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Measurement and Analysis of Triplet-State Lifetimes by Multifrequency Cross-Correlation Phase and Modulation Phosphorimetry

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In this paper we describe a novel approach to study the triplet-state lifetimes by a conventional multifrequency cross-correlation phase and modulation apparatus. The analysis of phase and modulation data of eosin-labeled band 3 erythrocyte ghosts revealed the existence of two phosphorescence lifetime values of 2700 and 750 μ s, with a fractional contribution of 78 and 22%, respectively, which are in good agreement with those reported in the literature. Differential polarization phase analysis, which facilitates the study of the rotational properties of band 3, provided data in good agreement with those reported in the literature. The method proposed in this paper to study the radiative emission from the triplet state may represent a convenient alternative to the pulse laser flash technique.

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In this last decade, an increasing number of biological applications of phosphorescence have been reported (1-3). In particular, the most popular approach to study the rotational diffusion properties of biological macromolecules in lipid bilayers has been by following the radiative emission decay from the triplet state of phosphorescent probes (1,4,5).

The common method to study radiative transitions of the triplet state is the pulse laser flash technique or time-domain phosphorimetry, which allows one to follow the decay of the triplet state of a given phosphores-

cent probe (6). The radiative emission is recorded between laser flashes using a gated photomultiplier which measures only those photons emitted during the phosphorescent event. In this way it is possible to get rid of both the laser flash scatter and the fast fluorescence. One of the major limitations of the time-domain approach, however, is the elevated cost of the required high-power pulse laser. In addition, the time resolution in the 1-10 μ s range may be heavily affected by the gating of the photomultiplier tube and recovery from the strong flash.

In this paper, we describe a new approach to study phosphorescence lifetimes based upon the frequency domain, a well-known technique utilized for the measurement and study of fluorescence lifetimes (7,8). The possible advantages of the frequency-domain approach are quasi-steady-state, low-level illumination and frequency response from very low (<1 Hz) to very high (>100 MHz), which allow the measurements of both fluorescence and phosphorescence signals. Frequency-domain methods have been previously used, notably in the work of Birmingham and Garland (9). However, these researchers used homebuilt equipment and specialized electronics. In this paper, we describe phosphorescence measurements performed with a commercially available instrument, the K2 frequency-domain fluorometer from ISS Inc. (Champaign, IL).

MATERIALS AND METHODS

Erythrocyte Band 3 Labeling and Erythrocyte Ghost Membrane Preparation

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes. Red blood cells

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were isolated by centrifugation at 4°C at 600g and washed four times with a buffer containing 5 mM sodium phosphate and 150 mM sodium chloride, pH 7.4. The buffy coat was carefully removed. Band 3 labeling of intact erythrocytes with eosin 5-isothiocyanate was carried out according to Nigg and Cherry (5). Ghosts were obtained from these red cells by hypotonic lysis using 20 vol of 5 mM phosphate buffer, 1 mM EDTA, pH 7.4, at 4°C. The hemolysate was centrifuged at 4°C and 25,000g. The resulting ghosts were resuspended in ice-cold hypotonic buffer and this process was continued until the red cell membranes were free of hemoglobin. SDS-PAGE² of the eosin-labeled ghost membranes showed that eosin fluorescence was almost completely localized in one band corresponding to the band 3 position. Erythrocyte ghost suspension utilized for phosphorescence studies contained 5–10 mM eosin-labeled band 3. All samples were flushed with argon for at least 20 min prior phosphorescence measurements to remove molecular oxygen.

Instrumentation

The phosphorescence lifetime instrument is a commercially available frequency-domain instrument (Model K2, ISS Inc.), equipped with an argon-ion laser (Spectra Physics, Model 164/9, Mountain View, CA). The light modulator is a Lasermatic (Model 1042 WA) Pockel's cell with a quarter-wave voltage of 75 V. The driving and measuring electronics are standard ISS electronics. Data collection and analysis were performed with software provided with the K2 instrument. The data analysis routine uses a nonlinear least-squares method based on the Marquardt algorithm (10,11). Data were collected in the frequency range from 1 Hz to 1 MHz. At each frequency, data were integrated until the standard deviation of the phase and modulation measurements were below 0.2° and 0.004, respectively.

