Pyridoxal-5′-phosphate as a probe for rotational diffusion

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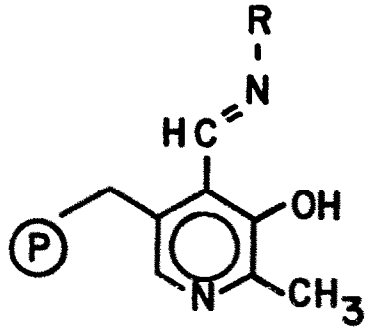
Pyridoxal-5′-phosphate, a metabolic derivative of vitamin B6, was successfully used as a probe for rotational diffusion. As the intrinsic cofactor in glycogen phosphorylase *b*, in binding with bovine serum albumin and in mixed micelles, the Schiff base adduct exhibited transient absorption dichroism at μs times. Its usefulness in measuring the slow rotation of proteins and micelles was demonstrated.

Pyridoxal-5′-phosphate *Rotational diffusion* *Protein* *Flash photolysis* *Micelle*

1. INTRODUCTION

The observation of rotational relaxation times in the microsecond region is always of interest to the study of protein and membrane dynamics. In [1] the flash photolysis and electron paramagnetic resonance methods which can provide this information was recently reviewed. We report here a new flash photolysis method which uses an intrinsic enzymic cofactor, pyridoxal-5′-phosphate (PLP), as a probe for the observation. For example, the PLP cofactor of glycogen phosphorylase was used to measure the relaxation time of the enzyme in glycerol solutions. PLP reacts with bovine serum albumin (BSA) to form a Schiff base which can be used to measure the relaxation time of the protein. The dodecylamine Schiff base of PLP easily associates with cetyltrimethylammonium (CTA) chloride to form mixed micelles. In these solutions rotation of the micelle and the probe was observed.

When PLP Schiff bases are excited with UV light they exhibit transient absorption which may last many μs [2,3]. The results largely follow those of salicylaldehyde Schiff bases. In the excited state the 3-hydroxyl proton migrates in several ps to the imine nitrogen [4,5]. Clear evidence for this is the abnormally large Stokes shift of the fluorescence, also observed in glycogen phosphorylase [6]. When the molecule returns to the ground state the transfer back of the proton is slower. This lower state with the proton still on the imine nitrogen exhibits transient absorption about 470 nm. The rate of decay of this transient depends on the Schiff base molecule and on the dielectric constant of its environment. It is unaffected by the presence of oxygen.



When the ultraviolet excitation beam is polarized the transient absorption of the analyzing light polarized parallel and perpendicular to the exciting flash is dichroic. The absorption dichroism decays with the randomization by the Brownian motion of the molecules. While this is a singlet–singlet transition, the lower singlet state is structurally altered

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and μs are required for its return to the original ground-state structure. The lower triplet state of fluorescein derivatives, also slow to return to the ground state, has been used in [7] to observe slow rotational diffusion of proteins. They used the decay of the absorption dichroism from the polarized lower triplet state. Authors in [8] used the depolarization of the phosphorescence from this lower triplet state to measure the rotational diffusion. Both techniques have been successful with the phosphorescence method obviously gaining in sensitivity.

2. MATERIALS AND METHODS

Photoexcitation of the PLP imine in the enzyme was achieved with a nitrogen laser [9] which emits 10-ns pulses at 337 nm. This is an ideal wavelength for those PLP imines in a hydrophobic environment wherein they absorb about 340 nm because the 3-OH is non-dissociated. After polarization with a Glan-Taylor prism, the UV pulse of 0.7 mJ was focused onto the sample with an aspheric lens to an area of 1 mm × 10 mm. The transient absorption in the 1 cm cell was measured with the 476.5-nm line from a Spectra Physics Model 165 laser equipped with an etalon to reduce the optical noise. The measuring light at 90° to the UV pulse was pulsed with a shutter. A ½ λ plate was used to rotate the polarized beam. The light intensity was recorded with a RCA 1P28 tube with a 820 Ω load resistance. The experimental RC time constant was 0.32 μs. The voltage signal was output on the vertical sweep of a Tektronix 7623 oscilloscope. Photographs of the traces were measured with a 7X comparator graduated in 0.1 mm or with a Hewlett-Packard 7074A plotter and digitizing sight. The trace was about 1 mm in width and the error in the measurement of the trace center at a given decay time is within ± 0.2 mm. This yields a 2% error in reading while the oscilloscope error is 3%. A 3% error in our measurement translates to a standard deviation of ± 0.004 for a rotational anisotropy of 0.143. The error in r_0 may be larger because of the facilities with which we had to make the measurements. $A_\parallel$ and $A_\perp$ were measured from separate traces because our beam was not split. Therefore, while we tried to get the same signal intensity at long time, r_0 may be off more than the standard deviation would indicate. The curves were fitted with the aid of the Hewlett-Packard 9825T calculator.

