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Fluorescence lifetime and polarization anisotropy studies of membrane surfaces with pyridoxal 5′-phosphate

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The fluorescence lifetime and depolarization of the pyridoxal 5′-phosphate label demonstrated different environments at the structure-solvent interface for micelles, liposomes, proteins and membranes. A short lifetime and rotational correlation time for the micelles and liposomes proved that the label was strongly associated with the water solvent and rotated freely about the covalent bond. The proteins provided a more buried or hydrophobic site as shown by an increase in the lifetimes. Rotational correlation times of 4–6 ns for sarcolemma and erythrocyte membranes suggested restricted rotation for the pyridoxal 5′-phosphate label. Lower values of the rotational correlation time for rod outer segment and myelin sheath proved that the protein ϵ-amino groups are at the solvent interface which allows for more rotation.

1. Introduction

Pyridoxal 5′-phosphate (PLP) binds to lysine residues through a Schiff base linkage with the ϵ-amino group. For this reason it has been employed successfully in specific investigations of protein structure [1–3]. Cornish and Ledbetter [4] used this label to measure the rotational diffusion of proteins. A hydrophobic binding site improves the stability by preventing the hydrolysis of the bond. For amino groups at the surface it may be necessary to reduce the C = N bond to improve the stability against water. With the P-pyridoxyl residues Irwin and Churchich [5] determined the degree of rotational mobility of the peptide chains. Williams and Churchich [6] used this approach in developing P-pyridoxyl phospholipid probes for interactions with proteins. Rifkin et al. [7] reduced the Schiff base with borotritide to form tritiated membrane proteins on viruses.

For the amino groups located at the surface of a liposome, membrane or protein, the P-pyridoxyl group provides for a measurement of the mobility at the surface through the rotation of the group about the 4′ covalent bond. Gottlieb and Wahl [8], using as a model a fluorescent group mobile about a rotation axis connected to a spherical macromolecule, first treated such a system. This model, also treated by Rigler and Ehrenberg [9], yields for the anisotropy decay

$$r(t) = Ae^{-(D_A - 6D_s)t} + Be^{-(4D_A + 6D_s)t} + Ce^{-6D_s t}, \quad (1)$$

where D_A and D_S are the diffusional constants for probe and macromolecular sphere, respectively. A, B and C are constants which depend on the orientation of the absorption and emission dipoles of the probe. Kinosita et al. [10] also obtained this result and demonstrated with others that, for a

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very large sphere, setting $D_s = 0$ yields

$$r(t) = Ae^{-D_A t} + Be^{-4D_A t} + C. \quad (2)$$

This would be applicable to a mobile probe on a protein embedded in a membrane. As Kinosita et al. further point out, if these three terms cannot be resolved with limited experimental precision, one may resort to a simple form, namely

$$r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty, \quad (3)$$

where ϕ is the rotational correlation time of the probe.

In this study the fluorescence lifetime of the P-pyridoxyl label was measured in micelles, liposomes, proteins and membranes. In these systems the fluorescence depolarization was measured and the rotational anisotropy fitted to an equation. These observations demonstrated that the mobility of the P-pyridoxyl probe was dependent on the environment. Smaller correlation times and lifetimes demonstrated that the lysine residues were near the aqueous interface in rod outer segments and myelin sheaths. Hydrophobic sites on protein and glycerol interfaces decreased the mobility with an increase in both the lifetime and rotational correlation time.

2. Experimental

A laboratory-constructed nitrogen laser designed by Schenck and Metcalf [11] excited the samples at 337 nm for the fluorescence. Removal of the rear mirror of the laser yielded the superradiance from only one direction and a shorter pulse with a width at one-half maximum of 8 ns. A fast lens system collected the fluorescence at 90° and focussed the emission onto an RCA 4526 photomultiplier tube in a shielded housing. Baffles and a cut-off filter removed the scattered exciting light. The 7A18 N vertical amplifier of a Tektronix 7623 storage oscilloscope measured the current output pulse across a 50 Ω load connected with 18 cm of RG 62A/U cable to the tube. The measured input capacitance, including that of the oscilloscope, was 52 pF. A dilute solution of rhodamine B in water yielded an emission lifetime of 2.2 ± 0.2 ns which for the apparatus means a working capacitance of 44 pF. Several decay curves were averaged by the storage capability of the oscilloscope at a repetition rate of about 10 Hz. A signal from an RCA 1P28 photomultiplier tube viewing a small portion of the excitation flash triggered the oscilloscope.

