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Aggregation of *Naja Nigricollis* Cardiotoxin: Characterization and Quantitative Estimate by Time-Resolved Polarized Fluorescence

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After purification to homogeneity by Bio-Rex 70 ion exchange chromatography, micromolar solutions of *Naja nigricollis* cardiotoxin were found to contain significant amounts of aggregates, as detected by time-resolved polarized fluorescence of its single tryptophan residue. The level of cardiotoxin aggregation depends strongly and reversibly on the protein concentration and pH. However, supplementary reverse-phase HPLC completely suppresses this aggregation, resulting in all cases in fluorescence anisotropy decays characteristic of the pure cardiotoxin monomer. The self-association properties of cardiotoxin, in the presence of a possible cofactor eliminated by the HPLC step, may be functionally relevant, and would deserve further investigation. The physical heterogeneity of the cardiotoxin samples required an appropriate model for the analysis of fluorescence depolarization, which was iteratively improved by comparison with experimental results. In this way, an approximate molar fraction of 10–15% aggregated cardiotoxin at a 90 μM total protein concentration, pH 7, was determined. The fluorescence of the partly aggregated samples is significantly perturbed as compared to the HPLC-treated monomer, indicating that the cardiotoxin aggregate must have an increased average fluorescence lifetime and a strongly decreased initial anisotropy. The decrease in initial anisotropy suggests either an increased mobility of the tryptophan residue upon aggregation or fast energy transfers between residues of different cardiotoxin molecules brought within a short distance in the aggregate. This study illustrates the high sensitivity of the time-resolved fluorescence technique, through both total fluorescence and anisotropy parameters, to low levels of physical or chemical heterogeneity in a protein sample.

KEY WORDS: Lifetime distributions; heterogeneities; associated dynamics.

INTRODUCTION

Cardiotoxins are potent cytotoxic proteins found exclusively in the venom of Elapid snakes [1]. They are small (MW 6800) and strongly basic proteins and share with postsynaptic neurotoxins a characteristic main chain folding along three loops rich in β-sheet, held together by a central core of disulfide bridges [2]. Although the detailed mechanism of cardiotoxin action *in vivo*, leading to cardiac arrest, is not fully understood, it is widely documented that the toxin binds strongly to cell membranes and induces various structural perturbations of the bilayer [3]. Depolarizing and lytic activities of *Naja nigricollis* cardiotoxin are well correlated with its lethality [4]. During a study of the time-resolved

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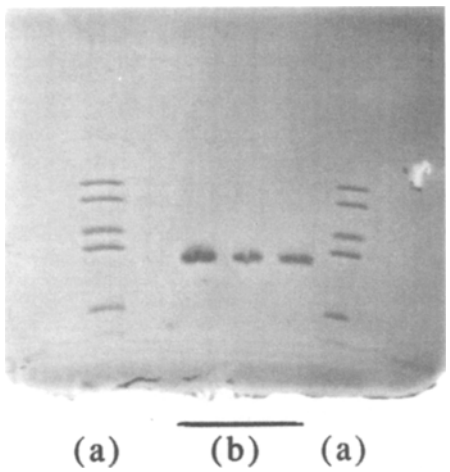

Fig. 1. SDS-PAGE pattern of cardiotoxin after Bio-Rex ion exchange chromatography: (a) molecular weight standards, 2512, 6214, 8159, 14,404, and 16,949 D; (b) cardiotoxin, dilution range 1 to 3. Twenty percent acrylamide, 30% ethylene glycol, Coomassie staining.

fluorescence of the unique tryptophan residue (Trp11) of this toxin [5], we found that, unless they were treated by additional RP-HPLC, homogeneous cardiotoxin samples exhibited significant amounts of aggregation. Self-association of cardiotoxin in solution has not, to our knowledge, been previously reported. A better control of the physical state of cardiotoxin may nevertheless be important, not only for the correct interpretation of spectroscopic data on the isolated protein, but also for both *in vivo* and *in vitro* studies of cardiotoxin activity.

Polarized fluorescence techniques are extremely sensitive methods for the detection of protein oligomers and aggregates at low concentration. This may be useful for the physical characterization of purified native or recombinant proteins [6–8] and in the study of protein association equilibria [9–11] and early steps of protein crystallization [12]. The reporter group may be either intrinsic, by exploiting the naturally fluorescent aromatic residues, or extrinsic, with fluorescent chemical derivatives of the protein. In principle, time-resolved experiments may provide further informations such as molecular axial ratios [13,14], stoechiometry of equilibria, and dissociation constants [9,11].

However, the method meets some limits when definite quantitative informations are sought. Problems arise when heterogeneities are simultaneously present in the fluorescence and the depolarization kinetics, which is most often the case in an aggregated protein mixture. In this case, the analytical form of the anisotropy decay must associate each depolarization process with its particular fluorescence kinetics [15]. The classical description of anisotropy decays does not allow this separate treatment. As shown by simulations and experimental studies [16–18], this may lead in some cases to large distorsions in the extracted parameters. For protein mixtures containing a limited number of components, some of them having been characterized in isolated form by independent measurements, simplified alternative models may be derived. In the case of cardiotoxin, we have exploited both time-resolved and steady-state information obtained on the pure monomer to reach a reasonable quantitative estimate of the aggregation level in cardiotoxin samples.

