Journal of Fluorescence, Vol. 1, No. 1, 1991

Distribution of Separations Between Groups in an Engineered Calmodulin

Robert F. Steiner,[1,3] Sharon Albaugh,[1] and Marie-Claude Kilhoffer[2]

Received November 9, 1990; revised December 13, 1990; accepted December 13, 1990

An engineered calmodulin (VU-9-CaM) has been prepared in which a tryptophan group is present at position 99 and a tyrosine at position 138. The tyrosine was converted to nitrotyrosine. Time-domain dynamic fluorescence measurements were made of energy transfer from the tryptophan donor to the nitrotyrosine acceptor. These were analyzed to yield the parameters characterizing the distribution of separations between the two groups, which are located on Ca^{2+}-binding domains III and IV. Their mean separation is in reasonable agreement with the crystallographic value.

KEY WORDS: Fluorescence; energy transfer; distance distributions; calmodulin.

INTRODUCTION

Two questions of major importance in comprehending the relation between structure and activity of biopolymers are the equilibrium distribution of conformations and the dynamics of their interconversion. One experimental approach to this problem is to examine the distribution of intramolecular separations between two specific groups by means of radiationless energy transfer. Methods have been developed for determining this distribution by both time- and frequency-domain measurements of donor decay [1–4] and of sensitized fluorescence of acceptor [5].

An approach of this kind requires some assumption as to the mathematical form of the distance distribution; this has often been taken to be Gaussian [2]. If donor decay only is measured, a simple interpretation requires that mutual diffusion of donor and acceptor be negligible during the time decay of the donor [1]. The effects of a distribution of mutual orientations of the transition dipoles of donor and acceptor are difficult to correct for and should be minimized by an appropriate choice of fluorophores.

In this report we consider the average separation and distribution of separations between a donor and an acceptor group located in Ca^{2+}-binding domains III and IV, respectively, in an engineered calmodulin, VU9-CaM [6,7]. In this variant of mammalian calmodulin, the tyrosine group at position 99 is replaced by a tryptophan, while the balance of the primary structure remains unchanged, including the tyrosine group at position 138. Nitration of Tyr-138 converts it into a chromophore whose absorption band shows extensive overlap with the emission band of Trp-99 and, hence, into an efficient acceptor group for the donor fluorescence of Trp-99 [8]. By the use of these two groups as a donor–acceptor pair, their separation and distribution of separations have been examined for different conditions, including the absence or presence of Ca^{2+} and neutral or acid pH.

EXPERIMENTAL

Materials

Calmodulin. VU-9 calmodulin was prepared as described elsewhere [7]. The protein was homogeneous by

[1] Department of Chemistry and Biochemistry, University of Maryland (Baltimore County), 5401 Wilkens Avenue, Baltimore, Maryland 21228.

[2] Laboratoire de Biophysique, Faculté de Pharmacie de Strasburg, UA CNRS 491, Université Louis Pasteur, BP24, 67401 Illkirch Cedex, France.

[3] To whom correspondence should be addressed.

1053-0509/91/0300-0015$06.50/0

the criterion of sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and high-performance liquid chromatography (HPLC) and had an activity equivalent to the wild type for the activation of myosin light-chain kinase. The protein concentration was determined by ultraviolet absorption spectroscopy, using a molar extinction coefficient of 7400 M^{-1} cm^{-1} for the protein in the absence of Ca^{2+} [6].

Methods

Nitration of Tyr-138. The nitration of Tyr-138 by tetranitromethane was carried out by a procedure basically equivalent to that of Richman and Klee [8]. To VU9-CaM (~100 μ*M*) in 0.05 *M* Tris, 0.2 *M* NaCl, 1 m*M* Ca^{2+}, pH 7.9, was added tetranitromethane (0.2%). The solution was allowed to stand for 20 h at 3°C. It was then freed from excess reagent by passage through a Sephadex G-25 column, eluting with the buffer of choice. The degree of nitration as determined from the ultraviolet absorption spectra at pH 6.5 and at pH 10 [9] was greater than 97%. Richman and Klee [8] have shown that, in normal bovine calmodulin, only tyrosine is modified by tetranitromethane. Both the native and the nitro forms of VU9-CaM were homogeneous by the criterion of gel electrophoresis; in particular, there was no indication of any aggregates.

Since tetranitromethane is also known to attack tryptophan in some proteins, although at a relatively slow rate [10], it was important to verify that only unmodified tryptophan was contributing to the emission spectra (data not shown). The normalized emission spectra of nitro-VU9-CaM were independent of excitation wavelength and equivalent to those of unmodified VU9-CaM, as were the excitation spectra. In particular, no fluorescence was excited upon irradiation at wavelengths outside the tryptophan absorption band (>325 nm). It thus appears likely that the only fluorescence observed arose from unmodified tryptophan.

