one molecule are each allowed to bind to each of the active-region peptides of the other molecule. Thus, the interaction of three α-bungarotoxin (α-BTX) synthetic loop peptides with four synthetic peptides representing the toxin-binding regions on human AcChoR permitted the determination of the region–region interactions between α-BTX and the human receptor. Based on the known three-dimensional structure of the toxin, the active peptides of the receptor were then assembled to their appropriate toxin-contact regions by computer model building and energy minimization. This allowed the three-dimensional construction of the toxin-binding cavity on human AcChoR. The cavity appears to be conical, 30.5 Å in depth, involving several receptor regions that make contact with the α-BTX loop regions. One AcChoR region (within residues 125–136) involved in the binding to α-BTX also resides in a known AcCho-binding site, thus demonstrating in three dimensions a critical site involved in both AcCho activation and α-BTX blocking. The validity of this approach was first established for three of four peptides corresponding to regions on the β chain of human hemoglobin involved in binding to the α chain. Thus, studying the interaction between peptides representing the binding regions of two protein molecules may provide an approach in molecular recognition by which the binding site on one protein can be described if the three-dimensional structure of the other protein is known.**

The biological activity of most proteins is initiated by binding either to a ligand or to another protein. Therefore, elucidation of protein molecular recognition is the key to the understanding of protein function and its relationship to structure and may lead to the rational design of protein molecules possessing desired biological functions. The nicotinic acetylcholine (AcCho) receptor (AcChoR) effects postsynaptic neuromuscular transmission by permitting ion flux across the cell membrane in response to binding of AcCho (1, 2). The α chain of AcChoR contains the AcCho binding site(s) (3–5). The regulatory effect of AcCho is inhibited by the binding of an α-neurotoxin to AcChoR. The toxin-binding site(s) also resides in the α subunit of AcChoR (6). Recent studies from this laboratory using synthetic uniform-sized overlapping peptides encompassing the entire extracellular parts of the α chain of human AcChoR and *Torpedo* AcChoR enabled the localization of the full profile of the toxin-binding regions on the *Torpedo* (7–9) and human (10) receptors. Conversely, the binding sites for AcChoR on α-bungarotoxin (α-BTX) were mapped by synthetic peptides representing each of the α-BTX loops (11, 12).

The present paper describes an approach for studying the details of protein–protein recognition. Each of the active peptides of one protein is allowed to interact with each of the active peptides of the other protein. Based on relative binding affinities of peptide–peptide interactions, the position of AcChoR peptides relative to the α-BTX molecule was assessed. The receptor peptides were docked onto the appropriate regions of α-BTX, whose three-dimensional (3-D) structure is known (13), by computer graphics and energy minimization, thus allowing a 3-D model to be constructed of the binding-site cavity for the toxin on human AcChoR.

MATERIALS AND METHODS

Materials. Synthesis, purification, and characterization of the human AcChoR α-chain peptides have been reported (10). The α-neurotoxin binding regions on human AcChoR reside (10) in the peptides shown in Fig. 1. These peptides were used in the present work. The receptor-binding regions on α-BTX are present in the three loop peptides shown in Fig. 1 (12). The synthesis, purification, and characterization of the monomeric forms of the three cyclic peptides (Fig. 1) have been described (12). α-BTX and its synthetic peptides were labeled with iodine-125 by using the chloramine-T method (14). Radioiodinated materials were used immediately after labeling. The specific activities of the labeled peptides were: LIN, 3.1×10^3 cpm/pmol; L2, 2.4×10^3 cpm/pmol; and L3E, 2.1×10^3 cpm/pmol.

