Biotecnología Aplicada, Vol. 8, No. 3, 278-297 (1991)

Protein internal dynamics: the time-resolved fluorescence approach

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Recibido en julio de 1991
Aprobado en julio de 1991

SUMMARY

The principles and data analysis of the time-correlated single photon counting fluorescence technique applied to protein fluorescence emission are summarized. Two major natural fluorophores in proteins: the Tyrosine and the Tryptophan residues are responsible for their fluorescence emission. Proteins usually contains both fluorphores and if it is the case, the signal is dominated by that of Tryptophan. Two principal modes of experimentantion are employed: i) excitation with unpolarized or ii) polarized light. Informations on the existence of local conformational substates of the protein, on local flexibilities of the indole ring, of the peptide backbone in the nanosecond or subnanosecond time domain and on the overall brownian motion of the protein which depends on its hydrated volume, its shape and also on the temperature/viscosity factor, are provided. Specific examples studied in our laboratory are presented like the distribution of conformations and state of aggregation of apocytochrome c in solution and the interactions of phospholipase A_2 with ligands.

RESUMEN

Se exponen los principios y el análisis dė datos de la técnica de conteo de la fluorescencia del fotón simple correlacionada en el tiempo. La señal procesada por esta técnica proviene de los dos principales fluoróforos en proteínas, los residuos de tirosina y triptófano. Las proteínas usualmente contienen ambos fluorófonos, y en este caso la señal que predomina es la debida a la emisión del triptófano. Dos modos experimetales son principalmente empleados: i) excitación con luz no polarizada, o ii) verticalmente polarizada.

Se obtienen informaciones sobre la existencia de subestados conformacionales locales de la proteína, sobrė flexibilidades locales del anillo indólico y del esqueleto peptdico en el dominio del tiempo de nanosegundos o subnanosegundos y sobre el movimiento browniano general de la partícula que depende de su volumen de hidratación, de su forma y también del factor de temperatura/viscosidad. Son presentados ejemplos específicos estudiados en nuestro laborato, así como la distribución de conformaciones y estados de agregación del apocitocromo C en solución y las interacciones de la fosfolipasa A_2 con ligandos.

INTRODUCTION

The correct understanding of how proteins function is a complex task for the cell biologist which must be grounded upon several and complementary approaches to include all the many aspects in which proteins are involved in the cellular economy of life. From the point of view of a molecular biologist, it is of primary importance to collect a wealth of

informations about the molecular nature o the considered protein in pure state in solution, extracted from the living cell environment, where its natural function is developped. Moreover, the knowledge of the intimate interactions of that precise protein with small molecules or ions or other proteins or even more complex multi-macromolecular structures like biomembranes, are needed for the description of its mechanism of action and of its functional place in the cellular life.

During the last 20 years, the functions of enzymatic proteins have been rationalized from mainly the determination of their 3-D structure by X-ray crystallography. This approach was undertaken first to understand a vital function in the living organisms: the fixation to and the liberation from hemoglobin and myoglobin molecules of molecular oxygen (Perutz & Mathews, 1966). However, it soon appeared from the crystal structure that the active site is inaccesible to the oxygen molecule without movements of the protein side-chains. Since that time, a large number of proteins has been crystallized and their 3D-structure has been solved, showing in all cases a comapct structure (McPherson, 1982; Feher & Zam, 1985).

However, although there is no doubt about the importance of the knowledge of the 3-D structure of proteins, these experiments provide an *essentially static picture of the protein molecular structure* and is a time-averaged or a frozen three dimensional structure of perhaps a more complex dynamic situation. Spectroscopic techniques such as NMR (Williams, 1989) and fluorescence (Munro *et al.*, 1979; Wahl, 1983; Beechem & Brand, 1985) have brought another concept which becomes now to be generally recognized, although its complexity does not facilitate its use for peptide design for isntance, that is the *essential dynamic nature of peptides and proteins.*

One of the first experimental arguments suggesting the existence of local flexibility inside a protein in the nanosecond time domain came in fact from fluorescence study of oxygen diffusion through proteins (Lakowicz & Weber, 1973). Since that time, the development of time-resolved technique, and essentially the pulsed fluorescence, has provided a large set of experimental data demonstrating the existence of fast motions inside proteins (Munro *et al.*, 1979; Lakowicz *et al.*, 1983; Ludescher *et al.*, 1985; 1988; Nicot *et al.*, 1985; Gallay *et al.*, 1987; Fleming *et al.*, 1987; Vincent *et al.*, 1988; Gentin *et al.*, 1990; Kuipers *et al.*, 1990; 1991).

Hydrogen-exchange experiments have also provided experimental arguments about these motions (Woodward & Hilton, 1979) as well as NMR studies (Williams, 1989). However, even the X-ray determination of protein 3-D structure in the crystal have provided experimental arguments for the existence of mobility and flexibility inside a protein molecule. From the examination of the temperature factor, one was led to conclude on the existence of fluctuations within the protein structure, essentially at the protein surface but also in other parts (Artimiuk, 1988).

These observations suggest that in order to function, the protein has to undergo some local or global rearrangements in its average structure. This is needed for the accomodation of the substrate molecule. For instance in the classical cases of hemoglobin and myoglobin, the active site is inaccessible to the oxygen molecule due to the compactness of the aminoacid side chains in the vicinity of the heme group, and one can wonder how oxygen can diffuse through the protein molecule to reach the iron atom where it is bound

for transportation from the lungs to the peripheral tissues. One is led to imagine movements of limited amplitudes of aminoacid side chains and of limited parts of the C α skeletton in order to allow the oxygen molecule to find a pathway to the central iron atom (Perutz & Mathews, 1966).

In a different physiological process, the protein-protein interactions occuring in the recognition mechanism by antibodies, it was hypothesized that one series of epitopes corresponded to flexible regions of the protein (Westhof *et al.*, 1984; Tainer *et al.*, 1984; Bloomer *et al.*, 1985). Although not based on 3-D structure but on indirect enzymatic activity studies, the necessity for a protein to be flexible for an efficient translocation through membranes is now becoming largely accepted (Eilers & Schatz, 1986). This phenomenom is of primary importance for the import or export processes of proteins into cell organelles like mitochondria (Randall & Hardy, 1986). The specificity of hormone-membrane receptor interactions may also involved the flexible character of the peptide hormone. In cases where for instance a given hormone molecule can display different target cells, selection of one of the potential structures may be possible on the specific receptor, membrane system in the plasma membranes.

Beside these experimental arguments (Frauenfelder *et al.*, 1988), the importance of flexibility and internal motions in biological molecules has been evidenced in the recent years by the development of theoretical techniques such as the molecular dynamics trajectories (Karplus & McCammon, 1981; McCammon & Harvey, 1987; Sessions *et al.*, 1988; Axelsen *et al.*, 1988; 1989). Recent works have demonstrated the relationship between the protein flexibility and their thermostability (Vihinen, 1987).