In the depolarization studies a Glan-Taylor prism (Karl Lambrecht) polarized the excitation pulse. The emission collection path included a film linear polarizer (Ealing) for observation of the parallel and perpendicular components. Photographs of the traces on the oscilloscope were digitized with a Hewlett-Packard 7074 plotter and digitizing sight and a Hewlett-Packard 9825T computer.

The following chemicals were obtained from Sigma: pyridoxal 5′-phosphate (PLP), pyridoxal hydrochloride (PL), synthetic dipalmitoyl-DL-α-phosphatidylethanolamine (DPPE), synthetic dipalmitoyl-DL-α-phosphatidylcholine (DPPC), sodium borohydride, bovine serum albumin and aldolase (EC 4.1.2.13) from rabbit muscle. Dodecylamine (DDA) came from Eastman. We prepared the red blood cell ghosts from recently outdated blood according to Cherry [12]. Other membrane preparations were generous gifts from our colleagues expert in their preparation: bovine rod outer segment disk membranes from Professor R.C. Crouch, rabbit spinal cord intact myelin sheath from Professor N.L. Banik and intact vesicles of canine ventricle sarcolemma from Professor G.E. Lindenmayer. When incubated over a period of time, pyridoxal 5′-phosphate reacts with available lysine residues in the membrane preparations and in the proteins. We followed this Schiff base formation through the spectral changes. After the reaction, the addition of $NaBH_4$ reduced the Schiff base forming the pyridoxyl CH_2-NH linkage which is not susceptible to hydrolysis when exposed to solvent. Dialysis removed excess reactants.

Preparations of vesicles of DPPE/DPPC were standard. We dissolved 0.7 mg PLP, 2.5 mg DPPE and 13.3 mg DPPC in 10:1 $CHCl_3/CH_3OH$. Added $NaBH_4$ reduced the DPPE-PLP Schiff base. We evaporated the solvent under vacuum and resuspended the solid in 150 mM NaCl and 5 mM

potassium phosphate at pH 7.4 and 45°C with stirring. Sonication converted these large multilayer vesicles to smaller bilayer vesicles. Addition of PLP to micelles of DDA formed the Schiff base which $NaBH_4$ readily reduced to the pyridoxyl form.

3. Analysis and results

The data were analyzed by three methods: the phase plane method of Wyker and Demas [13] for the total emission lifetime, the iterative convolution integral technique of Grinvald and Steinberg [14] for the lifetime and the iterative convolution integral technique of Kawato et al. [15] for the rotational diffusion time of the pyridoxyl-labeled molecule. The convolution integral for an observed sample decay, $D(t)$, from the observed excitation profile, $E(t)$, can be given by

$$D(t) = K \exp(-t/\tau) \int_0^t E(x) \exp(x/\tau)\, dx, \quad (4)$$

where K is the proportionality constant and τ the sample lifetime. The desired monoexponential impulse response is

$$i(t) = K \exp(-t/\tau). \quad (5)$$

The phase plane plot method involves taking the derivative of eq. 4 with respect to t and integrating between the limits of 0 and t to yield

$$D(t) = K \int_0^t E(x)\, dx - \frac{1}{\tau} \int_0^t D(x)\, dx. \quad (6)$$

Dividing both sides by $\int_0^t E(x)\, dx$ gives

$$W(t) = \left(-\frac{1}{\tau}\right) Z(t) + K \quad (7)$$

where $W(t)$ and $Z(t)$ are the obvious integral fractions. A plot of $W(t)$ vs. $Z(t)$ yields τ from the slope $(-1/\tau)$. The integrals for $E(t)$ and $D(t)$ are evaluated by the trapezoidal rule [16].