RESULTS AND DISCUSSION

There have been two groups which have studied phosphorescence lifetimes using frequency-domain methods, although their methods suffer several drawbacks (9,12): Basically, they used methods for phase and modulation measurements other than the well-established cross-correlation technique, which offers unique advantages over other phase detection techniques (7,8,13). In addition, Mousa and Winefordner used only single discrete frequencies taken on the portion of either the modulation or the phase curve with the greatest slope for the analysis of phosphorescence lifetimes (12). This simple approach has been recognized to be of limited use, especially regarding the resolution of phosphorescence lifetimes in a heterogeneous system (13). Birmingham and Garland (9), instead, described a rather complex and expensive homebuilt apparatus which employs the syn-

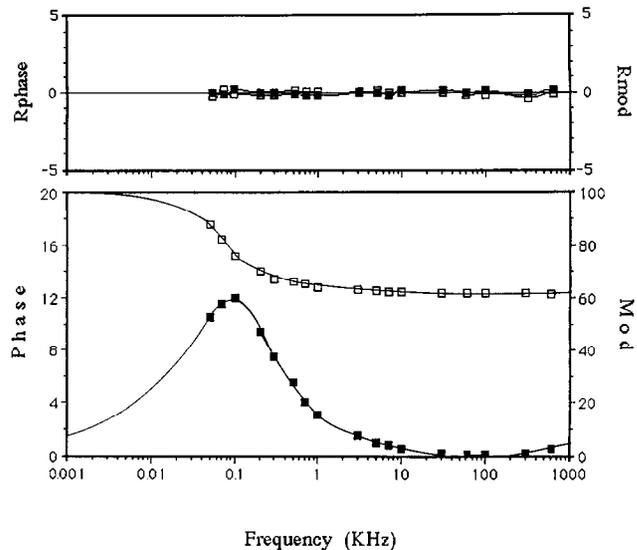


FIG. 1. Phase (■) and modulation (□) values for eosin 5-isothiocyanate covalently bound to band 3 in erythrocyte ghost membranes at 25°C (bottom panel). Excitation wavelength was 514 nm, emission wavelength 580 nm (Hoya 0-58). (Upper panel) The residuals for both phase and modulation data. Phase is expressed in degrees and modulation in percentage.

thesis of multiple frequencies and a chopper to eliminate scattered and fluorescent light.

Our system takes advantage of the multifrequency phase and modulation analysis, and its appropriate statistical evaluation, to determine phosphorescence decays other than single-exponential decay (8,10,11). The major advantages of our instrument are, on the one hand, the utilization of the proven cross-correlation technique to accurately measure the phase shift and the modulation ratio and, on the other hand, the low cost and availability of data analysis programs.

To test the validity of our instrumentation, we decided to adopt one of the most common biological applications of triplet probes, which is the study of rotational diffusion of protein, eosin-labeled band 3 in human erythrocyte membrane. Band 3, a major constituent of erythrocyte membrane protein, is involved in anion transport across the red blood cell membrane, and its rotational diffusion properties have been extensively investigated using pulse methods (4,5,14–16). One major concern at the beginning of this study was the contribution of the fluorescence signal in comparison to the phosphorescence signal derived from the triplet probe under examination. As can be seen in Fig. 1, however, the measurement of the triplet radiative transition from eosin-labeled band 3 in red cell membranes revealed a broad phase-shift band with a peak value of 12° at 50 KHz and a modulation ratio curve which reached a plateau value of 0.63 at 1 MHz. The remaining phase shift and demodulation contribution associated with the fluorescence signal appeared above 1 MHz (data not shown). These results indicate that with an eosin probe, even in

