QUENCHING OF FLUORESCENCE OF CHLOROPHYLL IN VIVO BY LONG-LIVED EXCITED STATES

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1. Introduction

Recent measurements of the fluorescence lifetime of chlorophyll *a* in vivo using picosecond techniques [1–4] give unusually short lifetimes as compared to the lifetimes obtained with more classical techniques [5–7]. This discrepancy can be accounted for by considering quenching of the fluorescence induced by bimolecular exciton annihilation processes [8,9] which can be important in view of the large density of excitons which is created upon high energy laser excitation. When a sample is excited with the output of a mode-locked laser (typically consisting of a train of 5–20 psec wide pulses spaced 5–10 nsec apart), at least two different types of quenching can occur. Singlet excited states created within a given picosecond pulse can be deactivated either by other singlet excitons created within the same pulse or by longer-lived triplet excitons which have been created within previous pulses. Evidence for singlet–singlet exciton quenching of the chlorophyll *a* fluorescence in *Chlorella* at room temperature has been recently reported by Campillo et al. [10] using single picosecond pulses of various intensities. Fluorescence quenching by triplet excited states in vivo has been proposed to account for some of the fluorescence properties of chlorophyll *a* in *Chlorella* [11]. Fluorescence quenching processes due to bimolecular exciton annihilation depend on the density and the mobility of the excitons. The bimolecular annihilation rate constants [8] are sensitive to the pigment composition since the diffusion of excitons from one point in the photosynthetic unit to another is inhibited in heterogeneous pigment systems such as photosystem II (PS II) [12]. The fluorescence of chloroplasts at room temperature arises primarily from PS II, but at lower temperatures the fluorescence from both photosystems can be resolved (for a summary, see [13]). The two major fluorescence bands at 685 and 735 nm can be attributed to antennae light harvesting-PS II, and PS I pigments, respectively [14]. The pigment compositions in these two photosystems are known to be very different [15] and consequently a difference in the mobility of the excitons can be expected [8,12]. These considerations have led us to investigate the excitonic quenching of the fluorescence in spinach chloroplasts both at room temperature and at 100 K using either single or multiple picosecond pulses of high energy. In this letter we report a quenching of the fluorescence which is more pronounced at low temperatures than at room temperature. At low temperatures the efficiency of the quenching is greater in PS I than in PS II and, on the basis of the kinetics of the build-up of this quenching process, we attribute these effects to the presence of relatively long lived quenchers which survive from one picosecond pulse to another when a series of pulses is used for excitation. These long-lived excited states give rise to the preferential quenching in PS I and we tentatively attribute these effects either to triplet excited states, or to ionized pigments which are formed by singlet–singlet annihilation.

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2. Experimental

The preparation of spinach chloroplasts has been described elsewhere [16]. A drop of a concentrated suspension was placed between two microscope coverslips squeezed together giving a sample thickness of 0.05–0.2 mm. The absorbance of the sample was kept in the range of 0.15–0.20 at 680 nm. Cooling was achieved by using a regulated nitrogen gas flow (stability ± 1°C).

The fluorescence was excited by a dye laser (Electro-Photonics Model 33) operated with the dye Rhodamine 6G. Mode locking was achieved with the dye DODCI (3,3'-diethyl oxadicarbocyanine iodide) and the output wavelength of the laser was close to 610 nm. Typically the output of the mode locked train was 1500–2000 nsec long and consisted of about 300 pulses spaced 5 nsec apart. A Pockels cell with 3, 20 and 100 nsec gates was used to select single or multiple picosecond pulses from a region within the pulse train where the pulses are narrow (5–10 psec) and of constant energy (5–10 μJ). The number of pulses selected, as well as their picosecond character was monitored for each flash by viewing the light transmitted through the sample with a streak camera (Electrophotonics ICC 512). The energy of the selected pulses was measured with a R 3230 energy meter (Laser Precision corp.) and a beam splitter. The light was focussed onto the sample (estimated area 0.02 cm^2). The microscope coverslips containing the sample were oriented with their planes at an angle of 45° with respect to the direction of the laser beam, while the fluorescence was viewed at a 90° angle with respect to the laser beam. Scattered laser light was eliminated by means of a red cut-off filter and the fluorescence was focussed on the entrance slit (0.5 mm) of a spectrograph coupled to an Optical Multichannel Analyzer (OMA). This device permits the recording of fluorescence emission spectra utilizing single or multiple picosecond pulses for excitation. The signal recorded by the OMA was presented in digital form and the fluorescence intensity at a given wavelength was recorded for each shot and divided by the energy meter reading to obtain a relative measure of the fluorescence quantum yield. Using scattered laser light we found that the OMA response was somewhat non-linear, being less sensitive at lower intensities. This non-linearity, which was estimated to be as much as 20% for a variation of an order of magnitude in the input energy, has been taken into account in the results presented here. The fluorescence light incident on the OMA detector head was attenuated when necessary by using spectrally flat optical density filters. The spectra presented here have not been corrected for the spectral response of the detector (1205 D) and the spectrograph. A low power helium-neon laser with a beam colinear to the one of the dye laser was used to monitor the fluorescence under conditions of relatively low continuous excitation (<2 mW/cm^2 incident on the sample).

