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Changes in the molecular topography of the light and heavy chains of type A botulinum neurotoxin following their separation

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Botulinum neurotoxin serotype A, an approx. 150 kDa protein, is composed of two subunits, the light and heavy chains (~ 50 and ~ 100 kDa, respectively). The neurotoxin's mode of action is believed to depend on coordinated but independent actions of the two subunit chains. The molecular environments of the aromatic amino acid residues of the dichain neurotoxin and the two isolated subunit chains were analyzed using near-ultraviolet circular dichroism (CD) (between 250 and 320 nm) and second-derivative ultraviolet absorption spectroscopy (between 240 and 320 nm) to investigate the conformational variations of the subunit chains in separated and conjugated forms. The mean residue weight ellipticities showed virtually no change (i.e., 1.7%) in the vicinities of Phe (268 nm), and only a small change (11%) around Tyr (279 nm) residues following dissociation of the subunit chains. However, significant changes (23–26%) at 286 nm as well as at 292 nm were noted, suggesting considerable alteration in the conformation of the subunits. Second-derivative ultraviolet absorption spectra indicated the degree of Tyr exposure in the dichain neurotoxin, isolated heavy and light chains at 70.7, 81.5 and 46.4%, respectively. A weighted mean of the degree of exposed Tyr residues in the separated heavy and light chains was 69.6%, virtually same as the 70.7% exposed Tyr residues observed in the intact dichain neurotoxin, indicating no difference in their Tyr exposure upon separation of the two chains. This was corroborated by the CD data which revealed only small changes in the CD signals of Tyr residues, and no alteration in those of the Phe residues following separation of the subunit chains. However, a change in the CD signal at 292 nm suggested that the conformations of Trp-containing segments of the two chains were significantly influenced upon their separation. The heavy and light chains of the neurotoxin therefore appear to exist as two semi-independent domains, in spite of being linked by disulfide and noncovalent bonds, and at least part of their conformations depends on interactions between them.

1. Introduction

Botulinum neurotoxin, a high molecular mass (~ 150 kDa) protein, causes flaccid muscle paralysis in the disease botulism by blocking the release of a neurotransmitter, acetylcholine, from the presynapses at the neuromuscular junctions. Studies with neuromuscular preparations indicate that the neurotoxin binds to the presynaptic membranes, then a portion or the entire neurotoxin goes across the membrane and, finally, inside the cytosol the protein induces the blockage of acetylcholine release [1,2].

The seven serotypes of the neurotoxin (called types A–G) have a common general structure. The neurotoxin molecules (~ 150 kDa) isolated from the bacterial cultures are found as a single chain (e.g., type E), a dichain (e.g., type A), or a mixture of single and dichain molecules (e.g., type B). The single-chain molecule is nicked endogenously, in the bacterial culture, at one-third the distance from the N-terminus into the dichain form (e.g., type A). The light (L, ~ 50 kDa) and heavy (H, ~ 100 kDa) chains are linked by -S-S- and nonco-

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valent bonds [3]. The separated and purified preparations of the L and H chains appear nontoxic but retain their potential biological activities. For example: (i) they can be reconjugated to form the neurotoxic dichain molecule [4–6]; (ii) the H chain bound to the 'receptors' on the susceptible cells antagonizes toxicity and binding of the parent neurotoxin (ref. 7 and references cited therein).

The separated L and H chains, administered in vitro to susceptible tissue only in a particular order (i.e., first the H chain and then the L chain), induce paralysis similarly to their parent neurotoxin [7,8]. It is not clear whether the two isolated chains reconjugate at the site of their action and then participate in the intoxication process or if they act independently in a functional coordination. A recent report, suggesting a requirement of the two chains on the cytosolic side of the neuronal plasma membrane for expression of toxicity [9], argues in favor of the former possibility.

