Probing Local Secondary Structure by Fluorescence: Time-Resolved and Circular Dichroism Studies of Highly Purified Neurotoxins

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ABSTRACT The relationship between β-sheet secondary structure and intrinsic tryptophan fluorescence parameters of erabutoxin b, α-cobratoxin, and α-bungarotoxin were examined. Nuclear magnetic resonance and x-ray crystallography have shown that these neurotoxins have comparable β-sheet, β-turn, and random coil secondary structures. Each toxin contains a single tryptophan (Trp) residue within its β-sheet. The time-resolved fluorescence properties of native erabutoxin b and α-cobratoxin are best described by triple exponential decay kinetics, whereas native α-bungarotoxin exhibits more than four lifetimes. The disulphide bonds of each toxin were reduced to facilitate carboxymethylation and amidocarboxymethylation. The two different toxin derivatives of all three neurotoxins displayed triple exponential decay kinetics and were completely denatured as evidenced by circular dichroism (random coil). The concentration (*c*) values of the three fluorescence decay times (time-resolved fluorescence spectroscopy (TRFS)) were dramatically different from those of the native toxins. Each neurotoxin, treated with different concentrations of guanidinium hydrochloride (GuHCl), was studied both by circular dichroism and TRFS. Disappearance of the β-sheet secondary structural features with increasing concentrations of GuHCl was accompanied by a shift in the relative contribution (*c* value) of each fluorescence decay time (TRFS). It was found that certain disulphide residues confer added stability to the β-sheet secondary structure of these neurotoxins and that the center of the β-sheet is last to unfold. These titrations show that Trp can be used as a very localized probe of secondary structure.

INTRODUCTION

After denaturation many proteins are able to refold under suitable conditions, implying that the amino acid sequence provides the necessary information that determines the tertiary structure of the protein. The rules that govern protein folding are required for the rational design of novel proteins, but they remain largely unsolved. The ability to monitor structural changes at a local site on a protein is often desirable when studying protein folding/unfolding.

Time-resolved fluorescence offers the advantage of high sensitivity allowing for experiments to be performed at in vivo concentrations. Tryptophan (Trp) is a useful intrinsic fluorophore due to its relatively low abundance in proteins and its ability to report information from a specific site. Although Trp fluorescence has the potential to probe the structure of its surrounding environment, data to support this relationship has not been clearly established. This has lead to an effort to correlate protein structural features with measurable fluorescence parameters. Recently, time-resolved fluorescence spectroscopy was utilized to establish a relationship between Trp fluorescence and local random coil/α-helical secondary structure in parathyroid hormone (PTH) and model peptides (Willis and Szabo, 1992; Willis et al., 1994). These studies showed that the relative contributions of the three decay time components were directly proportional to the α-helix content. It was proposed that the decay time values were associated with ground-state χ_1 rotamers of the Trp side chain and the observed changes in the relative proportions of the decay time components were the result of main chain conformational constraints on the χ_1 rotamer populations. The existence of three ground state Trp rotamers in crystalline erabutoxin b has been clearly demonstrated by time-resolved fluorescence spectroscopy in a recent study by Dahms et al. (1995). Calculations from this study suggested that the concentration (*c*) values found for crystalline erabutoxin b are closely related to those found in solution.

A preliminary study of several highly purified neurotoxins had revealed that the relative proportions for the fluorescence decay times were distinct from those observed for Trp in an α-helix and random coil (Dahms et al., 1992). In each case, the Trp residue was located near the center of an antiparallel β-sheet. Because by definition these neurotoxins exhibit no true tertiary structure (see Fig. 1, *A* and *B*), it was suggested that the *c* parameters obtained by fluorescence were indicative of Trp within a solvent exposed β-sheet secondary structure. If this were the case, the denatured neurotoxins should display relative decay time proportions similar to those observed for Trp in a random coil. Herein we report the complete results on the structural correlation. The effect of unfolding experiments and disulphide modification on the fluorescence parameters of a Trp residue located within the β-sheet of erabutoxin b, α-cobratoxin, and α-bungarotoxin will be discussed.

Received for publication 24 October 1994 and in final form 28 April 1995.

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0006-3495/95/08/569/08 $2.00

MATERIALS AND METHODS

Protein preparation

The crude venoms of *Lacticauda semifasciata*, *Naja naja kouathia*, and *Bungarus multicinctus* (Sigma Chemical Co., St. Louis, MO) were treated by batch separation using carboxymethylated (CM) cellulose (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM NaAc, pH 6.5. The CM cellulose was stirred for 1 h at 4°C with a mixture of the crude venom dissolved in 10 mM NaAc, pH 6.5. The resin was filtered and the adsorbed material was extracted from the resin using 190 mM NaCl, in 10 mM NaAc pH 6.5. The mixture was lyophilized to near dryness and dialysed against 10 mM NaAc, pH 6.5, in preparation for high performance liquid chromatography (HPLC) separation. All three toxins were purified by HPLC on a TSK CM-5PW column (Supelco, Mississauga, Ontario, Canada) using 10 mM NaAc, pH 6.5 with a salt gradient. α-bungarotoxin, α-cobratoxin, and erabutoxin b eluted at 0.06 M, 0.16 M, and 0.02 M NaCl, respectively. The purified proteins were analyzed by nebulization-assisted electro-spray ionization (ESI) mass spectrometry (API III quadrupole; Sciex, Mississauga, Ontario, Canada) using ~0.5 mg/ml protein solution in 10% acetic acid/water.