Binding Studies. The coupling of proteins and peptides to CNBr-activated Sepharose CL-4B was carried out under optimum conditions as described (15). The binding of ^{125}I-labeled α-BTX or α-BTX peptides to adsorbents of the human AcChoR peptides was determined by a quantitative solid-phase radiometric binding assay (12, 15, 16). All titrations were carried out in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing 0.1% bovine serum albumin. Nonspecific binding was determined by titrating equivalent volumes of uncoupled Sepharose and Sepharose coupled to unrelated proteins (bovine serum albumin, hen lysozyme) and peptides [synthetic peptides of myoglobin and a nonsense peptide [Glu-Ser-Ser-Gly-Thr-Gly-Ile-Glu-Ser-Ser-Gly-Thr-Gly-Ile (15)] under identical conditions. Dissociation constants of the binding of ^{125}I-labeled α-BTX or its peptides to each of the receptor peptides were calculated by Scatchard analysis (17) from titrations using a fixed amount

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Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; α-BTX, α-bungarotoxin of *Bungarus multicinctus*; Hb, human adult hemoglobin; 3-D, three-dimensional.

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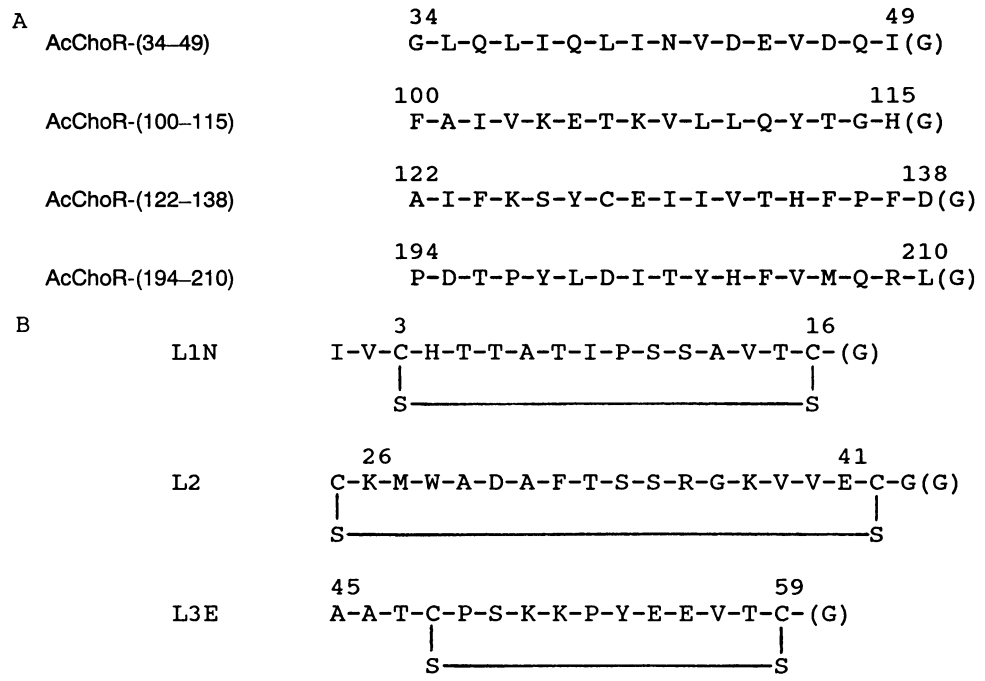

FIG. 1. Covalent structures of the synthetic peptides used in this work. (*A*) Peptides of the AcChoR α chain that are involved in binding to α-neurotoxins (10). (*B*) Peptides of the α-BTX molecule that are involved in binding to AcChoR (11, 12). Note that the disulfide bonds in LIN and L2 are artificial (for details, see ref. 12). The binding areas on the AcChoR peptides have been assigned (10) to residues 32–41, 100–110, 125–136, and 198–208. Note that the glycine residues in parentheses are not part of the α-BTX or the AcChoR sequences, but the peptides were synthesized on a Gly-resin for convenience.

(10 μl) of adsorbent suspension (1:1, vol/vol) in PBS/0.1% bovine serum albumin and increasing amounts of labeled ligand (Fig. 2). The specificity of the binding of the α-BTX peptides was confirmed by their inhibitory activity towards the binding of ^{125}I-labeled α-BTX to adsorbents of the receptor peptides.

