κ-Neurotoxins: Heterodimer Formation between Different Neuronal Nicotinic Receptor Antagonists†

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ABSTRACT: The κ-neurotoxins are a family of snake venom polypeptides that are competitive antagonists of acetylcholine at a variety of neuronal nicotinic receptors. We have previously determined that κ-bungarotoxin, purified from the venom of *Bungarus multicinctus*, exists in solution entirely as a dimer of identical subunits. We now report that the three other known κ-neurotoxins, namely, κ_2-bungarotoxin and κ_3-bungarotoxin from *Bungarus multicinctus* and κ-flavitoxin from *Bungarus flaviceps*, also self-aggregate in solution. Furthermore, when two different κ-neurotoxins are mixed, a heterodimer species spontaneously forms and reaches an equilibrium with the two homodimers after which 40–50% of the protein exists as the heterodimer. A cation-exchange high-pressure liquid chromatography procedure is described which readily separates κ-neurotoxin heterodimers from the homodimers. Sedimentation equilibria experiments give an M_r = 15 500 ± 1000 for κ-flavitoxin and an M_r = 14 500 ± 700 for a mixture of κ-bungarotoxin and κ-flavitoxin. Since the subunit molecular weights of κ-bungarotoxin and κ-flavitoxin are respectively 7313 and 7242, self-aggregation of these toxins in solution results in a preponderance of κ-neurotoxin dimers. The stoichiometry of the heterodimer formed by κ-bungarotoxin and κ-flavitoxin is 1:1, as determined by amino acid sequence analysis. After isolation, the κ-neurotoxin heterodimer partially dissociates and again reaches equilibrium with the homodimers, a process which requires 2–4 h at 23 °C. The ability to self-aggregate to form heterodimers and homodimers thus appears to be a common property of the κ-neurotoxins. This property distinguishes the toxins from the α-neurotoxin family of nicotinic antagonists, which are generally more potent at muscle nicotinic receptors than at neuronal nicotinic receptors. The α-neurotoxins examined did not spontaneously form dimers or heterodimers in solution.

The κ-neurotoxins are a family of basic polypeptides purified from the venom of elapid snakes (Chiappinelli et al., 1988). These neurotoxins share considerable sequence homology (47%) with the long-type α-neurotoxins, such as α-bungarotoxin isolated from *Bungarus multicinctus* venom. The α-neurotoxins are potent competitive antagonists at nicotinic acetylcholine receptors located in vertebrate skeletal muscle and in muscle-derived electric organ tissue, but they are ineffective at many neuronal nicotinic receptors (Chiappinelli, 1985). In contrast, the κ-neurotoxins are potent neuronal nicotinic receptor antagonists but show a much lower affinity for muscle nicotinic receptors (Ravdin & Berg, 1979; Chiappinelli, 1983; Loring et al., 1986; Wolf et al., 1988).

The first κ-neurotoxin to be completely sequenced (Grant & Chiappinelli, 1985) was κ-bungarotoxin, isolated from the venom of *Bungarus multicinctus* (Chiappinelli, 1983). κ-Bungarotoxin has a calculated subunit molecular weight of 7313, consisting of a single polypeptide chain with 66 amino acids internally cross-linked by 5 disulfide bonds. More recent data indicate that a polypeptide referred to as toxin F is identical in amino acid sequence with κ-bungarotoxin and a toxin named Bgt 3.1 may also be identical with κ-bungarotoxin (Loring et al., 1986).

Under physiological buffer conditions, κ-bungarotoxin exists entirely as a dimer of identical subunits (Chiappinelli & Lee, 1985). κ-Bungarotoxin dimers are noncovalently bound and can be dissociated by the denaturing agents sodium dodecyl sulfate and urea, or by nondenaturing conditions of high ionic strength and high pH. A complete reassociation of nondenatured κ-bungarotoxin monomers occurs following return to a physiological buffer (Chiappinelli & Lee, 1985).