In the popular fast iterative convolution method of Grinvald and Steinberg [14], assuming that the interval between points is small enough, the area defined by $E(t_j)\exp(t_j/\tau)$ and $E(t_{j+1})\exp(t_{j+1}/\tau)$ in eq. 4 can be calculated with the trapezoidal rule. Eq. 4 can be written as

$$D(t) = KE(x) \exp[-(t-x)/\tau]\, dx. \quad (8)$$

Trapezoid integration of eq. 8 gives

$$D(t_i) = tK \sum_{j=1}^{i-1} E(t_j) \exp[-(i-j)t/\tau] + 0.5tKE(t_i). \quad (9)$$

where t is the time increment for i discrete channels. The recursion formula for computer implementation of the integration is

$$D(t_{i+1}) = [D(t_i) + 0.5tKE(t_i)] \exp(-t/\tau) + 0.5tKE(t_{i+1}) \quad (10)$$

From the observed fluorescence intensities of I_v and I_H the following relations were calculated

$$D_T = I_v + 2I_H \quad (11)$$

$$D_D = I_v - I_H \quad (12)$$

$$r = I_D/I_T \quad (13)$$

Then, to obtain the total fluorescence emission lifetime, the calculated function $D(t)$ from eq. 10 was compared to the observed function $D_T(t)$ so as to minimize

$$\sum_{i=1}^{n} w_i \left[D_T^{calc}(t_i) - D_T^{obsd}(t_i)\right]^2 \quad (14)$$

where n is the number of channels and w_i the weighting factor.

After the emission parameters of K and τ for a system were determined for $D_T(t)$, the emission anisotropy was obtained by the convolution integral of Kawato et al. [15], namely

$$D_D(t) = \int_0^t r_0 E(x) D_T(t-x) \times \exp[-(t-x)/\phi]\, dx. \quad (15)$$

The values of r_0 and ϕ were determined from the Marquardt algorithm in order to minimize the function

$$\sum_{i=1}^{n} w_i \left[D_D^{calc}(t_i) - D_D^{obsd}(t_i)\right]^2. \quad (16)$$

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Table 1

Lifetimes and rotational parameters in mimetic systems

Values obtained from: 1, phase plane plot of Wyker and Demas [13]; 2, convolution integral of Grinvald and Steinberg [14].

System	Solvent	τ (ns)		ϕ (ns)	r_0
		1	2		
PLP	H_2O	1.1	0.9		
PLP/DDA	50% MeOH	2.4	2.4	0.22	0.20
	20% MeOH	2.4	2.3	0.77	0.13
	H_2O	2.4	2.3	1.4	0.13
	SDS	2.4	2.4	0.53	0.26
	DMF	4.2	3.6		
	glycerol	5.9	6.4	99.5	0.36
PL/DDA	H_2O	2.1	1.9		
PLP/DPPE/DPPC	H_2O	3.0	3.0		
PLP/DPPE/DPPC	(sonicated)	1.9	1.7	0.71	0.13
PL/DPPE/DPPC	(sonicated)	2.3	2.5		

$-r_0)e^{-t/\phi} + r_0$ and the function $r_1 e^{-t/\phi} + r_0$ were also tried in the convolution integral but only the single-exponential function yielded reasonable results.

The computer program for the phase plane plot was an adaptation for the Hewlett-Packard 9825T

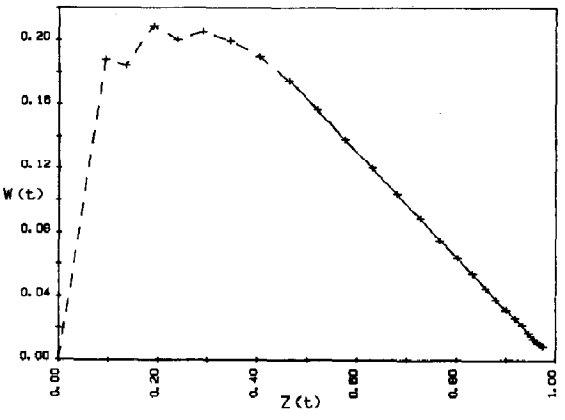

Fig. 1. Phase plane plot for P-pyridoxyl-labeled erythrocyte ghosts. The solid line is a least-squares fit from points 12 to 30.

computer of the EXPFIT program of Demas [17] which uses the Marquardt algorithm for nonlinear least-squares fitting for one or two exponentials. The data for emission lifetimes were fitted with one exponential. Fig. 1 presents results of the phase plane plot, $W(t)$ vs. $Z(t)$, for red blood cell ghosts. A least-squares analysis from point 12 to 30 yielded a fluorescence lifetime of 3.1 ns. Fig. 2