Model Building of the Toxin-Binding Cavity on AcChoR. For structural prediction algorithms, the computer program ALB (18) was used. Standard conditions (pH 7.0; ionic strength, 0.15 M; dielectric constant, 78.5; and temperature, 300 K) were used to predict the globular structure of the entire human AcChoR α chain from sequence information. The secondary structures for the peptides were then chosen based on this calculation. Model building was done by the graphics program PSFRODO (19) on an Evans–Sutherland PS300 graphics system connected to a VAX 8550 computer. The conformations of the predicted peptide secondary structures were constructed by replacing residues in similar peptides obtained from refined structures to match perfectly with the composition of the target peptides. The replaced residues were then regularized by using the REFINE option in PSFRODO. The resulting structures of the receptor peptides were docked with the appropriate loop(s) in the refined α-BTX structure (13). Initial docking was done to agree with the results of receptor peptide–α-BTX loop binding studies. Hydrogen bond and positive van der Waal's interactions were then maximized. Structural refinement of the receptor peptide segments was then accomplished by energy minimization, using the computer program YETI (20), while maintaining the x-ray coordinates for α-BTX.

RESULTS

Binding of ^{125}I-labeled α-BTX and Its Synthetic Peptides to AcChoR Peptides. The results of titrations of fixed amounts of human AcChoR peptide adsorbents with various amounts of ^{125}I-labeled α-BTX or its active (i.e., receptor-binding) loop peptides are summarized in Fig. 2. It was found that each of the receptor peptides comprising residues 34–49, 100–115, and 122–138 bound more than one α-BTX peptide. Conversely, a given α-BTX loop peptide bound to more than one receptor peptide. Thus, the receptor peptides exhibited the following binding activities: AcChoR-(34–49) hexadecapeptide bound α-BTX and its peptides L2 and LIN; AcChoR-(100–115) hexadecapeptide bound α-BTX and its peptides L2, L3E, and LIN; AcChoR-(122–138) heptadecapeptide bound α-BTX and peptides L2 and LIN; and AcChoR-(194–210) heptadecapeptide bound α-BTX and effectively only peptide LIN. However, the affinities of the toxin peptides that bound to a given AcChoR peptide differed. The dissociation constants (K_d) of the binding of ^{125}I-labeled α-BTX or its synthetic peptides to the AcChoR peptides are summarized in Table 1. In the binding to AcChoR-(34–49), the K_d value for the α-BTX peptide L2 was smaller by a factor of about 7.3 than that of peptide LIN, and the K_d of α-BTX was smaller by a factor of 6.5 than that of L2. In binding to AcChoR-(100–115), the K_d values were: α-BTX < L2 = L3E << LIN. For binding to AcChoR-(122–138), the K_d values increased in the following order: α-BTX < L2 << LIN. Finally, only toxin peptide LIN (and of course α-BTX itself) bound to AcChoR-(194–210).

Inhibition Studies with the Synthetic α-BTX Loop Peptides. The specificity of binding of the synthetic α-BTX peptides to the AcChoR peptides was confirmed by inhibition experiments. The binding of ^{125}I-labeled α-BTX to an adsorbent of a given AcChoR peptide was inhibited by the appropriate α-BTX loop peptide or by an equimolar mixture of the relevant α-BTX peptides (Fig. 3). The experiments showed that incubation of inhibitor peptides with AcChoR-(34–49) and AcChoR-(100–115) for 4 hr gave IC_{50} values of 0.8 and 1.5 μM, respectively. When the incubation time was extended to 14 hr, the IC_{50} values were improved to 0.5 μM for AcChoR-(34–49) and 0.2 μM for AcChoR-(100–115). The IC_{50} values of AcChoR-(122–138) and AcChoR-(194–210) were not improved by increasing the incubation time from 4 hr to 14 hr. Therefore, the appropriate optimum interaction time was