EXPERIMENTAL PROCEDURES

Purifications and Materials

Cardiotoxin B and BB

Cardiotoxin was isolated from the total venom of *N. nigricollis* (Pasteur Institute, Paris), as previously described [19,20]. The different steps of this purification are (i) filtration on a Sephadex G75 molecular sieve, (ii) one (cardiotoxin B) or two (cardiotoxin BB) chromatographies on a Bio-Rex 70 Na^+ columns, and (iii) a final lyophilization. After purification by Bio-Rex ion exchange chromatography, cardiotoxin samples are homogeneous in protein content, as checked by SDS-PAGE electrophoresis (Fig. 1) and are reported to contain less than 1% (w/w) of phospholipase A_2 (PLA_2) [20].

Cardiotoxin H

After two Bio-Rex chromatographies, the cardiotoxin samples were run through a HPLC chromatography which is a slight modification of the procedure of Gatineau *et al.* [21]. Approximately 0.7 mg of protein in 100 ml was injected on a Vydac TP-C4 column equilibrated with 0.1% (v/v) trifluoroacetic acid in water (solvent A). Elution is achieved with a gradient of 70% acetonitrile in solvent A (solvent B) from 0 to 60% of B in A during 45 min. The elution profile according to the ODs at 230 and 278 nm are reproducible and show a strongly dominant fraction (Fig. 2) which was collected and lyophilized. Reverse-phase HPLC is reported to reduce further the PLA_2 contamination, to less than 0.001% [22].

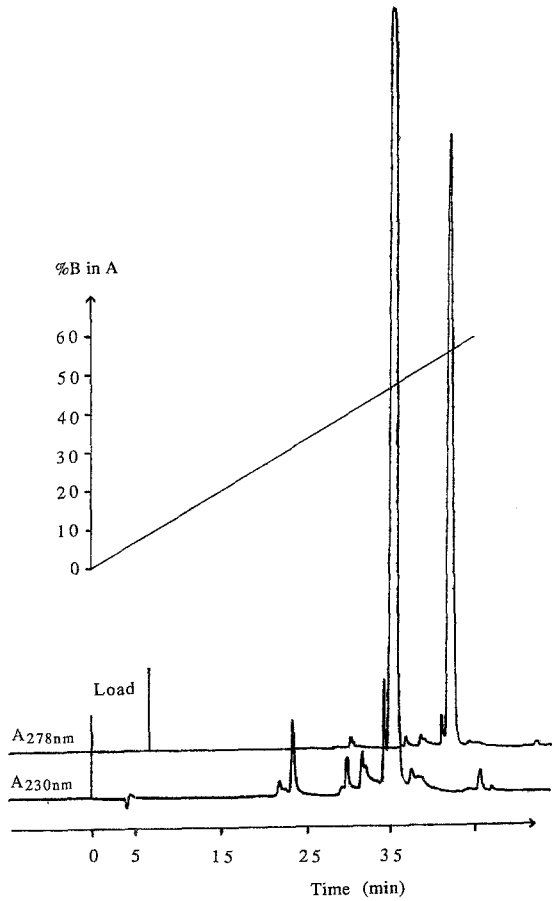

Fig. 2. Reverse-phase HPLC elution profile of cardiotoxin H, after two prior Bio-Rex ion exchange chromatographies.

Sample Preparation for Experiments

Before measurements, the toxins were redissolved and the samples were applied to a Sephadex G25M column equilibrated with the working buffer, a 10 m*M* cacodylate buffer, 1 m*M* NaCl, 0.1 m*M* EDTA, pH 7.0. The fluorescence of the eluant buffer immediately ahead of the protein fractions was less than 1% of the protein fluorescence and, thus, was not subtracted in the time-resolved fluorescence experiments.

Materials

HCl, NaCl, EDTA, and cacodylate were of the highest grades from Merck; the HPLC Vydac TPC_4 column was from Waters Associates; trifluoroacetic acid was from Fluka Chimie AG and acetonitrile from Hipersolv.

Steady-State and Time-Resolved Fluorescence Measurements

Steady-state fluorescence spectra, total fluorescence decays, and fluorescence anisotropy decays were recorded and analyzed as described in detail previously [5]. The excitation was set at 300 nm ($\Delta\lambda$ = 6 nm) and emission observed at 347 nm ($\Delta\lambda$=6 nm). Under these conditions, the relative level of scattered light (Rayleigh+Raman) in the fluorescence signal was lower than 1%, as checked with a Ludox solution. In the present case, we had to detect very small differences between complex fluorescence lifetime distributions. This requires the repeated collection of data to high counting statistics and free of any significant systematic noise. In addition, analysis of the fluorescence decays must correctly take into account small variations in optical delays between the instrumental function and the fluorescence [5]. Finally, the number of iterations performed under maximum entropy analysis using MEMSYS2 subroutines [23] must be controlled by a stopping criterion based on the effective improvement of the fit, such as variations in χ^2 [5,24]. These precautions are necessary to minimize the dispersion of recovered lifetime profiles due to the fit of different data sets under nonequivalent conditions. The different parameters given in the tables are obtained from the distribution profiles recovered from maximum entropy analysis by integration over separate peaks as described in Ref. 5.

Analysis of Fluorescence Anisotropy Decays

The classical analysis of fluorescence anisotropy decays usually assumes, explicitly or implicitly, the following form for the two polarized components of the fluorescence decay:

$$I_{vv}(t) = g(t) * \int_0^\infty \alpha(\tau)\, e^{-t/\tau} \left(1+2\, r(t)\right) d\tau \quad (1)$$

$$\beta I_{vh}(t) = g(t) * \int_0^\infty \alpha(\tau)\, e^{-t/\tau} \left(1-r(t)\right) d\tau \quad (2)$$

where $\beta(t)$ is an experimental weighting factor determined as described in [5], $g(t)$ is the instrumental function, $\alpha(\tau)$ is the distribution of amplitudes of the fluorescence lifetimes and $r(t)$ is the fluorescence anisotropy decay, assumed to be the same for all fluorescent species in the sample. This last hypothesis allows the anisotropy term to be factored out of the integral in (1) and (2) and thus simplifies greatly the analysis. This description is, however, not correct if the sample contains

several species having different fluorescence kinetics and anisotropy decays [see, for example, Eqs. (14) for the cardiotoxin case].