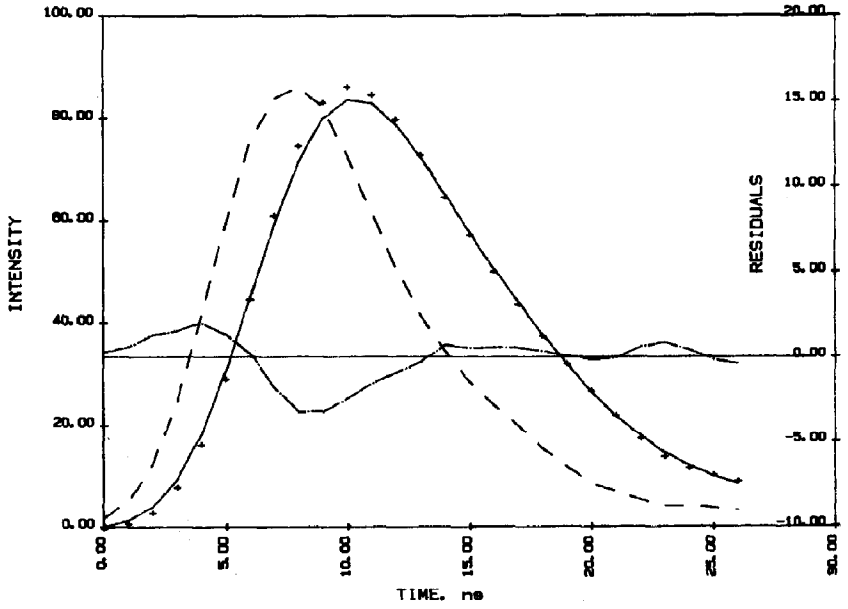

Fig. 2. Total fluorescence intensity (D_T) of P-pyridoxyl-labeled erythrocyte ghosts (+). (———) Fit by the method of Grinvald and Steinberg [14]; (·–·–·) residuals; (— — —) excitation flash.

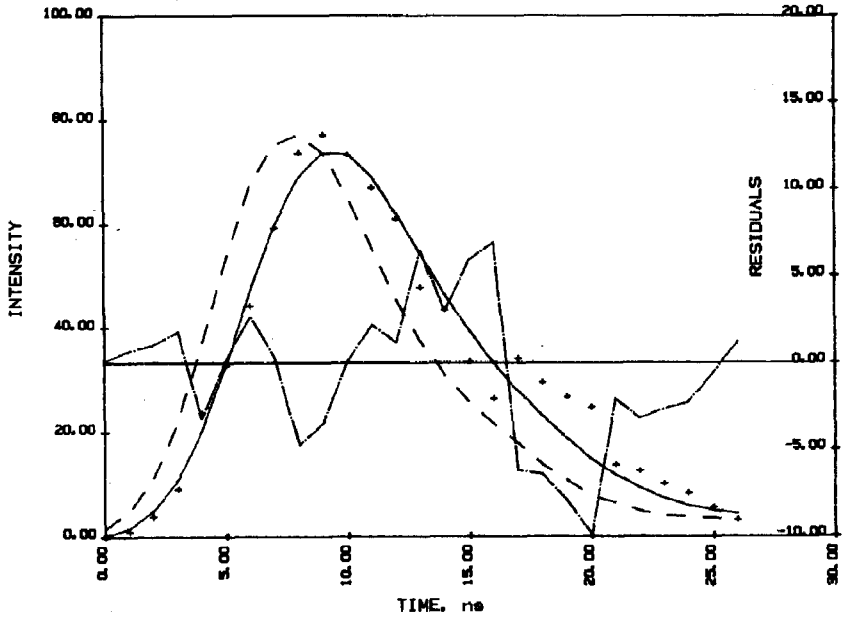

Fig. 3. Difference in the fluorescence intensity (I_D) from the polarization experiments on P-pyridoxyl-labeled erythrocyte ghosts (+). (———) Fit by the method of Kawato et al. [15]; (·–·–·) residuals; (— — —) excitation flash.

illustrates the results of the convolution method of Grinvald and Steinberg. The plus symbols represent the total observed fluorescence intensity. The data were fitted (unbroken line) with one exponential decay with a weighting factor of $1/I_T(t_i)$. A lifetime of 3.1 ns was obtained, in good agreement with the phase plane method. Tables 1 and 2 list the fluorescence lifetimes from both methods for other systems studied.

Table 2

Lifetimes and rotational parameters in protein and membrane systems

Values obtained from: 1, phase plane plot of Wyker and Demas [13]; 2, convolution integral of Grinvald and Steinberg [14].