The ability to self-aggregate into dimers distinguishes κ-bungarotoxin from α-bungarotoxin and other α-neurotoxins (Chiappinelli & Lee, 1985; Wolf et al., 1988). We now report that three other κ-neurotoxins exist in dimeric form. Furthermore, after two different κ-neurotoxins are mixed together, a new heterodimer species is formed. Since the dimeric forms of the κ-neurotoxins appear to be physiologically active, this property may account in part for the selectivity of these snake venom polypeptides for neuronal nicotinic receptors.

Materials and Methods

Purification of κ-Neurotoxins. Purification of κ-bungarotoxin was done as previously described (Chiappinelli, 1983). κ-Flavitoxin was purified from crude *Bungarus flaviceps* venom as described in Chiappinelli et al. (1987). κ_2-Bungarotoxin and κ_3-bungarotoxin were isolated from *Bungarus multicinctus* venom collected in the Guangdong province of China. Initial purification of these two toxins was as described for κ-bungarotoxin (Chiappinelli, 1983). This procedure yielded a single peak containing both polypeptides. The toxins were then separated by high-pressure liquid chromatography (HPLC)[1] using the method described below.

Purification of α-Neurotoxins. α-Bungarotoxin was purified by the method of Chiappinelli (1983). Toxin 3, the principal α-neurotoxin of *Naja naja siamensis*, was purified from crude

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[1] Abbreviation: HPLC, high-pressure liquid chromatography.

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venom (Miami Serpentarium, lot NS011STLZ) by a modification of the method of Karlsson et al. (1971), in which the cation-exchange column contained Cm-cellulose (Whatman CM52) in place of Bio-Rex 70. In both purification procedures, toxin 3 was collected as the second peak after initiation of the ammonium acetate gradient. This peak was the largest peak of the elution. After a further CM52 gradient purification, toxin 3 was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Chiappinelli, 1983). The purified material ran as a single band of protein at M_r 8000. Amino acid analysis of purified toxin 3 was consistent with the published amino acid sequence of this α-neurotoxin (Karlsson et al., 1972).

Cation-Exchange HPLC. A cation-exchange column (Bio-Rad TSK-SP-5-PW; 75 × 7.5 mm) was attached to a Varian Model 5000 equipped with an automatic gradient maker. Samples were loaded onto the preequilibrated column in start buffer [90% (v/v) 25 mM sodium phosphate (pH 6.0)/10% (v/v) acetonitrile]. Start buffer was pumped through the column for an additional 10 min; then a 120-mL linear gradient was run using 90% (v/v) 500 mM sodium phosphate (pH 6.0)/10% (v/v) acetonitrile as the end buffer. Flow through the column was maintained at 1 mL/min. Protein peaks were monitored by measuring the absorbance at either 210 or 280 nm, and 1-mL fractions were collected.

Sedimentation Equilibrium. Molecular weights were determined by sedimentation equilibrium measurements in a Beckman Model E analytical ultracentrifuge using the method described in Chiappinelli and Lee (1985). Sedimentation equilibria were conducted in 75 mM ammonium acetate at pH 5.0 and 20 °C. Loading concentrations were 115–230 μg/mL. Partial specific volumes of toxins were calculated by the method of Cohn and Edsall (1943) using the previously determined amino acid compositions (Grant & Chiappinelli, 1985; Grant et al., 1988). The calculated values were $\bar{v}$ = 0.704 mL/g for κ-flavitoxin and $\bar{v}$ = 0.708 mL/g for κ-bungarotoxin.

Sequence Analysis. N-Terminal amino acid sequences were determined by automated Edman degradation of the intact proteins as previously described (Grant & Chiappinelli, 1985; Grant et al., 1988).

Results

Sedimentation Equilibria of κ-Flavitoxin. Previous sedimentation equilibria experiments (Chiappinelli & Lee, 1985) indicated that κ-bungarotoxin has an apparent M_r = 14 000 ± 3000 (±maximum deviation), essentially double the subunit molecular weight of 7313 (Grant & Chiappinelli, 1985). In the present study and under similar buffer conditions, the apparent molecular weight for κ-flavitoxin was 15 500 ± 1000 (±maximum deviation). As previously observed with κ-bungarotoxin, there was a slight concentration dependence in molecular weight. The value given here is that extrapolated to an infinite dilution. Since the subunit molecular weight of κ-flavitoxin is 7242 (Grant et al., 1988), the sedimentation equilibria data indicate that κ-flavitoxin exists in solution as a dimer.