It is therefore becoming more and more clear that proteins cannot be considered anymore as rigid bodies. Recognition processes of any kind cannot be explained only by the lock and key model. Rather, proteins should be considered, as the etymological significance of their name: *i.e.* presenting many shapes, as pexible in nature, displaying many possible but well-defined conformations in a given time domain (Karplus & McCammon, 1981). The purpose of this article is to present the approach of the protein dynamics by the pulse fluorescence technique which can experimentally measure the time duration of the excited state populations and their rate of rotation.

TECHNICAL GROUNDS

The fluorescence phenomena will be only briefly presented since books and monographs have already dealt with this particular interaction of light with matter (Lakowicz, 1983; Demchenko, 1986).

A time-resolved fluorescence experiments in the polarized mode, as it is when sources such as argon-dye picosecond lasers or synchrotron radiation as available in Orsay are used, consists in exciting the fluorophore (Tryptophan or Tyrosine) with a vertically polarized light pulse. The emission light is collected through a monochromator and detected by a fast and sensitive photomultiplier (usually a microchannel plate). A polarizer prism which can abe oriented either parallel or perpendicular to the excitation polarizer is interposed in the optical path between the sample and the monochromator to collect the intensities emitted in a plane respectively parallel -$I_w(t)$- and

perpendicular -$I_{vh}(t)$- to the polarization plane of the excitation light. Two kinds of time-resolved fluorescence techniques are existing: the phase-modulation technique (Spencer & Weber, 1969) and the time-correlated single photon countring technique (Yguerabide, 1972).

The principle of the time-correlated single photon counting technique will only be presented since it is that which has been developed at LURE (Brochon, 1980). The principle of the technique is to measure the time lag between the excitation event, *i.e.* the light pulse of the synchrotron or the laser source, and the first photon of fluorescence emitted by the sample. To reach this purpose, fast electronics are needed (discriminators, fast amplifiers). The heart of the electronic chain is the time-to-amplitude-concerter (TAC) which delivers pulses proportional to the time lag between a start signal (the fluorescence photon) and a stop signal (photoelectron emitted by a fast photodiode which monitors the excitation pulses). The histogram of the number of counts as a function of time is recorded on a multichannel analyzer and the data can be transferred to a computer for analysis. A schematic drawing of a time-resolved fluorescence set-up is represented in figure 1.

Only the molecules presenting their transition dipole parallel to the electrical vector of the incident light will be excited *(photoselection process)*.

After absorption of a photon, an excited state [A*] is created which decays exponentially to the ground state by emitting a photon of lower energy. In the cases of aromatic molecules such as those studied here, the transitions to the excited state are π-π^*transition. The photochemical events leading to the formation and decay of the excited state are schematized in figure 2.

The emission of a photon is most often not the only way for the molecule to deactivate and to return to the ground state. Non radiative deactivation processes, in competition with the radiative decay, are taking place. Some of them are represented in the above scheme.

Intramolecular processes like thermal conversion (rate constant k_{nr}) or inter-system crossing to triplet state (rate constant k_{ic}) (which can lead to phospho-rescence) can occur. Intermolecular phenomena can also be involved like collision with other molecules during the excited state (rate constant k_q), charge transfer (k_{ct}), excimer formation and so on. For a discussion of these phenomena the reader is referred to Lakowicz's book already mentionned or to others (Birks, 1971; Demchenko, 1986).

Excited state decay

In the mode of excitation with natural light, or vertically polarized light and detection of the fluorescence through a polarizer oriented at the "magic angle" (Lakowicz, 1983) one has access to the excited state lifetime of the fluorophore which characterizes its photophysics. In the simple case where only one excited stat is formed, its decay is exponential:

$$[A^*]_{(t)} = [A_o^*] \exp(-t/\tau)$$

where τ is the experimental lifetime (usually in the nanosecond time range) and $[A_o^*]$ the concentration of excited state at time zero. The intensity decay is obtained by summing the parallel and the perpendicular components of the measured intensities:

$$I(t) = Ivv(t) + 2\, G_{corr} Ivh(t)$$

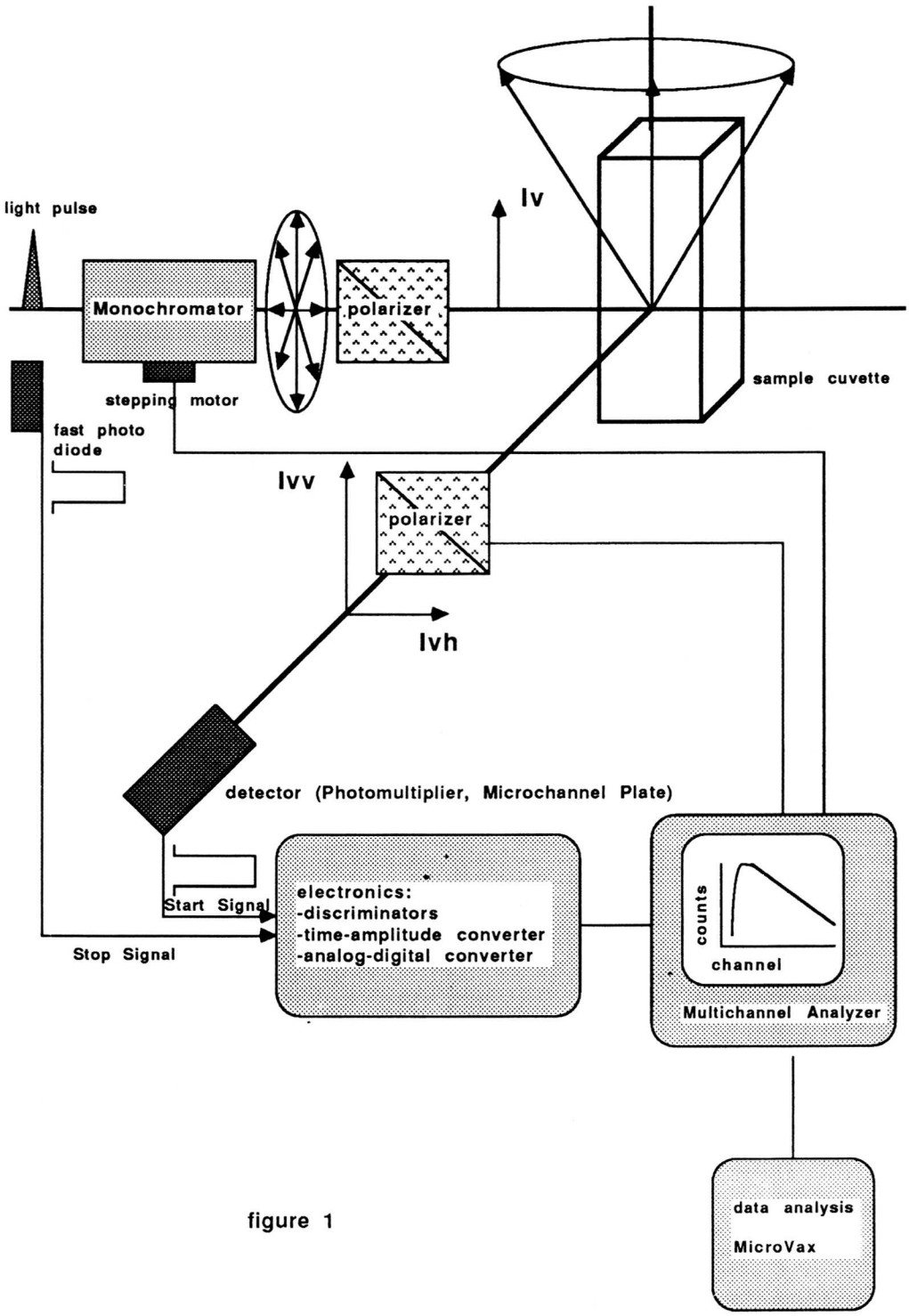
light pulse
Monochromator
polarizer
Iv
sample cuvette
stepping motor
fast photo diode
Ivv
polarizer
Ivh
detector (Photomultiplier, Microchannel Plate)
Start Signal
Stop Signal
electronics:
-discriminators
-time-amplitude converter
-analog-digital converter
counts
channel
Multichannel Analyzer
data analysis
MicroVax