	τ (ns)		ϕ (ns)	r_0
	1	2		
Protein				
BSA/PLP	3.0	3.1	26.4	0.21
BSA/PL	2.8	2.7	8.0	0.24
Aldolase/PLP	2.2	1.9	9.6	0.19
Membrane				
Rod outer segments	2.7	2.3	1.8	0.26
Sarcolemma	3.0	2.9	6.4	0.13
Myelin	2.7	2.8	0.73	0.22
Erythrocyte ghosts	3.1	3.1	4.44	0.09

The plus symbols in fig. 3 represent I_D values obtained by $I_v - I_H$ from the polarization experiment on ghosts. The convolution integral of Kawato et al. [15] was formed and the data fitted (unbroken line) to a single rotational correlation time, as in

$$r(t) = r_0 e^{-t/\phi} \tag{17}$$

with weighting factor of unity. The residual curve of fig. 3 shows a reasonable fit for this type of data. The rotational correlation times and r_0 values are given in tables 1 and 2 for the other systems.

4. Discussion

Use of the pyridoxyl label at the structure/solvent interface allows the determination of the fluidity at that point by fluorescence measurements. We have described in this paper the label-

ing specifications and the experimental hardware necessary for these studies. The availability of other hardware systems for fluorescence measurements gives to these observations some practical value. As long as a free amino group at the surface is available, which is likely the case because amino groups are hydrophilic, the pyridoxal molecule will react to form the Schiff base. This base can be readily reduced to form a water-stable covalent bond.

Using a fast detection line the fluorescence lifetime was measured by the phase plane and convolution integral techniques with good agreement. The lifetimes appear with lower values (1–2 ns) for systems in table 1 in which the pyridoxyl is exposed to water. Removal of the water increases the lifetime. The large liposomes, DMF and glycerol cause the lifetime to increase to 3–6 ns. Sonication of the liposomes reduces the lifetime as more water becomes associated with the pyridoxyl. In table 2 BSA demonstrates that over aldolase the pyridoxyl is bound in a more hydrophobic site. Such a distinction is not as evident among the membranes. Considering that the error in the values is about ± 0.2 ns, the sites of bound P-pyridoxyl for rod outer segments and myelin may harbor more water molecules. This means that the ϵ-amino groups are strongly associated with solvent water. More careful measurements with photon-counting techniques or even with a transient digitizer should be more fruitful.

The values of ϕ and r_0 in table 1 for PLP/DDA show clearly the rotation of the pyridoxal 5′-phosphate. A correlation time of 100 ns for ϕ with a limiting value for r_0 in glycerol assures that the technique responds to the rotation and that $r_0 e^{-t/\phi}$ is sufficient for description.

On the micelles the pyridoxyl 5′-phosphate rotates freely about the linkage. The short rotational correlational times of 0.2–1.4 ns demonstrate a small molecular species in a fluid medium such as water. Irwin and Churchich [5] found for P-pyridoxyl bound to lysozyme a time of 0.16 ns and attributed this motion to rotation of the pyridoxyl about the bond to the ϵ-amino group. On the protein and on the membrane the situation is quite different. In the BSA protein limited rotation of the label occurs with the pyridoxyl 5′-phosphate being slower than pyridoxyl, probably the result of interactions with the 5′-phosphate. The value of 26 ns for ϕ could also mean that rotation of the BSA ($M_r \approx 66\,000$) was observed. Wahl [18] observed a value of 58 ns for the apparent relaxation time for this protein. The value of 8 ns for the pyridoxyl label must represent limited rotation about the covalent bond and not simply rotation of the protein. For the larger protein ($M_r \approx 160\,000$) the value of 9.6 ns for ϕ must also represent limited rotation of the pyridoxyl 5′-phosphate. The site of the ϵ-amino group is less restrictive. The fluorescence lifetime demonstrates that the aldolase site contains more water molecules.

The membrane systems provide the most interest. The low values of ϕ for rod outer segments and myelin sheath suggest that the ϵ-amino groups are strongly associated with the water solvent allowing the pyridoxyl 5′-phosphate more freedom for rotation. The higher values of 6.4 and 4.4 ns for the ϵ-amino groups of sarcolemma and erythrocyte proteins suggest sites which restrict rotation. These values, together with those of τ, suggest that these sites are partially buried and certainly more remote. Wahl et al. [19] obtained a value of 3 ns for dansyl residues on membranes and interpreted this result for some restricted motion. Conversely, the amino groups of myelin sheath proteins appear on the surface without confinement.

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