Heterodimer Formation and Dissociation. Cation-exchange HPLC is the final step in the purification of κ-flavitoxin (Chiappinelli et al., 1987). Using an increasing ionic strength gradient, purified κ-flavitoxin elutes from the HPLC column as a single peak. Under identical conditions, purified κ-bungarotoxin elutes as a single peak at an earlier position in the gradient (Figure 1).

When a sample of κ-bungarotoxin is combined with a sample of κ-flavitoxin prior to being loaded onto the HPLC column, the elution pattern is more complex than that anticipated for a mixture of two noninteracting components. In addition to two peaks eluting at the positions expected for κ-bungarotoxin and κ-flavitoxin, a third peak appears between the pure toxin peaks (Figure 1). Several lines of evidence indicate that this peak consists of a heterodimer, containing one subunit of κ-bungarotoxin and one subunit of κ-flavitoxin. The height of this peak depends on the amount of time elapsed between mixing of the κ-neurotoxins and their elution from the column.

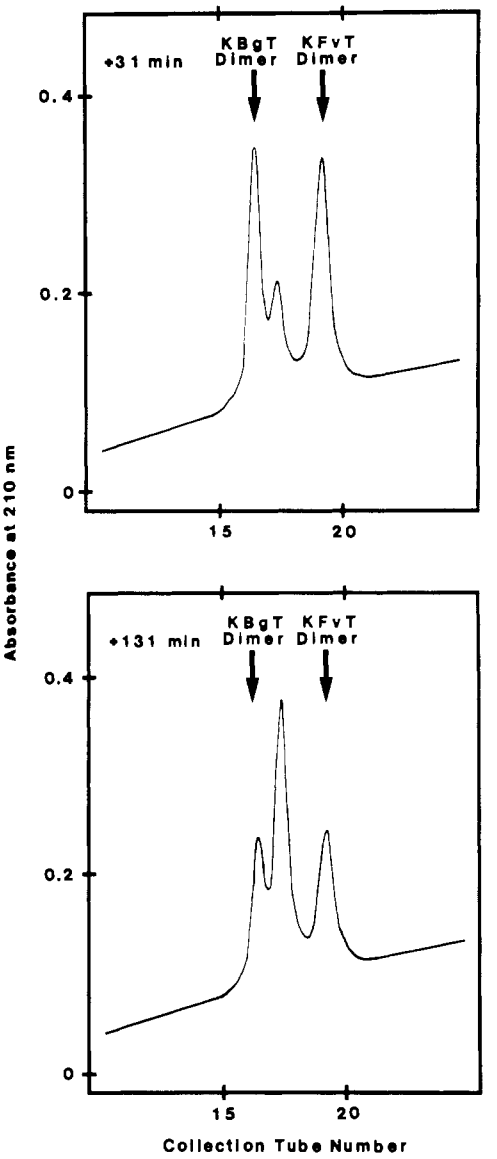

FIGURE 1: Formation of κ-neurotoxin heterodimers. Samples of pure κ-bungarotoxin and pure κ-flavitoxin were mixed together at a final protein concentration of 20 μg/mL in HPLC start buffer. Aliquots containing 20 μg of this mixture were loaded onto the HPLC column at various times after mixing and eluted from the column by using the linear ammonium acetate gradient described under Materials and Methods. The elution was followed by monitoring absorbance at 210 nm. Arrows indicate positions where pure κ-bungarotoxin (KBgT Dimer) and pure κ-flavitoxin (KFvT Dimer) eluted under identical conditions. Note that the mixture eluted in three peaks: one in the position expected for κ-bungarotoxin, one in the position expected for κ-flavitoxin, and one between these two peaks. The middle peak increased in size as the time between mixing and elution was increased from 31 min (top graph) to 131 min (bottom graph). At 131 min, the heterodimer peak contained 49% of the protein in the mixture. For clarity, only the portions of the elution profiles containing protein peaks are shown in this figure and in Figure 2. No peaks other than those shown were detected in the profiles. The slowly rising base line is due to slight absorption by the buffer at 210 nm.