Under this approximate model, we found previously [5] that the solutions obtained from maximum entropy analysis of the anisotropy decays of cardiotoxin could be equated in all cases to the simplified discrete form:

$$r(t) = r_i \left(\varphi_c\, e^{-t/\theta_c} + \varphi_\infty \right) = r_c\, e^{-t/\theta_c} + r_\infty \qquad (3)$$

where r_c and θ_c are the amplitude and relaxation time of the time-dependent part of the anistropy decay, and r_∞ is a constant term, discussed under Results. The total amplitude of the decay, $r_i = r_c + r_\infty$, corresponds to the fluorescence anisotropy extrapolated at zero time, which, in the absence of fast depolarization processes, should approach the fundamental value of 0.300 expected for tryptophan excited at 300nm [25]. For samples where r_∞ is zero, the anisotropy decay reduces to a single exponential term, and the relaxation time θ_c can be equated to the rotational correlation time for the tumbling of the cardiotoxin molecule, approximated to a rigid sphere, and given by the Einstein–Stockes relation:

$$\theta_c = \frac{\eta V}{kT} \qquad (4)$$

η being the viscosity of the solvent, V the hydrated molecular volume of cardiotoxin, k the Boltzmann constant, and T the temperature.

For illustration purposes exclusively, an experimental, undeconvoluted anisotropy decay function is defined as

$$r_{exp}(t) = \frac{I_{vv}(t) - \beta\, I_{vh}(t)}{I_{vv}(t) + 2\,\beta\, I_{vh}(t)} \qquad (5)$$

Description of Mixtures of Monomeric and Aggregated Cardiotoxin

Average Fluorescence Lifetime and Steady-State Anisotropy

Independently of any assumption about fluorescence kinetics and depolarization processes, if $\bar{\tau}_f$ is the first-order average fluorescence lifetime of a fluorescent solution and $\bar{r}$ its steady-state fluorescence anisotropy, defined by

$$\bar{r} = \frac{\int_0^\infty I_{vv}(t)\, dt - \beta \int_0^\infty I_{vh}(t)\, dt}{\int_0^\infty I_{vv}(t) + 2\,\beta\, I_{vh}(t)\, dt} \qquad (6)$$

the following additivity relationships may easily be shown, for a mixture of several non-interacting fluorescent components j:

$$\bar{\tau}_f = \sum_j c_j\, \bar{\tau}_j \qquad (7)$$

$$\bar{\tau}_f\, \bar{r} = \sum_j c_j\, \bar{\tau}_j\, \bar{r}_j \qquad (8)$$

were cj is the fractional amplitude of component j in the fluorescence kinetics, which may be taken in first approximation as its molar ratio in the mixture, assuming similar absorption coefficients, radiative lifetimes and emission spectra for all components.

Let $\bar{\tau}_M$, $\bar{r}_M$, $\bar{\tau}_A$, $\bar{r}_A$, $\bar{\tau}_X$, and $\bar{r}_X$ be the average fluorescence lifetimes and steady-state anisotropies of (M) the cardiotoxin monomer, (A) the cardiotoxin aggregate, and (X) a mixture of the monomer with an unknown proportion x of aggregated cardiotoxin molecules, respectively. The properties of the aggregate may be expressed as functions of the experimentally determined values of the monomer and the mixture, and of the unknown fraction x:

$$\bar{\tau}_A = \frac{\bar{\tau}_X - (1-x)\, \bar{\tau}_M}{x} \qquad (9)$$

$$\bar{r}_A = \frac{\bar{\tau}_X \bar{r}_X - (1-x)\, \bar{\tau}_M \bar{r}_M}{\bar{\tau}_X - (1-x)\, \bar{\tau}_M} \qquad (10)$$

The relative error $\Delta\bar{r}_A/\bar{r}_A$ is obtained by derivatizing (10) and expressed as a function of x, $\Delta\,\bar{\tau}_M$, $\Delta\,\bar{\tau}_X$, $\Delta\,\bar{r}_M$, and $\Delta\,\bar{r}_X$.

Time-Resolved Fluorescence

Polarized Fluorescence Decays of Monomeric Cardiotoxin. The fluorescence properties of cardiotoxin H at 20°C, pH 7, are taken as those of the pure monomeric protein. Since the anisotropy decay of cardiotoxin H is described by a single exponential term [5], the polarized fluorescence decays of the monomer may be written as

$$I^M_{vv}(t) = g(t) * \int_0^\infty \alpha_M(\tau)\, e^{-t/\tau} \left(1 + 2\, r_{0M}\, e^{-t/\theta_M}\right) d\tau \qquad (11a)$$

$$I^M_{vh}(t) = g(t) * \int_0^\infty \alpha_M(\tau)\, e^{-t/\tau} \left(1 - r_{0M}\, e^{-t/\theta_M}\right) d\tau \qquad (11b)$$

where $\alpha_M(\tau)$ is the lifetime distribution determined for cardiotoxin H at 20°C, pH 7, $r_{0M} = 0.263$ is the exper-

