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Fluorescence studies of the restricted motion of tryptophan in α-cobratoxin

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Abstract. The fluorescence lifetime and rotational correlation time of the single tryptophan residue in α-cobratoxin have been measured between pH 2 and 10. The fluorescence decays are non-exponential and give lifetimes that are shorter than normally observed in small proteins (0.3 ns and 1 ns). This emission is consistent with a model in which the tryptophan residue is in slightly different environments in the protein. Fluorescence anisotropy decays show that the tryptophan residue is almost completely immobilised by neighbouring groups in the protein. The range of the "wobbling" motion is slightly pH dependent and limited to between 5° and 10°.

Key words: Fluorescence lifetime, tryptophan residues, neurotoxins

1. Introduction

Elapidae venoms possess neurotoxins which block the nicotinic acetylcholine receptor specifically and with high affinity (Lee 1972). The α-cobratoxin is a single-chain peptide consisting of 71 amino acid residues but with only a single tryptophan (Trp) at position 29. Compined CD and NMR data from this toxin shows that, in solution, the β-sheet framework of the molecule is similar to that in the crystal (Walkinshaw et al. 1980). Some parts of the protein, such as in the vicinity of His_{18}, may possess different and quite mobile structures (Hilder et al. 1982). The toxin molecule is held in its rigid conformation by five disulphide bridges, four at the head and one at the tip of the long central loop. When such linkages are broken (Prutz et al. 1982), the β-sheet structure collapses and the polypeptide chain adopts an essentially unfolded form. The central loop contains the "functionally" invariant groups between different toxins and the properties of this loop are of importance in understanding the neurotoxicity of the whole molecule.

The Trp residue forms part of the structure that gives the toxin its curare mimicking properties. We have used fluorescence from this Trp residue to probe the local and environmental perturbation occuring in the toxin molecule.

2. Experimental

The α-cobratoxin (*Naja naja siamensis*) was isolated and purified as described previously (Hider et al. 1982); R. Hider also supplied an additional sample. The solutions were freshly prepared in 0.02 M borate buffer (pH 10), phosphate buffer (pH 7.4) and acetate buffer (pH 4.1). A toxin concentration of 0.01 mg/ml was used.

The fluorescence lifetimes and anisotropy decays were measured by time-correlated, single-photon counting (Tran et al.1982; Beddard et al. 1981), using a mode-locked, argon ion laser to synchronously pump a cavity-dumped, dye laser. The 15 ps dye laser pulses were frequency doubled to 310 nm, at a repetition rate of 10^5 Hz. A block diagram of the instrument is shown in Fig. 1. The fluorescence was measured at 90° to the vertically polarised excitation using interference filters at 314, 340, and 360 nm and laser cut-off filters. Fluorescence was detected through a polariser set at 54.7° to the vertical when fluorescence decays were measured, and set at 0° and 90° for anisotropy measurements. The change in signal between 0° and 90° polariser settings was measured using 2,5-diphenyloxazole in hexane as a calibrant. This molecule rotates rapidly compared to the tryptophan residue.

The anisotropy $r(t)$ was obtained from the observed fluorescence intensities, $I_{\parallel}(t)$ and $I_{\perp}(t)$ using

$$r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2\,I_{\perp}(t)]\,. \quad (1)$$