Specific interactions between the H and L chains are likely to maintain a conformation of the H chain critical for its biological activity. Consequently, the isolated H chain, following its separation from the L chain, may not retain the biologically active conformation present in the dichain neurotoxin. These considerations are relevant and significant because: (i) the isolated H chain, at concentrations more than 10-fold higher than that of the parent neurotoxin antagonized only incompletely the neuroparalytic activity of the neurotoxin [7]; and (ii) this antagonism was not observed in another laboratory [8] which, however, reported paralysis induced by sequential administration of the two isolated chains similar to that described earlier [7]. Possibly, the conformationally altered H chain does not bind to the receptors on the cells as efficiently as the dichain neurotoxin. Elucidation of the interactions between the two subunits of the dichain neurotoxin is, therefore, important for understanding the structural basis of the function of the neurotoxin.

This report examines the conformational state of the type A dichain neurotoxin and its separated L and H chains by probing the molecular environments of the aromatic amino acid residues using near-ultraviolet circular dichroic (CD) spectroscopy and second-derivative ultraviolet absorption spectroscopy. The results indicate significant changes in the environment of Trp but not those of Tyr and Phe residues of the L and H chains upon their separation and isolation.

2. Materials and methods

Type A neurotoxin was isolated, its subunit L and H chains being subsequently separated as described previously [10,11]. The neurotoxin, and isolated L and H chains were precipitated with ammonium sulfate (39 g/100 ml) and stored at 2°C. The isolated L chain is prone to fragmentation upon storage [12], and hence was used immediately after isolation and precipitation. The precipitated proteins were pelleted and dissolved in 10 mM sodium phosphate buffer (pH 8.1), containing 100 mM NaCl (buffer A). All protein solutions were filtered through 0.2 μm Acrodisc filters (Gelman Sciences) before optical measurements and were checked for purity by SDS-polyacrylamide gel electrophoresis as shown elsewhere [10,11]. Protein concentrations were determined as described before [13].

Ultrapure guanidine · HCl (Schwartz/Mann, Cleveland, OH) and all other chemicals of highest grade were obtained commercially. Buffer and solutions were made with deionized distilled water.

Absorption and derivative spectra were recorded on a Uvikon 860 (Kontron Instruments) at room temperature (23–25°C). The degree of tyrosine exposure, α, was estimated according to the method of Ragone et al. [14] using the equation

$$\alpha = (\gamma_n - \gamma_a)/(\gamma_u - \gamma_a)$$

where γ_n and γ_u denote the ratios of the second-derivative peaks (a/b; see fig. 4a) of proteins in the native and unfolded (with 6 M guanidine · HCl) states, and γ_a the second-derivative peak ratio of free tyrosine and tryptophan residues in the same molar ratio as in the given proteins. Tyr/Trp ratios were calculated from amino acid compositions of the neurotoxin, and L and H chains [11].

γ_a was calculated using the equation [14]:

$$\gamma_a = \frac{AX + B}{CX + 1}$$

where A, B and C are the constants -0.18, 0.64 and -0.04, respectively, taken from the data of Ragone et al. [14]. X represents the molar ratio of Tyr/Trp in a given protein. The values of X for the neurotoxin, L and H chains used were 4.18, 6.25, 3.06, respectively [11].

Near ultraviolet CD spectra were recorded between 250 and 320 nm with a step size of 0.5 nm on a modified Cary (model 60) spectropolarimeter (On-Line Instrument Systems, Jefferson, GA) using a 0.5 cm path length quartz cuvette and a protein concentration of 0.6–1.0 mg/ml. The recording speed used was 17.5 nm/min and the time constant was fixed at 3 s. The spectral bandwidth was set at 1 nm. Ten scans recorded for each sample at room temperature (23–25°C) were averaged and smoothed before plotting the mean residue weight ellipticities (θ_{MRW}) vs. wavelength. Mean residue weights of 115.2, 114.0 and 115.9 for the neurotoxin, L and H chains, respectively, calculated from their amino acid compositions [11], were used for computation of the θ_{MRW} value.