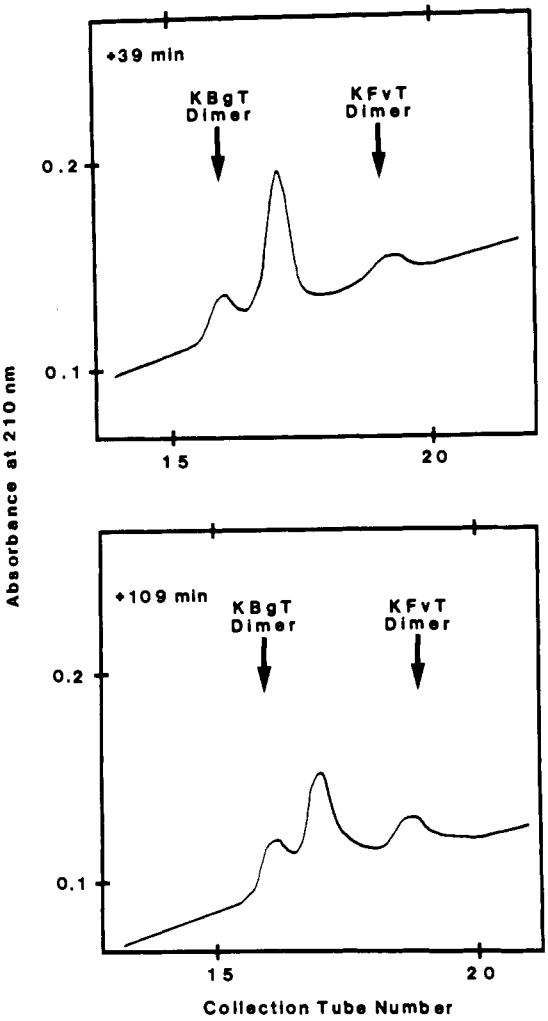

FIGURE 2: Dissociation of κ-neurotoxin heterodimers. The κ-bungarotoxin/κ-flavitoxin heterodimer peak was collected in experiments such as those shown in Figure 1. At various times after collection, aliquots of the heterodimer samples were loaded onto the HPLC column and eluted as before. The elution profile contained three peaks, two of which appeared at the positions expected for pure κ-bungarotoxin (KBgT Dimer) and pure κ-flavitoxin (KFvT Dimer). The heterodimer peak in between these peaks contained 68% of the protein in the mixture 39 min after collection (top graph) but contained only 54% of the protein in the mixture 109 min after collection (bottom graph).

With short incubation times, the peak is much smaller than the κ-bungarotoxin and κ-flavitoxin peaks (Figure 1). After 4 h of mixing, the height of the heterodimer peak reaches a maximum, which does not change after a further incubation of 20 h (Figure 3). The heights of the κ-bungarotoxin and κ-flavitoxin peaks are inversely proportional to that of the new peak, indicating that the concentrations of both homodimers decrease as the heterodimer concentration increases.

The heterodimer, formed with subunits of κ-bungarotoxin and κ-flavitoxin, is in equilibrium with the homodimers. This is confirmed by studying the dissociation of the newly formed heterodimer peak (Figure 2). Once separated from the two homodimers by the HPLC column, the heterodimer begins to dissociate, and both homodimers reappear. Equilibrium is again reached between heterodimer and homodimers, indicating that the reaction is reversible. When κ-bungarotoxin and κ-flavitoxin are mixed in equal amounts, 49% of the protein appears in the heterodimer peak at equilibrium, a value which is unchanged even after 6 months at 4 °C (Figure 3).

