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Prompt heat release associated with ΔpH-dependent quenching in spinach thylakoid membranes

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Abstract

Photosystem II, the oxygen-evolving photosynthetic reaction centre, is highly susceptible to photodamage. ΔpH-Dependent quenching is a physiological mechanism which protects Photosystem II by converting excess excitation energy to heat in response to acidification of the thylakoid lumen. The energy quenching has been proposed to occur either at quenching centres in the light-harvesting antenna or as a result of charge recombination processes at the Photosystem II reaction centre. These models have proved difficult to distinguish by conventional methods, but make radically different predictions for the timescale of the heat release. Here, we use laser-induced optoacoustic spectroscopy to show that the heat release associated with ΔpH-dependent quenching is complete within 1.4 μs. This indicates that charge recombination in the reaction centre is not involved.

Key words: Photosynthesis; Photosystem II; Energy quenching; Laser; Optoacoustic spectroscopy; Laser-induced optoacoustic spectroscopy

1. Introduction

A principal problem facing plants in their natural environment is their capacity to be damaged by excess sunlight. The major site of photodamage is the Photosystem II reaction centre and in particular the D_1 polypeptide [1,2]. However, plants have evolved a physiological protection mechanism which reduces the damage caused by excess irradiance by converting excitation energy to heat, which is harmlessly dissipated. This quenching of excitation energy occurs within the photosynthetic membrane in response to acidification of the thylakoid lumen [3] and is therefore termed ΔpH-dependent quenching (q_E). There have been two main proposals for the mechanism by which excitation energy is converted to heat.

(a) The energy quenching occurs as a result of charge-recombination processes at the Photosystem II reaction centre [4]. Several models of this type have been proposed. A common feature of these models is that an exciton is initially trapped by the Photosystem II (PS II) reaction centre, resulting in the transfer of an electron from the primary donor chlorophylls (P) to the pheophytin intermediate acceptor I and then to the bound quinone Q_A, as in normal photochemistry (Fig. 1). However, it is proposed that under quenching conditions electron transfer from the oxygen-evolving complex via the tyrosine residue Z to P is inhibited. This will increase the lifetime of P^+, allowing charge recombination to take place (Fig. 1). It has been further suggested that this charge recombination releases heat, either directly [5] or by generating the triplet excited state of chlorophyll, which may then transfer energy to generate the triplet excited state of a carotenoid, which subsequently decays with the release of heat [6].

(b) An alternative model for q_E envisages the formation of quenching centres in the light-harvesting antenna of PS II [7–9]. The quenching centres could be chlorophyll aggregates [10]; alternatively the quenching mechanism could involve the transfer of singlet [11] or triplet excited states from chlorophylls to carotenoids

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Abbreviations: E_0, laser pulse energy; H, optoacoustic signal amplitude; H_n, energy-normalised optoacoustic signal ($H_n = H/E_0$); I, pheophytin intermediate acceptor of PS II; LHC II, Light-harvesting Chl *a*/*b*-protein of PS II; LIOAS, laser-induced optoacoustic spectroscopy; P, primary donor chlorophylls of PS II; PS II, Photosystem II; Q_A, bound quinone acceptor of PS II; q_E, ΔpH-dependent quenching; Z, tyrosine electron donor of PS II.

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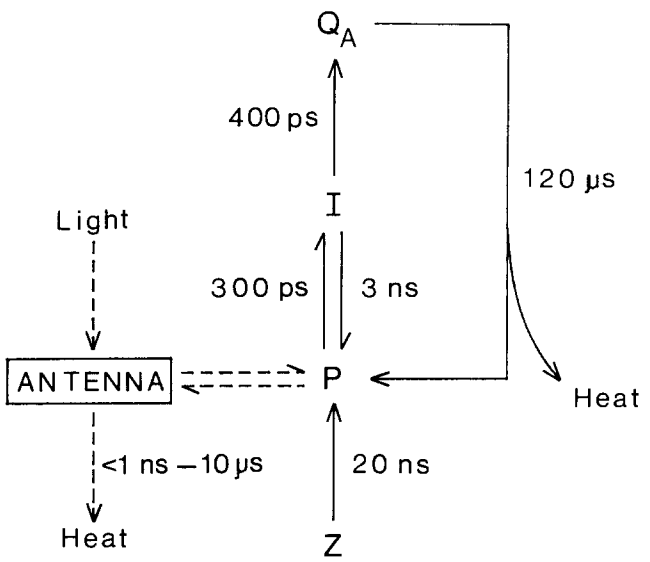