The PLP, dodecylamine (DD), and CTA chloride were of reagent quality. The BSA was purchased from Sigma. The glycogen phosphorylase was prepared by the method in [10]. Sample concentrations are given in fig. 1.

In the solutions using glycerol and water as the solvent, the water content was measured as in [11] by the intensity of the $\nu_2 + \nu_3$ band. We certainly recommend this procedure when viscosities of glycerol–water solutions are taken from the tables [12].

3. RESULTS

Equimolar quantities of BSA and PLP were mixed at neutral conditions and the reaction followed with the spectrophotometer. After equilibrium the high ratio of the 332-nm band to the 415-nm band demonstrated that the PLP binds

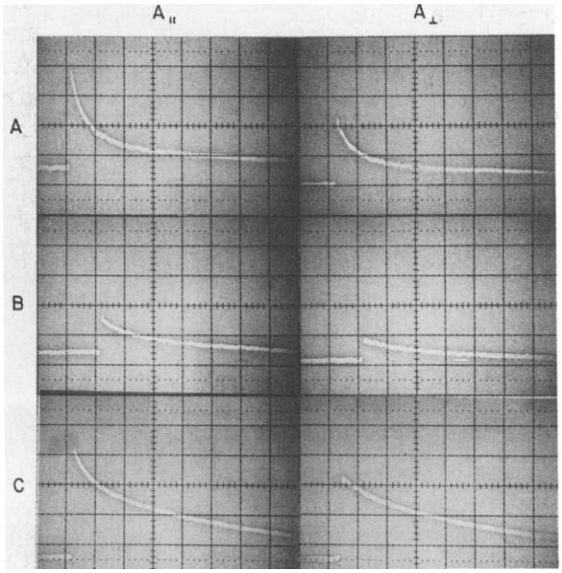

Fig. 1. Oscilloscope traces of the transient absorption dichroism. Dark-light deflections was 300 mV. (A) 2×10^{-3} M BSA and 2×10^{-3} M PLP at equilibrium in 94% glycerol–water at RT. Vert. 20 mV/div; Hor. 20 μs/div; (B) 10^{-3} M glycogen phosphorylase *b* in 88% glycerol–water at 273 K. Vert. 20 mV/div; Hor. 50 μs/div.; (C) 2×10^{-3} M PLP, 4×10^{-3} M dodecylamine, and 5×10^{-3} M CTA chloride in ~100% glycerol at 258 K. Vert. 20 mV/div; Hor. 20 μs/div.

to a probable ϵ-amino group as a Schiff base [13] in a hydrophobic pocket. As a result, a large transient absorption signal with a lifetime of 34 μs at room temperature was observed at 476.5 nm. Assuming a molar absorptivity of 10^4 for the transient species, we estimate a quantum yield of about 0.05. With the complex in 94% glycerol, the transient signal was dichroic.

The oscilloscope traces in fig. 1 show the transient absorption dichroism of the PLP probe in BSA, in glycogen phosphorylase *b*, and in the CTA-DD mixed micelle. The absorbance, A, at given time intervals were determined for each trace. The rotational anisotropy was then calculated according to:

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

The values for ln $r(t)$ are plotted against t for BSA in 94% glycerol-water at 4 temperatures in fig. 2. The linear plots demonstrate that the decay of the anisotropy can be expressed by a single first order decay constant. For the decreasing temperatures these values are 92 000 s^{-1}, 31 000 s^{-1}, 17 000 s^{-1} and 4800 s^{-1}. According to [4] the decay of $r(t)$ for a spherical molecule follows:

$$r(t) = r_0\, e^{-t/\phi} \quad (2)$$

where: ϕ is the rotational correlation time and r_0 is the value of $r(0)$. For BSA ϕ as a function of the solution viscosity agrees well with the values reported in [8].