If the newly formed peak is indeed a heterodimer, its apparent molecular weight should be ca. 14 500. To test this

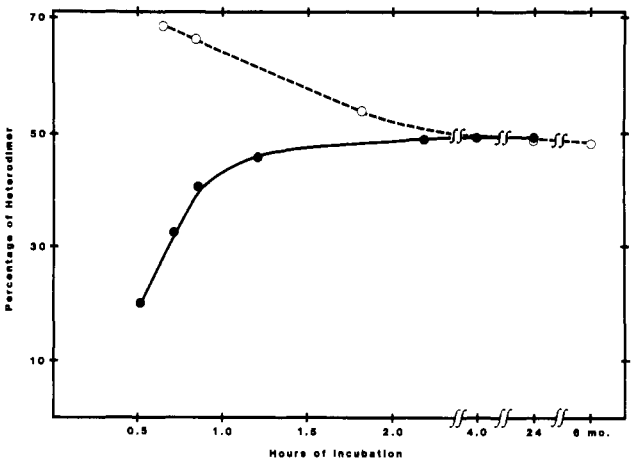

FIGURE 3: Rates of heterodimer association and dissociation. Equilibrium between the κ-bungarotoxin/κ-flavitoxin heterodimer and the homodimers can be reached from either direction. The percentage of heterodimer present at various times after mixing κ-bungarotoxin and κ-flavitoxin is shown for times ranging from 0.5 to 24 h (●—●). Dissociation of κ-bungarotoxin/κ-flavitoxin heterodimer is plotted for times after collection of 0.65–24 h (O---O). A final point shows that after incubation for 6 months at 4 °C, the equilibrium reached from heterodimer dissociation is unchanged from that seen after 24 h at 23 °C.

point, equal amounts of κ-bungarotoxin and κ-flavitoxin were mixed, and after a 4-h incubation, the mixture was subjected to sedimentation equilibrium analysis. The apparent molecular weight of the mixture was 14 500 ± 700 (±maximum deviation). This represents the apparent molecular weight of the heterodimer peak after it has attained equilibrium with the homodimers; i.e., 49% of the protein in the sample represents genuine heterodimer, and the remaining molecules are homodimers.

Identity and Stoichiometry of Heterodimer. The identity and stoichiometry of the heterodimer were determined by automated microsequencing of the HPLC-isolated material. The amino acid sequences of κ-bungarotoxin and κ-flavitoxin differ at a total of 12 positions. In the N-terminal regions of the molecules, these positions are residues 11, 16, 23, 26, 29, and 30. However, the amino acids present in both proteins are sufficiently stable for an accurate quantitative analysis only at positions 16 and 26. These residues are asparagine and lysine at position 16, and glutamine and phenylalanine at position 26. Position 11 in κ-flavitoxin is a serine, position 23 is a threonine, position 29 is an arginine, and position 30 is a tryptophan. Although these residues can be identified on a qualitative level, they are not generally satisfactory for precise analysis.

Quantitation of the results of automated Edman degradation of the putative heterodimer peak indicated that both asparagine and lysine were present in cycle 16 in approximately equimolar amounts (Asn = 157 pmol, Lys = 142 pmol). Furthermore, the proline in cycle 15 (Pro = 354 pmol) and the glycine in cycle 17 (Gly = 271 pmol) were the only residues identified in those cycles and were present at approximately twice the level of each individual residue in cycle 16. By cycle 26, the background and carry over interfered with a reliable quantitation. However, the serine and threonine residues expected for κ-flavitoxin were qualitatively identified at cycles 11 and 23, respectively, along with the expected proline and leucine residues of κ-bungarotoxin. The amino acid sequence analysis is consistent with a stoichiometry of 1:1 for the heterodimer.

κ_2-Bungarotoxin and κ_3-Bungarotoxin. Most commercially available *Bungarus multicinctus* venom is collected from snakes captured in Taiwan. We have recently obtained