Fluorescence. Measurements of the time decay of fluorescence intensity and anisotropy were made using the time-domain instrument of the Regional Laser and Biotechnology Laboratory at the University of Pennsylvania, with the kind assistance of Drs. Gary Holtom and Sam Abrash. This instrument is equipped with a mode-locked argon laser which drives a rhodamine laser. The frequency is 4 MHz. In these studies the excitation wavelength was 298 nm and the emission wavelength was 360 nm.

The time decay of fluorescence intensity was analyzed in terms of amplitudes and decay times using the least-squares fitting program developed by Dr. Gary Holtom [11]. The time decay of fluorescence anisotropy was analyzed in terms of amplitudes and rotational correlation times, also using a least-squares fitting program written by Dr. Holtom [11].

Frequency-domain measurements of the time decay of fluorescence intensity and anisotropy were made using the variable-frequency phase fluorometer located at the Center for Fluorescence Spectroscopy, School of Medicine, University of Maryland at Baltimore [12]. Measurements were made by Dr. Henry Malak. Data were analyzed using least-squares fitting programs developed at the Center for Fluorescence Spectroscopy.

The values of R_o, the separation for 50% transfer efficiency between tryptophan and nitrotyrosine, were determined by standard procedures [13,14] as described elsewhere [15]. Values for the quantum yield of Trp-99 were taken as equal to 0.19 and 0.15 in the absence and presence of Ca^{2+}, respectively [6]. Values for the quantum yield under other conditions were obtained using the former as a standard.

THEORY

If no acceptor group is present, the time decay of fluorescence intensity of a fluorescent label linked to a biopolymer is given by

$$i_D(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (1)$$

where $i_D(t)$ is the intensity of the donor fluorescence as a function of time, t, and α_i and τ_i are the amplitude and decay time, respectively, of the ith decay mode.

In the presence of an acceptor group for which there is a distribution of separations, the above is replaced by Eq. (2):

$$i_{DA}(t) = \int_o^\infty \sum_i \alpha_i \exp\left\{-(t/\tau_i)\,[1 + (R_o/r)^6]\right\} P_r dr \quad (2)$$

where r is the separation of donor and acceptor, R_o is the separation for 50% transfer efficiency, and P_r is the probability distribution function for the separation. While the form of P_r is generally unknown, it may plausibly be represented by a Gaussian function leading ultimately to the relation (4):

$$i_{DA}(t) = \frac{1}{\sqrt{\pi}} \int_{z^*}^\infty \sum_i \alpha_i \exp\left\{-(t/\tau_i)[1 + (\bar{u} + \sqrt{2}\,\sigma z)^{-6}]\right\} e^{-z^2} dz \quad (3)$$

where $z = (u - \bar{u})/\sqrt{2}\,\sigma$; $u = r/R_o$; $\bar{u} = \bar{r}/R_o$; $\sigma = \sigma'/R_o$; $z^* = -\bar{u}/\sqrt{2}\,\sigma$; and $\bar{r}$ and σ' are the most probable separation and the standard deviation, respectively, of the Gaussian distribution. (σ' is related to the half-width, w, of the distribution by $w = 2.35\,\sigma'$.)

The above assumes that R_o and $\bar{u}$ are the same for each of the decay modes. This may not be the case if the different decay modes arise from different configurations, and hence different microenvironments, of the fluorophore, whose duration is not short in comparison with that of the excited state. In this case Eq. (3) may be replaced by

$$i_{DA}(t) = \frac{1}{\sqrt{\pi}} \sum_i \alpha_i \int_{z^*}^{\infty} \exp\left\{-\left(\frac{t}{\tau_i}\right)[1 + (\bar{u}_i + \sqrt{2}\,\sigma_i z)^{-6}]\right\} e^{-z^2}\,dz \quad (4)$$

Here $\bar{u}_i = \bar{r}/R_{oi}$ and $\sigma_i = \sigma'/R_{oi}$, where R_{oi} is the value of R_o for the ith decay mode. It is reasonable to assume that R_{oi} is related to the average value of R_o, $\bar{R}_o$, by $R_{oi} = \bar{R}_o\,(\tau_i/\bar{\tau})^{1/6}$, where $\bar{\tau}$ is the average decay time; this would be the case if the different decay times arose from purely dynamic interactions of the excited state. Also, $\bar{u}_i = \bar{u}\,(\bar{R}_o/R_{oi})$.

Another potentially serious complication is the effect of a distribution of values of the orientation factor κ^2. The common assumption that $\kappa^2 = 2/3$ is strictly valid only if the two transition dipoles have a fixed orientation equivalent to the random or if they undergo a dynamic reorientation which is rapid in comparison to the decay of the excited state. It is possible to treat this case, provided that an assumption is made as to the form of the distribution function for κ^2.

