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PICOSECOND FLUORESCENCE KINETICS AND POLARIZATION ANISOTROPY FROM ANTHOCYANIN PIGMENTS

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SUMMARY

Picosecond fluorescence kinetics and polarization anisotropy measurements were obtained from anthocyanin pigments extracted from *Streptocarpus holstii* and *Anthurium andreanum* flower petals, as well as from the in vivo samples. Evidence for quenching of the fluorescence and for a non-random orientational order of the pigments in vivo is indicated by measurements of a reduced fluorescence lifetime and a smaller value for the polarization anisotropy as compared to the in vitro condition.

INTRODUCTION

The understanding of many photobiological processes has been greatly enhanced by the application of picosecond spectroscopic techniques. In particular, invaluable information has been gained on the primary energy conversion processes in photosynthesis [1,2] as well as the primary photochemical conversions in vision [3,4]. A great deal of interest has recently been shown in the understanding of the energy transfer properties of the accessory pigments in photosynthetic systems [5,6] and their relationship to the morphological structure of these pigments in vivo.

Anthocyanin pigments are found in the petals and leaves of flowers, and the skins of fruits. These pigments are responsible for their varied coloration which also serves to attract birds and insects for plant pollination and seed dispersal. In this paper, we report on the first in vivo and in vitro picosecond fluorescence kinetics and polarization measurements of anthocyanin pigments from flowers. Information on the orientational distribution of these chromophores in the in vivo molecular environment can be obtained by measurements of the fluorescence polarization anisotropy. A non-random orientational order of the anthocyanin pigments in their naturally occurring vacuoles is indicated by comparison of the in vivo and in vitro initial values of the fluorescence polarization anisotropy. Evidence for fluorescence quenching was also found in the in vivo samples.

MATERIALS AND METHODS

The experimental setup [7,8] used in this research is schematically shown in Fig. 1. A mode-locked Nd:glass laser system with single pulse selection apparatus was used to obtain a single pulse at the laser fundamental wavelength of 1.06 μm. The selected pulse was amplified and frequency doubled to obtain an actinic pulse of 8 ps duration at 530 nm. The kinetics of the fluorescence emission beyond 550 nm were measured with a Hamamatsu streak camera (with 12 ps time resolution) coupled with a Princeton Applied Research Optical Multi-channel Analyzer (OMA). The data was analyzed on a Digital Equipment Corp. PDP 11/03 minicomputer. For the polarization measurements two orthogonally polarized 530 nm excitation pulses with approximately equal intensity, and separated by one nanosecond delay, were used to excite the sample. An analyzer (Polaroid HN-38) was placed between the sample and the streak camera, thus allowing for the simultaneous measurement of the fluorescence signal both parallel to and perpendicular to the incident polarization direction. The system was calibrated by equalizing the fluorescence emission from a 2×10^{-4} M solution of erythrosin in water excited by the two pulses with the analyzer removed.

Samples of *Streptocarpus holstii* and *Anthurium andreanum* were obtained from the Bronx Botanical Gardens of New York. An anthocyanin extract was prepared by placing the flower petals in a methanol solution

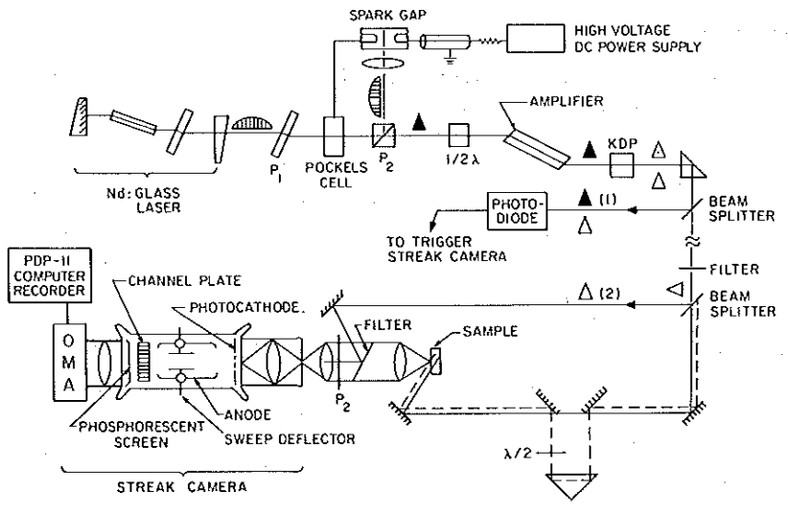

Fig. 1. Experimental setup used to measure the fluorescence kinetics from two delayed orthogonally polarized, 530 nm excitation pulses. The output pulse train from the Nd: glass laser is passed through a single pulse selection apparatus consisting of a Pockels cell situated between two crossed polarizers P_1, P_2. A 17 kV voltage pulse is performed on a transmission line and is switched onto the Pockels cell by a laser triggered nitrogen spark gap cell.