All the experiments were repeated with the type A neurotoxin isolated from two separate batches of the bacterial cultures. The subunit L and H chains were also separated from the two batches of neurotoxin.

3. Results

3.1. Near-ultraviolet CD

The near-ultraviolet CD spectrum of type A neurotoxin (fig. 1) shows several well-resolved negative CD peaks between 250 and 320 nm. Strong peaks were observed at 261, 268, 286 and 292 nm (table 1) with respective θ_{MRW} values of -7.6, -12.0, -17.9 and -15.8 degree cm^2 dmol^{-1}. In addition, clear shoulders were evident at 279–282, 310 and 316 nm. A second-derivative CD spectrum of the neurotoxin (not shown) further resolved spectral peaks at 261, 269, 278, 286, 295 and 313 nm (table 1).

The near-ultraviolet CD spectrum of the H chain (fig. 1) also exhibited major peaks at 261, 268, 286 and 292 nm (table 1) with respective θ_{MRW} values of -6.7, -9.5, -15.1 and -15.5 degree cm^2 dmol^{-1}. A second-derivative spectrum of the H chain (not shown) indicated no further resolution of the CD spectrum, and positive peaks were observed at 260, 268, 285 and 295 (table 1).

The near-ultraviolet CD spectrum of the L chain (fig. 1) showed major negative CD peaks at 261, 268 and 276–279 nm. In contrast to the neurotoxin and H chain, the L chain did not show any band at 286 and 292 nm (table 1). Furthermore, the L chain showed small positive CD bands above 300 nm. The observed θ_{MRW} values for the L chain CD peaks were significantly higher than for the H chain. The θ_{MRW} values at 261, 268 and 276 were -8.0, -14.3 and -18.3 degree cm^2

Table 1

Near-ultraviolet CD analysis of the type A botulinum neurotoxin and its light and heavy chain subunits (peak positions in nm)

The major CD bands observed were assigned as follows: 261 and 268 nm for Phe, 278–283 nm for Tyr, 286 nm for Tyr and/or Trp, 292–295 nm for Trp, and 307–315 nm for Cys. (s), shoulder.

Neurotoxin		Light chain		Heavy chain	
Extrema	2nd-derivative peaks	Extrema	2nd-derivative peaks	Extrema	2nd-derivative peaks
261	261, 269	261	261, 268, 278(s)	261	260, 268
268	278, 286, 295	268	283, 296(s), 307, 316	268	285, 295
286	313	276–279		286	
292				292	

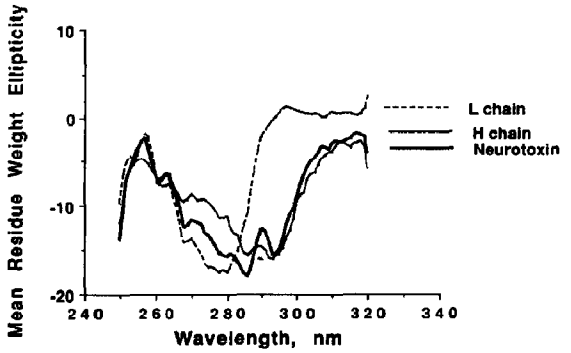

Fig. 1. CD spectra of the type A botulinum neurotoxin and its light and heavy chain subunits dissolved in 10 mM sodium phosphate (pH 8.1), containing 100 mM NaCl (buffer A). Spectra were recorded at room temperature (23–25° C) as described in section 2. Ordinate: mean residue weight ellipticity (in degree cm^2 $dmol^{-1}$).