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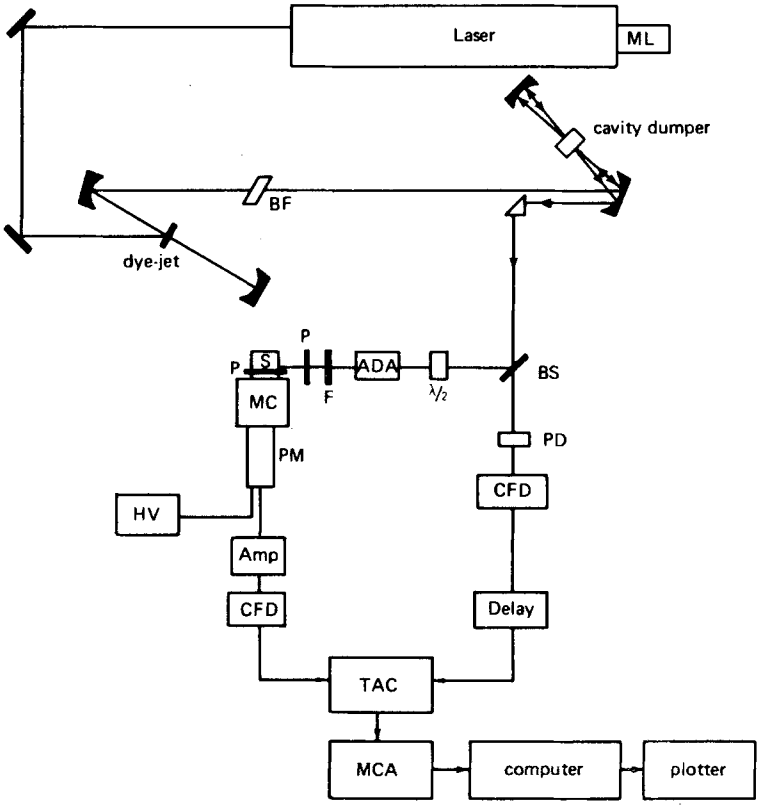

Fig. 1. The optical and electronic arrangement of the single photon counting instrument. *ML* – mode-locker; *BF* – birefringent tuning filter; *MC* – monochromator; *CFD* – discriminator; *TAC* – time to amplitude converter; *MCA* – multichannel analyser; *BS* – beam splitter; *P* – polariser; *PD* – photodiode; *ADA* – frequency doubling crystal; *s* – sample

A two exponential function for $r(t)$, Eq. 2, was used to model the experimentally observed anisotropy $r(t)$.

$$r(t) = r_0 [f \cdot \exp(-t/\varphi_a + (1-f) \cdot \exp(-t/\varphi_b)] . \quad (2)$$

The correlation time, φ_a, arises from the rotational motion of the protein and φ_b derives from the comtined motion of the Trp residue and the rotational diffusion of the whole protein. When the emission dipole in the chromophore (Trp in this case) is parallel to the rotational symmetry axis, the shorter measured correlation time, φ_b, is related to the rotational correlation time of the protein, φ_a, and of the Trp residue (φ_t) by

$$1/\varphi_t = 1/\varphi_b - 1/\varphi_a . \quad (3)$$

The correlation times from three sets of data were averaged and the results are listed in Table 2. A non-linear, least-squares algorithm was used (Tran et al. 1982; Beddard et al. 1981). Analysis using convolution was not necessary for the anisotropy data since the width of the excitation function is much less than the smallest correlation time measured.

The rotational diffusion of the Trp residue is restricted by those residues surrounding it. A related model has been proposed by Kinosita et al. (1977) for probes in (immobile) membranes. In this model, the anisotropy of a probe molecule $r(t)$ is given by

$$r(t)/r_0 = A_\infty + (1 - A_\infty) \cdot \exp(-t/\varphi_t) ,$$

where

$$A_\infty = 1/4 [\cos\alpha (1 + \cos\alpha)^2] . \quad (4)$$

The parameter A_∞, the limiting anisotropy, is equated with f which is measured from the experiment using Eq. 2. In the Kinosita model the semi-angle of the cone through which the probe molecule diffuses is denoted by α. We use α as a measure of the relative freedom of motion of the Trp within the protein.

Fluorescence lifetime data, $I_{54.7}$ (t), were also analysed by an equation of the same form as Eq. 2 and are shown in Table 1, with lifetimes τ_1 and τ_2 replacing the correlation times φ_1 and φ_2. Allowance for background counts and "lamp-shift", if needed, were also incorporated into the data analysis. In each experiment the χ^2 parameter was smaller for two exponential fitting (Eq. 2) than for a single exponential. This was true of both the anisotropy and fluorescence lifetime data. The reduced χ^2 for fluorescence lifetime data was less than 1.2 with fitting over all data points.

