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Trapping and annihilation in the antenna system of Photosystem I

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The primary charge separation in Photosystem I of pea chloroplasts was measured as a photovoltage in the pico- and nanosecond time range by applying laser flashes at 532 nm of variable energy and different duration (12 ns and 30 ps, respectively). Contributions to the photovoltage from Photosystem II was eliminated by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and preillumination. The dependence of the photovoltage amplitude on the excitation energy could be described by an exponential saturation law when the excitation flash had a duration of 12 ns. Nearly the same dependence was found when the excitation source was the train of a mode-locked laser (approx. ten 30-ps flashes spaced by 7 ns; highest energy of a single flash, 80 $\mu J/cm^{-2}$). Even with single 30-ps flashes the photovoltage was only slightly smaller than the one elicited by 12-ns flashes of the same energy. These findings demonstrate that trapping of excitation energy by the reaction center of Photosystem I is much more effective than losses by annihilation and other loss processes. The photovoltage yield was nearly independent of the fraction of closed traps, thus demonstrating that the absorption cross section of Photosystem I is not altered by the closing of its reaction centers. By recording the rise time of the photovoltage with our highest time resolution we found that the trapping rate of the excitation energy in Photosystem I depended on the energy of the 30-ps flashes: at low excitation energies (less than 10^{14} photons/cm^2 per pulse) trapping occurred within 90 ± 15 ps and at high excitation energy (10^{15} photons/cm^2 per pulse) trapping and charge stabilization occurred within the time resolution of the apparatus, i.e., up to 50 ps. The trapping rate at low energies is in agreement with the one determined by fluorescence decay kinetics. Up to 50 ns there was no further detectable electrogenic phase (neither forward nor backward reactions). This demonstrates that all the electrogenicity, produced by the charge separation, takes place in less than 50 ps.

Introduction

The traditional way of studying the membrane potential due to the primary charge separation in the photosynthetic reaction centers is the measurement of the electrochromic absorption change at 515–520 nm. The maximal time resolution of this method appears to be limited to approx. 20 ns because of the strong background absorption of

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS I, Photosystem I of green plants; PS II, Photosystem II of green plants; RC, reaction center; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; P-700, primary donor of Photosystem I; P-680, primary donor of Photosystem II.

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antenna pigments [1]. A better time resolution can be achieved by photoelectric techniques as for example the light-gradient method [2].

The light-gradient effect is a method to measure electrically with two electrodes the primary photosynthetic charge separation in chloroplasts or photosynthetic bacteria [2–7]. Briefly, the effect is based on the asymmetry in pigmented vesicular membranes introduced by the light-gradient from a non-saturating flash. The asymmetric excitation is connected with an anisotropic distribution of the dipole moments arising from the primary charge separation. In this way the suspension becomes electrically polarized in the direction of the incident light. This flash-induced dielectric polarization can be picked-up by metal electrodes and recorded as a photovoltage.

Under appropriate experimental conditions the amplitude of the photovoltage has been shown to be a measure of the dipole strength and the time-course to reflect the kinetics of dipole generation [7,8]. It has been also reported that the photovoltage is subject to several experimental parameters like the ionic conductivity of the medium and the geometry of the electrodes [9]. This problem was recently solved by constructing the electrodes as a planar capacitor in which the chloroplast suspension forms the dielectric. For this geometry and for a given medium conductivity, a time window could be characterized in which the photovoltage is independent of the conductance of the medium. In this time window the kinetics of the photovoltage are a measure of the kinetics of the electrogenic reaction and the amplitude of the photovoltage is a measure of the strength of its electrogenicity [8]. The time window extends from approx. 30 ps to 300 μs. Here we apply the light-gradient method to study the primary reactions in Photosystem I of green plants.

Under low intensity excitation conditions, the first reaction in photosynthesis is the absorption of a photon by antenna pigments, since these occur in a more than 100-fold abundance over the photochemically active pigments in the reaction centers. The excited singlet state is then rapidly transferred via other antenna pigments to the RC, where it is photochemically trapped by a redox reaction between an electron donor and electron acceptor. During the transfer time to the trap part of the excitation energy can be lost by different deactivation processes, one of them being the emission of fluorescence. The fluorescence intensity is proportional to the concentration of excited states in the antenna system. Thus measurements of the fluorescence decay kinetics provide information on the transfer time (trapping time) [10–15].

Since the photosynthetic apparatus of higher plants contain the two photosystems, PS I and PS II, the emitted fluorescence is heterogeneous: 80–90% originate from PS II and only 10–20% from PS I [16]. Due to the higher fluorescence yield of PS II, we know more on the pigment organization of PS II than of PS I (for a review, see Ref. 15). Only in recent fluorescence decay studies with the single photon timing technique, a 40–80 ps component could be assigned to the mean trapping time in PS I [12–14].