Table I. Parameters of the Fluorescence Anisotropy Decays of Cardiotoxin After Different Final Purification Steps[a]

Cardio-toxin	$r_i \pm$ 0.006	r_i/r_o (%)	θ_c (ns) $\pm$ 0.2	$r\infty/r_i$ (%)	$\bar{r} \pm$ 0.004	χ^2
H[b]	0.263	88	3.6	< 0.2	0.180	1.12
B[c]	0.252	84	2.5	26	0.174	1.01
BB[d]	0.262	87	2.8	21	0.180	1.06
B, diluted[e]	0.135	84	2.4	17	0.084	1.06

[a]Toxin concentrations 80–90 μ*M*, 20°C, pH 7, λ_{exc} = 300 nm, and λ_{em} = 347 nm, unless otherwise specified.
[b]Two Bio-Rex chromatographies followed by a reverse-phase HPLC.
[c]One Bio-Rex, no HPLC.
[d]Two Bio-Rex, no HPLC.
[e]Toxin concentration 5.4 μ*M*, pH 4, λ_{exc} = 290 nm.

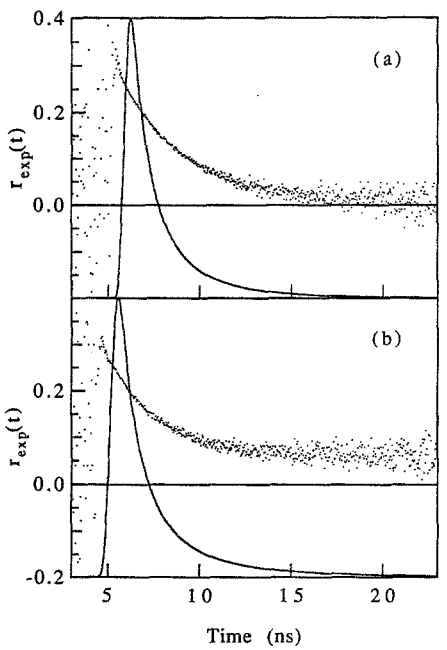

Fig. 3. Experimental anisotropy decays (points) of (a) cardiotoxin H and (b) cardiotoxin B at 20°C, λ_{exc}=300 nm, linear scale. Continuous lines are the corresponding normalized fluorescence decays. Other experimental conditions as in Table I.

imental initial anisotropy of cardiotoxin H, and θ_M = 3.59 ns is the measured rotational correlation time of cardiotoxin H [5].

Polarized Fluorescence Decays of Aggregated Cardiotoxin. Since the rotation of the aggregate is too slow to induce a significant depolarization of the fluorescence during the lifetime of the excited state, the anisotropy is described by a single constant term $r_A(t) = r_{0A} = \bar{r}_A$. Therefore, the polarized fluorescence decays of the aggregate are

$$I^A_{vv}(t) = g(t) * \int_0^\infty \alpha_A(\tau)\, e^{-t/\tau} \left(1+2\, r_{0A}\right) d\tau \quad (12a)$$

$$I^A_{vh}(t) = g(t) * \int_0^\infty \alpha_A(\tau)\, e^{-t/\tau} \left(1 - r_{0A}\right) d\tau \quad (12b)$$

On the other hand, the fluorescence lifetime distribution of aggregated cardiotoxin $\alpha_A(\tau)$ is assumed to be shifted by a factor K, compared to the monomer:

$$\alpha_A\left(\tau \cdot K\right) = \alpha_M(\tau) \quad (13)$$

Mixture of Aggregated and Monomeric Cardiotoxin. Given an aggregation ratio x, the corresponding average fluorescence lifetime $\bar{\tau}_A$ and initial anisotropy $r_{0A} = \bar{r}_A$ of the aggregate are computed from experimental data using (9) and (10). This in turn gives the factor K for the computation of $\alpha_A(\tau)$ in (13). The polarized fluorescence decays of the mixture are then computed as weighted sums of the polarized emissions of the monomer and the aggregate:

$$I^X_{vv}(t) = (1-x)\, I^M_{vv}(t) + x\, I^A_{vv}(t) \quad (14a)$$

$$I^X_{vh}(t) = (1-x)\, I^M_{vh}(t) + x\, I^A_{vh}(t) \quad (14b)$$

Preparation and Analysis of the Synthetic Data. The appropriate distributions of relaxation terms for each polarized component are convoluted with a measured instrumental function. The convolutions are then scaled to give a final number of counts of 20×10^6 in the total decay, to approach realistic experimental statistics, and finally, a quasi-Gaussian noise is added [24]. These synthetic data are then analyzed by the maximum entropy programs in the same way as experimental data.

RESULTS

Aggregation in Cardiotoxin Samples

Table I gives the parameters of the fluorescence anisotropy decays obtained on different cardiotoxin samples at 20°C. The anisotropy decay of cardiotoxin H, after a final reverse-phase HPLC, is described by a single relaxation term, θ_c=3.6 ns, corresponding approximately to the expected rotational correlation time of the monomeric protein [5]. In cardiotoxin B, in which this last HPLC step has been omitted, the experimental anisotropy clearly does not decay to zero but includes an additional constant amplitude (Fig. 3). This constant term r_∞ amounts to about 25% of the total amplitude of the decay, while simultaneously the apparent relaxation