figure 1

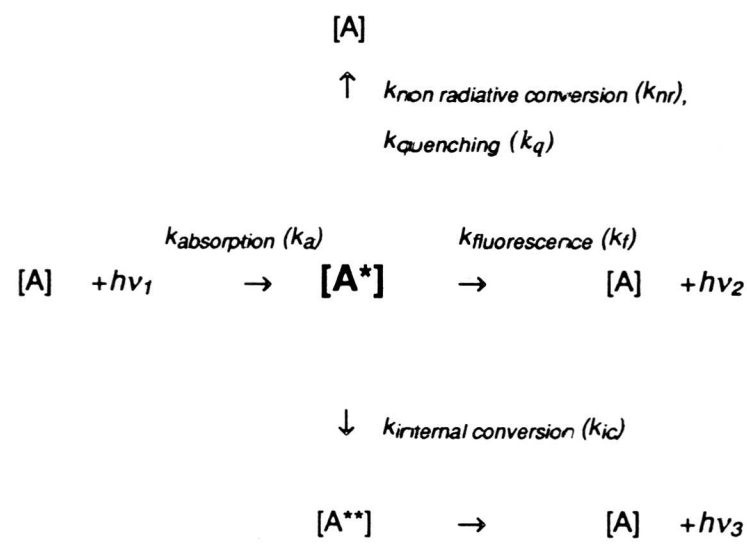

figure 2

where G_{corr} is a correction factor for the differential transmission of the vertical and horizontal components of the emitted light by the emission monochromator (Wahl, 1979). The decay of the intensity follows the exponential law:

$$I_{(t)} = I_o \exp(-t/\tau)$$

where I_o is the intensity at time zero of the excitation.

The different parameters are described in the following. The excited state lifetime is the recirpocal of the sum of all the kinetic contants:

$$\tau = (k_f + k_{nr} + k_{ic} + k_q + \Sigma k_{nf})^{-1}$$

Q is the quantum yield which is defined as the ratio:

Q = number of emitted photons / number of absorbed photons that is:

$$Q = \tau / \tau_o$$

where τ is the experimental excited state lifetime and τ_o the natural lifetime which can be calculated from the strength of the transition and is the lifetime value without any other deactivation process except emission (Strickler & Berg, 1962):

$$\tau_o^{-1} = 2.88 \times 10^{-9} n^2 \langle \bar{\nu}^{-3} \rangle_{av} \int \frac{\varepsilon}{\bar{\nu}} d\bar{\nu}$$

where

$$\langle \bar{\nu}^{-3} \rangle_{av} = \left(\int \bar{\nu}^3 f(\bar{\nu})\, d\bar{\nu} \right) / \left(\int_?^? f(\bar{\nu})\, d\bar{\nu} \right)$$

and n is the refractive index.

Anisotropy decay

In the second type of analysis, one define the anisotropy parameter as:

$$r(t) = \{I_{vv}(t) - G_{corr}\, I_{vh}(t)\} / I(t)$$

which provides the informations on the rotational correlation time of the fluorophore characterizing its brownian rotational motion.

The decay of the anisotropy $r_{(t)}$ is described in the simple case of a rigid rotating sphere by:

$$r_{(t)} = r_o \exp(-\tau/\theta)$$

where r_o is the intrinsic anisotropy which characterizes a transtion and 0 is the rotational correlation time of the fluorophore describing the brownian rotation of the particle.

The intrinsic anisotropy r_o is a function of the angle Φ between the absorption and the emission oscilators:

$$r_o = 0.2\,(3\cos^2\Phi - 1)$$

Two extreme cases are met:

when the oscillators are colinear, $\Phi = 0°$ and $r_o = 0.4$

when the oscillators are perpendicular, $\Phi = 90$ and $\rho_o = -0.2$

The rotational correlation time is a function of the hydrated volume V_h, the shape of the particle and the temperature-viscosity factor. In the case of a rotating sphere:

$$\Phi = \eta V_h / RT$$

For the cases of ellipsoids of revolution, the reader is referred to Wahl (1983) and reference therein.

In the case of internal motions of the fluorescence moiety within the protein matrix, the situation is more complex. The autocorrelation function describing the motions is a product of the specific autocorrelation functions associated to the different motions if they are independent (Ichiye & Karplus, 1983). The fluorescence anisotropy decay is multiexponential in these cases but owing to the present accuracy of the measurements, only three correlation times can be resolved:

$$r(t) = \sum_{i=1}^{i=3} \beta_i \exp(-\tau / \tau_i)$$

The angular amplitude of the fastest rotation caracterized by the wobbling angle 0_{max}, which is the semi-angle of the cone of rotation, can be calculated (Kinosita *et al.*, 1977) as:

$$\sum_{i=2}^{i=3} \beta_i = \left[\frac{\cos\theta_{max}(1 + \cos\theta_{max})}{2}\right]^2$$

It should be emphasized that the rotational motion is but only one of the physical phenomena leading to the depolarization of the incident light. The sencond cause of depolarization is the resonance energy transfer for the theory of which the reader is referred to the following references: Förster, 1948; Stryer, 1978.