If it is assumed that the distribution of values of κ^2 is rectangular; i.e., the probability of occurrence of a particular value of κ^2 is constant within the limits κ_1^2 and κ_2^2 and equal to zero outside these limits. Subject to these assumptions, we have

$$i_{DA}(t) = \frac{1}{\sqrt{\pi}\Delta\beta} \sum_i \alpha_i \int_{\beta_1}^{\beta_2} \int_{z^*}^{\infty} \exp\left\{-\left(\frac{t}{\tau_i}\right)[1 + \beta(\bar{u}_i + \sqrt{2}\,\sigma_i z)^{-6}]\right\} e^{-z^2}\,dz d\beta \quad (5)$$

where $\beta = (3\kappa^2/2)$ and equals the factor by which R_o^6 is altered by the given value of κ^2; β_2 and β_1 correspond to the maximum and minimum values of κ^2, respectively.

The theory of Haas *et al.* [16] provides a means of estimating the limiting values of κ^2; it is especially relevant to the case where donor and acceptor correspond to multiple transitions. If the acceptor is a fluorophore and the polarizations are known for the immobilized fluorophores, the tables of Haas *et al.* yield the range of values of R_o.

A potential complication is the occurrence of mutual diffusion of donor and acceptor, which when present, may distort the measured separation and distribution [5]. However, the effects of diffusion can be minimized by carrying out measurements under conditions of high viscosity.

CALCULATIONS

An iterative least-squares search procedure [17] was used in which $\bar{u}$ and σ were systematically varied so as to obtain an optimum fit of the observed time decay [4]. The stepwise procedure was as follows.

(i) A set of α_i, τ_i was computed by standard least-squares fitting procedures to yield the optimum fit to the time decay of intensity for the donor in the absence of an acceptor [13,14].

(ii) For trial values of $\bar{u}$ *and* σ, $i_{DA}(t)$ is computed using Eq. (3) [or Eq. (4)] and then convolved with the excitation pulse, $E(t')$, to yield computed values, $I_{DA,c}$, of the intensity of donor in the presence of acceptor as a function of time:

$$i_{DA,c}(t) = \int_o^t E(t')\, i_{DA}(t - t')\,dt' \quad (6)$$

For the Ar^+-rhodamine laser source used in this study the excitation pulse (hw $\simeq$ 20 ps) is distributed over a very few time channels. Equation (6) may be replaced by

$$i_{DA,c}(t_i) = \sum_{j=0}^{j=i} E_j\, i_{DA}(t_{i-j}) \quad (7)$$

where $i_{DA,c}(t_i)$ is the computed intensity for time channel i, E_j is the fraction of the excitation occurring in channel j, and $i_{DA}(t_{i-j})$ is the intensity as computed from Eq. (3) [or Eq. (4)] for a time equal to $(i-j)$ multiplied by the time per channel. Since the position of zero time is not precisely defined in time-domain measurements, zero time for emission was taken as the time channel for maximum emission; the units of the α_i were chosen so that $\sum_i \alpha_i$ was equal to the number of single photon counts in the latter channel. (Alternatively, $\sum_i \alpha_i$ was treated as a floating variable; however, the optimum fit was always obtained for the above value.)

Although the spectroscopic determination of the extent of nitration indicated that the degree of tyrosine modification was 100% within experimental uncertainty, the precision of the determination was not sufficient to rule out the persistence of a small fraction (<3%) of unsubstituted tyrosines. Because of the greatly reduced quantum yield of the nitrated calmodulin, the unsubstituted molecules may exert a disproportionate influence upon the time decay. Equation (3) was accordingly modified to allow for the presence of detectable amounts of unmodified calmodulin.

$$i_{DA}(t) = \epsilon i_D(t) + (1 - \epsilon) i_{DA}(t) \qquad (8)$$

where $i_D(t)$, the intensity of donor in the absence of acceptor, is given by Eq. (1) and ϵ is the fraction of the intensity at zero time which is contributed by the unsubstituted material. In practice, the fitting procedure was carried out for a series of assumed values of ϵ, using Eq. (8) and those values of ϵ, $\bar{u}$, and σ selected which correspond to the minimum value of χ^2.

RESULTS AND DISCUSSION

Native VU9-CaM

In agreement with the findings of Chabbert *et al.* [18], the time decay of intensity for the tryptophan fluorescence of VU9-CaM, as obtained from time-domain measurements, was nonexponential for all the conditions examined, reflecting the occurrence of multiple decay modes. For excitation at 300 nm and emission at 360 nm, a reasonable fit was obtained by assuming two decay modes; however, a significant improvement in χ^2 resulted from an increase in the assumed number of components from two to three in both the absence and the presence of Ca^{2+} (Table I and Fig. 1). The parameters obtained from the three-component fit were therefore employed in fitting data with Eq. (3). The values of the two lifetimes obtained assuming two decay modes were similar, although not identical, to those of the earlier study [18]. There was a difference in that the latter obtained an improvement in fit using a third component only in the presence of Ca^{2+} at emission wavelengths below 330 nm. It is possible that these minor differences can be attributed to the different pH's (6.5 vs 7.5) and different excitation wavelengths (300 vs 297 nm) of the two studies.