CM material was prepared by dissolving 1 mg of toxin in 1 ml of 8 M urea, 1 mM TRIS pH 9.3 (buffer A). The vessel was flushed with N_2, and the reaction was left to stir for 1 h. To this solution, 0.25 ml of 100 mM 2,3-dihydroxy-1,4-dithiobutane (dithioerythritol) in buffer A was added. Again the vessel was flushed with N_2 and the reaction left to stir. After 3 h, a threefold excess (with respect to the number of sulfhydryl (SH) groups) of iodoacetic acid in buffer A was added, the vessel was flushed with N_2 and the reaction stirred for 10 min in the dark. Absence of free SH was detected using 5′,5′-dithiobis-2-nitrobenzoic acid. The mixtures were dialysed against Milli-Q water, lyophilized and repurified by diethylaminoethanol-5PW HPLC (isocratic elution: 10 mM NaAc pH 6.5 with 99 mM NaCl).

The amidocarboxymethylated (ACM) material was prepared as above by substituting iodoacetamide for iodoacetic acid. Because the pI of the protein is not altered by amidocarboxymethylation, the modified toxins could be purified in the same manner as the native toxins. The identity of the CM- and ACM-modified toxins was determined by ESI mass spectrometry as previously described.

Circular dichroism (CD)

CD measurements were made with a JASCO 6000 spectropolarimeter at 20°C. The instrument was calibrated with aqueous D-10-camphorsulfonic acid. Protein concentration in 1 mM phosphate buffer (pH 6.5) was determined from the absorbance at 280 nm using an extinction coefficient of 6766 $M^{-1}\,cm^{-1}$ for erabutoxin b and α-cobratoxin and 7953 $M^{-1}\,cm^{-1}$ for α-bungarotoxin. Denaturation was facilitated by successive addition of guanidinium hydrochloride (GuHCl, Pierce sequinal grade). CD measurements are reported as mean residue molar ellipticity.

Fluorescence spectroscopy

Steady-state fluorescence spectra were measured on an SLM 8000C spectrofluorimeter at 20°C. The optical density of the protein solutions was <0.10 at the excitation wavelength. Emission spectra were taken with excitation and emission bandpasses of 4 nm, and each was corrected for (negligible) blank contribution. Time-resolved fluorescence was measured by time-correlated single photon counting. The excitation source was a sync-pumped dye laser, and fluorescent photons were detected by a microchannel plate after passing through a polarizer oriented at 57°, filter (glass, 3 mm), and monochromator (Willis and Szabo, 1989). Data from multiple emission wavelengths with a 295 nm excitation were analyzed using global methods. Procedures for global (simultaneous) analysis (Willis and Szabo, 1992; Knutson et al., 1983) and the criteria for assessing the quality of fit of the model to the data (Durbin and Watson, 1971) are given elsewhere (Willis and Szabo, 1989).

RESULTS

Protein preparation

ESI mass spectrometry was used to establish both the identity and purity of the native and modified toxins. The observed molecular weights for the HPLC-purified toxins were in good agreement with the calculated molecular weights as determined by the protein sequence (Table 1).

Circular dichroism

The CD spectra of each neurotoxin at μM concentrations indicated a high β-sheet content. Erabutoxin b displayed the greatest amount of β-sheet spectral characteristics, as predicted from the crystal structures (Walkinshaw et al., 1980; Love and Stroud, 1986; Basus et al., 1988). The native toxins treated with 6 M GuHCl, and CM or ACM, displayed CD spectral features consistent with random coil secondary structure (Fig. 2). The β-sheet CD spectral features were fully recovered upon dialysis of the GuHCl-treated native toxins, indicating complete refolding of the proteins. The CD spectral maxima at 202 nm and 228 nm (Fig. 2) are indicative of β-sheet secondary structure. The spectral maxima at 228.6 nm was chosen as a measurement of β-sheet content, because the larger maxima at 200 nm was obscured by the absorption of GuHCl.

Fluorescence spectroscopy

Steady-state and time-resolved fluorescence values for erabutoxin b, α-cobratoxin, and α-bungarotoxin are summarized in Tables 2, 3, and 4, respectively. The steady-state λ_{max} for each of the neurotoxins becomes progressively longer with successive additions of GuHCl and is furthest red-shifted for the CM and ACM toxins. This trend is consistent with an increasingly solvent-exposed Trp.