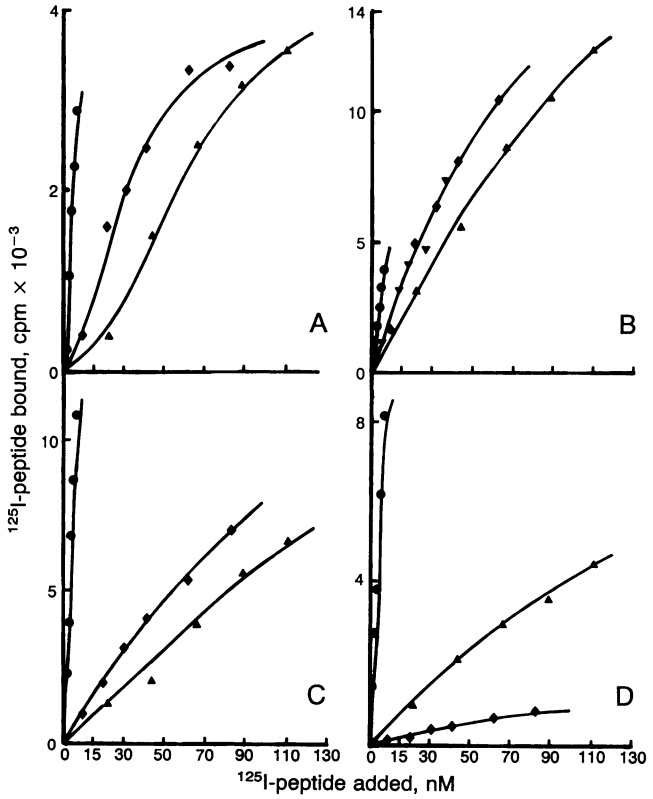

FIG. 2. Concentration-dependent binding of ^{125}I-labeled α-BTX and its receptor-binding synthetic peptides (^{125}I-peptides) to each of the α-BTX-binding peptides of human AcChoR. Fixed amounts (10 μl) of suspension (1:1, vol/vol) of each AcChoR peptide adsorbent were incubated with increasing amounts of ^{125}I-labeled α-BTX or its peptides LIN, L2, and L3E at room temperature for 14 hr. The binding was done in a reaction volume of 40 μl in PBS containing 0.1% bovine serum albumin. After reaction, the adsorbents were washed four times with PBS, transferred to clean tubes, and assayed for radioactivity on a Beckman 4000 γ counter. (*A*) Binding to AcChoR-(34–49). (*B*) Binding to AcChoR-(100–115). (*C*) Binding to AcChoR-(122–138). (*D*) Binding to AcChoR-(194–210). ▲, LIN; ◆, L2; ▼, L3E; ●, α-BTX.

used for each of the AcChoR peptides. Under these optimum conditions, the following inhibition results of α-BTX binding were obtained: binding to AcChoR-(34–49) was inhibited completely by an equimolar mixture of α-BTX peptides LIN and L2 (IC_{50}, 0.5 μM), to AcChoR-(100–115) by an equimolar mixture of α-BTX peptides L2 and L3E (IC_{50}, 0.2 μM), and to AcChoR-(122–138) by α-BTX peptide L2 only (IC_{50}, 2 μM). Finally, α-BTX binding to AcChoR-(194–210) was inhibited only by toxin peptide LIN (IC_{50}, 5 μM). It should be noted that an unrelated nonsense peptide, bovine serum albumin, and hen lysozyme had no inhibitory effect (Fig. 3) on this binding, even when used at 0.1 mM, thus further confirming the specificity of the interactions of the toxin peptides with the AcChoR peptides.

Table 1. Dissociation constants of the binding of ^{125}I-labeled α-BTX and its peptides to the human AcChoR peptides

Labeled ligand	K_d of binding to human AcChoR peptides, M			
	AcChoR-(34–49)	AcChoR-(100–115)	AcChoR-(122–138)	AcChoR-(194–210)
BTX	8.6×10^{-9}	4.7×10^{-9}	2.2×10^{-8}	4.0×10^{-8}
BTX peptides				
LIN	4.1×10^{-7}	2.3×10^{-7}	1.0×10^{-6}	2.0×10^{-7}
L2	5.6×10^{-8}	6.2×10^{-8}	2.9×10^{-7}	NB
L3E	NB	6.9×10^{-8}	NB	NB

Dissociation constants of the binding of ^{125}I-labeled α-BTX or its active synthetic loop peptides to adsorbents of each of the four active human AcChoR peptides were determined as described in the text. NB, no binding was obtained between these two peptides.