titrated to pH 0.3 with concentrated HCl. The low pH maintained a reducing environment, stabilizing the anthocyanins, which become oxidized and break down at higher pH concentrations [9]. At this pH the integrity of the purple color of the *Streptocarpus* anthocyanins as well as the red color of the *Anthurium* anthocyanins were maintained. Fluorescence from anthocyanin extracts were measured in a 2 mm optical cell at room temperature. The intact flower petals were mounted on a flat piece of aluminum, and a portion of the sample selected for homogeneity of color and physical appearance, such as uniformity of texture and the absence of structural folds, was illuminated.

Absorption spectra of the anthocyanin extracts were measured by using a G.C.A. McPherson spectrophotometer. The spectra showed an absorption maximum at 533 nm for both extracts. These results are consistent with those obtained by Song et al. [10] for cyanidin and pelargonidin extracts.

EXPERIMENTAL RESULTS AND DISCUSSION

The time-resolved polarized fluorescence emission measured parallel and perpendicular to the incident polarization direction is shown in Fig. 2 for the *Streptocarpus* flower, and in Fig. 3 for the *Streptocarpus* extract. Our measurements of the fluorescence decay kinetics and polarization anisotropy at $t = 0$ are displayed in Table I. All results are reported as the average and mean standard deviation of the specified number of measurements. The

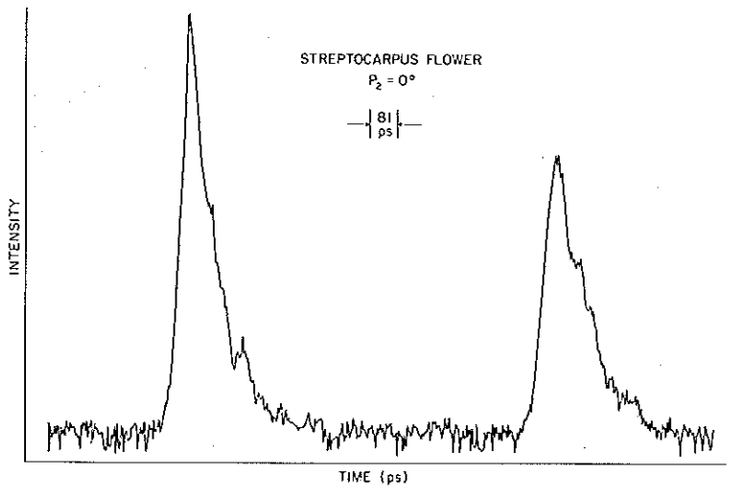

Fig. 2. In vivo polarized fluorescence emission kinetics from *Streptocarpus holstii* flower. The first fluorescence intensity decay profile is a measure of the fluorescence signal polarized parallel to the incident polarization direction, while the second fluorescence intensity decay profile measures the fluorescence signal polarized perpendicular to the incident polarization direction.

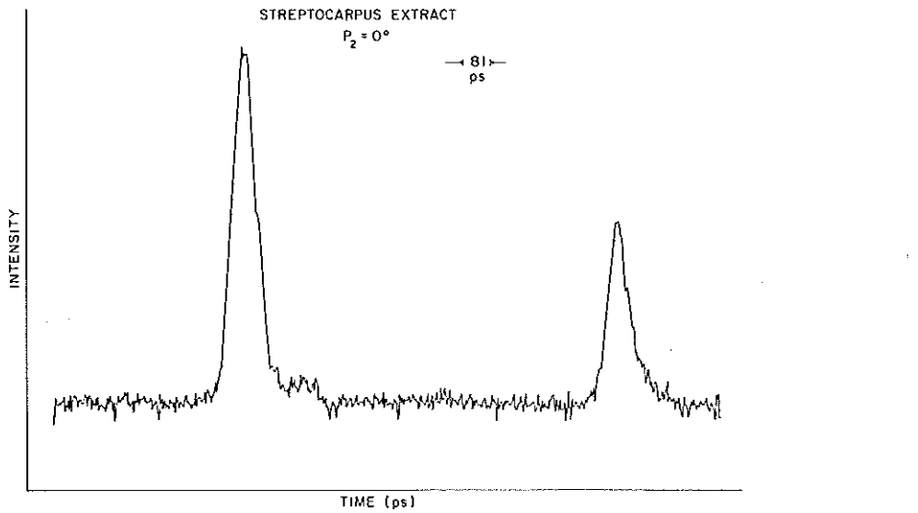

Fig. 3. In vitro polarized fluorescence emission kinetics from *Streptocarpus holstii* extract dissolved in methanol (pH 0.3).

fluorescence lifetime for the *Streptocarpus* extract was 128 ± 33 ps (15 measurements), while for the flower it was 59 ± 13 ps (20 measurements). The fluorescence lifetime for the *Anthurium* extract was 18 ± 8 ps (5 measurements), while for the flower it was 10 ± 3 ps (7 measurements).