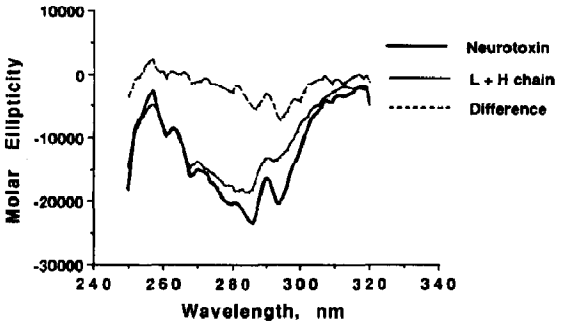

Fig. 3. CD spectra of type A botulinum neurotoxin based on molar ellipticity. The calculated composite spectrum of light and heavy chains (obtained by adding the CD spectra of the light and heavy chains, shown in fig. 2) is also shown along with the difference between the composite spectrum and the CD spectrum of the neurotoxin. Ordinate: as in fig. 2.

$dmol^{-1}$, respectively, for the L chain. A second-derivative CD spectrum of the L chain (not shown) resolved a few positive CD peaks that were not resolved in the zero-order spectrum (fig. 1). The derivative peaks were observed at 261, 268, 283, 307 and 316 nm with shoulders at 278 and 296 nm (table 1). It is interesting to note that most of these peaks were also observed in the zero-order and/or second-order CD spectra of the neurotoxin and the H chain (table 1).

The CD spectra of the L and H chains were plotted based on molar ellipticity to take into account the molecular sizes of the two polypeptides (fig. 2). The spectral profiles are similar,

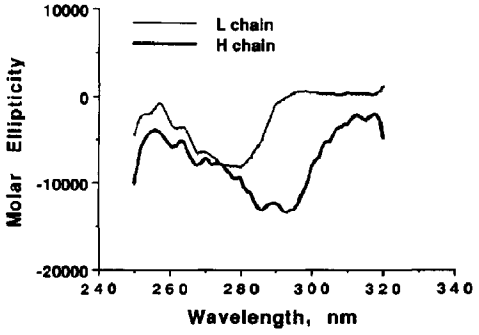

Fig. 2. CD spectra of the light and heavy chains of type A botulinum neurotoxin based on molar ellipticity. Ordinate: molar ellipticity (in degree cm^2 $dmol^{-1}$).

as expected, to the spectra based on mean residue weight ellipticity. The 53 kDa L chain shows, as expected, a lower molar ellipticity than the 97 kDa H chain although the L chain yielded higher signals based on the mean residue weight (vide supra). The composite spectra were obtained by adding the molar ellipticities of the L and H chains at intervals of 1 nm between 250 and 320 nm (fig. 3). Comparison of the composite and recorded spectra of the neurotoxin (150 kDa) revealed significant differences at 286 and 295 nm (fig. 3).

3.2. Second-derivative ultraviolet spectroscopy

The absorption spectra of the neurotoxin, and the separated L and H chains recorded between 240 and 320 nm were derivatized to the second order. The spectral region of interest for the determination of exposed tyrosine residues is 280–300 nm [14].

The absorption and second-derivative spectra of the neurotoxin dissolved in buffer A are shown in fig. 4a. The derivative spectrum of the neurotoxin between 280 and 300 nm showed two negative peaks at 284.5 and 292 nm, and two positive peaks at 289 and 296 nm. Upon denaturation with 6 M guanidine · HCl (30 min incubation at 23° C), the peak positions remained essentially the same (spectrum not shown) but the relative intensities changed, resulting in an increased a/b ratio (for