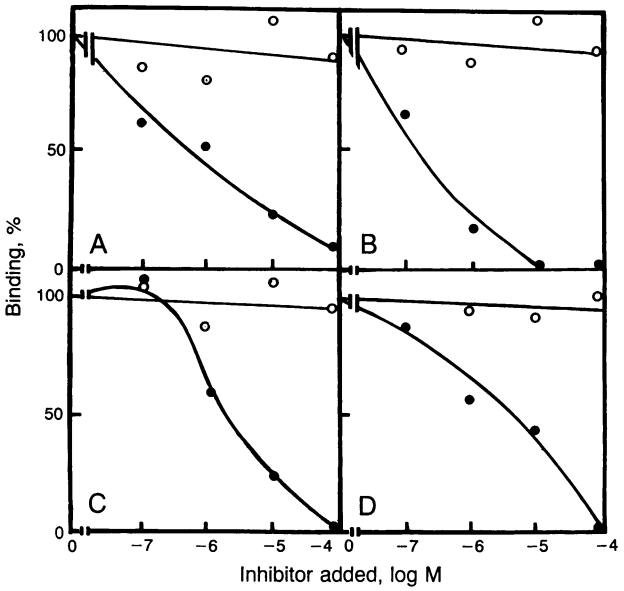

FIG. 3. Inhibition of the binding of ^{125}I-labeled α-BTX to AcChoR peptides by the synthetic α-BTX peptides. Fixed amounts (10 μl) of suspension (1:1 vol/vol) of each AcChoR peptide adsorbent were mixed with 10 μl 0.2% bovine serum albumin in PBS (4°C, overnight). Aliquots (10 μl) containing increasing amounts of the appropriate unlabeled α-BTX peptide (or controls) in PBS containing 0.1% bovine serum albumin were added, and the mixture was incubated with gentle agitation at room temperature for 4 hr. ^{125}I-labeled α-BTX (2.5×10^5 cpm) was then added, and the binding reaction was allowed to take place with gentle agitation at room temperature [4 hr for AcChoR-(122–138) and AcChoR-(194–210) or 14 hr for AcChoR-(34–49) and AcChoR-(100–115)]. The binding was done in PBS containing 0.1% bovine serum albumin in a final reaction volume of 40 μl. The adsorbents were then washed and assayed as described in Fig. 2. (*A*) Inhibition of the binding of ^{125}I-labeled α-BTX to AcChoR-(34–49) by an equimolar mixture of α-BTX peptides LIN and L2. (*B*) Inhibition of α-BTX binding to AcChoR-(100–115) by an equimolar mixture of L2 and L3E. (*C*) Inhibition of binding to AcChoR-(122–138) by peptide L2. (*D*) Inhibition of binding to AcChoR-(194–210) by peptide LIN. ●, Inhibition by the α-BTX peptides; ○, control inhibitors, which included hen egg lysozyme and an unrelated nonsense peptide with the amino acid sequence Glu-Ser-Ser-Gly-Thr-Gly-Ile-Glu-Ser-Ser-Gly-Thr-Gly-Ile.

DISCUSSION

The recent mapping of the full profiles of the α-neurotoxin-binding regions on the α chains of *Torpedo californica* (7–9) and human (10) AcChoR by synthetic overlapping peptides encompassing the entire extracellular parts of each of these subunits revealed a complex toxin-binding site on each receptor. The *Torpedo* receptor has five toxin-binding regions. In the human receptor, four of these regions retain the ability to bind α-BTX and cobratoxin, while the binding activity of the fifth region (residing within residues 1–16) is lost because of adverse amino acid replacements (10). The four toxin-binding regions of human AcChoR reside within, but do not necessarily include all of, the four α-chain peptides comprising residues 34–49, 100–115, 122–138, and 194–210. Previously, adsorbents of AcChoR-(100–115) were found (10) to have low capacity for α-BTX. Exhaustive analysis carried out in the present work with several different ^{125}I-labeled α-BTX preparations confirmed both the previously reported (10) low-binding activity of AcChoR-(100–115) and the profile