FUNDAMENTAL PROPERTIES OF TRYPTOPHAN

These properties have been recently reviewed (Creed, 1984). In buffer solution pH7, tryptophan is under a zwitterionic form. The fluorescence decay is the result of two decaying excited state populations of respective lifetime 0.5 and 3.2 ns (Szabo & Rayner, 1980; Petrich *et al.*, 1983; Chang *et al.*, 1983). This finding has been rationalized by the rotamer hypothesis (Szabo & Rayner, 1980; Colucci *et al.*, 1990). Three major rotamers around the C_α - C_β bond are possible (Dezube *et al.*, 1981). They are schematized as Newman projection in figure 3.

The lifetimes exhibit respective values of 0.5 and 3.2 ns with proportions of 28% and 72% (Petrich *et al.*, 1983). It is

assumed that the shortest component is due to the interaction of the NH_3^+group with the indole ring in rotamers A and C. A charge transfer occurs between the excited state indole ring and the ammonium group. At alkaline pH only one long lifetime (τ = 8.2 ns) is displayed (Petrich *et al.*, 1983) showing the influence of the ammonium group.

In proteins, one would expect that in the time scale of the excited state duration, tryptophan can undergo interactions with the aminoacid side chains in its close surrounding. If only one conformation were present, *i.e.* one conformer, or if the conformers display the same excited state lifetime, one would observe a single component decay. In many if not all cases, heterogeneous decays are observed. Therefore, it is of primordial importance to analyze accurately the data in order to extract the conformational distribution of indole in proteins from the lifetime distribution.

DATA ANALYSIS

In complex cases where several excited state populations are coexisting at the same time, displaying different excited state lifetimes and rotational behaviors, like in a mixture of fluorophores or in an heterogeneous system, one get the following expressions of the parallel and perpendicular components:

$$I_{vv}(t) = \frac{1}{3} E_\lambda(t) * \int_0^{\infty}\int_0^{\infty}\int_{-0.2}^{0.4} \gamma(\tau,\theta,A)\, e^{-t/\tau}(1 + 2A\, e^{-t/\theta}) d\tau\, d\theta\, dA$$

and

$$I_{vh}(t) = \frac{1}{3} E_\lambda(t) * \int_0^{\infty}\int_0^{\infty}\int_{-0.2}^{0.4} \gamma(\tau,\theta,A)\, e^{-t/\tau}(1 - A\, e^{-t/\theta}) d\tau\, d\theta\, dA$$

where $E_\lambda(t)$ is the temporal shape of the excitation flash and * denotes a convolution product $\gamma(\tau,\theta,A)$ represents the number of fluorophores with fluorescence lifetime τ, rotational correlation time θ and intrinsic anisotropy A.

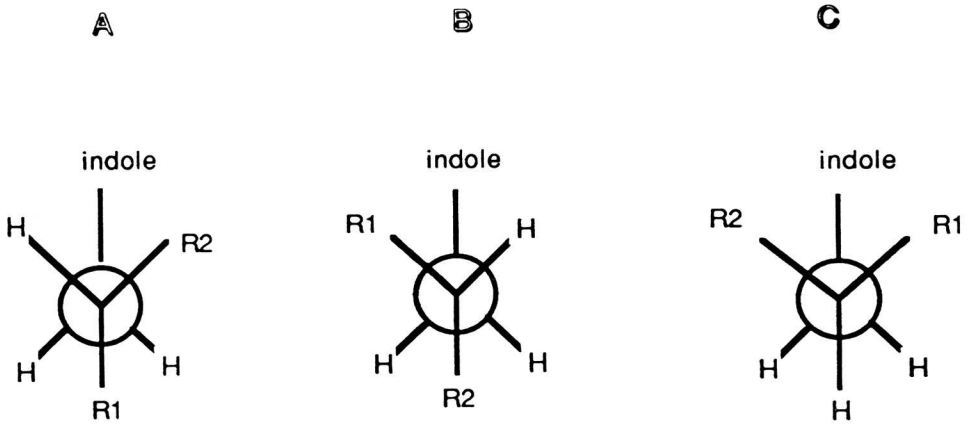

figure 3

with $R_1 = COO^-$; $R_2 = NH_3^+$

From these intensities, the excited state lifetimes and the correlation times must be extracted by mathematical tools.

The convolution problem comes from the fact that the instrumental response function E_λ (t) is not infinitely narrow but has a certain finite width. The experimetnal curve $I_{vv}(t)$ or $I_{vh}(t)$ is a convolution product which can be simply written as:

$$I(t) = E_\lambda (t) * F_\lambda (t)$$

$$I(t) = \int_0^t E_\lambda(t).\exp\left[\frac{-(t - T)}{\tau}\right] dT$$

Since the experimental data are noisy and finite in extent, there is strictly an infinite set of $\gamma(\tau,\theta,A)$ solutions within the experimental error. The classical analysis by non linear least square regression (Wahl, 1979) is not powerful enough for managing a high number of parameters such as needed for a distribution analysis.

A new method have been applied to the fluorescence data in LURE by J-C. Brochon and A.K. Livesey (Livesey & Brochon, 1987): the Maximum Entropy Method. The lifetime distribution which maximises the *Skillin-Jaynes entorpy function* (Jaynes, 1983; Livesey & Skilling, 1985) is chosen:

$$S = \int_0^{\infty}\int_0^{\infty}\int_{0.2}^{0.4} \gamma(\tau, \theta, A) - m(\tau, \theta, A) - \gamma(\tau, \theta, A) \log \gamma(\tau, \theta, A)/m(\tau, \theta, A)\, d\tau\, d\theta\, dA$$

where $m(\tau,\theta,A)$ is a starting model for the distribution which is flat in log (τ,θ) and A space. It has been shown that this choice has many compelling features: i) it introduces the minimum correlation between τ,θ and A, ii) the recovered $\gamma(\tau,\theta,A)$ is smooth, positive, unique and robust to noise (Livesey & Brochon,1987). In the studies dealing with a single chromophore, a fixed value of A is chosen equal to the fundamental anisotropy value measured in vitrified medium. A global analysis of $I_{vv}(t)$ and $I_{vh}(t)$ is performed.

Such an analysis allows in principle to detect the possible cross-correlations occurring between the excited state lifetime of the fluorophore in a certain protein conformational substrate and its correlation time in this particular transient subconformation. However, one has to point out that there is an inherent limit to the experimental method since the parrallel and the perpendicular components of the polarized decay (expression 1 and 2) are functions of two exponential series, the first one with time constant τi, the excited state lifetime of the fluorophore i and the second one with a time constant κi depending on both τi and θi (the rotational correlation time):

$$1/\kappa_i = 1/\tau_i + 1/\theta_i$$

One can see that τ and θ can be exchanged without any modification in the resulting expressions of the parallel and perpendicular components of the polarized decay. This leads to construction of iso-kappa curves on which exchanging τ and θ gives the name κ value. This limitaiton has been clearly pointed out in simulation studies (Brochon & Livesey, in preparation).