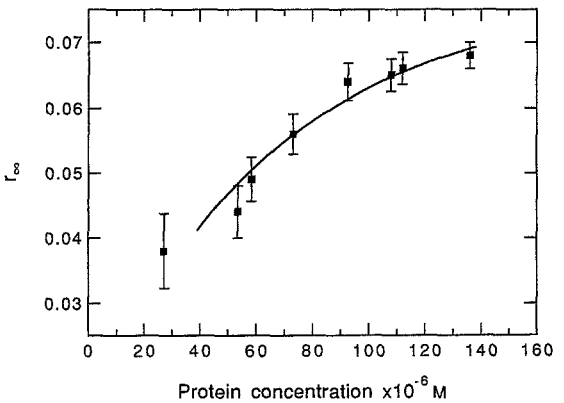

Fig. 4. Amplitude of the constant term r_∞ in the fluorescence anisotropy decays of cardiotoxin B, as a function of the protein concentration.

time θ_c is strongly decreased (Table I). Repeating twice the Bio-Rex ion exchange chromatography (cardiotoxin BB) does not significantly modify these results.

A constant anisotropy indicates very slow depolarization by the tumbling of high molecular weight components, described by very long (unmeasurable) rotational correlation times. Therefore, cardiotoxin B and BB contain significant proportions of a large component, presumably some cardiotoxin aggregates. In practice, the maximum measurable correlation time is about 15 to 20 times the average fluorescence lifetime [26], giving in the present case more than 20 ns, which would correspond to the rotation of cardiotoxin oligomers of at least five or six molecules. However, these aggregate samples did not show any eye-detectable turbidity. Their absorption spectra were identical to those of cardiotoxin H, the ratio of optical densities at 240 and 280 nm being in all cases about 0.50, indicating very weak light scattering.

Dependence of Cardiotoxin Aggregation on Concentration and pH

The partial amplitude r_∞/r_i, as obtained from a fit of the data to Eqs. (1), (2), and (3), reflects in first approximation the fraction of tryptophan chromophores attached to the contaminant aggregate. However, the use of the above equations is fully valid only if the different tryptophan species have identical fluorescence kinetics, which is not the case here (see below). In this situation, r_∞ becomes a complex function of the aggregation molar ratio. Nevertheless, the apparent value of r_∞ clearly increases with protein concentration, for different experiments performed on cardiotoxin B between 30 and 140 μM (Fig. 4). The low concentration points in this curve were obtained by direct dilution of the high concentration ones, showing, in addition, that this aggregation could be readily reversed.

Very low concentrations were difficult to study because of the low fluorescence signal. A sample of cardiotoxin B was prepared at a concentration of 5.4 μM (corresponding to a dilution factor of 15) and pH 4 (the cardiotoxin fluorescence decays are unchanged from pH 8 to pH 3 [27]). The excitation wavelength was set to 290 nm instead of 300 nm, to compensate for the lower fluorescence intensity of this sample. At this excitation wavelength, with a bandwidth $\Delta\lambda=6$ nm, the average fundamental anisotropy r_0 of tryptophan is 0.160 instead of 0.300 [25]. As a consequence, the absolute values of the initial and steady-state anisotropies are decreased, while the ratio r_i/r_0 remains unchanged (Table I). In this experiment, the relative amplitude of the infinite term is significantly decreased but still not completely suppressed.

On the other hand, the level of Rayleigh scattering of cardiotoxin B, as obtained during steady-state fluorescence measurements, is significantly higher than that of cardiotoxin H and shows a strong pH dependence, with a sharp and reversible increase across the isoelectric point of cardiotoxin, near pH 10 (Fig. 5). This shows further that a reversible pH-dependent aggregation takes place in cardiotoxin B, giving rise to detectable dynamic light scattering, although too weak under normal pH's to induce a significant increase in absorbance.

Fluorescence Properties of Aggregated vs Monomeric Cardiotoxin

Fluorescence Intensities and Lifetimes

The steady-state fluorescence properties of cardiotoxins B, BB, and H are undistinguishable (Table II). In particular, the heterogeneity of cardiotoxin B samples does not give rise to any detectable increase of the FWHM of their fluorescence spectra, showing that the emission of the aggregated contaminant is not strongly shifted in wavelength as compared to that of the monomer. The major kinetic components of the fluorescence decays appear also mostly identical, except for an increase in the longest lifetime, which induces a slightly higher average fluorescence lifetime for cardiotoxins B and BB (Table III).

However, under maximum entropy analysis, a systematic decrease in resolution is observed in the profile of fluorescence lifetimes of heterogeneous cardiotoxin B, compared to the highly purified cardiotoxin H (Fig. 6). Such a small difference could easily escape detection

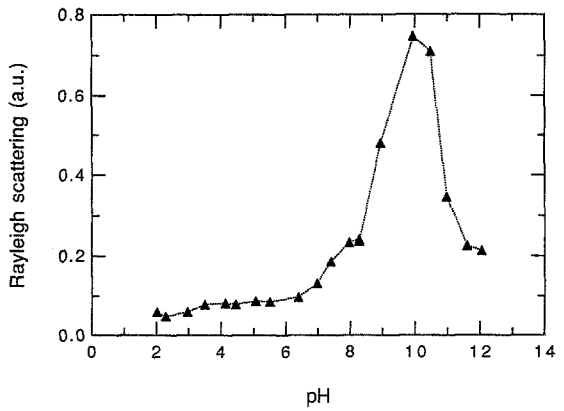

Fig. 5. Rayleigh scattered intensity of cardiotoxin B as a function of pH. Protein concentration 36 μM, $\lambda_{exc}=\lambda_{em}=300$ nm, $\Delta\lambda=4$ nm.