of the α-neurotoxin-binding regions on the α chain of human AcChoR. However, in spite of the low capacity for α-BTX of AcChoR-(100–115) adsorbents, α-BTX binds to AcChoR-(100–115) with high affinity (Table 1). The binding activity of AcChoR-(100–115) was highly dependent on the labeled α-BTX preparation. Perhaps, radioiodinations may have resulted in various degrees of modification on α-BTX of residues that are essential for binding to the receptor. Modification reactions that can occur during radioiodination of proteins by the chloramine-T method have been reviewed (21). By sequence comparison of the α chains of AcChoR from different species, the α-neurotoxin-binding regions were further narrowed down (10, 22) to the following residues: 32–41, 100–110, 125–136, and 194–208. These assigned regions were used in the present structural prediction and model-building studies.

The AcChoR-binding regions on α-BTX have been recently mapped by using synthetic peptides corresponding to the various loops and most of the surface areas of the toxin (12). It was found that α-BTX has three main AcChoR-binding regions within the loop peptides (Fig. 1): LIN (residues 1–16), L2 (residues 26–41, with an artificial disulfide bond between the two ends of the peptide; see ref. 12), and L3E (residues 45–59).

The binding studies reported here between α-BTX peptides and receptor peptides revealed that there are extensive contacts between the two molecules. Each of the AcChoR peptides was bound to the intact α-BTX molecule with greater affinity than to any of its loop segments. One interpretation of this would imply that the AcChoR segments have interactions with more than one loop. The major interaction would be with the loop that displayed the higher affinity. Indeed, except for the α-BTX loop L3E, each of the other α-BTX loops that participates in the binding makes contact with more than one peptide region on the receptor. Conversely, each active AcChoR peptide, except for AcChoR-(194–210), makes contact with more than one α-BTX loop. The relative importance of these contacts to complex formation has been ordered arbitrarily on the basis of the binding affinities displayed by a given peptide towards the peptides of the other molecule (Table 1). It is assumed that the greater the affinity (i.e., the smaller the K_d), the better the fit. Thus, from Table 1, AcChoR-(34–49) would make better contact with α-BTX peptide L2 than with LIN. Similarly, α-BTX peptide L2 interacts better with both AcChoR-(34–49) and AcChoR-(100–115) than with AcChoR-(122–138).

Knowledge of the regions involved in the interactions between two protein molecules and cross-binding studies between the correlate synthetic peptides should provide binding-affinity data indicative of specific interactions between the native molecules. In addition, if the three-dimensional structure of one molecule is known, then the binding areas formed by the other molecule can be appropriately fitted to yield a tentative 3-D description of its binding site. The 3-D structure of the α-BTX molecule as determined by x-ray crystallography (13) displays surface features that can be utilized in combination with peptide-binding studies to construct a possible active-site model for AcChoR.

Before applying this approach to modeling the AcChoR binding cavity for α-BTX, it was necessary to test the approach on two interacting polypeptides, both having known 3-D structure—i.e., the α and β subunits of human