If we are only interested in the determination of the total intensity decay parameters (lifetime distribution), we can considerably simplify the analysis by summing the parallel and the perpendicular components:

$$T(t) = I_{vv}(t) + 2\, G_{corr} I_{vh} = E_\lambda(t) * \int_0^{\infty} \alpha(\tau) \exp\left(-\frac{t}{\tau}\right) d\tau$$

where G_{corr} is the correction factor (Wahl, 1979) and $\alpha(\tau)$ is the lifetime distribution given by:

$$\alpha(\tau) = \int_0^{\infty} \int_{-0.2}^{0.4} \gamma(\tau,\theta,A)\, d\theta\, dA$$

In order to ensure our recovered distribution agrees with our data, we maximise S:

$$S = \int_0^{\infty} \alpha(\tau) - m(\tau) - \alpha(\tau) \log \frac{\alpha(\tau)}{m(\tau)}\, d\tau$$

subjected to the following constraint:

$$\sum_{k=1}^{M} \frac{(T_k^{calc} - T_k^{obs})^2}{\sigma_k^2} \leq M$$

where T_k^{calc} and T_k^{obs} are the k_{th} calculated and observed intensities. $\sigma^2 k$ is the variance of the kth point ($\sigma^2 k = \sigma^2 k,vv + 4\, G_{corr}{}^2 \sigma^2 k,vh$, Wahl, 1979). M is the total number of observations.

The center τj of a single class j of lifetimes over the $\alpha(\tau i)$ distribution is defined as:

$$\tau_j = \sum_i \alpha_i \tau_i / \sum_i \alpha_i$$

the summation being performed on the significant values of the $\alpha i(\tau i)$ for the j class. C_i is the normalized contribution of the lifetime class j. A lifetime domain spanning 150 values logarithmically spaced between 0.1 and 20.0 ns are routinelly used in the analyses. Simulations have demonstrated that the method can discriminate between discrete and continuous lifetime distributions (Vincent *et al.*, 1988).

I shall now present some of the results obtained in our laboratory on fluorescence of proteins containing a single tryptophan.

RESULTS

The internal dynamics and aggregation state of Apocytochrome c in aqueous solution

The single tryptophan residues (Trp-59), as well as one out of the four tyrosine residues (Tyr-48) of horse heart cytochrome c, play an import role in the interactions of the peptide chain with the heme moiety as they have been shown to be hydrogen-bonded respectively to each of the two heme propionic acid side-chains (Dickerson *et al.*, 1975; Wand & Englander, 1985). This situation in the holoprotein explains its extremely strong quenching (Fisher *et al.*, 1973). X-ray diffraction studies as well as recent NMR data have evidenced the high degree of structural organization of the holoprotein (Dickerson *et al.*, 1975; Wand & Englander, 1985).

The removal of the heme group evokes two main effects. Firstly, the secondary structure is lost as shown by circular dichroism as well as by ^{1}H-NMR measurements (Fisher *et al.*, 1973; Cohen *et al.*, 1974). Secondly, the intrinsic protein fluorescenc is strongly enhanced (Fisher *et al.*, 1973).

In the apoprotein, the "priviledged" situation of Trp-59 and its strong t-fluorescence make it a very atractive

intrinsic probe for studying the protein flexibility in different environments, at a specific and essential location. Such a flexibility is expected to be of great importance for the accomodation of the apoprotein to changing environments during the translocation form the cytoplasmic compartment where it is synthesized to the mitochondrion where the heme group is enzymatically bound. We have first studied the protein in solution (Vincent *et al.*, 1988).

The total fluorescence intensity decay of Trp-59 was analyzed as a sum of three of four exponentials by the non linear least square method, the last model always providing a slight but significant decrease in the chi-square values. Maximum entropy analysis strongly suggests the existence of a distribution including four separated classes of lifetimes. The center values were respectively around 0.1-0.2, 1, 3 and 5 ns, in agreement with the lifetime values obtained by non linear least square regression analysis (Table 1).

Temperature increase led essentially to a redistribution of the fractional amplitudes, affecting mostly that of the 5 ns component which almost totally disappeared at high temperature (35-40°C). The lifetime values were not significantly affected except for the 3 ns component which decreased by about 15 per cent in the temperature range studied. These observations strongly suggest that the protein exists under different conformational substates in thermal equilibrium. A continuum of conformations is not likely, even though the protein in solution is highly flexible.

Time-resolved fluorescence anisotropy measurements evidenced the existence of restricted angle of rotation of around 55° was calculated for the fast internal rotation of the Trp residue. A second internal motion, slower by one order of magnitude, corresponding likely to a local motion of the peptide chain involving the Trp-59 residue, was detected on the anisotroy decay curve.

Finally, the longest correlation time (5 ns) should correspond to the average brownian rotation of the overall protein. Its value doubled as a function of the protein concentration, revealing an association process leading most likely to a dimer in the concentration range studied (2-139 μM). The detection of this correlation time

Tabla 1
TOTAL FLUORESCENCE INTENSITY DECAY PARAMETERS OF Trp-59 OF APOCYTOCHROME C AS A FUNCTION OF THE EXCITATION WAVELENGTH

	$\tau_{1(ns)}$	$\tau_{2(ns)}$	$\tau_{3(ns)}$	$\tau_{4(ns)}$	α_1	α_2	α_3	α_4	χ^2
a	0.43	2.25	4.67	-	0.34	0.43	0.23	-	1.64
b	0.21	1.05	2.78	5.02	0.26	0.22	0.37	0.15	1.52
c	0.21	1.07	2.86	5.12	0.27	0.23	0.37	0.13	1.33

Protein concentration: 139 μM. Temperature : 20°C. Excitation wavelenght: 300 nm. Emission: $CuSO_4$ 1M filter (2 cm optical path). Excitatin bandwidth: 1 nm a) triple exponential analyzed by non linear least square regression; b) quadruple exponential model analyzed by non linear least square regression; c) maximum entropy analysis starting with a flat distribution of 150 lifetime values (Vincent *et al.*, 1988).