Fig. 1. Exciton trapping and electron transfer at the Photosystem II reaction centre: models for energy dissipation. Solid lines: electron transfer. Broken lines: exciton transfer. Excitation energy may be converted to heat either by reverse electron transfer from Q_A^- to P^+ or by the internal conversion of the excited states of antenna pigments. Lifetimes for electron transfer steps from [14,25,26]. The 300 ps lifetime for primary charge separation includes exciton transfer from the antenna [26].

and subsequent decay by internal conversion. The carotenoid zeaxanthin has been implicated in non-radiative energy quenching [12].

Most studies of q_E have been based on chlorophyll fluorescence measurement. The induction of q_E leads to a decrease in fluorescence yield [3] and a faster overall decay of PS II fluorescence [5]. However, the predictions of the various models for the effects of q_E on chlorophyll fluorescence are only subtly different and are heavily dependent on additional assumptions about the kinetics of energy transfer and exciton trapping by the PS II reaction centres [13]. The two models described above have therefore proved very difficult to distinguish. However, the two models make radically different predictions for the timescale of the heat release. The charge recombination of P^+ I Q^- is a relatively slow process which normally occurs with a lifetime of about 120 μs [14]. It is conceivable that the rate of recombination could be increased under inhibitory conditions. However, under these conditions, even the fastest phase of P^+ re-reduction has a lifetime of 10 μs, and the phase most likely to reflect charge recombination has a lifetime of 150–200 μs [15]. The charge-recombination model therefore predicts a relatively slow release of heat (Fig. 1). In the case of the antenna-quenching model, the timescale for heat release could range from a few nanoseconds or below (decay of singlet excited states) to a few microseconds (decay of carotenoid triplet excited states) [16], depending on the precise mechanism of de-excitation.

In view of the disparity on the timescales of heat release predicted by the two models, a time-resolved measurement of the heat release resulting from q_E is of great interest. This has recently become possible using laser-induced optoacoustic spectroscopy, in which the sample is excited by a short laser pulse and the pressure wave resulting from heat release by the sample is detected by piezoelectric transducers [17]. The development of resonant ceramic transducers has allowed the detection of heat release with high sensitivity down to sub-microsecond timescales [18]. We have used LIOAS to measure the total heat release occurring within the first 1.4 μs following a 10 ns laser pulse. We show that the additional heat release associated with q_E is complete within this time, and therefore that charge recombination in the reaction centre is not involved.

2. Materials and methods

Sample preparation. Spinach leaves were light-treated to maximise the extent of quenching [19] and chloroplasts were isolated [20]. The chloroplasts were suspended to 30 μg chlorophyll ml^{-1} in a buffer mixture containing 2.3 mM $MgCl_2$, 22 mM sorbitol, 0.17 mM EDTA, 33 mM Hepes and 33% (v/v) glycerol. Glycerol was found to increase the coefficient of thermal expansion of the buffer mixture and therefore the LIOAS signal size. The outer chloroplast membranes were initially ruptured by exposure to 5 mM $MgCl_2$. To prevent closure of PS II reaction centres and other effects of repeated exposure to the laser pulse, the suspension (absorbance at 650 nm 0.314 cm^{-1}) was pumped at 150 ml min^{-1} through a 3 ml, 1 cm pathlength square glass flow cuvette (Hellma, Müllheim-Baden, Germany) and returned to a stirred, darkened reservoir at 20°C. The rate of flow was sufficiently fast to ensure that no part of the sample was exposed to more than one laser pulse in each cycle.

LIOAS measurement. 10-ns pulses were generated at a frequency of 1.25 Hz by an excimer-pumped dye laser (EMG 50E and FL1002, Lambda Physik, Göttingen, Germany). The dye laser was tuned to 650 nm with 4-dicyanomethylene-2-methylene-6-(*p*-dimethylaminostyrene)-4H-pyrane (DCM) as laser dye. The beam was expanded to 2 mm diameter with two biconvex lenses before passing through the centre of the flow cuvette. Pulse energy was measured with an Ealing laser power meter and reduced with combinations of Balzers neutral density filters. The LIOAS signal was detected by two 4 mm thick, 400 kHz resonant piezoelectric transducers (constructed by the workshops of the Max-Planck-Institut für Strahlenchemie, Mülheim an der Ruhr, Germany with PZT ceramic, Vernitron) [18] attached to opposite walls of the cuvette. The signals

were added, further amplified (Analog Modules 351) and stored with a computer-linked 20 MHz transient recorder (R2000 from Rapid Systems, Seattle, WA, USA). The measurement was triggered by a reflection of the laser pulse detected by a photomultiplier. At low pulse energies the signals from 100 laser pulses were averaged; the number of repeats was decreased to 16 at high pulse energies.