Time-resolved fluorescence decay measurements of native erabutoxin b and α-cobratoxin in buffered solution at

TABLE 1 ESI mass spectral analysis of HPLC-purified toxins and carboxymethylated toxins

Identification	Calculated mol/wt (g/mol)	ESIMS mol wt (g/mol)	Standard deviation	% Difference
Ebtx b*	6860.8	6860.1	0.2	0.01
α-Cotx*	7821.1	7821.0	0.8	0.001
α-Butx*	7984.3	7984.9	0.1	0.007
Ebtx b CM‡	7334.9	7333.6	0.3	0.02
α-Cotx CM‡	8413.7	8412.4	1.0	0.02
α-Butx CM‡	8576.9	8575.0	1.0	0.02
Ebtx b ACM§	7325.0	7323.1	0.7	0.02
α-Cotx ACM§	8402.6	8400.1	1.2	0.03
α-Butx ACM§	8564.2	8562.3	0.8	0.02

* Native toxins: erabutoxin b (Ebtx b), α-cobratoxin (α-Cotx), α-bungarotoxin (α-Butx).

‡ Toxins treated by reduction and carboxymethylation.

§ Toxins treated by reduction and amidocarboxymethylation.

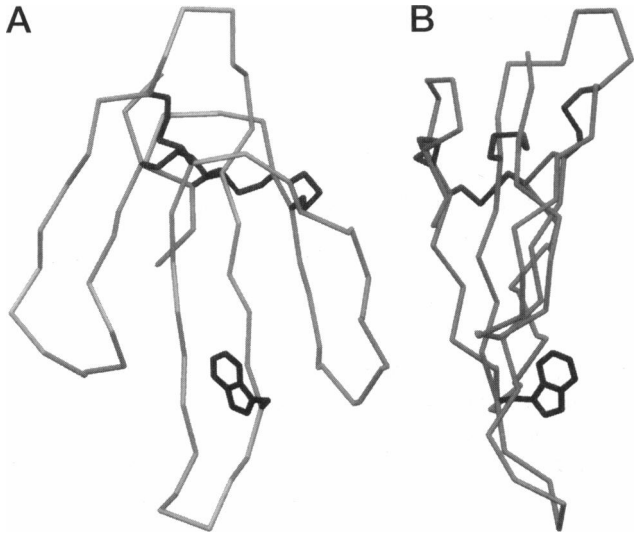

FIGURE 1 (*A*) "Front-face" representation of erabutoxin b as determined by x-ray crystallography (3EBX.pdb; Smith et al., 1988). Backbone, light gray; Cys-Cys side chains, medium gray; Trp side chain, black. (*B*) "Side view" representation of erabutoxin b. Backbone, light gray; Cys-Cys side chains, medium gray; Trp side chain, black.

12 individual emission wavelengths (305–420 nm) displayed three exponential decay components, whereas α-bungarotoxin was best described by four decay times. In all three cases, the decay times were similar at each wavelength justifying the global analysis of these data sets to provide the decay times displayed in Tables 2–4. In the case of erabutoxin b and cobratoxin, the statistical fits for three discrete exponential decay times (τ_i) were good under all conditions ($1.05 \leq \chi^2 \leq 1.11$ and $1.77 \leq$ serial variance ratio (SVR) ≤ 1.93). The native bungarotoxin showed only an adequate fit to four exponential decay times ($\chi^2 = 1.18$ and SVR = 1.64). The added complexity of this system is explained by the presence of two low energy conformations (alternate backbone geometry) for α-bungarotoxin in solution (Walkinshaw et al., 1980). Thus, as well as the Trp side chain rotamers, two added Trp environments would be contributed by backbone heterogeneity.

The fluorescence decay times for all three toxins did vary (≤25%) with denaturation (Tables 2–4), reflecting a change in the Trp environment.

It is possible to obtain a decay-associated spectra (DAS) by combining the steady-state and time-resolved fluorescence data (see Fig. 3). Assuming 1) that the decay kinetics arise from ground-state conformational heterogeneity and 2) that the ground-state conformers of the Trp residue have very similar extinction coefficients and radiative lifetimes, then the relative proportions of the three conformers in the ground state (c) can be determined (Willis and Szabo, 1992). Each c value is calculated by integrating the area under each spectral curve that is associated with a particular decay time (DAS). The integrated value is then divided by the respective decay time (τ_i) and normalized giving a relative c value for each rotamer.

The relative contributions of the three decay times observed for erabutoxin b and α-cobratoxin varied with [GuHCl] and were dramatically altered by disruption of the disulphide bonds (CM/ACM toxins). Transition from the β-sheet to random coil state in erabutoxin b resulted in 94%, 55%, and 58% changes in the long, intermediate, and short decay time-relative proportions, respectively. This change was achieved with CM, ACM, and GuHCl treatment. CM and ACM modification of α-cobratoxin showed similarly dramatic changes of 77%, 41%, and 20%. However, even at 6.6 M GuHCl the cobratoxin did not display "complete random coil" fluorescence values. The progression of β-sheet to random coil in bungarotoxin is complicated by a shift in the number of decay time components, and therefore the change in decay time relative proportion cannot be quantitated. Both the decay times and their relative proportions were restored to native toxin values upon the removal of GuHCl by dialysis (data not shown). For all three toxins, the CM- and ACM-treated material was best described by triple exponential decay kinetics, and the c values were consistent with those values obtained for the helical monomer (Willis et al., 1994) but not consistent with denatured PTH (Willis and Szabo, 1992).