However, information is lacking on the competition between trapping and annihilation when several excitons are simultaneously present in the antenna system [17,18]. Furthermore, it is not known whether the mean trapping time is closer to 40 ps or 80 ps.

In the present study we made use of the electrogenicity of the primary charge separation in PS I to determine (or to give limits to) (i) the number of antenna pigments per RC, (ii) the kinetics of primary photochemistry and exciton arrival at the trap, and (iii) the yield of charge separation when the photons in a flash are distributed over a 12-ns or a 30-ps time interval. In addition we studied (iv) the trapping efficiency and (v) the trapping kinetics as a function of the fraction of closed traps.

The advantage of the electrical method used here to study excitation energy transfer over the more common fluorescence analysis is twofold. First, the electric method is more direct as it detects only those excitons that are successfully trapped. In comparison, the fluorescence method detects only those excitons that have not, or not yet, been trapped by photosynthetic reaction centers. Second, the two photosystems present in chloroplasts of higher plants can more easily be separated in the electrical method than in the fluorescence assay. On the one hand, the small relative contribution of PS I to the overall fluores-

cence at room temperature and its short life time, which mixes with the fast phase of the decay of the PS II fluorescence, hampers a precise study of the trapping in PS I by fluorescence. On the other hand, the fluorescence which probes mostly processes taking place in the antenna, may not necessarily reflect the photochemical state of the reaction center. In contrast, photoelectric measurements are directly sensitive to both the trapping process and the redox state of the reaction center components as previously demonstrated by chemical or photochemical modification of bacterial reaction centers [7,20].

Materials and Methods

Chloroplasts were prepared from 10- to 14-days-old pea leaves (*Pisum sativum*) according to Ref. 21. Stock solutions were stored until use at liquid nitrogen temperature with 30% ethylene glycol in a suspension medium containing 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tricine buffer (at pH 7.8) and 100 mM sorbitol.

The chlorophyll concentration of the given chloroplast preparations was determined photospectrometrically according to Ref. 21 using an acetone/water mixture (80%/20%) as solvent. The absorption spectrum of aqueous suspensions of chloroplasts was measured under low scattering conditions. By knowing the chlorophyll concentration of a given chloroplast preparation and its extinction, we calculated the molar absorption cross section at 532 nm to be $1.6 \cdot 10^{-17}$ cm^2, in agreement with Ref. 22.

The measuring cuvette for the photoelectric measurements consisted of two capacitatively coupled metal electrodes [2,7]. The cuvette was miniaturized and designed as a coaxial cell (Fig. 1). The semitransparent upper electrode consisted of a platinized square mesh grid used for electron microscopy. The grid was separated from the platinum ground plate by a 100 ± 10 μm plastic spacer. The electric connection of the grid to the screwcap is accomplished by mechanical pressure. From the dimensions of the reaction compartment and the dielectric constant of water ($\varepsilon = 80$) a cell capacitance of 33 pF can be calculated. To avoid concentration changes of the chloroplast suspension by evaporation during an experiment and to prevent light absorption by chloroplasts lying above the grid, the cell was closed with a cylindric plexiglass stopper which protruded exactly to the upper surface of the grid.

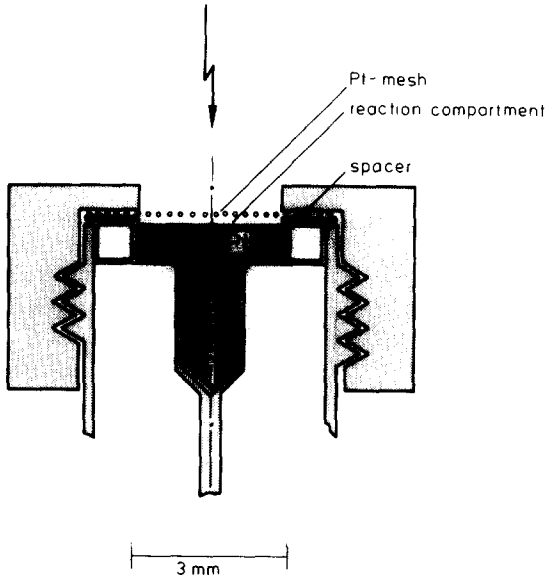

Fig. 1. Scaled drawing of the microaxial measuring cell used for the present experiments.