Calculation of energy storage. The energy normalised heat release (H_n) was calculated by dividing the LIOAS signal amplitude by the laser pulse energy (E_0). Since at high E_0 virtually all the absorbed energy is released as heat, the fraction of energy released as heat with open reaction centres is given by:

$$H_n \text{ (low } E_0)/H_n \text{ (high } E_0)$$

The fraction of absorbed energy lost as fluorescence with open reaction centres is assumed to be about 0.02, based on an average exciton lifetime under these conditions of 0.3 ns [5] and a radiative rate constant of 0.071 ns^{-1} [21]. Energy storage is the fraction of absorbed energy not released as heat or fluorescence within 1.4 μs. This is given by:

$$1 - 0.02 - H_n \text{ (low } E_0)/H_n \text{ (high } E_0).$$

Measurement of pH-induced quenching. Thylakoids were suspended in buffer at either pH 7.6 or pH 5.0 [19]. The sample (volume 60 ml) was kept in the dark during the measurement. The relative fluorescence yield of the sample with open or closed PS II reaction centres was measured with a Walz fluorimeter [20].

Measurement of light-induced quenching. Thylakoids were suspended at pH 7.6 in the presence of 1 mM methyl viologen and 100 μM sodium azide as electron acceptor. In one control measurement, nigericin was added to 3 μM. The sample was dark-adapted or adapted to white light at 50 $\mu E\ m^{-2} s^{-1}$ by illumination of the stirred sample reservoir. Prior to passing through the LIOAS measurement cuvette the sample (volume 120 ml) was pumped for 10 s through darkened tubing to allow the PS II reaction centre to re-open. To check that there was no residual PS II reaction centre closure, the fluorescence yield of the sample was measured with a modulated fluorescence measurement system (Hansatech, King's Lynn, UK) in a 100 μl fluorescence flow cuvette (Hellma) connected immediately before the LIOAS measurement cuvette.

Calorimetric standard. $CuCl_2$ was dissolved in the same buffer mixture as the thylakoid membranes to give the same absorbance at 650 nm ($\pm 1\%$) as the sample. LIOAS signals were measured under similar conditions as for the thylakoids, except that the sample was stationary; pumping was unnecessary since the calorimetric standard was unnaffected by repeated exposure to the laser pulse.

3. Results

The LIOAS signal amplitude is a measure of the integrated heat release during a short time-window following the laser pulse. The length of the time-window is equal to the acoustic transit time across the diameter of the laser beam [18]. In practice the beam diameter cannot be reduced below about 2 mm with photosynthetically active samples without causing excess photon flux density and the closure of reaction centres. We have used a 2 mm beam and suspended the sample in a buffer mixture including 33% glycerol in which the speed of sound was experimentally determined to be about 1400 m s^{-1}. Under these conditions, the amplitude of the initial pressure wave detected by the resonant piezoelectric transducers is proportional to the integrated heat release occurring during the first 1.4 μs following the laser pulse. Slower processes of heat release make no detectable contribution to the LIOAS signal amplitude [18].

Fig. 2 shows LIOAS signals at low pulse energy from spinach thylakoid membranes and a calorimetric standard which releases all the absorbed energy as heat. Excitation was at 650 nm, absorbed predominantly by chlorophyll *b* and therefore by the antenna system of PS II. Assuming about 1 PS II reaction centre per 360 chlorophylls [22], there should be about 0.6 absorbed photons per reaction centre at this pulse energy. The initial pressure wave can be seen about 3.9 μs after the laser pulse: the delay is due to the time taken for the pressure wave to travel from the centre of the cuvette to the detectors. The latter part of the signal reflects the ringing of the resonant piezoelectric