For each toxin, the molar ellipticity at the CD spectral maximum (228.6 nm) was plotted versus the time-resolved fluorescence c values with progressive denaturation by GuHCl (Figs. 4–6). The relationship between the loss of structure as detected by CD and time-resolved fluorescence was not linear.

DISCUSSION

A problem in protein folding is to determine whether local areas of a protein have the propensity to act as initiators of global folding. Nuclear magnetic resonance (NMR) and CD have been used routinely to determine the structure of peptide fragments that represent local areas of a protein;

TABLE 2 Time-resolved fluorescence parameters for erabutoxin b (Ebtx b) determined from the DAS (λ_{ex} = 295 nm, λ_{em} = 12 wavelengths) at different denaturant (GuHCl) concentrations

Identification	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	c_1	c_2	c_3	λ_{max}	SVR	σ
Native Ebtx b	3.85 ± 0.02	1.180 ± 0.002	0.258 ± 0.003	0.04	0.86	0.10	340	1.80	1.10
2 M GuHCl	3.30 ± 0.02	1.264 ± 0.003	0.263 ± 0.004	0.08	0.79	0.13	340	1.85	1.07
3.7 M GuHCl	3.26 ± 0.01	1.408 ± 0.005	0.306 ± 0.005	0.14	0.71	0.16	343	1.92	1.06
5.2 M GuHCl	3.437 ± 0.007	1.45 ± 0.01	0.277 ± 0.004	0.36	0.39	0.24	350	1.83	1.08
CM	3.676 ± 0.009	1.58 ± 0.01	0.354 ± 0.006	0.33	0.43	0.24	350	1.93	1.04
ACM	2.916 ± 0.008	1.264 ± 0.009	0.290 ± 0.004	0.29	0.43	0.28	348	1.92	1.05

TABLE 3 Time-resolved fluorescence parameters for α-cobratoxin (α-Cotx) were determined from the DAS (λ_{ex} = 295 nm, λ_{em} = 12 wavelengths) at different denaturant (GuHCl) concentrations

Identification	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	c_1	c_2	c_3	λ_{max}	SVR	σ
Native α-Cotx	3.57 ± 0.01	1.079 ± 0.003	0.267 ± 0.003	0.07	0.68	0.25	343	1.77	1.11
2 M GuHCl	3.21 ± 0.01	1.067 ± 0.003	0.285 ± 0.002	0.09	0.67	0.24	344	1.85	1.07
3.7 M GuHCl	2.99 ± 0.01	1.097 ± 0.002	0.300 ± 0.003	0.12	0.68	0.20	347	1.77	1.09
6.6 M GuHCl	3.263 ± 0.007	1.202 ± 0.006	0.280 ± 0.007	0.24	0.53	0.23	346	1.83	1.07
CM	3.692 ± 0.008	1.49 ± 0.01	0.350 ± 0.004	0.30	0.40	0.30	347	1.92	1.05
ACM	2.937 ± 0.005	1.080 ± 0.006	0.251 ± 0.003	0.26	0.41	0.33	348	1.86	1.05

however, these methods can be challenged by low levels of structure. Furthermore the ability to measure the structural details at a specific site of a protein (in solution) is not possible by CD and is difficult by NMR. The time-resolved fluorescence decay parameters of Trp offer the potential to directly report structural information from a local site on a protein at in vivo concentrations.

Several groups have established significant correlations between side chain dihedral angle probabilities and backbone ϕ,ψ values (Janin et al., 1978; Piela et al., 1987; McGregor et al., 1987; Schrauber et al., 1993; Dunbrack and Karplus, 1993). If the distribution of amino acid side chain conformers is dependent upon the local protein backbone ϕ,ψ angles, then rotameric populations would provide a sensitive probe of local secondary structure.

The fluorescence of Trp in proteins is often best described by triple exponential decay kinetics (Beechem and Brand, 1985; Eftink, 1991). This result has been rationalized in terms of alternate conformational states of the Trp side chain due to rotation about the C_α-C_β (χ_1 rotamer) and/or C_β-C_γ (χ_2 rotamer) bonds (Donzel et al., 1974; Szabo and Rayner, 1980). Several methods have provided direct evidence for this model (Philips et al., 1988; Tilstra et al., 1990; Colucci et al., 1990; Ross et al., 1992) and most recently, time-resolved fluorescence spectroscopy has been used to demonstrate the existence of Trp side chain rotamers in erabutoxin b protein crystals (Dahms et al., 1995). Using this model, the fluorescence decay time values (τ_i) would be associated with the different Trp rotamers and the normalized pre-exponential amplitudes (α_i) or fractional concentrations (c_i, in the case of data from a DAS experiment) would reflect the relative proportion of each rotamer. Therefore, the c values should vary as a function of local secondary structure.