Table 1. Fluorescence lifetimes of α-cobratoxin at different pH, excited at 310 nm

pH	λ (nm)	τ_1 (ns)	f	τ_2 (ns)	τ_{av} (ps)
2.0	314	1.17 ± 0.10	0.54	350 ± 60	794
	340	1.17 ± 0.12	0.55	350 ± 75	799
	360	1.19 ± 0.10	0.56	380 ± 90	832
4.1	314	1.27 ± 0.11	0.48	370 ± 60	795
	340	1.26 ± 0.09	0.49	350 ± 70	800
	360	1.32 ± 0.11	0.50	400 ± 70	860
7.4	314	1.16 ± 0.10	0.45	240 ± 70	660
	340	1.16 ± 0.09	0.48	240 ± 50	682
	360	1.25 ± 0.16	0.49	260 ± 60	748
10.0	314	1.28 ± 0.08	0.31	360 ± 20	647
	340	1.25 ± 0.12	0.31	350 ± 40	626
	360	1.38 ± 0.13	0.31	400 ± 40	703

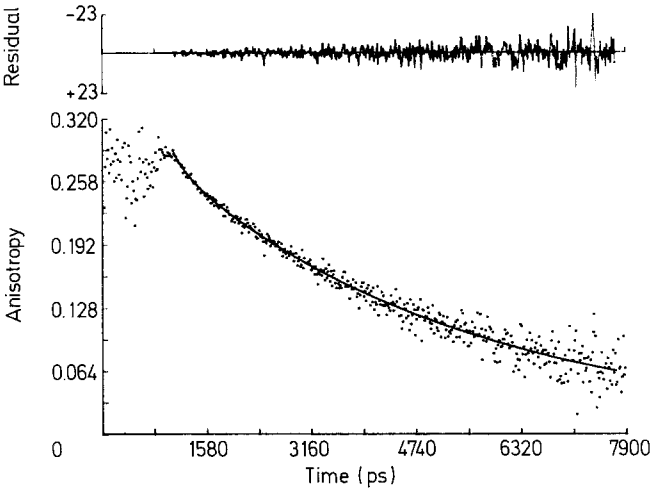

Fig. 2. Fluorescence anisotropy decay at pH 10. The solid line is the fitted curve of two exponentials of 258 ps and 4.5 ns. The top curve shows the residuals between the data and the calculated fit

3. Results and discussion

As with other proteins containing a single Trp, the fluorescence decays are not described by a single exponential function but, rather, by the sum of two exponentials. In the cobratoxin the two lifetimes measured were approximately 1 ns and 0.3 ns. The values are listed in Table 1. Both of these lifetimes are considerably shorter than for other small single-tryptophan containing proteins, such as glucagon (1.1 ns, 3.5 ns) (Tran et al. 1982) and melittin (0.7 ns, 3.1 ns) (Tran and Beddard 1984), and indicate very effective quenching mechanisms in the toxin. By comparison, the fluorescence lifetime of the non-perturbed chromophore in indole is 9.6 ns at pH 7 (Beddard et al. 1981).

The mean fluorescence lifetime τ_{av} is proportional to the fluorescence quantum yield. Values of τ_{av} are also listed in Table 1. At above pH 7 a slight quenching, of about 15%, is observed relative to the value at pH 2. Similar quenching was also observed by Menez et al. (1980) who measured relative yields in the toxin from *Naja nigricollis*.

The theory of fluorescence depolarisation has previously been described by Rigler and Ehrenberg (1973) who show that the motion of both the probe (Trp residue) and of the whole protein is reflected in the fluorescence anisotropy decay. Figure 2 shows some anisotropy data taken at pH 10. As the measured rotational correlation times (φ_a, φ_b) are different by a factor of 10, we assign the longer lifetime (φ_a) to the motion of the whole protein and the shorter to the restricted motion of the Trp residue, $\varphi_t = \varphi_b$.