Table 2
TIME-RESOLVED FLUORESCENCE ANISOTROPY DECAY PARAMETERS OF Trp-59 IN APOCYTOCHROME C AS A FUNCTION OF PROTEIN CONCENTRATION

Apo C (μM)	β_1	β_2	β_3	$\theta_{1(ns)}$	$\theta 2_{(ns)}$	$\theta 3_{(ns)}$
1.8	0.080	0.078	0.080	0.14	1.09	5.33
8.5	0.088	0.077	0.083	0.18	1.37	6.25
139	0.107	0.106	0.065	0.17	1.72	9.41
139[a]	0.127	0.110	0.043	0.18	1.52	6.99
				0.15[c]	1.28[c]	5.87[c]
139[b]	0.139	0.109	0.041	0.15	1.73	7.19
				0.09[c]	1.04[c]	4.33[c]

Emission: cut-off filter $CuSO_4$ 1M (2 cm optical path). Triple exponential model:

$$r(t) = \beta_1 \exp(-t/\theta_1) + \beta_2 \exp(-t/\theta_2) + \beta_3 \exp(-t/\theta_3)$$

Temperature: 20°C
[a] in presence of 3.7 M urea
[b] in presence of 8 M urea
[c] values corrected for the viscosity effect of urea (viscosity = 1.19 and 1.66 centipoise for 3.7 and 8 M respectively)

indicates that the apocytochrome c protein does not behave as a worm-like polymer. It must display some transient organized structure. The flexibility of the peptide chain was more restrained in the associated than in the monomeric form but not the fast internal rotation of the Trp residue (Table 2).

The urea effect points to the existence of only weak interactions between the monomers. Such results demostrate that the technique can provide informations on the aggregation state of a protein at low concentration. It is usefull to have a precise knowledge of this aggregation state not only from a fundamental point of view but also for practical purposes in cases where other techniques are employed such as NMR, in which the aggregation state of the protein may be critical for a correct interpretation of the connectivities.

Effects of ligand binding on the conformation and internal dynamics in specific regions of porcine pancreatic phospholipase A_2 with tryptophan as a probe

The exploration of the effect of ligand-protein interactions on conformational substates and internal dynamics in different regions of phospholipase A_2 from porcine pancreas (PLA_2), was performed by combining site-directed mutagenesis and time-resolved fluorecence measurements (Kuipers *et al.*, 1990; 1991). The single tryptophan residue (Trp-3) in the wild type protein was replaced by a phenylalanine

residue, whereafter Trp was substituted either for leucine-31, located in the calcium binding loop, or for phenylalanine-94, located at the "back side" of the enzyme, in α-helix E (Dijkstra *et al.*, 1981).

Analysis by the Maximum Entropy Method (MEM) of the total fluorescence intensity decays, provides in each case a distribution of separate lifetime classes, which can be interpreted as reflecting the existence of discrete conformational substates in slow exchange with respect to the time-scale of the decay kinetics. The fluorescence decay of the W94 mutant is dominated by an extremely short excited state lifetime of $\sim$ 60 ps, probably arising from the presence of two proximate disulfide bridges.

Time-resolved fluorescence anisotrypy studies show that the Trp residue near the NH_2 terminus (Trp-3) undergoes a more limited rotational motion than the Trp-31 located in the calcium binding loop. The widest angular rotation is observed at position 94, in α-helix E (Table 3).

There is virtually no effect of calcium binding on the lifetime distribution of the Trp residue at the 3 or the 94 position (figure 4 and 6). Binding of the monomeric substrate analog *n*-dodecylphosphocholine (C12PN) in the presence of calcium hardly affects neither the Trp-3 excited state population distribution (figure 4), nor its rotational dynamics (Table III). By contrast, the binding of the micellar substrate analog *n*-hexadecyl phosphocholine (C16PN) in the presence of calcium is very efficient in modifying the lifetime distribution (figure 4) and the protein dynamics.

A strong slowing down of the internal motion of the Trp-3 residue, which now displays a value around 0.2 ns, and a large reduction of its amplitude, suggested by the increased contribution of the long

Table 3
EFFECTS OF LIGAND BINDING ON THE ANISOTROPY PARAMETERS OF THE WILD TYPE, THE W31 AND THE W94 MUTANT PLA_2s

Enzyme	β_1	β_2	θ_1 (ns)	θ_2 (ns)	θ_{max}(°)
W3	-	0.221	-	7.67	26
+Ca^{2+}-	-	0.214	-	7.21	27
+C12PN (monomer)	0.023	0.188	0.14	7.84	31
+C16PN (micelle)	0.030	0.273	0.23	28.5	14
W31	0.040	0.170	0.69	7.40	34
+Ca^{2+}	0.056	0.129	0.84	5.02	43
+C12PN (monomer)	0.042	0.169	1.20	6.46	35
+C16PN (micelle)	0.062	0.185	1.21	24.4	32
W94	0.132	0.113	0.57	7.50	44
+C16PN (micelle)	0.130	0.081	1.74	24.9	54

The anisotropy decay was assumed as a sum of exponentials: $r(t) = \sum \beta_i \exp(-t/\theta_i)$. θ_{max} was calculated according to Kinosita et al. (1977).