Based on this reasoning, the relationship between Trp fluorescence parameters and protein secondary structure was investigated (Willis and Szabo, 1992; Willis et al., 1994). The most recent study utilized α-helical model peptides in which a Trp residue had been placed at the center of the helix. In one case, the peptide monomer was nonhelical, whereas the peptide dimer displayed an α-helical structure. By altering the peptide concentration it was possible to monitor the change in fluorescence parameters with a change in local secondary structure. The other model peptide was α-helical as a monomer, and loss of structure was conferred by titration with GuHCl. Tetrafluoroethylene (TFE) was utilized to induce further helical character. These studies showed that the relative contribution of each fluorescence decay time was correlated with α-helix content.

The three neurotoxins, erabutoxin b, α-cobratoxin and α-bungarotoxin are composed primarily of β-sheet, β-turn, and random coil secondary structure as determined by CD, NMR, and x-ray crystallography (Tsernoglou and Petsko, 1976; Bernstein et al., 1977; Walkinshaw et al., 1980; Love and Stroud, 1986; Le Goas et al., 1992; Inagaki et al., 1980, 1985; Hider et al., 1982; Ménez et al., 1980; Low et al., 1973). Erabutoxin b is classified as a short chain neurotoxin and displays a higher β-sheet content than the two "long chain" neurotoxins (α-cobratoxin and α-bungarotoxin). There is significant homology between the tertiary structures of the short and long chain neurotoxins. The additional amino acids found in the long chain neurotoxins are manifest mainly as an N-terminal random coil tail. The invariant Trp residue that has been found to be necessary for interactions with the acetylcholine receptor is located in the center of an antiparallel β-sheet for all three neurotoxins. By definition (Fig. 1, *A* and *B*) these neurotoxins display little or no tertiary structure but rather are defined by a simple

TABLE 4 Time-resolved fluorescence parameters for α-bungarotoxin were determined from the DAS (λ_{ex} = 295 nm, λ_{em} = 12 wavelengths) at different denaturant (GuHCl) concentrations

Identification	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4	c_1	c_2	c_3	c_4	λ_{max}	SVR	σ
Native α-Butx	2.52 ± 0.02	0.551 ± 0.007	0.201 ± 0.002	0.057 ± 0.001	0.01	0.10	0.54	0.34	339	1.64	1.18
2 M GuHCl	2.92 ± 0.02	0.656 ± 0.009	0.274 ± 0.003	0.080 ± 0.001	0.03	0.22	0.65	0.10	341	1.69	1.17
3.7 M GuHCl	3.15 ± 0.01	0.99 ± 0.02	0.373 ± 0.004	0.115 ± 0.002	0.06	0.16	0.66	0.12	341	1.88	1.07
5.2 M GuHCl	3.051 ± 0.006	0.995 ± 0.007	0.281 ± 0.003	0.058 ± 0.003	0.18	0.31	0.41	0.10	347	1.82	1.09
5.2 M GuHCl	2.987 ± 0.004	0.875 ± 0.005	0.231 ± 0.002		0.21	0.42	0.36		347	1.59	1.16
CM	4.05 ± 0.02	1.94 ± 0.01	0.445 ± 0.004		0.20	0.47	0.33		350	1.85	1.05
ACM	3.05 ± 0.01	1.405 ± 0.007	0.251 ± 0.002		0.25	0.43	0.32		348	1.82	1.08

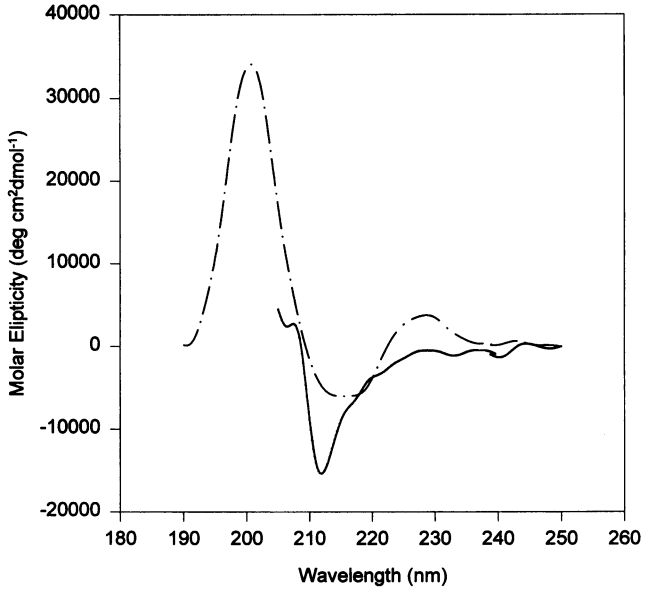

FIGURE 2 Far-uv CD spectrum of native erabutoxin b (— · —, 1 mM phosphate, pH 6.5) and denatured erabutoxin b (——, 1 mM phosphate, pH 6.5 with 6 M GuHCl).