3. Results and discussion

The fluorescence spectrum of spinach chloroplasts at 100 K using a mode locked train of 10 mJ energy is shown in fig.1a together with the fluorescence spectrum recorded with the helium-neon excitation. This last spectrum is identical if taken before or after the laser flash thus showing that there are no irreversible changes which are produced with the laser flash. The spectra have been normalized at 685 nm. We report elsewhere [17] that the fluorescence quantum yield at 100 K decreases by more than one order of magnitude when the flash energy is increased by four orders of magnitude within the range of 10^{-4}–10^{0} J/cm^2/pulse train; possible thermal quenching effects, bleaching effects and effects of photochemical reactions are also considered in detail and it is shown that the contribution of these processes is negligible [17]. From a comparison of the two spectra shown in fig.1a it is concluded that the fluorescence quenching is more pronounced in PS I than in PS II.

Using a single picosecond pulse for excitation, the fluorescence spectrum is quite similar to the one obtained using low intensity continuous excitation with the helium-neon laser (fig.1b). However, the spectrum changes when series of 4 and 22 pulses are used for excitation. The fluorescence spectrum is gradually modified (fig.1b). A progressive increase in the relative fluorescence quenching is observed in PS I as compared to PS II. In this experiment the energy per picosecond pulse was kept constant. Singlet excited state lifetimes are $\sim$ 0.9–1.5 nsec at 100 K [7] and are probably even smaller under conditions of high excitation [9]; thus few singlet excitons

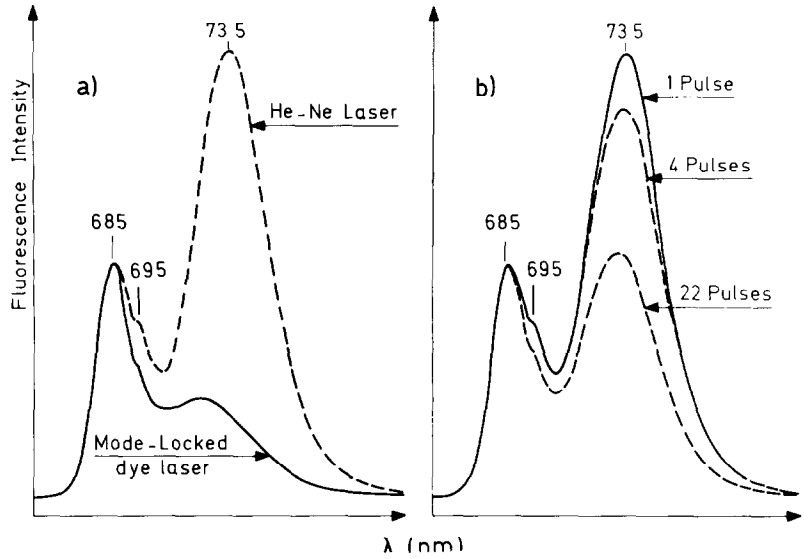

Fig.1. Fluorescence emission spectra at 100°K of spinach chloroplasts for various types of excitation. All curves have been normalized at 685 nm. (a) Helium-neon laser continuous low intensity excitation and high energy (10 mJ) Mode-locked dye laser excitation. (b) Single and multiple picosecond pulse excitation. The average energy of each individual picosecond pulse was 10 μJ.

survive from one pulse to the next within the pulse train. Accordingly the progressive and preferential build-up of the quenching of the PS I fluorescence as compared to the PS II fluorescence at 100 K must be accounted for by long lived quenching state(s). The build-up time of this quencher appears to be in the range of tens of nanoseconds.

Long lived quencher(s) are operative in both PS I and in PS II, but are more effective in PS I. This is demonstrated in table 1 where the relative fluorescence quantum yield at 685 and 735 nm at 100 K is given for different modes of excitation. Results at 300 K, where only the PS II 685 nm fluorescence is observable, are also shown. In all cases, the fluorescence quantum yield decreases as the number of pulses is increased. The quenching effects are more pronounced at 100 K than at room temperature.