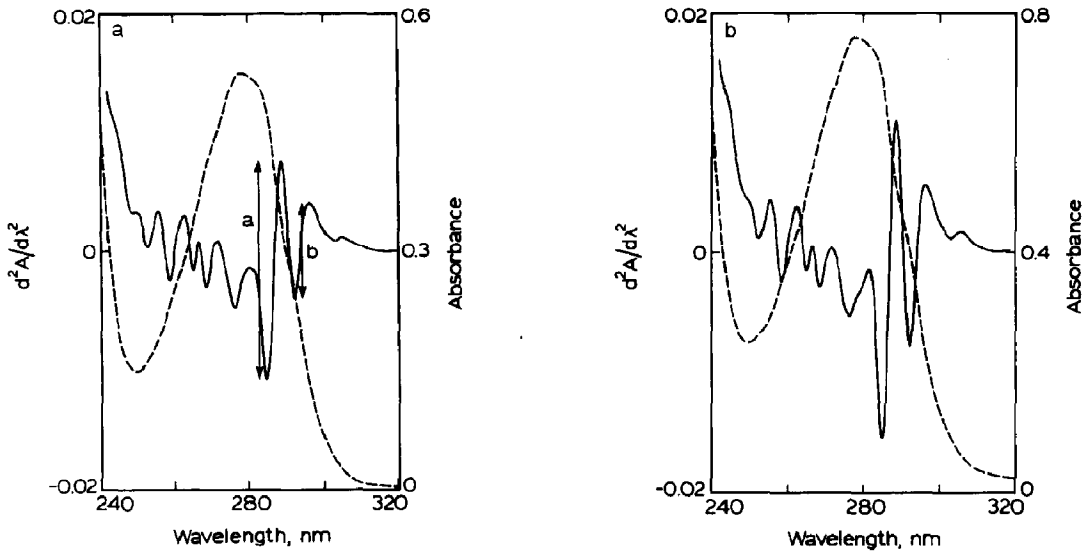

Fig. 4. Absorption (------) and second-derivative (———) spectra of (a) type A botulinum neurotoxin, and (b) heavy chain of botulinum type A neurotoxin dissolved in buffer A. Spectra were recorded at room temperature (23–25° C). Notation a represents the arithmetic sum of $d^2A/d\lambda^2$ at 284.5 and 288.5 nm. Notation b represents the arithmetic sum of $d^2A/d\lambda^2$ at 292 and 296 nm.

peak notations, see fig. 4a). The calculated degree of tyrosine exposure, α, of the neurotoxin was 70.7% (table 2).

The second-derivative spectrum of the H chain dissolved in buffer A (fig. 4b) shows negative peaks at 284.7 and 292.0 nm, and positive peaks at 288.5 and 296 nm, very similar to the spectrum of neurotoxin (fig. 4a). Upon treatment with 6 M guanidine · HCl (30 min at 23° C), the peak positions remained similar (negative peaks at 283.5 and 291.0 nm; positive peaks at 287.2 and 295.5 nm), and their intensities changed slightly (spectrum not shown). The degree of tyrosine exposure in the H chain was 81.5% (table 2).

The second-derivative spectrum of the L chain dissolved in buffer A shows only one negative peak at 284 nm (fig. 5a). Two positive peaks were observed at 288 and 295.5 nm with a positive trough at 291 nm between them. Denaturation of the protein with 6 M guanidine · HCl (30 min at

Table 2

Degree of tyrosine exposure in type A neurotoxin and separated L and H chains as determined by second-derivative spectroscopy

Sample [a]	γ_n	γ_u	X	γ_a	α (%)	Mean α (%)
A neurotoxin	2.26	3.23	4.18	−0.13	71.1	70.65
	2.25	3.23	4.18	−0.13	70.2	
H chain	1.96	2.32	3.06	0.10	83.9	81.45
	2.02	2.53	3.06	0.10	79.0	
L chain	4.52	10.16	6.25	−0.64	47.8	46.35
	4.13	10.00	6.25	−0.64	44.9	
Weighted mean [b]	–	–	–	–	–	69.64

[a] Two separate batches of neurotoxin, L chain and H chains were analyzed.

[b] Weighted mean tyrosine exposure in 150 kDa neurotoxin (calculated as (25 × L chain + 49 × H chain)/74, since L chain, H chain and neurotoxin have 25, 49 and 74 Tyr residues, respectively) = (25 × 46.4 + 49 × 81.5)/74 = 69.64.