The X-ray data (Walkinshaw et al. 1980) show that the protein appears to be more disc-like than

Table 2. Fluorescence anisotropy decay parameters of α-cobratoxin at different pH, detected at 340 nm, excited at 310 nm

pH	φ_b (ps)	$1-f$	φ_a (ns)	α (degrees)
2.0	330 ± 100	0.01	4.69 ± 0.69	5
4.0	300 ± 60	0.03	5.19 ± 0.65	8
7.4	540 ± 100	0.02	5.38 ± 0.30	7
10.0	250 ± 20	0.05	4.43 ± 0.37	10

spherical and measures about 3.0 by 2.5 by 1.5 nm. The backbone of the molecule in D_2O is similar to that in the crystal (Hider et al. 1982). The Trp and surrounding residues protrude from near the centre of the disc. Because of its position the Trp residue's emission dipole is parallel to the rotational symmetry axis and the fluorescence will not be depolarised by rotation of this disc about this axis and only a tumbling motion normal to it will have any effect. Using formulae for an oblate spheroid (Tao 1969) a rotational correlation time in the region of 4 ns is calculated which is in agreement with the measured φ_a values in Table 2.

Using the "wobbling in a cone" model for restricted motion (Kinosita et al. 1977; Lipari and Szabo 1980), the cone semi-angle (α) is related to the fraction ($1-A_\infty$) of the fluorescence anisotropy decay belonging to this local motion. Values of this angle are very small in the α-cobratoxin and increase slightly with pH (5°–10°) (Table 2). Similar measurements on other small proteins give much larger values of α, for example, in glucagon α = 30°–50° and in melittin α = 45°–55°. The theory on which the estimates of α are based was formulated for membranes and may not be exactly applicable to proteins, as lipid membranes and proteins have differing

inter-molecular potentials. The restricted motion that the Trp residue experiences, is observable directly from the anisotropy data as the small fraction of the total anisotropy decay that has a short correlation time. If the Trp residue was able to rotate more freely, much more of the initial anisotropy would be lost in this process alone.

The limiting anisotropy (r_0) which has a value of 0.28–0.3 shows that no depolarisation occurs faster than the instrumental time resolution: the maximum r_0 value for N-acetyltryptophanamide in glycerol is 0.3 (Lakowicz et al. 1983). The viscosity of the glycerol was 1,750 cp and no depolarisation due to Brownian motion occured at the temperatures used.

The origin of the non-exponential fluorscence decays in tryptophan and related Trp containing peptides has been extensively investigated (Chang et al. 1983; Beddard et al. 1981). In these compounds two models have been favoured; firstly, one in which the amino acid backbone alters the quenching rate in a dynamic manner, depending upon the distance and orientation of the quencher from the excited moleculs; and secondly a static model in which conformations about the $C_\alpha-C_\beta$ bond in tryptophan do not interconvert during the excited state lifetime.

Evidence has recently been obtained from tryptophan derivatives in solution which gives support to the conformer model (Chang et al. 1983). In solution tryptophan derivatives can exist in three conformers about the $C_\alpha-C_\beta$ bond. The shorter of the two fluorescence lifetimes arises from the conformer(s) with electrophilic groups nearest to the indole chromophore. The excited state quenching is due to charge-transfer from the indole to suitable acceptors on the amino acid backbone. A theory of charge-transfer from electronically excited molecules has recently been proposed by Hopfield (1977). The rate of charge-transfer is, by analogy to dipole energy-transfer, dependent upon the overlap of "electron removal" and "eleltron insertion" spectra of the donor and acceptor groups respectively, as well as their separation.

Models explaining the fluorescence behaviour of tryptophans in solution do not necessarily provide a complete description of the quenching of Trp residues in a protein. The conformation about the $C_\alpha-C_\beta$ bond is no longer of such importance, as quenching is not due to the immediate amino acid backbone, but to some electrophilic groups on adjacent residues. The distance apart and orientation of these other groups will be different from one protein to another and both could change as a result of, for example, random Brownian motions, pH, temperature or protein aggregation effects. Additionally, the rotational correlation times in proteins have to be reconciled with the two observed fluorescence lifetimes (Munro et al. 1979; Beddard et al. 1981; Tran et al. 1982; Lakowicz et al. 1983).