Table Ib cites values of decay times obtained from frequency-domain measurements. An optimal fit required the assumption of three decay components. The values of the corresponding decay times show some quantitative differences from those determined by time domain measurements. This is to be expected if there is a (possibly multimodal) distribution of decay times, rather than discrete values, since the weighting of different decay modes is different for the two procedures.

The time decay of fluorescence anisotropy could, in the absence of Ca^{2+}, be fit assuming two rotational modes; the more rapid of these clearly reflected a localized rotation, while the longer arose from a more global motion. In the presence of excess Ca^{2+} no short correlation time was detected, while the magnitude of the long correlation time was substantially increased (Table II). However, the zero-time anisotropy, A_o, corresponding to the summed amplitudes, was remarkably low in both cases and much lower than the theoretical limit of 0.4 (Table II). This must reflect either a rotation too fast to be resolved or an intrinsically low limiting anisotropy because of the presence of mixed transitions.

Table IIb cites the results of anisotropy decay analysis using frequency-domain measurements. While quantitative differences from the time-domain studies exist with respect to the magnitudes of the correlation times, the primary differences are that the zero-time anisotropy, A_o, is significantly greater for the frequency-domain measurements (0.31 vs 0.23–0.25) and that a short (98-ps) correlation time is detected for Ca^{2+}-liganded VU9-CaM by frequency measurements but not by time domain. In addition, the amplitudes of the more rapid rotational modes (β_1) relative to the slower (β_2) are greater for the frequency-domain measurements than for the time domain.

It is probable that these differences arise from the greater sensitivity of GHz dynamic fluorometry to short (<100-ps) correlation times. This results in a greater recovery of anisotropy, as reflected by increased values of A_o and the detection of rapid rotational modes which, in the time domain, are expressed only by a reduced value of A_o. It is likely that the short correlation time is actually a poorly defined average corresponding to more than one kind of molecular rotation.

Nitro-VU9-CaM

The time decay of tryptophan fluorescence for the NO_2-Tyr-138 derivative of VU9 was dramatically more rapid than that of the native form (Fig. 2). It was also more heterogeneous, as indicated by the elevated value of χ^2 for the optimum two-component fit, as compared with native VU9-CaM (Table I). The average decay times were substantially reduced in both the absence and the presence of Ca^{2+} (Table I), suggesting the occurrence of radiationless energy transfer. Moreover, the relative

Table I. Parameters for Intensity Decay (a) of VU9-CaM and NO_2-VU9-CaM[a] and (b) of VU9-CaM[a]

(a) From time-domain measurements

Sample	Ca^{2+}	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\bar{\tau}^b$	χ^2
VU9-CaM	−	—	—	0.464	1.35	0.536	5.83		2.5
	−	0.164	0.28	0.410	1.82	0.426	6.19	3.37	1.1
	+	—	—	0.683	1.60	0.317	7.54		3.0
	+	0.127	0.22	0.609	1.78	0.264	7.99	3.20	1.5
NO_2VU9-CaM	−	—	—	0.805	0.47	0.195	5.11		12.4
	−	0.661	0.20	0.232	1.30	0.107	6.39	1.09	1.5
	+	—	—	0.869	0.51	0.131	4.98		63.2
	+	0.673	0.18	0.268	1.05	0.058	6.39	0.77	2.7

(b) From frequency-domain measurements

Ca^{2+}	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	χ^2
−	0.12	0.59	0.51	2.58	0.37	7.26	1.6
+	0.21	0.19	0.45	2.36	0.33	7.40	1.1

[a]The conditions are those for Fig. 1.
[b]$\bar{\tau} \equiv \sum_i \alpha_i\tau_i / \sum_i \alpha_i$. This definition of average lifetime is cited, rather than the more standard $\langle\tau\rangle \equiv \sum_i \alpha_i\tau_i^2 / \sum_i \alpha_i\tau_i$, because, for dynamic quenching, it is proportional to the quantum yield.