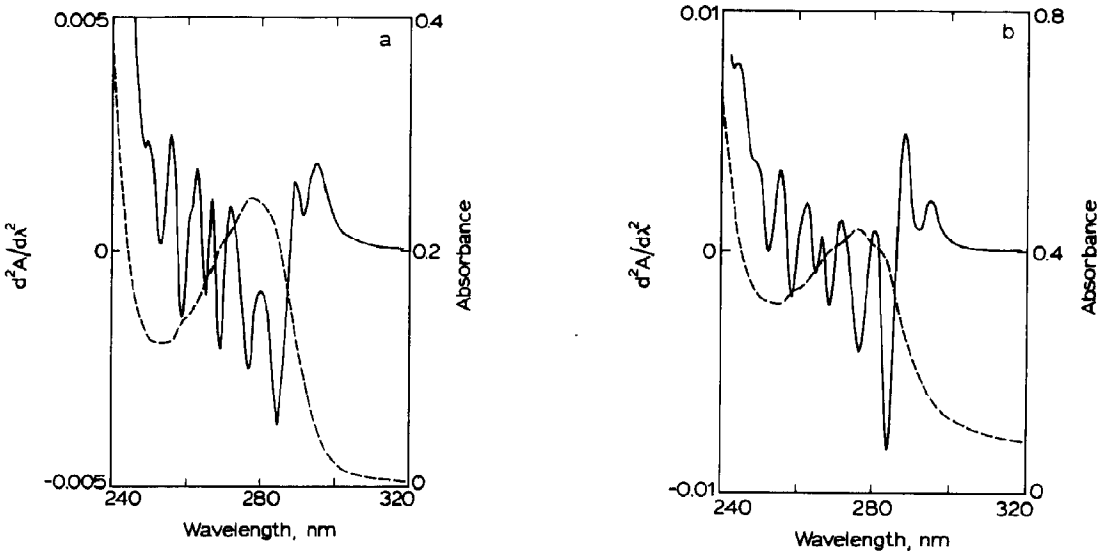

Fig. 5. Absorption (- - - - -) and second-derivative (———) spectra of the light chain of type A botulinum neurotoxin dissolved (a) in 10 mM sodium phosphate (pH 8.1), containing 100 mM NaCl and (b) in 6 M guanidine·HCl. Other details as in fig. 4.

23° C) resulted in a quite marked alteration in the relative peak intensities at 288 and 295 nm (fig. 5b). The calculated degree of tyrosine exposure in the L chain was 46.4% (table 2).

It is significant to note that the weighted mean of the exposed tyrosines in the separated L and H chains, viz., 69.6%, is virtually the same as that of the intact dichain neurotoxin, 70.7% (see legend to table 2).

4. Discussion

The near-ultraviolet CD spectra of proteins mainly arise from four types of chromophores; Phe, Tyr, Trp and Cys. The numbers of these residues in the neurotoxin are 68, 71, 17 and 10, respectively [11]. Various spectral bands could arise not only from the different chromophores but also from the same type of chromophore in various states (rigid vs. flexible) and environments (i.e., vicinal groups) which result in various vibronic transitions and dipole-dipole coupling (for a review, see ref. 15). The near-ultraviolet CD bands resolved in the second-derivative spectra of the neurotoxin and their subunit chains are the results of these factors.

Based on the studies of model compounds and proteins with a predominating type of side chain chromophore [15–20], we assigned the major bands of the neurotoxin, and the separated L and H chains as follows: 261 and 268 nm for Phe; 278–283 nm for Tyr; 286 nm for Tyr and/or Trp; 292–295 nm for Trp; and 307–315 nm for Cys. The weak negative CD bands between 307 and 316 nm found in the neurotoxin and the separated L chain suggest the presence of disulfide(s) in these protein preparations. This interpretation is supported as judged from the presence of one interchain disulfide bridge between the L and H chains and one intrachain disulfide near the C-terminus of the H chain [3,21]. Out of the 10 half-cystine residues of the dichain neurotoxin [11], only one -SH group was titratable, at most, employing four different analytical methods [22] indicating the presence of more disulfides, although their locations are not yet known.