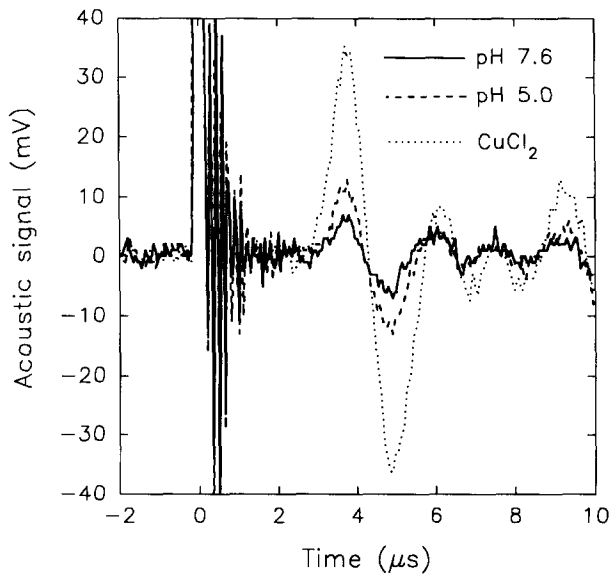

Fig. 2. LIOAS signals from spinach thylakoid membranes at pH 7.6 and 5.0 and $CuCl_2$ as a calorimetric standard. Pulse energy was 690 nJ. The signals from 100 laser pulses were averaged.

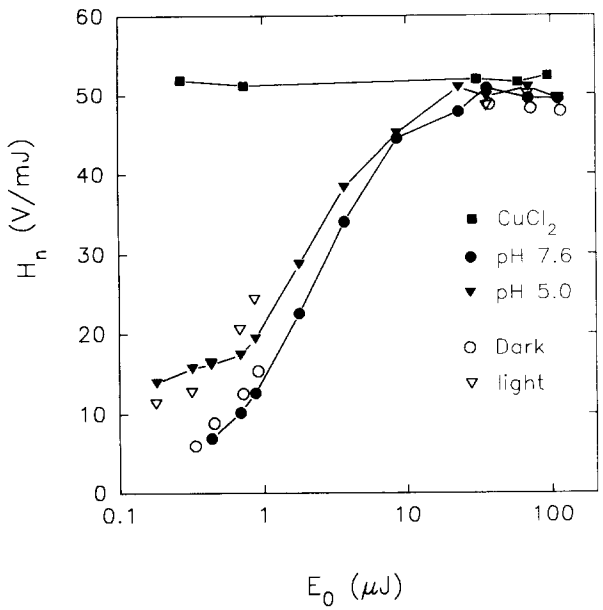

Fig. 3. Dependence of LIOAS signal on pulse energy. H_n is the energy-normalised optoacoustic signal obtained by dividing the amplitude of the initial pressure wave (Fig. 2) by the laser pulse energy (E_0). Filled symbols: thylakoids at pH 7.6 and pH 5.0 and $CuCl_2$ calorimetric standard. Open symbols: results of a separate experiment to measure light-induced energy quenching. Thylakoids at pH 7.6 were dark-adapted or adapted to white light as described in Section 2.

transducer and acoustic reflections within the cuvette [18]. The LIOAS signal from the thylakoid membranes under these conditions is far smaller than the signal from the calorimetric standard: the difference mainly reflects energy storage due to charge separation in the reaction centres, since the loss of energy due to fluorescence under these conditions is expected to be only about 2% (see Section 2). We assume that any volume changes accompanying charge separation in the reaction centres had a negligible effect on the signal, since the buffer/glycerol mixture had a high cubic expansion coefficient which selectively amplifies the contribution of heat release to the signal [18]. We further assume that our experimental time-window of 1.4 μs was sufficiently short to exclude any contribution to the signal

Table 1
Fluorescence and heat evolution from thylakoid membranes at pH 5.0 and 7.6

pH	Relative fluorescence yield		H_n (V/mJ)		Energy storage (low E_0)
	PS II open	PS II closed	low E_0	high E_0	
7.6	1.00	2.94	7.0	49.9	0.84
5.0	0.83	2.00	13.9	50.2	0.70

Relative fluorescence yields and values for energy storage are both accurate to within about ±0.02. Values for H_n are obtained from the data plotted in Fig. 3, and energy storage is calculated as described in Section 2.

Table 2
Changes in heat evolution induced by illumination of thylakoid membranes at pH 7.6

Illumination conditions	Heat evolution	Energy storage
Dark	0.14	0.84
Light	0.22	0.76
Dark-adapted after illumination	0.17	0.81
Dark + nigericin	0.15	0.83
Light + nigericin	0.15	0.83

Thylakoid membranes were illuminated to induce q_E, and heat evolution and energy storage were calculated from the optoacoustic signal amplitudes (H_n) at low and high pulse energy as described in Section 2. Values for heat evolution and energy storage are accurate to within about ±0.02.

by secondary electron transport processes and coupled proton movement.