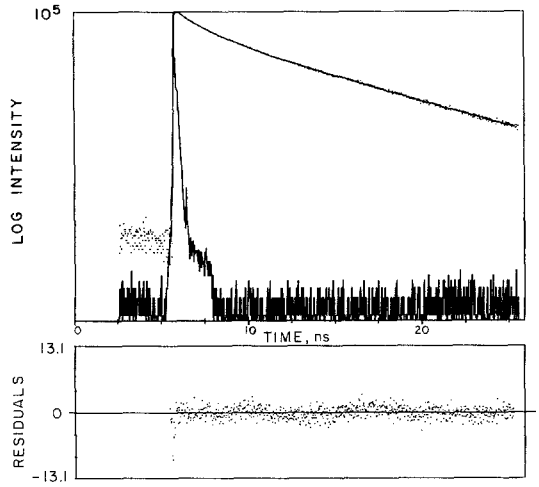

Fig. 1. Comparison of experimental and computed curves for VU9-CaM in 50 m*M* Mops, 1 m*M* EGTA, pH 6.5, 25°C. The excitation and emission wavelengths are 300 and 360 nm, respectively. The computed curve is generated using the parameters for a three-component fit cited in Table Ia. The lower curve shows the distribution of residuals.

amplitude of the component of long decay time was greatly reduced in both cases.

Table II. Parameters for Anisotropy Decay of VU9-CaM[a]

Ca^{2+}	β_1	θ_1 (ns)	β_2	θ_2 (ns)	A_o^b	χ^2
	(a) From time-domain measurements					
−	0.04	0.76	0.21	8.22	0.25	1.48
+	—	—	0.23	10.49	0.23	1.35
	(b) From frequency-domain measurements					
−	0.07	0.082	0.24	7.14	0.31	2.5
+	0.07	0.098	0.24	7.85	0.31	2.7

[a]The anisotropy as a function of time, $A(t)$, is fitted to a decay function of the form $A(t) = \sum_i \beta_i \exp(-t/\theta_i)$, where β_i and θ_i are the amplitude and rotational correlation time, respectively, corresponding to rotational mode i. The conditions are the same as for Fig. 1.
[b]Anisotropy at zero time; $A_o = \beta_1 + \beta_2$.

Analysis according to Eq. (3) indicated that it was impossible to fit the time decay with the assumption that $\sigma = 0$ (Fig. 3). Acceptable fits required finite values of σ (Fig. 3 and Table III). It is of interest that the value of $\bar{r}$ obtained using Eq. (3) was, for Ca^{2+}-liganded calmodulin, in reasonable agreement with the crystallographic separation, 13–15 Å (Table III) [19].

Equation (3) assumes that all decay modes of tryp-

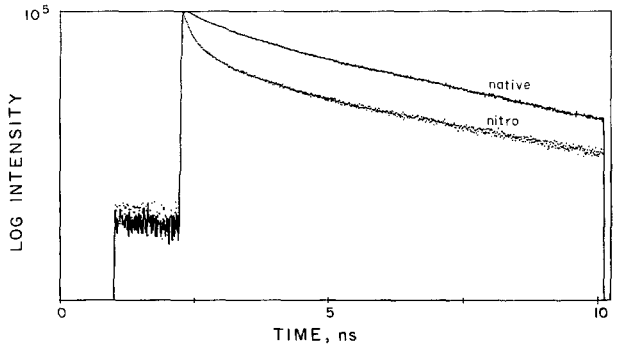

Fig. 2. Time decay of fluorescence intensity for VU9-CaM and NO_2-VU9-CaM (~100 μ*M* each) under the conditions for Fig. 1.

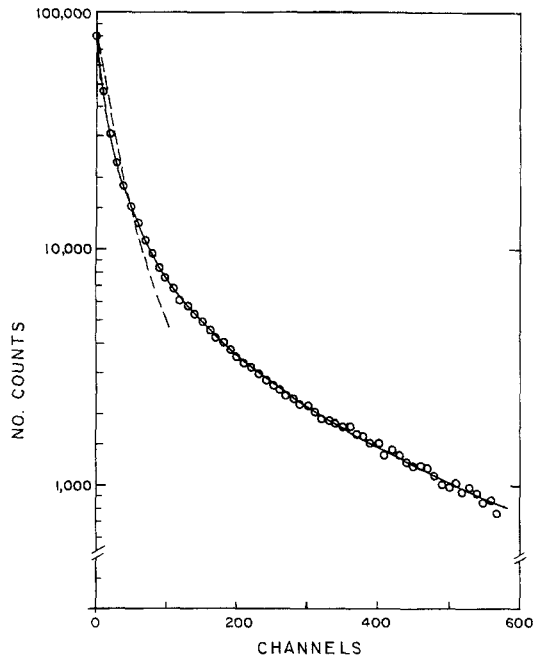

Fig. 3. Comparison of fits to experimental data using Eq. (3) and allowing σ to vary (——) and assuming σ = 0 (---) for NO_2-VU9-CaM under the conditions for Fig. 1, except that 2 m*M* Ca^{2+} is present instead of 1 m*M* EGTA.

tophan have the same value of R_0. If this assumption is removed and the data are fitted using Eq. (4), significantly shorter separations and broader distributions are obtained, as expected [4]. However, there was no significant improvement in goodness of fit (Table III).