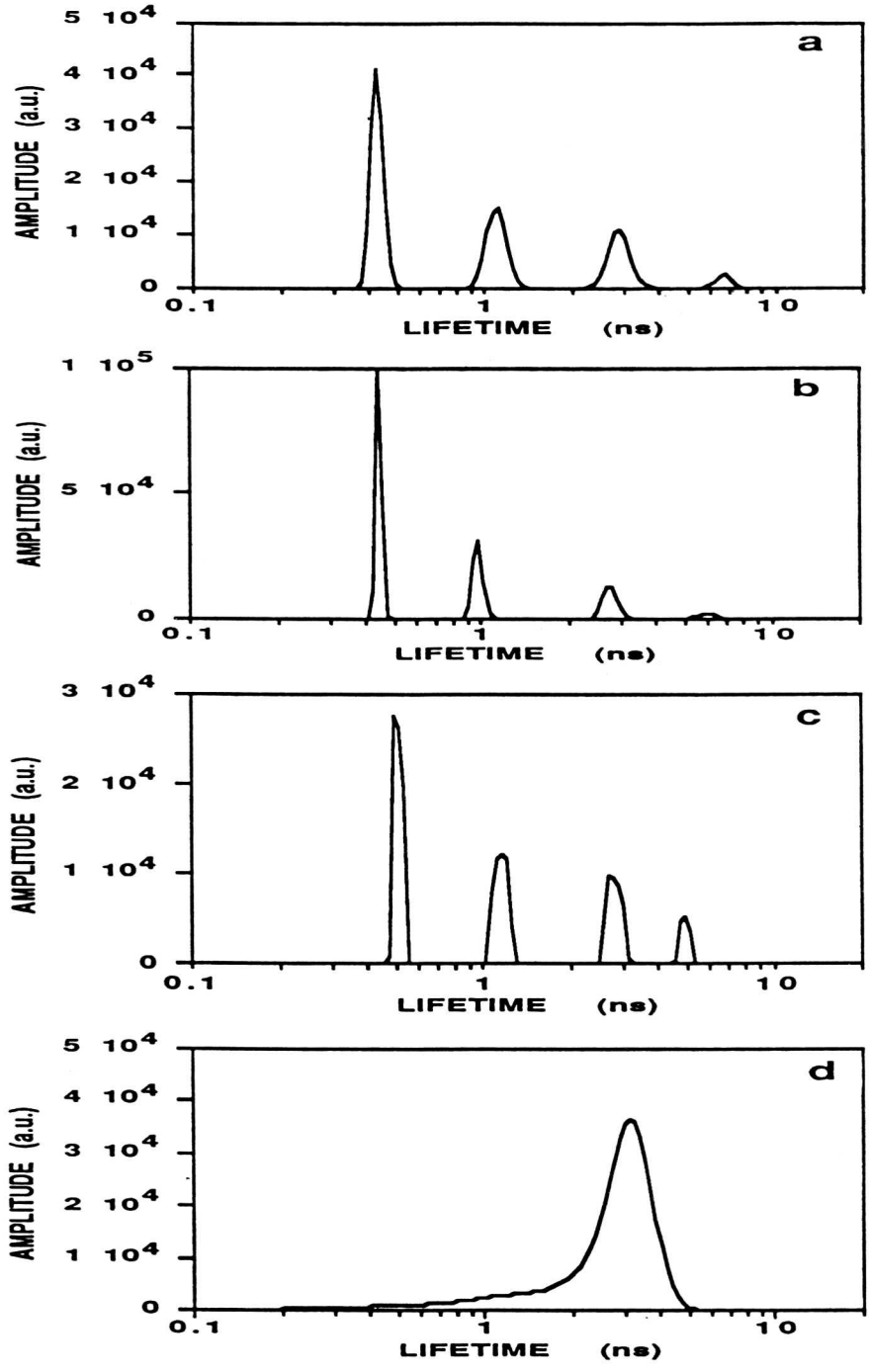

FIG. 4. Amplitude profiles of W3-PLA2 total fluorescence intensity decay recovered by MEM in buffer (a), in presence of Calcium 50 mM (b), in presence of Calcium 50 mM plus monomeric C12PN (c) and in presence of Calcium 50 mM plus C16PN micelles (d).

correlation time, are observed (Table 3). The value of this correlation time is considerably enlarged. This corresponds to the large size increase of the rotating body, comprising two protein molecules and about 80 phospholipid molecules per micelle, as has been found before with gel-filtration experiments (de Araujo *et al.*, 1979). The initial anisotropy value reaches 0.3 in the double exponential model, indicating that the Trp rotation is fully resolved.

Calcium binding displays a strong influence on the lifetime distribution of Trp-31: a major local conformation corresponding to a lifetime class with a barycenter value of ~5.5 ns and contributing to ~50% of the decay is selected (figure 5). The conformations giving rise to the short lifetimes (c_1 and c_2 lifetime classes) become less important. The contribution of the third lifetime class (c_3) stays at a constant value of 30%. In the presence of calcium, the amplitude of motion is wider than without the ion (Table 3).

Binding of the monomeric substrate analog *n*-dodecylphosphocholine (C12PN) in the presence of calcium affects only slightly the Trp-3 excited state population distribution and its rotational dynamics. The binding of C12PN monomers to the W31 mutant further increses the contribution of the c_4 lifetime class at the expense of c_2 (figure 5). A more restricted rotation of the Trp-31 residue is also induced (Table III). The interaction with C16PN micelles slightly modifies the lifetime distribution as compared to that described in the presence of monomeric substrate analog (figure 5). A small reduction of the wobbling angle is observed in the protein/micelle complex but not to the same extent as observed for the Trp at position 3 (Table 3). The rate of the internal motion is not changed. The long correlation time value is largely enhanced as the size of the whole rotating body is largely increased, but it is lower than the one measured for position 3 (Table 3).

For the W94 mutant, no large effect of C16PN micelle binding was observed on the lifetime distribution (figure 6). A moderate decrease of the contribution of the long correlation time is noticeable whereas the rate of internal rotation is slowed down. The long correlation time reflects the overall motion of the protein/micelle complex (Table 3).

In conclusión, by placing a single Trp-residue at strategic positions along the peptide chain of PLA_2, relevant to the binding of biological ligands, and excelent model system for the study of selective perturbations of conformational substates and internal dynamics is provided.

We can propose that in the enzyme micelle complex the substrate molecules are not distributed homogeneously around the protein molecule. The micellar domain of the substrate is more concentrated in the proximity of the N terminus region, whereas monomeric substrate is preferentially bound in that part of the recognition site near the calcium site, and therefore in the proximity of the catalytic site. The 31 position could be at the edge of the micellar region in the mixed micelle. The perturbation of the N-terminal part of the protein by micelle binding can be transmitted via the H-bond network (Dijkstra *et al.*, 1983) to the catalytic site. This could explain the increase in the catalytic activity towards aggregated substrates by several orders of magnitude as compared to dispersed substrates.