Table II. Steady-State Fluorescence of Cardiotoxin After Different Final Purification Steps[a]

Cardiotoxin	λ_{max} (nm) ± 0.5	FWHM (nm) ± 0.5	Approximate quantum yield (%)[b]	$\overline{\tau_f}$ (ns)	τ_r (ns)[b] ± 1.5
H	347.7	57.6	5.6±0.6	1.21±0.02	21.6
B	346.6	56.8	5.9±0.6	1.34±0.05	22.5
BB	347.7	57.4	5.4±0.6	1.29±0.05	23.9

[a]Experimental conditions as in Table 1.
[b]Determined as previously described [5], assuming a quantum yield of 0.14 for NATA [30], τ_r being the apparent radiative lifetime.

with data of insufficient quality or even under inappropriate analysis of good data (see Experimental Procedures). This broadening of the lifetime distribution of cardiotoxin B is to be ascribed to the aggregate contaminant, which must therefore have *a significantly different fluorescence kinetics* compared to the cardiotoxin monomer.

Initial Anisotropy

Surprisingly, despite a slower anisotropy decay, the steady-state fluorescence anisotropy of cardiotoxin B is not higher but, on the contrary, slightly lower than that of the pure monomer (Table I). Therefore, independently of any model of cardiotoxin fluorescence, one must conclude that the initial anisotropy r_{0A} of the aggregate is significantly lower than that of the cardiotoxin monomer. This can be shown quantitatively by the following line of argument: If x is the assumed aggregation level in cardiotoxin B, Eq. (10) shows that $r_{0A} = \overline{r_A}$ is a monotonous function of x, which, for our given set of experimentally determined quantities $\overline{\tau_m}$, $\overline{r_M}$, $\overline{\tau_X}$, and $\overline{r_X}$, varies between two finite limiting values given in Table IV. In the most extreme hypothetic cases, when $x=1$, or when $x=0$ with maximal unfavorable propagation of errors, r_{0A} cannot be higher than 0.223 (Table IV), which is still much lower than the 0.263 value of the monomer.

Model of the Polarized Fluorescence Decays of Cardiotoxin B

To account for the observed profile and average lifetime of cardiotoxin B, the fluorescence lifetime distribution of the contaminant must be broad, otherwise more important distorsions would be observed as compared to homogeneous cardiotoxin H and shifted to higher values than the monomer. The lower the assumed fraction x of aggregates present in cardiotoxin B, the longer the actual average fluorescence lifetime of the pure aggregate should be [Eq. (9)]. One can simply assume, for example, a lifetime distribution shifted from that of the monomer by a constant proportionality factor $K = \overline{\tau_A}/\overline{\tau_M}$. K being linked to x through Eq. (9), the only free variable of this model is thus x. Figure 7 shows the resulting lifetime distribution of the mixture for this model with $x=0.1$.

Assuming a given fraction x of aggregation for cardiotoxin B, a corresponding value of the initial anisotropy of the cardiotoxin aggregate is obtained from Eq. (10) and the synthetic polarized decays of the model mixture may be computed via Eqs. (14). These synthetic decays are in turn analyzed for recovery of fluorescence and anisotropy parameters by the usual programs. Figure 8 shows the fluorescence lifetime profile recovered from the synthetic decays constructed on the model of Fig. 7. The shape and resolution of this profile are fully compatible with the observed lifetime distribution of cardiotoxin B. On the other hand, the results of the classical anisotropy decay analyses, for different hypothetical values of x in the mixture, are in many respects quite instructive.

Estimate of the Aggregation Level in Cardiotoxin B Samples

Analyses of the above simulations amounted in all cases to the simplified analytical form of Eq. (3), with corresponding parameters given in Table V. When very low aggregation levels (5%) are assumed, the corresponding value of $\overline{\tau_A}$ must exceed $\overline{\tau_M}$ by a very large factor. Since the classical analysis performs a nondistinctive normalization of the depolarization by the total fluorescence decay of the mixture, this is a situation where this analytical description is particularly inade-

Table III. Fluorescence Decay Parameters of Cardiotoxin After Different Final Purification Steps[a]

Cardiotoxin	Lifetime (ns) and amplitude			τ_f (ns)	χ^2
H	0.71 ± 0.04 69 ± 3%	2.05 ± 0.08 28 ± 3%	4.8 ± 0.1 3 ± 1%	1.21 ± 0.02	1.18
B	0.67 ± 0.04 62 ± 3%	2.01 ± 0.05 34 ± 3%	5.7 ± 0.3 4 ± 1%	1.34 ± 0.05	1.04
BB	0.68 ± 0.04 64 ± 3%	1.98 ± 0.10 31 ± 3%	5.1 ± 0.2 5 ± 1%	1.29 ± 0.05	1.07
B, diluted	0.71 ± 0.04 65 ± 3%	2.14 ± 0.05 32 ± 2%	5.7 ± 0.2 3 ± 1%	1.30 ± 0.03	1.02

[a]Experimental conditions as in Table 1.