Chabbert *et al.* [18] have found that the relative amplitudes of the decay times of the tryptophan fluorescence of VU9-CaM are wavelength dependent and that a time-dependent spectral shift occurs, which may arise

Table III. Parameters Characterizing the Distribution of Separations Between Trp-99 and NO_2-Tyr-138

pH	Sucrose	Ca^{2+}	R_0	Eq. used	$\bar{u}$	σ	$\bar{r}$	hw	χ^2
6.5	–	–	26.3	(3)	0.62 ±0.01	0.09 ±0.01	16.3 ±0.3	5.6 ±0.6	1.9
				(4)	0.56 ±0.01	0.14 ±0.01	14.7 ±0.3	8.7 ±0.6	2.0
6.5	50%	–	26.3	(3)	0.64 ±0.02	0.11 ±0.02	16.8 ±0.5	6.8 ±1.3	2.1
6.5	–	+	25.3	(3)	0.66 ±0.01	0.08 ±0.01	16.7 ±0.3	4.8 ±0.6	1.85
				(4)	0.55 ±0.01	0.14 ±0.01	13.9 ±0.3	8.3 ±0.6	1.8
	50%	+	25.3	(3)	0.65 ±0.01	0.13 ±0.04	16.4 ±0.3	7.7 ±2.4	3.2
4.5	–	–	26.3	(3)	0.62 ±0.01	0.07 ±0.02	16.3 ±0.3	4.3 ±1.3	3.1

either from dipolar relaxation [20] or from the occurrence of conformations with different spectra whose interconversion is not on a much more rapid time scale than the fluorescence decay. Behavior of the former kind has been reported for indole derivatives at low temperatures and for single tryptophan-containing mutants of T4 phage lysozyme [21], whose behavior has some resemblance to that of the present system. It is plausible that the observed wavelength dependence of decay time may arise from the combined effects of both factors.

Values of $\bar{u}$ and σ computed using Eq. (4) represent limiting values corresponding to the case where the transition of the tryptophan between different microenvironments is slow in comparison with its intensity decay. In view of the evidence for substantial mobility of Trp-99 from the anisotropy decay results, this is unlikely to be literally the case; it is more probable that the rate of interconversion is at least comparable to, rather than much slower than, the decay time. For this reason, as well as the possible contribution of dipolar relaxation, Eq. (4) probably corresponds to an overcorrection; the values obtained from Eq. (3) are probably closer to the correct ones.

An estimate of the uncertainties associated with the values of $\bar{u}$ and σ obtained using Eq. (3) may be made by allowing either $\bar{u}$ or σ to vary while holding the second parameter constant for a series of values of the latter. From the dependence of χ^2 upon $\bar{u}$ or σ (Fig. 4) and taking a value of 1.2 for normalized χ^2 as corresponding to the 67% confidence limits, the uncertainties cited in Table III are obtained. Figure 5 shows a distribution function computed according to Eq. (3).

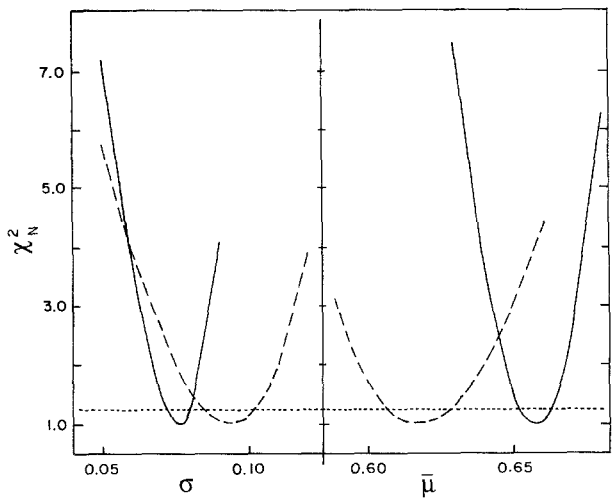

Fig. 4. Dependence of goodness of fit upon the value of $\bar{u}$ or σ in 2 m*M* Ca^{2+} (——) or 1 m*M* EGTA (---).

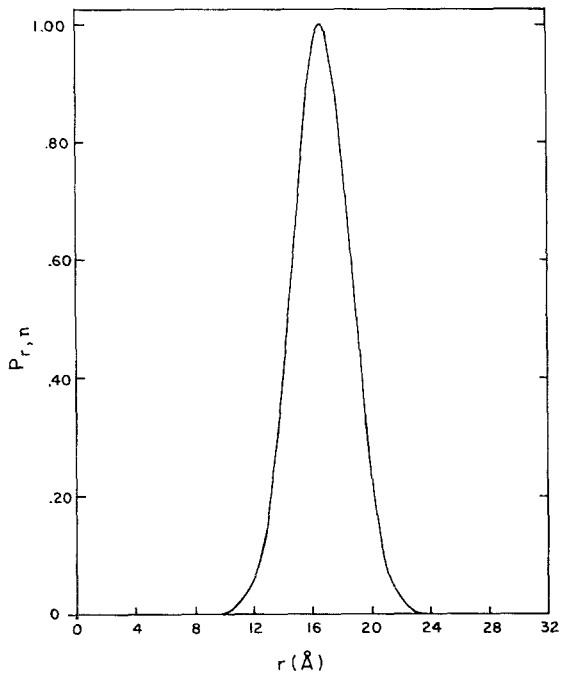

Fig. 5. Probability distribution function for donor–acceptor separation for NO_2-VU9-CaM in 2 m*M* Ca^{2+}. The conditions are otherwise the same as for Fig. 1.