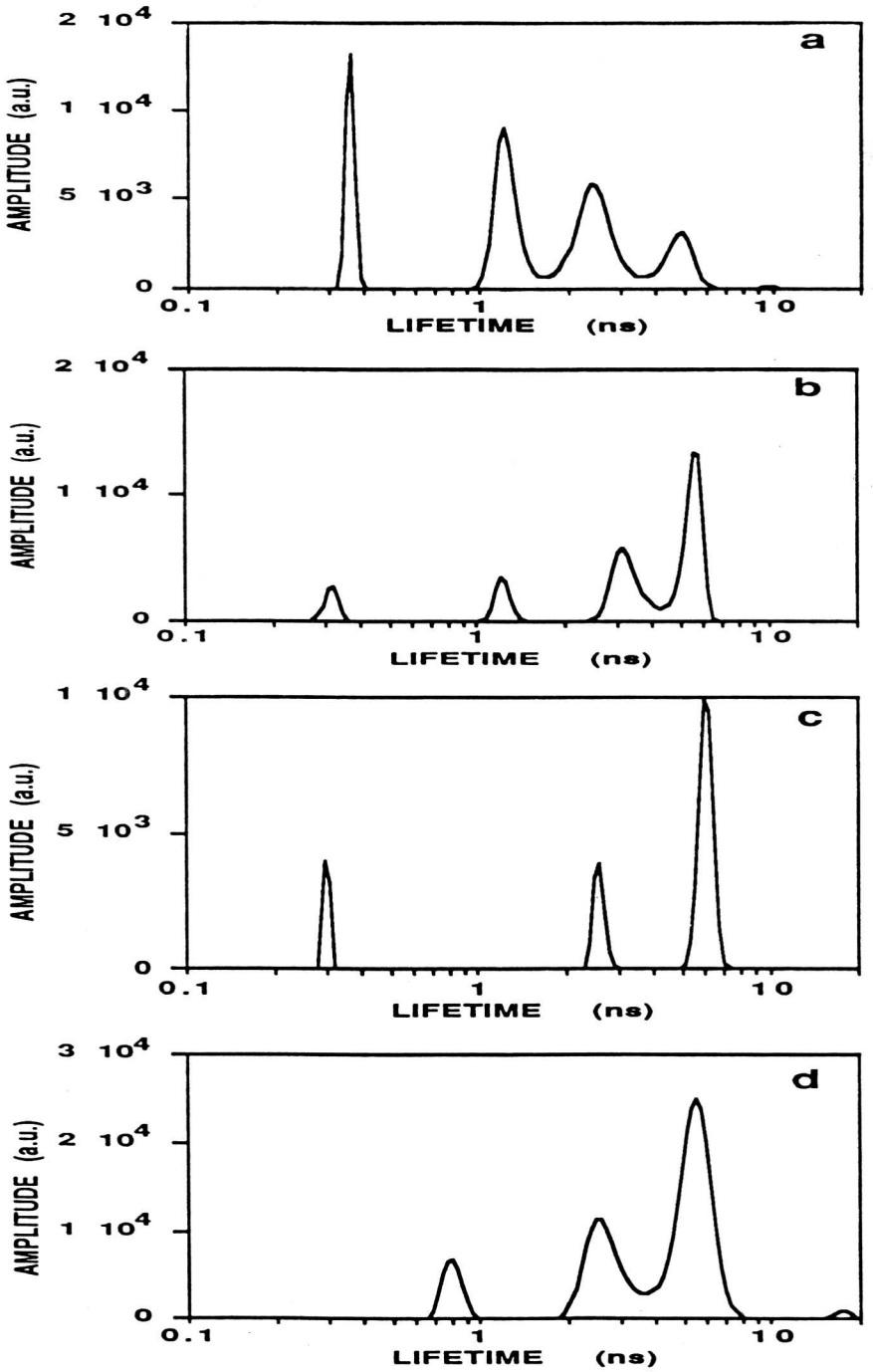

FIG. 5. Amplitude profiles of W31-PLA2 mutant total fluorescence intensity decay recovered by MEM in buffer (a), in presence of Calcium 50 mM (b), in presence of Calcium 50 mM plus C12PN (c) and in presence of Calcium 50 mM plus C16PN micelles (d).

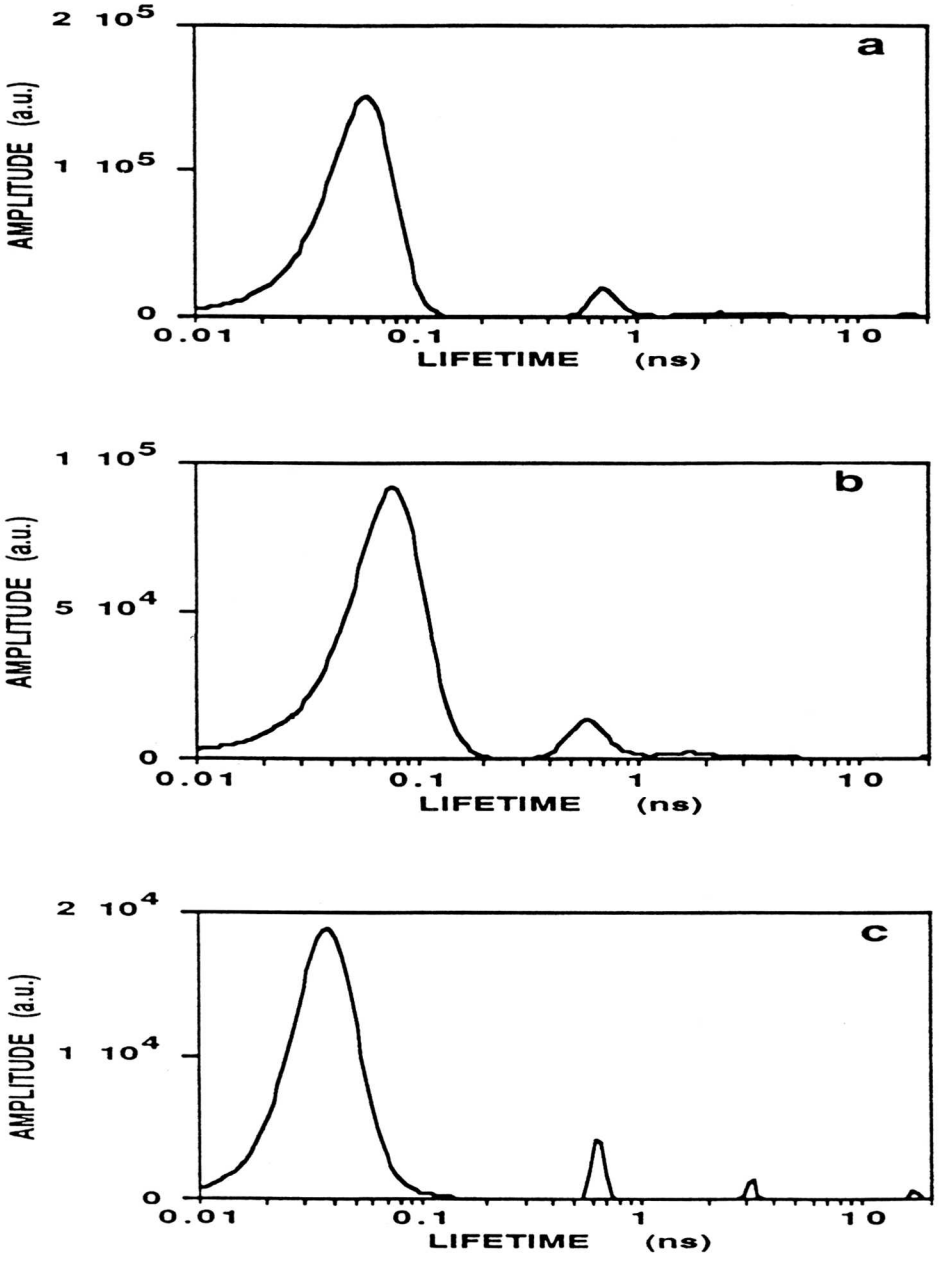

FIG. 6. Amplitude profiles of W94-PLA2 mutant total fluorescence intensity decay recovered by MEM in buffer (a); in presence of Calcium 50 mM (b) and in presence of Calcium 50 mM plus C16PN micelles (d).

CONCLUSIONS

The purpose of this short introductory review was to present the possibilities offered by the time-resolved fluorescence technique for the investigations of protein dynamics owing to the recent improvements both in the quality of the data obtained on synchrotron and laser sources (the presented examples were performed on the synchrotron at Orsay) and in the analysis. The method allows to get insight into the existence of local conformational substates of proteins and to measure their internal dynamics as well as their overall rotational rate. Informations on their aggregation state can then be gathered. The coupling of this technique to protein engineering increases its domain of application to more specific questions about the effect of ligands binding on specific regions of the protein.

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