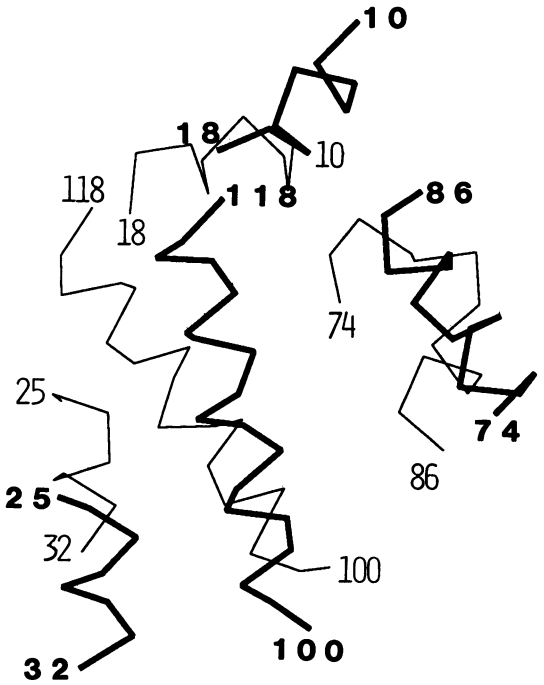

FIG. 4. Computer graphic drawings of the calculated (heavy lines) α-carbon backbones of human hemoglobin β-chain peptide regions comprising residues 10–18, 25–32, 74–86, and 100–118 [which have been shown to bind in solution to the intact α chain of Hb (23)]. The β-chain regions were fitted by the approach as described onto the appropriate regions of the α chain by using its known 3-D structure within Hb. The calculated conformations are compared with those expected (thin lines) for these β-chain regions within Hb, as obtained from the x-ray structure of the crystalline Hb tetramer.

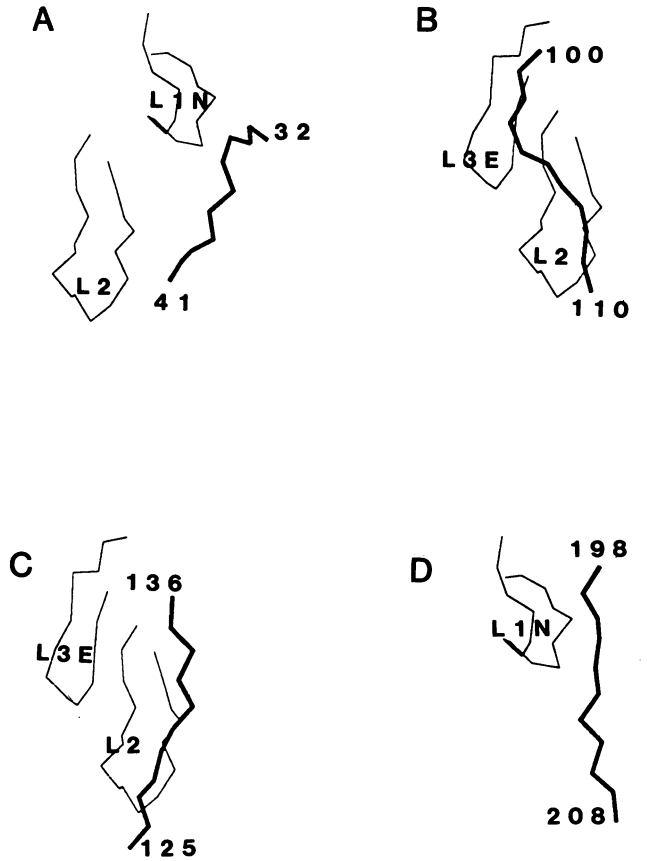

FIG. 5. A computer graphics drawing of the α-carbon backbones showing the association of the various AcChoR regions (heavy lines) with the appropriate part(s) of the 3-D structure of α-BTX. (*A*) Receptor residues 32–41 bound to α-BTX loops LIN and L2. (*B*) Receptor residues 100–110 bound to α-BTX loops L2 and L3E. (*C*) Receptor residues 125–136 bound to loop L2. (*D*) Receptor residues 198–208 bound to loop LIN.