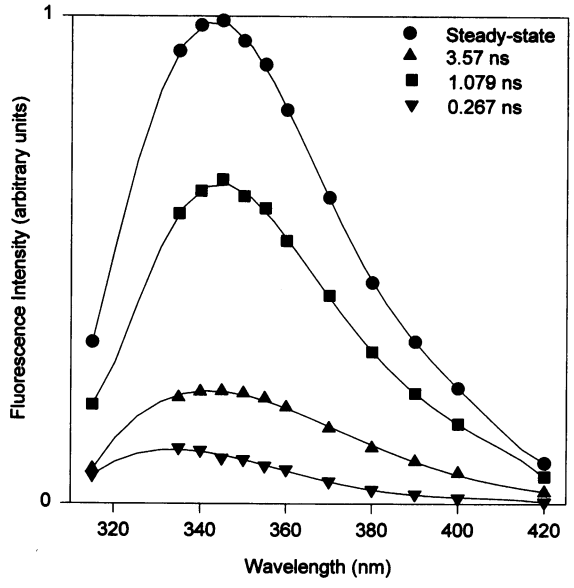

FIGURE 3 DAS of native α-cobratoxin. Data were collected using an excitation wavelength of 295 nm (20°C, pH 7.5). The DAS were obtained from the global analysis of a multiple wavelength experiment (12 data sets). The standard errors associated with τ_1, τ_2, τ_3 were ±0.01, ±0.003, ±0.002 (SVR = 1.8, σ = 1.1).

β-sheet secondary structure, which is maintained via strategically placed disulphide linkages. The Trp can be considered to be completely solvent-exposed.

Time-resolved fluorescence values for each toxin are summarized in Tables 2–4. The three fluorescence decay times of erabutoxin b are very comparable to that of α-cobratoxin, under all conditions, indicating a similar environment for the Trp residue (Tables 2 and 3). Subtle differences in the decay time values reflect small differences in the local Trp environment (surrounding residues within a 7 Å radius of the indole ring). The two toxins display nearly identical fluorescence decay times for the CM- and ACM-treated material. In each case, shorter fluorescence decay times are observed for ACM as compared with the CM-treated protein. This observation is in accordance with previous studies in which amide groups were found to be more efficient quenchers of Trp than carboxylate (Cowgill, 1976). The fluorescence decay kinetics of α-bungarotoxin are more complex than those observed for the other two toxins. The fluorescence decay curve of α-bungarotoxin cannot be fit to three exponentials, and the statistical criteria indicate that four decay time values provide only an adequate fit (Table 4; SVR = 1.64, and χ^2 = 1.18). The structure of α-bungarotoxin determined by x-ray crystallography (Love and Stroud, 1986) is distinct from that determined by NMR (Basus et al., 1988; Inagaki et al., 1985). The NMR structure places the conserved Trp residue in a position similar to that of all other postsynaptically acting neurotoxins, whereas the crystal structure reports less β-sheet and an unusual orientation of the invariant Trp. It has been proposed that the difference in the two structures arises from crystal packing (Basus et al., 1988) and that there are two low energy conformations with different backbone geometry. Since the crystal-packing forces are sufficient to bring about this conformational change, it was concluded that there must be a relatively low barrier to interconversion. It is reasonable to propose that the high sensitivity of fluorescence spectroscopy allows for the detection of Trp in this alternate backbone conformer. Hence, it might be expected that the Trp residue would display different decay times for each backbone conformation. This would potentially lead to six decay time components. It has been shown previously that it is difficult or impossible to resolve decay times of similar value (i.e., within a factor of 1.5), much less five or six discrete decay time values.

The *c* values for native erabutoxin b and α-cobratoxin are distinct from those observed for Trp in a random coil or α-helix (Tables 2 and 3; Figs. 4 and 5), and the Trp residue of each toxin is found on a β-sheet (NMR and x-ray crystallographic data). It was not our intention to provide kinetics of the folding or unfolding of the proteins nor to provide a detailed structural titration curve with denaturant. Rather, the main purpose of this study was to establish whether the *c* values observed for the toxins were indicative of a Trp local β-sheet secondary structure and whether those values would change upon denaturation with GuHCl or by elimination of the structurally important disulphide bonds.

The *c* values of erabutoxin b and α-cobratoxin are dominated by the intermediate decay component (0.86/0.68) with relatively small contributions from the long and short decay components (Tables 2 and 3). These results are similar to those observed for melanocyte-stimulating hormone in acidic lipids, and in this case the hormone is thought to exist in a β-turn-type structure (Ito et al., 1993). This is in contrast to the helical dimer (α-helical structure) that dis-