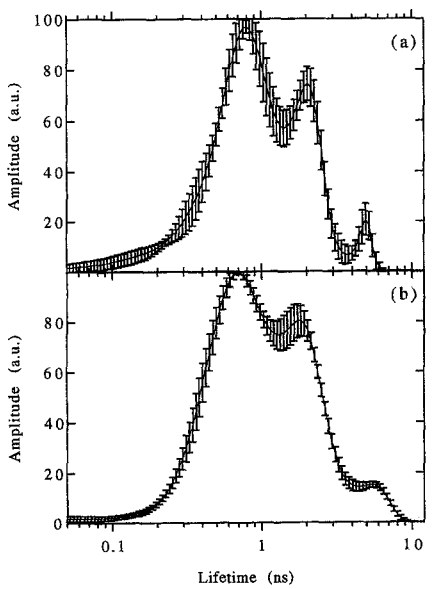

Fig. 6. Fluorescence lifetime distributions of the single tryptophan of cardiotoxin, averaged from repeated experiments at 20°C, with the corresponding standard deviations on the profiles: (a) cardiotoxin H, five determinations; (b) cardiotoxin B, four determinations. Other experimental conditions as in Table I.

quate. Indeed, the fit of the synthetic data is not satisfactory, resulting in large χ^2 and nonrandom residuals (Fig. 9a). In addition, as was observed experimentally, the apparent relaxation time θ_c is much lower than the monomer rotational correlation time (Table V). For intermediate aggregation levels of 10–15%, the fit of the data becomes nearly acceptable with a χ^2 of 1.13–1.08 (Fig. 9b), giving apparent values of θ_c, r_i, and r_∞ which all reproduce well the experimental values observed on cardiotoxin B (compare with Table I). Finally, when x is further increased to 20%, τ_A becomes closer to τ_M, which allows an almost-exact description of the noisy synthetic data by the classical but inadequate formulation. The quality of the fit becomes excellent, with a χ^2 of 1.04, while the slight nonrandom distribution of the residuals (Fig. 9c) would probably not be detected in the presence of low levels of real experimental noise. The recovered parameters remain, however, significantly distorted compared to the inputs of the model, while they begin to diverge from those experimentally observed on cardiotoxin B (Tables I and V). The best agreement of all parameters of the simulations with experimental observations is thus simultaneously obtained for cardiotoxin aggregation ratios of about 10–15%. Under this estimation, we find correlatively that the average fluorescence lifetime of the aggregate should be around 2.1–2.5 ns and that its initial anisotropy should be about 0.15 (Tables IV and V).

We have tried some other models such as a single exponential fluorescence decay for the aggregate or initial anisotropy of the aggregate imposed to be equal to that of the monomer. None of them gave satisfactory agreement with the experimental observations. We have also analyzed separately the synthetic decays of the pure monomer and the pure aggregate, to check the ability of the method to correctly retrieve these simple inputs. While the parameters of the monomer were perfectly recovered, the fluorescence lifetime profile obtained for the aggregate was unresolved, although its anisotropy parameters were correctly recovered. This was due to the fact that the time range of the synthetic data was kept identical to that used for the monomer, the mixture, and the experiments. This time range was, however, insufficient for the complete measurement of the longer fluorescent decay of the aggregate. This shows that the analysis is not very sensitive to the exact shape of the fluorescence lifetime distribution of the aggregate, which is probably equally true in the case of real experimental data.

Table IV. Range of Possible Values for the Initial Anisotropy $r_{0A} = \overline{r_A}$ of the Cardiotoxin Aggregate, According to Experimental Data and Eq. (10)

x^a	0.00	0.01	0.05	**0.1**[b]	0.5	1
$\Delta\overline{r_A} / \overline{r_A}$	91%	77%	47%	**30%**	6%	2%
$\overline{r_A}$ min	0.010	0.028	0.073	**0.103**	0.158	0.170
$\overline{r_A}$	0.117	0.122	0.137	**0.147**	0.169	0.174
$\overline{r_A}$ max	**0.223**[c]	0.217	0.201	**0.192**	0.179	0.177

[a]Assumed relative fraction of aggregated molecules in cardiotoxin B.
[b]Value corresponding to 10% aggregation, giving the best agreement with experimental data.
[c]Maximum value of $\overline{r_A}$ under the most extreme hypotheses.

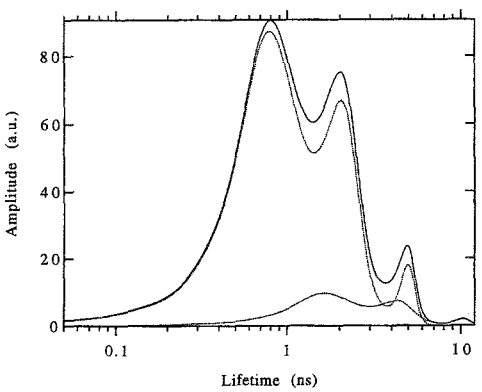

Fig. 7. Construction of a model for the fluorescence lifetime distribution of cardiotoxin B assuming $x=10\%$ aggregate: lifetime distributions of the monomer and the aggregate (dashed lines), and sum of 0.9*monomer + 0.1*aggregate (continuous line), used for the convolution of synthetic data.

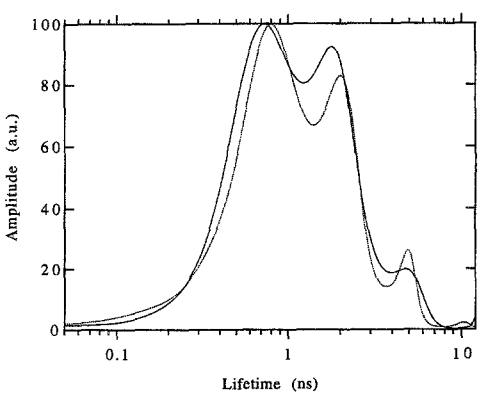

Fig. 8. Maximum entropy analysis of the synthetic unpolarized data $I^X_{vv}(t)+2*I^X_{vh}(t)$, computed from the model with $x=10\%$ aggregate in Fig. 7: (dashed line) initial model; (solid line) distribution recovered by the MEM analysis.