In general, the CD bands of the L chain were stronger than those of the H chain. For example, θ_{MRW} of the L chain was 50.5% higher at 268 nm

and 64.2% higher at 279 nm than that of the H chain. This suggests that the Phe (268 nm) and Tyr (279 nm) residues of the L chain are in more rigid segments of the protein than those of the H chain. In other words, a significant number of the Phe and Tyr residues of the L chain are buried within the protein matrix. The CD spectrum of L chain (fig. 1) did not show any band at 292 nm (Trp) even though the L chain has four Trp residues [11]. This suggests that Trp residues are either in the flexible domains of the protein, or their opposite CD signals cancel one another. The second interpretation seems more likely, since a fluorescence study of the L chain has revealed [13] that its fluorescent Trp residues are in relatively hydrophobic environments, suggesting their presence in the buried state in the protein matrix.

As mentioned above, the CD band at 286 nm of the neurotoxin and the H chain could arise from Tyr or Trp residues (see ref. 15). Since the 286 nm band is absent in the L chain along with the 292 nm band (Trp), it is reasonable to interpret the 286 nm band as arising from Trp residues.

The near-ultraviolet CD signals of aromatic rings in proteins are sensitive to topographical and environmental changes in their vicinity [15,23] and, therefore, can be used to monitor changes occurring due to interaction between the L and H chains in the dichain neurotoxin. This approach has been successfully utilized to determine the interactions between A- and B-chains of the toxic protein ricin [24]. θ_{MEW} values of the neurotoxin, L and H chains at the designated wavelengths corresponding to the transitions of Phe, Tyr and Trp listed in table 3 show virtually no change in the vicinities of Phe (at 261 and 268 nm) of the L or H chains upon their separation. The number of Phe residues in the L and H chains are 34 and 37, respectively [11]. The segments of the L and H chains having Tyr residues appeared to change a little (10.9%, sample calculation: $(14.7 - 13.1) \times 100/14.7 = 10.88$) as monitored by the vibronic transition of Tyr at 279 nm (^1L_b bands). However, there are significant changes at 286 nm (23–26%) and 292 nm (23%) which probably arise from the ^1L_a and ^1L_b transitions of Trp residues. This is supported by the difference spectrum calculated from the composite CD spectrum of the L and H chains and the CD spectrum of the neurotoxin (fig. 3). Such changes in the microenvironments of Trp residues have also been observed using fluorescence spectroscopy [13]. The band at 286 nm can also arise from Tyr, or may stem from both Tyr and Trp side chains. If the Tyr residues of the L and H chains contribute significantly to the 286 nm band of the neurotoxin, it is likely that such Tyr residues would be buried within the protein matrix. Exposed Tyr residues do not contribute significantly to the near-ultraviolet CD transitions due to their flexibility [15].

The degree of Tyr exposure calculated from the second-derivative spectra of the proteins indicated

Table 3

Mean residue weight ellipticities (θ_{MRW}, in degree cm^2 dmol^{-1}) of type A neurotoxin, and separated L and H chains in the near-ultraviolet region

Wavelength (nm)	Assigned amino acid residue	Neurotoxin	H chain	L chain	Weighted mean [a]	(θ_{MRW} of A neurotoxin) / (θ_{MRW} of weighted mean)
261	Phe	-7.5 ± 0.2 [b]	-7.3 ± 0.8 [b]	7.9 ± 0.1 [b]	−7.6	0.99
268	Phe	-12.0 ± 0.1	-9.5 ± 0.4	-14.3 ± 0.0	−11.8	1.02
279	Tyr	-14.7 ± 0.7	-10.8 ± 0.1	-17.7 ± 0.3	−13.1	1.12
286	Tyr	-17.6 ± 0.4	-14.4 ± 1.1	-10.4 ± 0.4	−13.0	1.35
	Trp	-17.6 ± 0.4	-14.4 ± 1.1	-10.4 ± 0.4	−13.6	1.29
292	Trp	-15.4 ± 0.5	-14.6 ± 1.3	-0.5 ± 0.4	−11.8	1.31