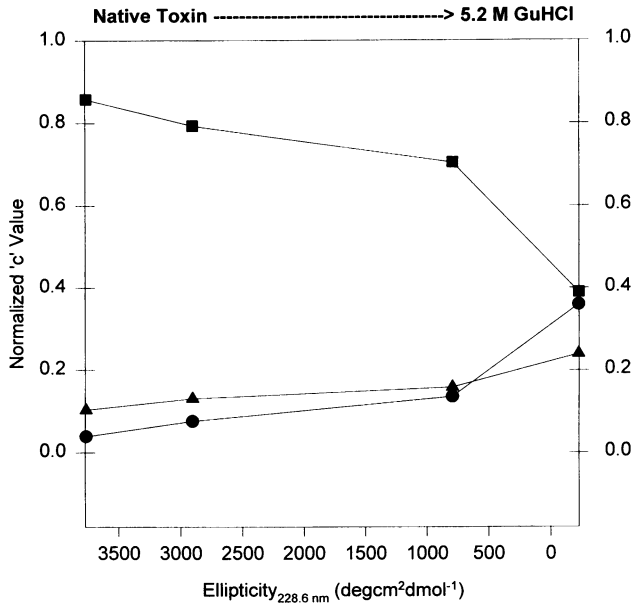

FIGURE 4 Relationship between CD (228.6 nm) and c values for erabutoxin b with varied GuHCl concentrations (0, 2, 3.7, and 6 M). (●) C_1; (■) C_2; (▲) C_3.

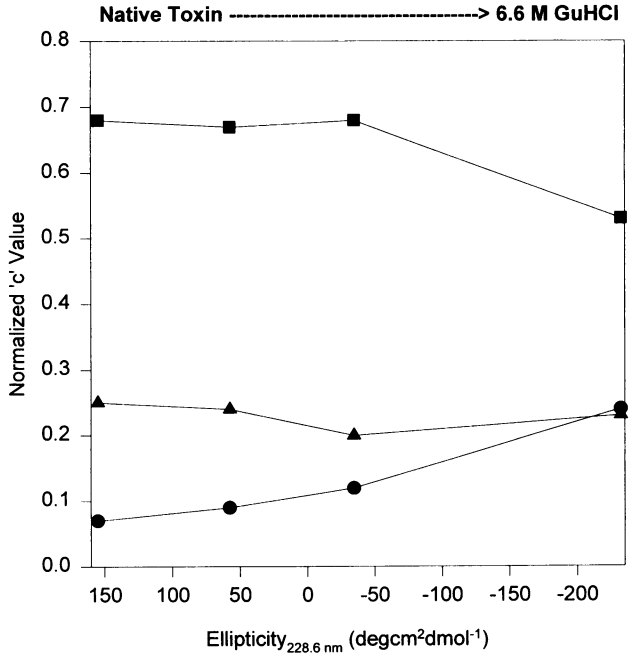

FIGURE 5 Relationship between CD (228.6 nm) and c values for α-cobratoxin with varied GuHCl concentrations (0, 2, 3.7, and 6.6 M). (●) C_1; (■) C_2; (▲) C_3.

plays relative contributions of 0.43, 0.38, and 0.19 for the long, intermediate, and short decay times, respectively (Willis et al., 1994). In its complete monomeric form (non-helical), the c values become 0.28, 0.45, and 0.27, respectively. On the other hand, the observed c values of PTH in the presence of 35% TFE (α-helical) were 0.33, 0.53, and 0.14. Native PTH displayed c values of 0.36, 0.44, and 0.20, and in the presence of 6 M GuHCl the values observed were 0.48, 0.39, and 0.13.

Several different methods were used to denature the toxins. GuHCl was initially used such that a titration at different molar concentrations of the denaturant could be performed. Previous studies (Willis and Szabo, 1992) have shown that GuHCl has a negligible effect on fluorescence parameters. Two other methods of denaturation were also examined. Because the toxins are small proteins, the four (erabutoxin b) or five disulphide linkages seem to be very important in maintaining structural integrity. Reduction and carboxymethylation of the disulphide linkages was performed on each toxin. This modification has the advantage of adding only a small group to the protein, but results in the addition of 8 (or 10) negative charges to the protein. For this reason reduction/amidocarboxy-methylation was also utilized, allowing the protein to maintain its native pI.

For all three toxins, disruption of the disulphide bonds by either method resulted in total loss of secondary structure by CD (data not shown). The fluorescence of each toxin is best described by three decay times with c values (Tables 2–4) comparable to those found for the (non-helical) helical monomer (Willis et al., 1994). Since the interaction of neighboring residues with Trp will affect the fluorescence decay time values, the characteristic relative proportion (c) may not always be associated with the same lifetime for all proteins. The c values that were reported for denatured PTH, PTH fragments (Willis and Szabo, 1992), and nuclease A (Eftink and Wasylewski, 1992) are different from those of both the helical monomer and the denatured toxins. This implies that there is not a unique set of c values that represent the denatured state for all proteins. However, these values may still be used to monitor protein unfolding for a given protein.