The peptide group is a poor electrophile and in the Trp residue induces little charge-transfer from the adjacent indole chromophore. This is demonstrated in human serum albumin (HSA), where the Trp is buried in a hydrophobic region, and the fluorescence lifetimes are long, 9.4 ns ($f = 0.95$) and 2.8 ns (Munro et al. 1979). The fluorescence anisotropy had a 31.4 ns component due to the motion of the whole protein and a faster one ($\varphi = 0.7$ ns at 316 K) due to restricted motion of the Trp. The cone semi-angle is about 26° which is a little larger than that seen in the cobratoxin. In glucagon the Trp fluorescence lifetimes are pH and concentration independent ($\tau = 1.1$ ns and 3.5 ns) (Beddard et al. 1981), and with φ equal to 0.4 ns and 1.8–2.8 ns depending upon temperature. On the other hand, in melittin the fluorescence lifetimes are pH and concentration dependent ($\tau =$ 0.6–0.7 ns and 2.9–3.1 ns) and $\varphi = 0.5$–0.7 ns and 1.7–2.3 ns (Tran and Beddard 1985). In these last two examples, as in cobratoxin, charge-transfer to electrophiles other than the peptide group is apparently occurring. Of the groups available in a protein the amino groups are likely to be good charge acceptors, particularly when protonated. Amide groups and to a lesser extent $-CH_3$ and $-COO^-$ groups may also participate. From a consideration of a number of sequences related to α-cobratoxin (Dufton and Hider 1983), the conserved residues, lysine 23 and arginine 33, could quench the Trp fluorescence and the large degree of quenching is consistent with close packing of the Trp with adjacent residues, as implied by the anisotropy data.

A model of the Trp residue photophysics should be consistent with both the dual nature of the fluorescence lifetime and the single rotational correlation time (φ_b) of the Trp in the protein. The changes in absorption and emission spectra between hydrophilic and hydrophobic sites should also be explained. The radiative rate of tryptophan changes slowly with temperature (10 °C–70 °C) (Kirby and Steiner 1970) and hence any temperature dependences of τ and φ are caused by protein folding. Similarly, changes of τ or φ with protein concentration are primarily due to aggregation effects on the Trp environment.

The fluorescence lifetime and rotational correlation time per se are independent of one another since the molecular mechanisms giving rise to them are different. Short-range repulsions between atoms on adjacent residues and solvent molecules give rise to the rotational correlation time. In some small proteins, such as glucagon, solvent water molecules are adjacent to the Trp residue but this is not so in HSA. The fluorescence lifetime is determined by longer

range interactions than occur during collisions, such as proton-, energy-, or charge-transfer.

Even though τ and φ are independent per se, the measured fluorescence anisotropy can be a function of the excited state decay. This occurs when one or more chromophores are in distinct environments in the protein. The measured anisotropy for the case where two tryptophans in a single protein have different τ and φ is

$$r_m(t) = [f \cdot \exp(-t/\tau_1) \cdot r_1(t) + (1-f) \cdot \exp(-t/\tau_2) \cdot r_2(t)] / [f \cdot \exp(-t/\tau_1) + (1-f) \cdot \exp(-t/\tau_2)] .$$

Species 1 and 2 have anisotropy $r_1(t)$ and $r_2(t)$ and fluorescence lifetimes τ_1 and τ_2. In order to apply this model to the cobratoxin data, or even that of glucagon or melittin, the Trp must have two or more radically different local conformations. In one of these the Trp would exhibit rapid rotational relaxation while in the other(s) it would be held fixed for periods longer than several fluorescence lifetimes. This model may be valid for some proteins where there is compelling evidence other than fluorescence data, but it is unsatisfactory as a general explanation.