[a] Weighted means are calculated as follows: $((A_L \times \theta_L) + (B_H \times \theta_H))/(A_L + B_H)$ where A_L and B_H are the respective numbers of a given amino acid in L and H chains; θ_L and θ_H are the mean residue weight ellipticities of L and H chains at a given wavelength (corresponding to the transition of the noted amino acid side chain).

[b] Refers to the standard deviation of the mean of two independent experiments.

relatively large numbers (13 out of 25) of Tyr residues are buried in the L chain whereas in the H chain only 9 (out of 49) Tyr residues are buried. This suggests that the 286 nm CD band would be stronger in the L chain than in the H chain if it were to arise from the vibronic transitions of Tyr residues. However, the contrary observations (table 3) suggest the origin of the 286 nm band from Trp residues. The degree of tyrosine exposure (α) also does not seem to change with chain separation because the weighted mean of the α values of the L and H chains is 69.6% (table 2) which is virtually the same as that of the dichain A neurotoxin (70.7%).

A 1.75-fold higher Tyr exposure in the H chain compared to the L chain (table 2) suggests that the peptide segments containing exposed Tyr residues are more accessible to the surface in the H chain than in the L chain. Since Tyr residues are hydrophobic in nature, their greater accessibility may reflect more flexibility in the polypeptide folding of the H chain relative to the L chain. This inference is supported by a significantly higher (> 64%) CD signal assigned to Tyr residues (vide supra) in the L chain than in the H chain, which indicates relative rigidity of the polypeptide folding in the L chain.

A higher degree of Tyr exposure in the H chain relative to the L chain is consistent with the accessibility of Tyr to the reagent tetranitromethane. When type A dichain neurotoxin was modified with a 500-fold excess of tetranitromethane, about one-third of its 72 Tyr residues were modified and most of the modification appeared to occur on the H chain [25].

Summary of the data suggests the absence of strong interactions between the subunit L and H chains of the type A neurotoxin, since upon their separation: (i) no changes occurred in the near-ultraviolet CD of Phe; (ii) only small changes took place in the near-ultraviolet CD of Tyr; and (iii) no changes were detectable in the degree of tyrosine exposure of the L and H chains. The above conclusion is reasonable because the Tyr and Phe residues that make up 145 of the total 1259 amino acid residues of the neurotoxin [11] are not likely to be clustered in segments of the polypeptide. Four widely separated segments of the neurotoxin (i.e., two in the L and two in the H chain) have been partially sequenced; these limited data do not reveal any clustering of Phe, Trp or Tyr [12,26]. Their random distribution throughout the polypeptide backbone thus offers to serve as a monitoring marker. These data are consistent with earlier findings [13] where no significant changes in the secondary structure (estimated from far-ultraviolet CD spectra) of L and H chains were observed upon their separation. Nevertheless, the polypeptide folding of the L and H chains exhibited alterations in the vicinity of Trp residues as demonstrated by changes in the near-ultraviolet CD transitions corresponding to Trp residues. This is also consistent with our earlier observation that the fluorescence quantum yields of the L and H chains change upon their separation [13].

The data accumulated from the earlier work [13] and the present study indicate that virtually no changes occur in the secondary structure, and that only very slight modifications take place in the environment of the Phe and Tyr residues of the L and H chains upon their separation. These observations provide support for the idea that the L and H chains of type A botulinum neurotoxin exist as two independent domains (bound by disulfide bond(s) and weak non-covalent interactions), but the data on Trp residues indicate that the conformations of at least certain segments (containing Trp residues) of the two chains are significantly influenced by the interactions between them. The two subunit chains, therefore, appear to represent two quasi-independent domains of the neurotoxin.

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