² Abbreviation used: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

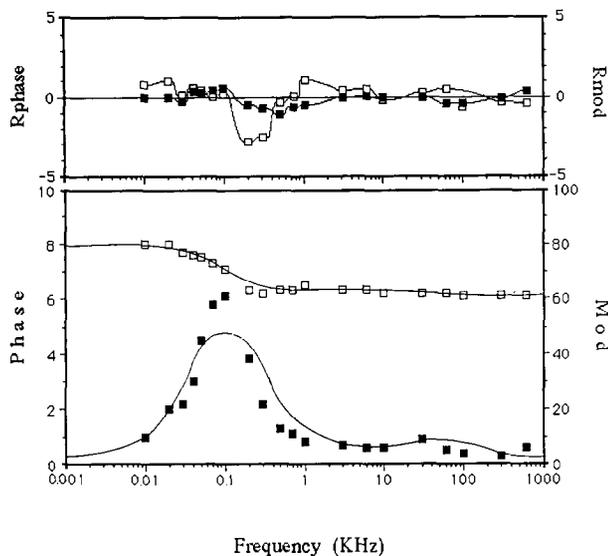


FIG. 2. Differential polarized phase (■) and modulation (□) values for eosin 5-isothiocyanate covalently bound to band 3 in erythrocyte ghost membranes at 25°C (bottom panel). Excitation wavelength was 514 nm, emission wavelength 580 nm (Hoya 0-58). (Upper panel) The residuals for both phase and modulation data. Phase is expressed in degrees and modulation in percentage.

the presence of a strong fluorescence signal, it is possible to detect enough phosphorescence signal to provide reliable frequency-domain data to measure phosphorescence lifetimes. Of course the sample was carefully deoxygenated. The standard deviation of each phase measurements was in the range of 0.2° whereas the modulation standard deviation was 0.004. As expected, the readmission of oxygen in the cuvette promptly quenched the phosphorescence signal. The analysis of the phase and modulation data using a nonlinear least-squares routine, described elsewhere (10,11), allowed us to resolve at least two lifetime components due to phosphorescence decay which produced the broad phase-shift band described above. In this analysis we assigned, as fixed parameters, a fast lifetime value in the nanosecond range to describe the fluorescence component and its fractional contribution considering the overall radiative transition (fluorescence plus phosphorescence). In other words, we used three exponential components in order to obtain a satisfactory fit of the data. The two long lifetime values obtained were 2700 ± 500 and $750 \pm 50 \mu\text{s}$, with a fractional contribution of 78 ± 1 and $22 \pm 1\%$, respectively. The fractional contributions to the phosphorescence signals of the two slow components, which are calculated considering the overall radiative transition, were normalized taking into account only the phosphorescence emission.

The results of differential polarized phase and modulation measurements of eosin-labeled band 3 ghosts are shown in Fig. 2. The solid lines correspond to a fit using two rotational correlation times: $\phi_1 = 2570 \mu\text{s}$, $r_{01} = 0.156$; and $\phi_2 = 2.8 \mu\text{s}$, $r_{02} = 0.011$. These data are in

good agreement with those reported in the literature (5,16). In fact, it is well known that erythrocyte membranes contain at least two band 3 populations identifiable by their diverse mobilities. These two different band 3 populations most likely stem from self-aggregation of band 3 dimers, and the cytoskeletal network localized on the cytoplasmic surface of the red cell membrane imposes restrictions on both rotational and translational diffusion of band 3 (4,5,16). An important feature of the rotational diffusion properties of band 3 is the remarkable sensitivity to temperature. Experiments conducted with our apparatus on the influence of temperature on eosin-labeled band 3 ghosts provided data which are in good agreement with this view (data not shown).

In conclusion, we have demonstrated that the conventional frequency-domain fluorometer can be used for measurement of phosphorescence lifetime and for the determination of slow rotational motion of proteins in membranes.

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