Bungarus multicinctus venom from snakes captured in the Guangdong province of China. Guangdong is separated from Taiwan by 250 km of open ocean, which would appear to be an effective genetic barrier for this terrestrial snake. Using the method for purifying κ-bungarotoxin from Taiwanese venom (Chiappinelli, 1983), we have isolated two new κ-neurotoxins from Guangdong venom. κ_2-Bungarotoxin and κ_3-bungarotoxin exhibit considerable sequence homology with κ-bungarotoxin and have similar pharmacological effects (Chiappinelli et al., 1988). They are also capable of heterodimer formation, such that a considerable amount of κ_2-bungarotoxin/κ_3-bungarotoxin heterodimer exists naturally within the venom. Separation of homodimers from the heterodimer is accomplished by the HPLC method described above. κ-Bungarotoxin has not yet been found in venom containing κ_2-bungarotoxin and κ_3-bungarotoxin, but it readily forms heterodimers with either toxin. At equilibrium, the heterodimer peak formed by κ-bungarotoxin and κ_3-bungarotoxin contains 41% of the protein in the mixture. As with the heterodimer formed between κ-bungarotoxin and κ-flavitoxin, this new heterodimer peak partially dissociates after collection to re-form both homodimers, reaching equilibrium after several hours at 23 °C.

α-Neurotoxins. Since the long-type α-neurotoxins are structurally related to the κ-neurotoxins, we examined whether they were capable of forming heterodimers. No heterodimer formation was detected following mixing of α-bungarotoxin and toxin 3 from *Naja naja siamensis*. This was true even when these α-neurotoxins were incubated together for 24 h prior to HPLC elution.

Discussion

The self-association of snake venom κ-neurotoxins has been examined. All four known κ-neurotoxins exist as dimers in solution. κ-Bungarotoxin and κ-flavitoxin are initially purified as homodimers. κ_2-Bungarotoxin and κ_3-bungarotoxin, which are present within the same batches of crude snake venom, exist naturally as a mixture of heterodimers and homodimers. A cation-exchange HPLC gradient elution procedure is described which can separate κ-neurotoxin homodimers from heterodimers.

Heterodimer association occurs spontaneously upon mixing of two different κ-neurotoxins, and steady-state equilibrium between the heterodimer and the two homodimers is reached in 4 h at 23 °C. Heterodimer formation is reversible. Newly collected heterodimer reestablishes an equilibrium with the two homodimers in which 40–50% of the protein is in the heterodimer form. In contrast, there is no indication of heterodimer formation between snake venom α-neurotoxins.

Self-association thus appears to be a common property of the κ-neurotoxins. Since monomeric species of κ-neurotoxins are undetected in solution even at protein concentrations as low as 0.009 μg/mL (Chiappinelli & Lee, 1985), it appears likely that the dimeric forms of these neurotoxins are physiologically active. Furthermore, we have determined that mixtures of κ-neurotoxins have potencies for the blockade of nicotinic transmission in chick ciliary ganglia that are comparable to those observed for the respective homodimers (Chiappinelli and Wolf, unpublished results). Thus, the κ-neurotoxin heterodimers produced artificially in this study, as well as those which occur naturally in venom containing κ_2-bungarotoxin and κ_3-bungarotoxin, appear to retain the pharmacological activity of κ-neurotoxin homodimers. The ability to reversibly form heterodimers implies that the three-dimensional structures of the κ-neurotoxins are quite similar. In fact, these toxins exhibit 80–85% sequence homology, thus supporting this conclusion.

It is not yet clear why the self-association of κ-neurotoxins is so overwhelmingly favored while the α-neurotoxins lack this property. The amino acid sequences of the four known κ-neurotoxins show considerable homology (~47%) with the long-type α-neurotoxins, but there are at least four invariant sequence positions in the α-neurotoxins at which the κ-neurotoxins exhibit different amino acid residues (Chiappinelli et al., 1988; Grant et al., 1988). These or other structural differences between the two families of neurotoxins must account for the differences in their tendencies to self-aggregate. The relatively short COOH-terminal tails of the κ-neurotoxins (2 residues versus 4–11 residues in the long-type α-neurotoxins) might be important for dimer formation. However, L.s.III, an α-neurotoxin with a short COOH-terminal tail, does not form dimers in solution (Wolf et al., 1988), indicating that this property alone is not sufficient for dimer formation. In addition, the family of short-type α-neurotoxins, which have two-residue COOH-terminal tails comparable to those of κ-bungarotoxin and κ-flavitoxin, do not spontaneously form dimers in solution. Karlsson (1979) has produced small amounts of α-neurotoxin dimers by repeated cycles of lyophilization. These artificially produced α-neurotoxin dimers exhibit reduced potencies for muscle nicotinic receptors. It has been proposed, therefore, that the dimeric nature of the κ-neurotoxins may in part account for their weak affinities for muscle nicotinic receptors (Chiappinelli & Lee, 1985).