A more feasible explanation is that each fluorescence lifetime is the result of specific interactions of the Trp in each conformation. In each of these positions the Trp is not held rigidly, but undergoes limited rotational relaxation with a lifetime of about 0.5 ns. The repositioning of the Trp from one conformation to another could be large when brought about by a movement of the backbone $-C-CONH-$ group but smaller when caused by rotation about its own $C_\alpha - C_\beta$ bond. The energy barrier to movement is provided by the surrounding residues. The exponentially decreasing dependence of charge-transfer with separation of the donor and acceptor groups would explain the different excited state lifetimes observed in different conformers. As two distinct fluorescence lifetimes are observed the movement from one conformer position to another must be slow compared to these lifetimes. If the movement between conformer positions were fast, then a single averaged lifetime would be observed, containing components from each conformer in proportion to its stability.

The influence that pH has on the cobratoxin conformation has been investigated by NMR and CD (Hider et al. 1982; Dufton and Hider 1983). They observed that His_{18} is sensitive to pH but is too far away to influence the Trp directly, and also that there is a change in the backbone near to one, or more, of the disulphide bridges and Tyr_{21}. The disulphide bridge adjacent to the Trp is not implicated directly in the pH dependent changes (Hider et al. 1982). It seems therefore, that in this instance the fluorescence from the Trp is more sensitive to conformational changes than are CD or NMR. The mean lifetime (τ_{av} in Table 1) decreases above pH 6, not as a result of changes in τ_1 and/or τ_2, but as a result of the change in the fraction(f) of Trp residues in each conformation.

A change in fluorescence lifetimes was also observed at different emission wavelengths. The mean lifetime increases slightly as the wavelength increases and is mainly the result of changing the proportions of species with lifetimes τ_1 and τ_2 in the total emission. The Trp with the longer fluorescence lifetime also fluoresces at the longer wavelength. The changes with wavelength as well as pH (Table 1) support our model of Trp in different conformational sites in the protein.

Wavelength-dependent fluorescence lifetimes imply a time-resolved emission spectrum. This can have a trivial origin, such as a change in the proportions of species emitting at different wavelengths, or a more complicated origin caused by relaxation of the excited state due to interactions with the environment. The trivial case occurs for Trp in proteins and is evident in the data from α-cobratoxin. The extent to which the latter effect is also present is unclear, but may be estimated from a theory of time-resolved changes in fluorescence spectra recently proposed by Bagchi et al. (1984). The time-resolved change in the fluorescence spectral maximum was formulated in terms of the dipolar interaction between a solute and a surrounding continuum of solvent molecules. The stabilization energy of the excited state was calculated and related to the change in fluorescence maxima. The stabilization energy depends upon the change in magnitude and orientation of the dipole moment between the ground and excited states, the solvent dielectric constants and Debye relaxation time, and the solute rotational diffusion coefficient.

When the excited molecule is in a low dielectric cavity, only small shifts in the fluorescence maxima are observed compared to the vapour. The Trp residues with shorter lifetimes are in this class as their emission is to shorter wavelengths. The fact that the excited state stabilization energy depends upon the rotational correlation time could affect both the fluorescence lifetime and anisotropy data. When the time scale for stabilization is comparable to the time for fluorescence emission, the wavelength sweep of the fluorescence emission could distort the measured fluorescence decays. In water, however, the dynamics of the fluorescence shift are calculated to be very rapid (< 1 ps) and should probably not affect the present fluorescence data. When the Trp is in a hydrophobic site, such as in HSA, or where there is

high viscosity the excited state stabilisation should take longer and could therefore influence the fluorescence decays and hence anisotropy.

4. Summary

The fluorescence from the Trp residue in α-cobratoxin decays bi-exponentially with lifetimes of about 0.3 ns and 1.1 ns. These fluorescence lifetimes are slightly pH and emission wavelength dependent. The large excited state quenching causing these lifetimes is due to charge-transfer to nearby electrophilic groups such as arginine_{33} and lysine_{23}. The two lifetimes are explained in terms of a conformer model in which limited rotational diffusion is allowed in each site.

Fluorescence anisotropy measurements show that the Trp is held within 5°−10° during the fluorescence decay. The rigidity of the tryptophan might confer biological function; since the Trp residue is in the "active site" of the toxin, the rigidity of this region may contribute to the high affinity of the toxin for its receptor.

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