If we assume that the c values of these toxins reflect the secondary structural features for the protein backbone in the vicinity of the Trp residue, we can monitor unfolding at a very specific site. Since the CM and ACM toxins show only random coil structure by CD (data not shown), the c values observed under these conditions are used as the values expected for the completely denatured toxins. Fig. 4 shows the denaturation of erabutoxin b with increasing concentrations of GuHCl. The c values change only slightly between the native state and 3.7 M GuHCl, even though the CD shows marked loss of β-sheet. However, when the CD results are plotted versus [GuHCl] (data not shown), a linear relationship was observed. Although there was global unfolding in the protein in this [GuHCl] range, the local structure surrounding the Trp residue was maintained. This implies that there is an equilibrium intermediate at 3.7 M GuHCl with very little secondary structure other than that which is in direct contact with the Trp residue. These results are in agreement with recent studies of barnase, which showed that the center of a β-sheet was the first to fold and the last to unfold (Fersht, 1993); for these toxins, the Trp residue is located near the center of the β-sheet.

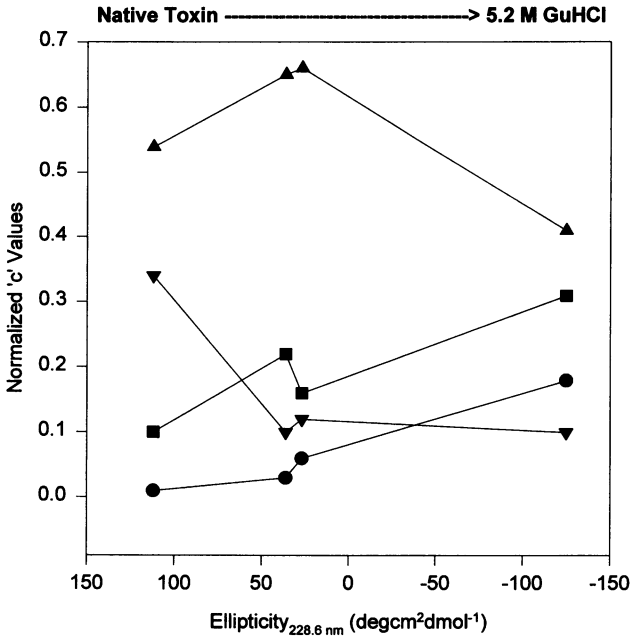

FIGURE 6 Relationship between CD (228.6 nm) and c values for α-bungarotoxin with varied GuHCl concentrations (0, 2, 3.7, and 5.2 M). (●) C_1; (■) C_2; (▲) C_3; (▼) C_4.

A similar phenomenon is observed for α-cobratoxin (Fig. 3) and α-bungarotoxin (Fig. 4), but the effect is more dramatic in these two cases. With GuHCl treatment the α-cobratoxin Trp c values begin to converge toward those observed for the modified toxins (random coil); however, total loss of secondary structure does not occur even at 6.6 M GuHCl. The global analysis of the α-bungarotoxin fluorescence at 5.2 M GuHCl was best fit to four exponential decay times (SVR = 1.82, χ^2 = 1.09) as compared with triple exponential decay kinetics (SVR = 1.59, χ^2 = 1.16) indicating that the local area surrounding the Trp residue was not yet completely denatured and backbone heterogeneity is still observed. (CM- and ACM-treated α-bungarotoxin displayed triple exponential decay kinetics with c values similar to CM- and ACM-treated erabutoxin b and α-cobratoxin). Structural data show both the α-bungarotoxin and α-cobratoxin to have an added disulphide bridge located at the β-turn between the third and fourth β-strand, whereas the erabutoxin does not. It seems that the close proximity of the disulphide to the Trp residue preserves some of the local backbone conformation, even in the presence of high GuHCl concentrations. Protein stability due to the disulphide linkages was also observed in the recovery of CD and fluorescence values (refolding) for each protein (data not shown) due to elimination of GuHCl (6 M) by dialysis. The stability conferred by the disulphide linkage, in the case of α-bungarotoxin and α-cobratoxin, is observed in combination with the effect from the Trp residue being at the center of the β-sheet.

It is evident from this study that care should be exercised in the use of fluorescence c values (rotamer relative proportions) as a tool for predicting structure, in the absolute sense. The evidence presented in this paper strongly suggests that the c values for Trp in a β-sheet are dramatically different from those observed for Trp in an α-helical or random coil type structure. This study confirms the laboratory's earlier proposal that there is a connection between the secondary structure of protein segments containing a Trp residue and the c values from the fluorescence of that Trp (Willis et al., 1994). Not surprisingly, it appears that the relationship is more complex than first assumed, likely because of subtle effects caused by the interactions of the Trp side chain with alternate neighboring residues. This further interaction of the Trp side chain could be studied using single Trp model peptides in which the residues directly next to the Trp residue are varied.

We have shown that the dependence of the fluorescence decay relative proportions on secondary structure can be used to monitor very localized structural changes in a protein at in vivo concentrations. This technique has the potential to be a powerful tool for helping to unravel the rules that govern protein folding.

The authors gratefully acknowledge the technical support of D. T. Krajcarski. We also wish to thank Dr. M. Yaguchi for performing the ESI mass spectrometry and Dr. K. J. Willis for insightful discussions. T. E. S. Dahms is a predoctoral student and was funded by the National Science and Engineering Research Council during this work.

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