Table 1
Dependance of the relative fluorescence quantum yield (normalized to 100 at 685 nm) as a function of the number of exciting picosecond pulses in a pulse train (the pulses are spaced 5 nsec apart)

Wavelength, nm	1 pulse	4 pulses	22 pulses
685 (300 K)	100 ± 10	77 ± 6	56 ± 3
685 (100 K)	100 ± 10	34 ± 5	23 ± 1
735 (100 K)	192 ± 20	57 ± 8	24 ± 1

Triplets states have been observed in chloroplasts following high energy laser excitation [18]. There is a rapid triplet–triplet energy transfer from chlorophyll *a* to carotenoids in vivo on time scales of 30–50 nsec [19]. The long-lived quenchers observed both at room temperature and at 100°K could thus be triplet states of either chlorophyll *a* or carotenoids. Rahman and Knox [20] have shown that quenching of chlorophyll *a* singlets by chlorophyll *a* triplet excitons is feasible, thus indicating that this hypothesis is valid. Furthermore, the ∿ 60 nsec rise time in the fluorescence quantum yield following a brief actinic flash observed by Mauzerall [21] could also be related to the decay of triplets which were generated by the actinic flash.

However, other quenching species can also be formed during each picosecond flash. In particular, singlet–singlet exciton annihilation can in principle lead to the generation of electron-hole pairs within the antenna pigment systems. The efficiency of this ionization quenching channel ($S_1 + S_1 \rightarrow$ ion pairs) as compared to the quenching channel in which one of the

singlets is quenched while the other one is promoted to a higher excited state $n(S_1 + S_1 \rightarrow S_0 + S_n)$, depends on the polarity of the local environment. The lifetime of the ion pairs is probably longer at lower temperatures and thus the singlet–ion quenching mechanism is also consistent with our results and cannot be ruled out. The effects are discussed in detail elsewhere [17].

Our experiments demonstrate that in multiple pulse experiments singlet–triplet as well as singlet–ion quenching effects must be considered. The preferential quenching within photosystem I may indicate that the excitons are more mobile in this pigment system, which would be in agreement with Swenberg's hypothesis.

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References

[1] Seibert, M. and Alfano, R. R. (1974) Biophys. J. 14, 269–283.
[2] Kollman, V. H., Shapiro, S. L. and Campillo, A. J. (1975) Biochem. Biophys. Res. Comm. 63, 917–923.
[3] Breton, J. and Roux, E. (1975) in: Lasers in Physical Chemistry and Biophysics (J. Joussot-Dubien, ed), pp. 379–388, Elsevier, Amsterdam.
[4] Beddard, G. S., Porter, G. and Tredwell, C. S. and Barber, J. (1975) Nature 258, 166–168.
[5] Müller, A., Lumry, R. and Walker, M. S. (1969) Photochem. Photobiol. 9, 113–126.
[6] Mar, T., Govindjee, Singhal, G. S. and Merkelo, M. (1972) Biophys. J. 12, 797–808.
[7] Hervo, G., Paillotin, G. and Thiery, J. (1975) J. Chim. Phys. 72, 761–766.
[8] Swenberg, C. E., Geacintov, N. E. and Pope, M. (1976) submitted for publication.
[9] Campillo, A. J., Kollman, V. H. and Shapiro, S. L. (1976) Science, in press.
[10] Campillo, A. J., Shapiro, S. L., Kollman, V. H., Winn, K. R. and Hyer, R. C. (1976) Biophys. J. 16, 93–97.
[11] Duysens, L. N. M., den Haan, G. A. and Van Best, J. A. (1975) in: Proceedings of the Third International Congress on Photosynthesis (M. Avron, ed), Vol. 1, pp. 1–12, Elsevier, Amsterdam.
[12] Swenberg, C. E., Dominijanni, R. and Geacintov, N. E. (1976) Photochem. Photobiol., in press.
[13] Papageorgiou, G. (1975) in: Bioenergetics of Photosynthesis (Govindjee, ed), pp. 319–371, Academic Press, New York.
[14] Butler, W. L. and Kitajima, M. (1975) Biochim. Biophys. Acta 396, 72–85.
[15] Thornber, J. P. (1975) Ann. Rev. Plant. Physiol. 26, 127–158.
[16] Breton, J., Roux, E. and Whitmarsh, J. (1975) Biochem. Biophys. Res. Comm. 64, 1274–1277.
[17] Geacintov, N. E. and Breton, J. (1976) Submitted for publication.
[18] Breton, J. and Mathis, P. (1970) C.R. Acad. Sci. (Paris) 271, 1094–1098.
[19] Mathis, P. (1969) in: Progress in Photosynthesis Research (H. Metzner, ed), Vol. 2, pp. 818–822, Tübingen.
[20] Rahman, T. S. and Knox, R. S. (1973) Phys. Stat. Sol. (b)58, 715–720.
[21] Mauzerall, D. (1972) Proc. Natl. Acad. Sci. USA 69, 1358–1362.