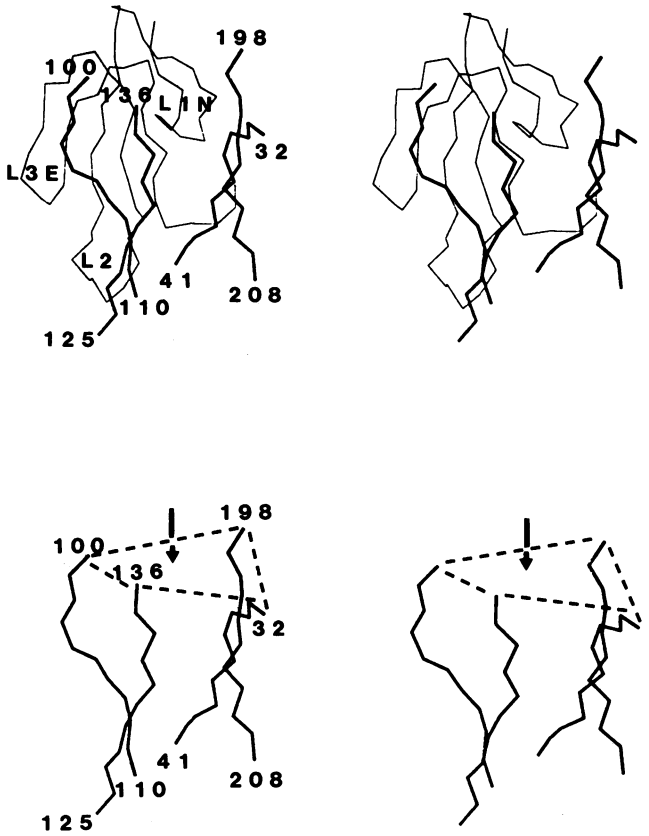

FIG. 6. A stereo drawing of a 3-D construction of the toxin-binding cavity in AcChoR, with the α-BTX molecule (backbone only) bound in the cavity (*Upper*) and without the α-BTX molecule (*Lower*). The somewhat conical cavity has the following dimensions: residues 100–136, 21.32 Å; residues 136–32, 35.01 Å; residues 32–198, 16.06 Å; and residues 198–100, 22.13 Å. The depth of the cavity is 30.48 Å.

adult Hb. Previously, using synthetic uniform-sized overlapping peptides encompassing the entire Hb subunits, we determined the regions involved in the interacting surfaces of the α and β subunits in solution (23) and compared them with those expected from the x-ray structure of Hb (24). Four regions of the β region of Hb (residues 10–18, 25–32, 74–86, and 100–118), which bind to the Hb α chain in solution (23), were fitted by the approach described in *Materials and Methods* onto the appropriate regions of the α chain by using the known 3-D structure of the α chain within the Hb molecule (24). The calculated structures of the α-carbon backbone of the three β-chain regions (residues 10–18, 25–32, and 100–118) were in good agreement with those expected for these regions from the known 3-D structure of Hb (Fig. 4). However, for β-chain residues 74–86, the calculated structure for the free peptide in solution showed reversed polarity and poor resemblance to the shape expected for this region within the x-ray-derived structure of Hb (Fig. 4). This divergence could be due to the freedom of the peptide in solution and might indeed reflect its true conformation when the free peptide is allowed to bind to the α chain of Hb. Also, it should be noted that the orientation of the side chains could not be reliably determined by this method.

With this limitation in mind, the approach was used to derive a tentative model for the 3-D backbone structure of the α-BTX-binding cavity on AcChoR. Based on considerations of binding affinity and with the knowledge of the x-ray coordinates of α-BTX at 2.5-Å resolution (13), the AcChoR-binding peptides were fitted onto the appropriate regions of α-BTX. While the modeled AcChoR structure is not a unique solution, it represents an energetically favorable model that satisfies both the binding data and structural constraints. The main interactions are summarized region by region in Fig. 5. The binding site is shown in a stereo drawing in Fig. 6, with and without the α-BTX backbone. The site comprises a deep conical cavity (30.5 Å in depth), the dimensions of which are indicated in Fig. 6. The binding between α-BTX and AcChoR involves several areas of contact on both molecules. It is important to point out that one of these areas of contact on the receptor (residues 125–136) resides in the AcCho binding site (25). Since the affinity of α-neurotoxin to the receptor is several orders of magnitude higher than that of AcCho, the binding of toxin will be expected to prevent that of AcCho (and thus disrupt receptor function) completely, even in the presence of a large excess of the latter.

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