The shorter lifetimes measured in the in vivo condition as compared to the in vitro case may be indicative of concentration quenching in the flowers. Although the values for the pigment concentrations in flowers have not yet been established, these concentrations must be greater than any which can be possibly obtained in vitro. From the observed lifetime changes in vivo and in vitro, the quenching rate is estimated to be 9×10^9 s^{-1} for the *Streptocarpus* flower and 4×10^{10} s^{-1} for the *Anthurium* flower.

The polarized fluorescence intensity peaks were used to determine the

TABLE I

MEASUREMENTS OF THE FLUORESCENCE DECAY KINETICS AND POLARIZATION ANISOTROPY AT $t = 0$

Sample	Fluorescence lifetime (ps)	Polarization anisotropy
Streptocarpus flower	59 ± 13	0.13 ± 0.05
Streptocarpus extract	128 ± 33	0.31 ± 0.03
Anthurium flower	10 ± 3	—
Anthurium extract	18 ± 8	0.35 ± 0.16

fluorescence polarization anisotropy, $R(t)$, at $t = 0$ (i.e. within the resolution time of the apparatus). In vitro, $R(0)$ for the *Streptocarpus* extract was 0.31 ± 0.03 (14 measurements) and for the *Anthurium* extract it was 0.35 ± 0.16 (5 measurements). In vivo, however, the measured value of $R(0)$ for the *Streptocarpus* flower was 0.13 ± 0.05 (7 measurements). Polarization measurements in the *Anthurium andreanum* flower were not possible due to its weak fluorescence emission. It is apparent that the in vivo lifetime is shorter than that in solution.

The fluorescence polarization anisotropy, $R(t)$, is an experimentally accessible parameter which characterizes the polarized fluorescence emission in terms of the intensity of fluorescence measured parallel to and perpendicular to the incident polarization direction ($I_{/\!/}(t)$ and $I_{\perp}(t)$ respectively) [11—13]:

$$R(t) = \frac{I_{/\!/}(t) - I_{\perp}(t)}{I_{/\!/}(t) + 2I_{\perp}(t)}. \tag{1}$$

Picosecond fluorescence polarization measurements allow for easier access to the determination of the orientational order of molecules in their local environment when compared to steady-state polarized fluorescence measurements which require knowledge of both the fluorescence decay time and correlation time for rotational diffusion. The emission anisotropy at $t = 0$ for an oriented molecular distribution, for the case where the absorption dipole is parallel to the unique symmetry axis of the system, is given by [11]:

$$R(0) = \frac{(2\langle P_2^2\rangle + \langle P_2\rangle)\, P_2(\cos\delta)}{1 + 2\langle P_2\rangle}, \tag{2}$$

where $\langle P_2\rangle$ is the expectation value of the second order Legendre polynomial for the distribution function $f(\theta)$, and is given by:

$$\langle P_2\rangle \equiv \langle P_2(\cos\theta)\rangle = \int_0^{\pi} \sin\theta\, d\theta f(\theta)\, P_2(\cos\theta) \tag{3}$$

where θ is the angle between the long axis of the molecule and the major symmetry axis of the system, and δ is the angle between the absorption and emission dipole.

The calculated values of $R(0)$ for two different orientational distribution functions are presented as follows:

$f(\theta)$	$R(0)$
1/2	0.4
$3/2\ \cos^2(\theta)$	0.1039

For *Streptocarpus*, the in vitro measured value for $R(0)$ was 0.31 ± 0.03, which is in fair agreement with the theoretical value of $R(0) = 0.4$ for a ran-

dom distribution. The in vivo measured value of $R(0) = 0.13$ indicates that the alignment of these pigment molecules in their vacuoles is non-random. As a model for the orientational order of the pigment molecules in their vacuoles we have chosen the normalized orientational distribution function $3/2 \cos^2 \theta$, giving a calculated value of 0.1039 for the emission anisotropy. This agrees quite well with the measured value of 0.13.

Our kinetic measurements in the in vivo *Streptocarpus* flower present evidence for a strong non-radiative decay which can arise from either concentration quenching, molecular orientational order (which can increase the energy transfer efficiency among the pigments to a non-radiative trap), or some specific in vivo interaction with protein or other molecules. Evidence is also found for orientational order of the anthocyanin pigment molecules in their vacuoles. The highly polarized emission is clear evidence of the local molecular order. Further research is needed to establish the exact morphological order of these pigment molecules in vivo.

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