There remained the question of the contribution of any nonrandomness of orientation to the form of the time decay [16]. The latter workers have evaluated the range of values of κ^2 consistent with a given pair of limiting polarization (or anisotropy) values for donor and acceptor. From Table II, the limiting anisotropy for Trp-99 is ~0.24 in both the absence and the presence of Ca^{2+} (corresponding to a limiting polarization of 0.32). While NO_2-Tyr-138 is of course nonfluorescent, for the purposes of this calculation it may be represented by tyrosine, for which the limiting anisotropy is ~0.28 [22]. From Table 3 of Haas *et al.* [16], the predicted range of values of κ^2 is 0.91–1.12. The calculations of Haas *et al.* [16] are actually for a collection of immobilized fluorophores. For a case where the limits were 0.91 and 1.08, Haas *et al.* have shown that the effect upon the distribution of donor–acceptor separations was negligible. In the present case, the fluorophores are not immobilized; this is especially the case for the acceptor [22]. In the case of the thrombic 107–148 fragment, which contains only Tyr-138, the fraction of the anisotropy decay arising from purely localized motion of the fluorophore, corresponding to a correlation time less than 500 ps, is 0.70 in the absence of Ca^{2+} and 0.23 in its presence [23]. Since there is no obvious reason for nitration to interfere seriously with tyrosine mobility, it is likely that the mobility persists to some degree for NO_2-Tyr-138.

In view of the considerations cited above, it is not unreasonable to expect that any distribution of dipole orientations may not contribute significantly to the apparent distribution of donor–acceptor separations. The use of Eq. (5), which allows for a distribution of orientations, assuming a rectangular form for the distribution, failed to produce any improvement in fit and in fact resulted in an increase in χ^2 (Table IV).

To gain further insight into the possible contribution of a distribution of orientations, as well as mutual diffusion of donor and acceptor, the measurements were repeated in 50% sucrose in both the absence and the presence of Ca^{2+}. At 25°C this increases the solvent viscosity 15-fold. (The use of low temperatures was avoided, as this might modify the structure.) In the absence of Ca^{2+} there is no significant change in either $\bar{u}$

Table IV. Separation Distributions Computed Assuming a Distribution of Orientations[a]

Ca^{2+}	β_2	β_1	$\bar{u}$	σ	χ^2
–	1.1	0.9	0.60	0.09	2.0
+	1.05	0.95	0.65	0.08	2.4
	1.10	0.90	0.65	0.08	2.4
	1.20	0.80	0.65	0.04	2.7
	1.30	0.70	0.62	0	36.0

[a] $\bar{u}$ and σ are computed using Eq. (5). The conditions are the same as for Fig. 1.

or σ, the minor changes observed being easily within experimental uncertainty (Table III). In the presence of Ca^{2+}, both parameters are likewise unchanged within experimental uncertainty, although, in the case of σ, the difference approaches the limits of uncertainty. If mutual diffusion were a significant factor in determining the efficiency of transfer, it would be expected that the apparent average separation would be greater in the more viscous medium [5]. As this proved not to be the case, there is an implication that diffusion is not a major influence for this system. These results also are relevant to the question of the influence of a distribution of values of the orientation factor κ^2 upon the apparent distribution function for separations. An increase in viscosity would be expected to reduce the efficiency of randomization of orientations by rotational motion of the donor and acceptor [1] and hence to increase the apparent width of the distribution of separations. While there is a marginal increase in σ in the viscous medium (Table III), it does not exceed experimental error, with the implication that the observed distribution is not distorted to a major degree by a distribution of orientations.

Measurements were also carried out at pH 4.5, which is close to the crystallographic conditions. There was not a major change in separation or distribution width from neutral pH, except for a small decrease in both which was not outside of experimental error (Table III).