Fig. 3 shows the dependence of the energy-normalised LIOAS signal (H_n) on laser pulse energy. The pulse energies used range from 180 nJ (about 0.16 photons per reaction centre) to 110 μJ (about 100 photons per reaction centre). At high pulse energies, there is virtually no energy storage, due to saturation of the reaction centres and exciton annihilation processes in the antenna [18]. At the lowest pulse energies, multiple photon hits are unlikely and the LIOAS signal therefore reflects heat release with open, photosynthetically competent reaction centres. Under these conditions, the heat release from thylakoids at pH 5.0 is approximately twice the heat release from thylakoids at pH 7.6 (Figs. 2 and 3). Decreasing the pH from 7.6 to 5.0 also induced a decrease of about 17% in the fluorescence yield with open reaction centres and a decrease of about 32% in the fluorescence yield with closed reaction centres (Table 1). We found that an increase in heat release at low pulse energies could also be induced by illumination of coupled thylakoid membranes suspended in buffer at pH 7.6 (Fig. 3). The illumination conditions were carefully controlled so as to induce q_E without closing PS II reaction centres. The light-induced effect was reversible in the dark and was completely inhibited by the uncoupler nigericin (Table 2).

4. Discussion

We have used LIOAS to measure the heat release from thylakoid membranes during the first 1.4 μs following a laser pulse. Excitation was at 650 nm, which will be absorbed predominantly by Chl *b* and therefore by the light-harvesting system of PS II. PS II reaction centres should be in the state (Z^+ P I Q^-) after 1.4 μs (Fig. 1). We find that an increase in the prompt heat release with open reaction centres can be induced by

two treatments which mimic the effect of ΔpH-dependent quenching in vivo:
(a) Decreasing the buffer pH from 7.6 to 5.0 [19,23].
(b) Illumination of thylakoid membranes in the presence of electron acceptors so as to induce photosynthetic electron transport coupled with the acidification of the thylakoid lumen [3,23]. This effect was inhibited by the uncoupler nigericin (Table 2), confirming that it was induced by a light-induced transmembrane proton gradient.

At pH 7.6, about 14% of absorbed energy is released as heat within 1.4 μs (Table 1). We note that heat release in our spinach thylakoid membranes is considerably lower than the 45% observed under similar conditions in detergent-solubilised cyanobacterial PS II reaction centres [18]). The difference could be explained in part by electric depolarisation in the solubilised sample. Other possibilities include a difference in the enthalpy gap between the ground and charge-separated states of the two systems, or a proportion of inactive reaction centres in the detergent-solubilised preparation. Our experimentally-measured energy storage of 86% (Table 1) is considerably higher than that which would be expected on the basis of energy levels calculated from steady-state redox titrations [18]. It is possible that the difference is due to a relaxation process slower than our experimental time-window of 1.4 μs, but this point requires further investigation.

When the pH is decreased to 5.0, the proportion of absorbed energy released as heat within 1.4 μs approximately doubles (Table 1). This corresponds to a 17% decrease in energy storage by the reaction centres (Table 1). Decreasing the pH to 5.0 also decreases the relative fluorescence yield with open reaction centres by about 17% (Table 1).

Since the decrease in energy storage correlates with the fluorescence quenching induced by low pH (Table 1), it appears that all the energy dissipated by the quenching mechanism is released as heat within 1.4 μs. This indicates that charge recombination from the state P^+ I Q^- of the PS II reaction centre cannot be involved in the quenching mechanism which we observe, since the lifetime for charge recombination is about 120 μs (Fig. 1). The involvement of carotenoid triplet excited states also appears unlikely, since these generally have lifetimes of several microseconds [16]. This leaves the non-radiative decay of the singlet excited states of quenching chromophores in the light-harvesting antenna as the most likely mechanism for ΔpH-dependent quenching of PS II. Such a mechanism would provide optimum protection against excess irradiance by preventing the accumulation of excitation energy at the Photosystem II reaction centre, which is the principal site of photodamage [1,2]. A quenching process which can be observed in isolated aggregates of LHC II has been postulated to be involved in q_E in vivo [9]: it is interesting to note that this quenching process also results in prompt heat release [24].

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6. References

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