DISCUSSION

While *N. mossambica mossambica* cardiotoxin V^{II}4 crystallizes as a dimer in the asymmetric unit [2], *N. nigricollis* cardiotoxin was shown recently by the same authors to crystallize as a trimer with a very different topology (B. Rees, personal communication). We have found that cardiotoxin samples, as purified through a widely used procedure, may contain in solution significant proportions of a higher molecular weight aggregate, depending on the concentration and pH. Considering the homogeneity of cardiotoxin B samples after Bio-Rex chromatography, heterogeneous protein

Table V. Analysis of the Simulations for Different Assumed Fractions x of Aggregated Cardiotoxin

x	$\overline{\tau_A}(x)$ (ns)[a]	Anisotropy decay parameters			
		r_i	θ_c (ns)	$r\infty/r_i$	χ^2
5%	3.61	0.263	2.16	31%	1.50
10%	2.51	0.253	2.47	26%	1.13
15%	2.08	0.247	2.54	29%	1.08
20%	1.86	0.241	2.64	31%	1.04

[a]Average fluorescence lifetime of the aggregate, estimated from Eq. (9).

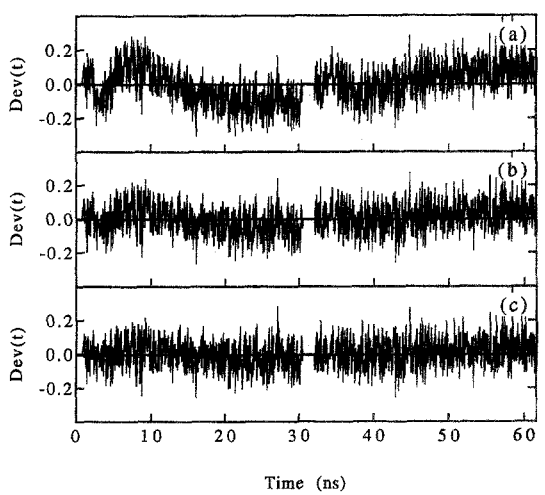

Fig. 9. Residuals of classical analysis of anistropy decays from the synthetic polarized data $^X_{vv}(t)$ and $I^X_{vh}(t)$: (a) $x=$ 5% aggregate; (b) $x=10\%$ aggregate; (c) $x=20\%$ aggregate.

contamination, including the reported PLA_2 contamination (less than 1%), cannot account for the levels of aggregation observed. Therefore, it must be concluded that this aggregation is a property of the cardiotoxin molecule itself.

The observed aggregation could correspond to some irreversible aging of the cardiotoxin samples. However, although the Bio-Rex ion exchange chromatography alone is expected to lead a highly homogeneous and active toxin [1,19,20], it is not efficient in separating these "aged" aggregates. The RP-HPLC, on the contrary, completely eliminates them. Furthermore, HPLC definitely suppresses the self-association ability of cardiotoxin, since no trace aggregation of the HPLC-treated samples was observed up to several months after their initial preparation. This is in contrast with the fact that aggregation is found readily reversible in cardiotoxin B. Therefore, it may be suggested that the HPLC step has influenced cardiotoxin aggregation in a different way, by the separation of an essential cofactor of the aggregation process, for example, PLA_2, some negatively charged ions, or some phospholipids, strongly linked to the native protein. It may be also that the RP-HPLC has induced some subtle but irreversible structural perturbations of the cardiotoxin molecule, on which its self-association ability would depend. In all cases, one should consider the question whether the RP-HPLC purified cardiotoxin H, although physically homogeneous, may well not represent the most native and active form of cardiotoxin.

The very low estimate for the initial anisotropy of the cardiotoxin aggregate (0.15), compared to monomeric cardiotoxin (0.263) or the fundamental value expected for tryptophan (0.300), shows that some specific, very fast depolarization process takes place in the aggregate. This could reflect an increase in local flexibilities around the tryptophan residue induced by aggregation. However, these flexibilities would have to be of very high amplitude, since the value is lower than that observed on denatured proteins [28] or random coil oligopeptides [29]. A high degree of flexibility is also usually associated with intermediate subnanosecond relaxations in the anisotropy decay, which are completely absent from our data. It is therefore more likely that such a large depolarization of the fluorescence arises from efficient intermolecular energy transfers. Aggregation would thus bring some tryptophan residues within sufficient proximity for such transfers, which would also perturb the fluorescence kinetics of the chromophore.

Our simulations give some striking and realistic examples of the differences which may lie between the true hydrodynamic parameters of a heterogeneous system and those retrieved through the usual formulation of fluorescence anisotropy decays. These distorsions may remain unsuspected if the small differences in fluorescence properties of heterogeneous vs homogeneous samples are not properly detected. Beyond the problem of quantifying protein aggregates, this approach has broader implications in all cases of chemical, physical, or dynamical heterogeneities of a chromophore. In these cases, a compartmental analysis associating correctly each fluorescent state with its proper rotational dynamics [30,31] is clearly necessary to arrive at physically meaningful values of both initial anisotropies and rotational correlation times. A completely free two-dimensional treatment of independent lifetimes and correlation times such as that proposed by Brochon et al. [18] could, in principle, deal correctly with such heterogeneous data. However, the problem here is very complex since both cardiotoxin monomer and aggregate have broad and overlapping lifetime distributions. The limited, although respectable statistics of our data would probably forbid the retrieval of such a high number of free parameters. This calls for further improvements in the overall accuracy of fluorescence measurements.

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