For a sphere with isotropic rotation ϕ is related to the radius and to the viscosity of the solution by the following expression [15,16]:

$$\phi = \frac{4\pi a^3 \eta}{3\,kT} = \frac{1}{6D} \quad (3)$$

D is the rotational diffusion coefficient. Using the relationship, the calculated radius for a spherical BSA molecule was 38 Å. For comparison the radius is 21 Å for the unhydrated sphere, 36 Å from the measured diffusion coefficient, and 33.7 Å from the intrinsic viscosity [17].

In glycogen phosphorylase PLP is bound to the ϵ-amino group of Lys-679 [18] as the Schiff base. The high ratio of 100/7.5 for the absorbance of the 333-nm and 425-nm bands at pH 7.0 show the hydrophobic nature of the cofactor site [6]. The lifetime of the transient band in fig. 1B was 119 μs and the absorption was dichroic.

The ln $r(t)$ values at 3 temperatures for phosphorylase *b* at pH 6.8 in 88% glycerol-water are plotted against time in fig. 3. Again, the plots are linear with rate constants of 57 000 s^{-1}, 26 000 s^{-1} and 5000 s^{-1}. Other percentages of glycerol, 79% and 70%, were used at various temperatures. The log of ϕ was plotted against the log of viscosity in fig. 4. The slope of this plot is 0.81, rather than unity, yet confirms that we are measuring the rotation of the molecule. The calculated value of the radius was 49 Å, compared to 36 Å [19] assuming

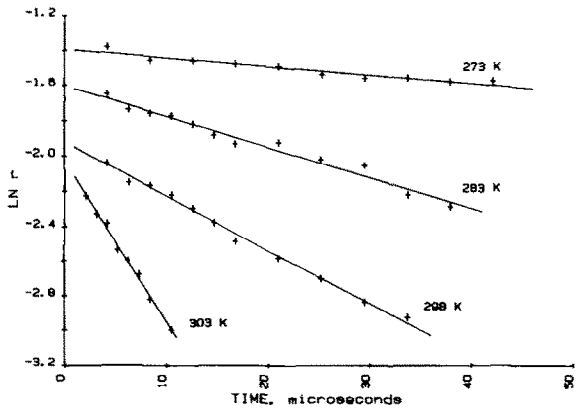

Fig. 2. Plots of ln $r(t)$ against t for BSA with probe in 94% glycerol-water at the temperatures shown. The plot at 283 K has 0.42 added to ln r; at 303 K, −0.75 added.

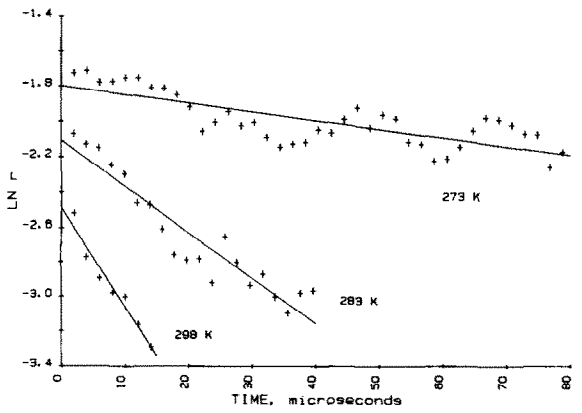

Fig. 3. Plots of ln $r(t)$ against t for glycogen phosphorylase *b* in 88% glycerol-water at the temperatures shown. The plot at 298 K has −0.6 added to ln r.

a sphere for the unhydrated molecule.

Demonstration of the dichroism of this particular absorption by placing the probe in poly(methylmethacrylate) was not possible because of the low solubility. However, the salicylaldehyde Schiff base of dodecylamine was soluble and showed the dichroism for over 1 ms at room temperature. This molecule has a slower decay and can, therefore, be used for longer ϕ's. In order to slow the rotation of the PLP probe we were able to place it in a micelle in glycerol. The PLP-dodecylamine Schiff base was prepared in a few drops of methanol and the CTA added. When the solvent evaporated, glycerol was added. The reaction was easily followed with the spectrophotometer. When the temperature of the glycerol solution of the PLP-dodecylamine Schiff base-CTA mixed micelle was lowered to about 0°C, absorption dichroism became evident. Figure 5 shows the plot of ln $r(t)$ against time at -15°C. Obviously not linear, this curve can be fitted with the following equation:

$$r(t) = 0.073\ e^{-120\,000\ t} + 0.037\ e^{-9500\ t} \quad (4)$$

In order to understand better the rotations involved, the location of the PLP probe can be inferred from other data. In a hydrophobic environment the ratio of the 340-nm and 420-nm absorption band of the Schiff base is high. For PLP-DD in glycerol it shifted from 0.7–1.7 with CTA. While association with the CTA is apparent the 1.7 ratio still shows PLP exposed to the glycerol. Further CTA will increase the ratio, indicating that PLP moves within the micelle. At this point there is a loss of dichroism due to the lower viscosity of the aliphatic chains. From this we conclude that our measurements are taken when the probe is on or near the surface of the mixed micelle. The slow rotation has a correlation time of 105 μs and is congruent with a sphere of 11 Å radius. This number is certainly reasonable for the mixed micelle. The fast component (ϕ = 8.3 μs) might be associated with restricted rotation of the probe about its long axis. Under the conditions of the experiment the dichroism is not observed in the absence of the CTA.

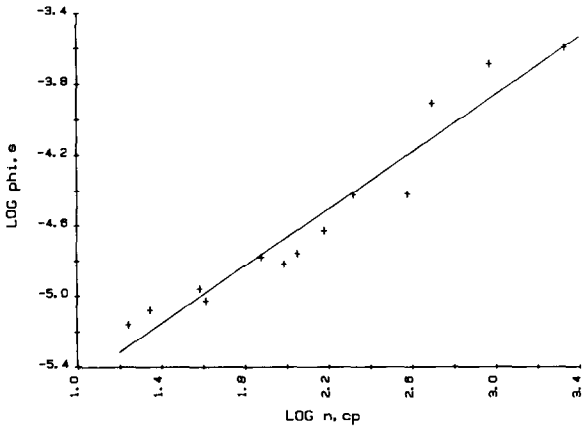

Fig. 4. Plot of log ϕ against log η for glycogen phosphorylase *b* in glycerol-water.

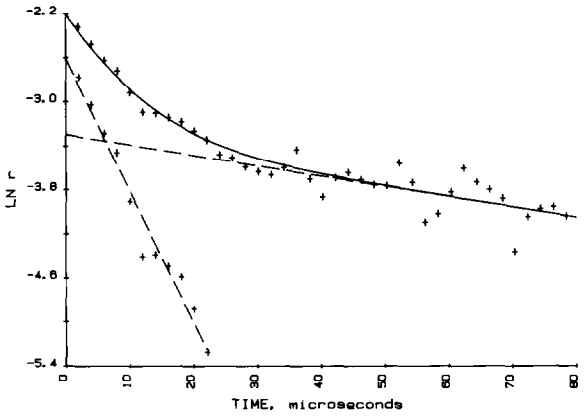

Fig. 5. Plot of ln $r(t)$ against t for mixed micelle in 1C at 258 K. Solid line represents data fitted with eq. (4). Broken lines represent respective first order decays.

4. DISCUSSION

When excited by UV light the PLP imines in a hydrophobic setting showed transient absorption dichroism. The time decay of the dichroism allowed the measurement of the rotational correlation time in the μs domain. Since the transient can be observed in the first few ns [2] or possibly sooner [5] and may last 1 ms, we have the capability of time measurements over 6 orders of magnitude. Here, we have used it to measure the rotation of two proteins, one of which requires the PLP cofactor for enzymatic activity, and the rotation of a micelle. This, we believe is the first use of PLP and the intrinsic cofactor to measure rotational diffusion. Its use requires available ϵ-amino groups on the protein and some hydrophobic character at the binding site. For those proteins bound within a

membrane, the hydrophobic character of the membrane may allow its use. One drawback is certain to be the concentration required. However, hydrophobicity will enhance the signal. Another possibility is the development of other *o*-hydroxyl Schiff bases for increased sensitivity.

While r_0 will contain more error than ϕ because of our method, the generally low values must be assumed. This may be due to the instrumental errors or to the value of γ, the angle between the transition moments of excitation and measurement. Although the measurement involves a singlet transition, it is from a molecularly altered lower state. It is reasonable to assume a change in the molecular geometry of the metastable state. We cannot, therefore, assume a γ of 0. From the experiment with the salicylaldehyde–dodecyl imine in the methyl methacrylate polymer r_0 is 0.15, which might be expected to be the maximum. Other values of r_0 were typically 0.1.

The observation of the dichroism and the agreement of ϕ with other methods is reassuring. We can conclude that this transient state of PLP Schiff bases can be used as a probe for rotational diffusion of macromolecules.

ACKNOWLEDGEMENT

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