X-ray diffraction studies of crystalline α-bungarotoxin reveal a dimeric state in which the triple-stranded β-sheets of each monomer unite into a six-stranded β-sheet held together by van der Waals contacts and by hydrogen bonding (Love & Stroud, 1986). However, the solution structure of α-bungarotoxin differs significantly from the crystal structure, indicating that the dimeric conformation is not energetically favored in solution (Inagaki et al., 1985; Love & Stroud, 1986; Basus et al., 1988). The structurally related neurotoxin, cardiotoxin $V^{II}4$, also exhibits stable dimers in the crystalline state with the formation of a six-stranded antiparallel β-sheet between the monomers (Rees et al., 1987). Dimer formation in κ-neurotoxins may involve similar interactions between toxin monomers, which in the case of the κ-neurotoxins are energetically favored in solution.

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Influence of Globin Structure on the Heme in Dromedary Carbonmonoxyhemoglobin†

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ABSTRACT: By use of X-ray absorption near-edge structure (XANES), circular dichroism, and visible absorption spectroscopies, dromedary carbonmonoxyhemoglobin has been characterized structurally and functionally. By consideration of the experimental results the following view emerges: (i) the quaternary structure is not the unique factor determining the tertiary environment around the heme, and (ii) the multiplicity of interactions between hemoglobin and solvent components induces a large number of globin conformations, which somehow affect the conformation of the heme such that the structural parameters (i.e., the doming of porphyrins, the movements of the iron relative to the heme plane, the distortion of the ligand field, and the change in the Fe–C–O angle) can be uncoupled.

Cooperativity in hemoglobin, i.e., the increase in binding energy of the fourth heme ligand relative to the first (Antonini & Brunori, 1971; Perutz et al., 1987), arises from a linkage between the stereochemistry at the heme and various protein conformations, which differ in the tertiary structure of the α and β subunits and in the quaternary structure of the tetramer (Ackers & Smith, 1987; Perutz et al., 1987). A large body of equilibrium and kinetic data supports the idea that hemoglobin exists in two main quaternary structures, T (tense) and R (relaxed) state(s), which are different for heme–ligand affinity and that cooperativity is associated with a switch from the T to the R state(s) at some point on the binding curve (Perutz, 1979; Brunori et al., 1985). X-ray diffraction studies have revealed that in the unligated derivative the porphyrins are domed and, regardless of the quaternary structure, the iron atoms are displaced by 0.04 nm from the plane of the porphyrin nitrogens toward the proximal histidines. On ligand binding, the irons move toward the porphyrin planes, which remain domed in the T structure but flatten on transition to the R structure (Perutz et al., 1987). Such perturbations at the hemes are coupled to a net movement of the FG corner together with a portion of the F helix (i.e., with tertiary conformational modifications), thus triggering changes in the quaternary structure (Baldwin & Chothia, 1979; Gelin et al., 1983; Arnone et al., 1986). Therefore, as hemes are successively bound to a ligand molecule, the interactions that stabilize the T quarternary structure are lost (due to the strain at the active site), relieving the protein constraints and consequently increasing the affinity for heme ligands. On the other hand, interactions between hemoglobin and solvent components may bring about tertiary and quaternary structural variations, which propagate to the hemes and to intersubunit interfaces modulating in this way the reactivity of hemoglobin for heme ligands. In general, each quaternary state (T or R) may differ in tertiary conformation of the subunits (t or r), depending on the lower or higher intrinsic heme–ligand affinity of the subunits, and therefore, the system may be described in terms of (at least) four alternative states [denoted as follows: Rr and Rt, for conformations at high and low affinity for heme ligands of the subunits within the R structure; Tt and Tr, for low- and high-affinity conformations of chains within the T structure (Perutz, 1972; Santucci et al., 1985, 1986a,b; San-

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