Comparison with Other Results

Within experimental uncertainty the average separation of Trp-99 and NO_2-Tyr-138 is consistent with the crystallographic separation of Tyr-99 and Tyr-138 (~15Å) [19], as well as the average separation determined by transfer between Tyr-99 or Tyr-138 and the nitro derivative of the second tyrosine [9]. The present results are thus counter to a major change in the mutual positions of Ca^{2+}-binding domains III and IV in solution, as compared with the crystallographic conditions. In an earlier publication [6] approximate estimates of the separation of Trp-99 and Tyr-138 were made using the static efficiency of transfer from tyrosine to tryptophan. While the value obtained in the absence of Ca^{2+}, 13.5 Å, is in reasonable agreement with the results cited above, the value obtained for Ca^{2+}-liganded VU-9, 25 Å, is unreasonably high, as recognized in the earlier publication [6]. However, the determination of transfer efficiencies by static measurements encounters severe difficulties for this system because of the low fluorescence intensity of tyrosine as compared with that of tryptophan, with whose emission spectrum it overlaps. For this reason transfer efficiencies and apparent separations determined by tyrosine → tryptophan transfer are subject to substantially greater uncertainties than the transfer determinations described in the present paper, for which there is only a single fluorophore of much greater intensity.

It is of interest that a significant distribution of separations exists for all conditions examined, implying a significant mutual mobility of domains III and IV. There is thus a small ($\sim 10^{-4}$), but nonzero, probability of a close approach of the two residues to van der Waals contact. This would be consistent with the finding [24] that Tyr-99 and Tyr-138 can undergo a photochemical combination.

REFERENCES

1. E. Haas, M. Wilchek, E. Katchalski-Katsir, and I. Z. Steinberg (1975) *Proc. Natl. Acad. Sci. USA* **69,** 2273–2277.
2. J. R. Lakowicz, M. L. Johnson, W. Wiczk, A. Bhat, and R. F. Steiner (1987) *Chem. Phys. Lett.* **138,** 587–593.
3. H. C. Cheung, C. Wang, I. Gryczynski, M. L. Johnson, and J. R. Lakowicz (1988) *Proc. Int. Soc. Opt. Eng. (SPIE)* **909,** 163–169.
4. R. F. Steiner and S. Albaugh (1989) *J. Phys. Chem.* **93,** 8013–8016.
5. J. M. Beechem and E. Haas (1989) *Biophys. J.* **55,** 1225–1236.
6. M. C. Kilhoffer, D. M. Roberts, A. O. Adibi, D. M. Watterson, and J. Haiech (1989) *Biochemistry* **28,** 6086–6092.
7. M. C. Kilhoffer, D. M. Roberts, A. O. Adibi, D. M. Watterson, and J. Haiech (1988) *J. Biol. Chem.* **263,** 17023–17029.
8. P. G. Richman and C. B. Klee (1978) *Biochemistry* **17,** 928–935.
9. R. F. Steiner and M. Montevalli-Alibadi (1984) *Arch. Biochem. Biophys.* **234,** 522–530.
10. P. Cuatrecasas, S. Fuchs, and C. B. Anfinsen (1968) *J. Biol. Chem.* **243,** 4787.
11. R. F. Steiner, G. Holtom, and Y. Kubota (1990) in J. P. Fouassier and J. F. Rabek (Eds.), *Lasers in Polymer Science and Technology: Applications, Vol. 4,* CRC Press, Boca Raton, FL, p. 171.
12. J. R. Lakowicz and G. Laczko (1990) *Proc. Int. Soc. Opt. Eng. (SPIE)* **1204,** 13–20.
13. J. R. Lakowicz (1983) *Principles of Fluorescence Spectroscopy,* Plenum, New York.
14. R. F. Steiner (1983) *Excited States of Biopolymers,* Plenum, New York.
15. R. F. Steiner, L. Marshall, and D. Needleman (1986) *Arch. Biochem. Biophys.* **246,** 286.
16. E. Haas, E. Katchalski-Katsir, and I. Z. Steinberg (1978) *Biochemistry* **17,** 5064–5070.
17. P. R. Bevington (1969) *Data Reduction and Error Analysis for the Physical Sciences,* McGraw-Hill, New York.
18. M. Chabbert, M. C. Kilhoffer, D. M. Watterson, J. Haiech, and H. Lami (1989) *Biochemistry* **28,** 6093–6098.
19. Y. S. Babu, J. S. Sack, T. J. Greenhough, C. E. Bugg, A. R. Means, and W. J. Cook (1985) *Nature (London)* **315,** 37.
20. B. Bagchi, D. W. Oxtoby, and G. R. Fleming (1984) *Chem. Phys.* **86,** 257–296.
21. B. Hudson and D. Harris (1990) *Proc. Int. Soc. Opt. Eng. (SPIE)* **1204,** 80–91.
22. I. Gryczynski, J. R. Lakowicz, and R. F. Steiner (1988) *Biophys. Chem.* **30,** 49–59.
23. I. Gryczynski, J. R. Lakowicz, and R. F. Steiner (1990) *Biophys. Chem.,* in press.
24. D. A. Malencik and S. R. Anderson (1988) *Biochemistry* **27,** 419–428.