The excitation source was a frequency-doubled Nd-YAG laser operated either Q-switched (flash duration, 12 ns) or under mode-locking conditions. The mode-locked train consisted of about 10 flashes (Fig. 4b), each having a duration of 30 ps. A pulse-clipping system selected a single 30-ps pulse from the train, which was subsequently amplified by a factor of 10. The fundamental at 1064 nm was suppressed from the first harmonic at 532 nm by a dichroitic mirror and two band-pass filters (Schott, BG 18 and BG 39) to less than 10^{-3}.

The laser flashes were sent through a light pipe with scrambled fibers in order to achieve homogeneous illumination of the sample. The energies quoted refer to the plane of the upper surface of the grid.

The flash-induced dielectric polarization of the chloroplast suspension was measured either with a custom-built impedance converter of high-input impedance (limiting frequency, 500 MHz; gain 1) connected to a 50-Ω preamplifier (Nucletudes S.A., Orsay, France; frequency range, 10 MHz–6GHz;

voltage gain, 21) or with the 50-Ω preamplifier alone. Therefore, the photovoltage amplitudes measured with the different recording devices can be directly compared (Fig. 2b and c).

The signals were recorded either with a 1-GHz oscilloscope (Tektronix, 7104) equipped with a digitizing camera (Thomson CSF, model TSN 1150), or with a digital 7-GHz oscilloscope (Intertechnique, IN 7000).

Preillumination was achieved using a commercial slide projector equipped with a wide band interference filter (WB 550, Ditric Optics). The resulting wavelengths, 530–570 nm, fell within the absorption minimum of chlorophyll and secured that the light penetrated to all chloroplasts in the cuvette.

The photovoltage signals were stable within 10% over the time-course of an experiment, which lasted typcially less than 30 min. All experiments were carried out at room temperature, $20 \pm 2°$ C.

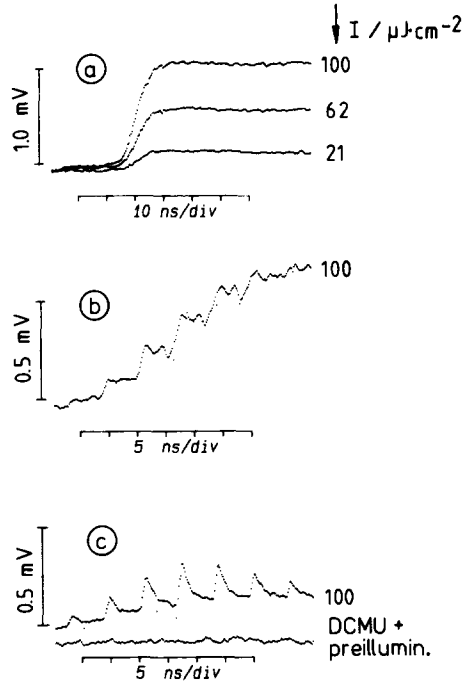

Fig. 2. (a) Time-course of the photovoltage measured under high impedance conditions (impedance converter) and excited by 12-ns flashes of different energy. The traces in (a) and (b) have been deconvoluted by the RC discharge time constant of 60 ns. (b) Time-course of the photovoltage measured under high impedance conditions and excited by a train of 30-ps flashes. (c) Upper trace: time-course of the photovoltage measured with 50-Ω impedance and excitation by a train of 30-ps flashes. Lower trace: control experiment in which a saturating preflash from a ruby laser (60 ns duration; 3 mJ/cm^2 per pulse) was applied 5 µs prior to the train. Bandwidth of the oscilloscope in a, b and c, 1 GHz.

Results

Except for experiments to determine the dependence of the photovoltage on the chloroplast concentration, all following experiments were made at a chlorophyll concentration of 3.5 mM. At this concentration the absorbance at 532 nm is 0.17 (for the given 0.01 cm pathlength of the experimental cell). At this absorbance, the chloroplasts in the rear layer are exposed to 30% smaller photon fluxes than chloroplasts in the front layer. This yields a mean heterogeneity in the excitation of 15% which is not corrected for in the following.

Excitation with 12-ns flashes

Fig. 2a shows examples of the time-course of the photovoltages measured under high-impedance measuring conditions when a suspension of pea chloroplasts was exposed to 12-ns laser flashes at 532 nm and different energies as indicated. Since the intrinsic rise times of the impedance converter and the oscilloscope used for registration were shorter than 1 ns, the kinetics of the rising phase displayed the integrated time-course of the laser flash. Due to the electrolyte conductance within the capacitative measuring cell, a self-discharging occurs as discussed in Ref. 8. In the present case of cell geometry and ionic strength of the suspension medium the decay had a time constant of 60 ns. For presentation this decay was eliminated by deconvolution of the traces in Fig. 2a. The constant photovoltage after completion of the rise therefore indicates the absence of backreactions.

The photovoltage, V, evoked by 12-ns flashes increased with increasing excitation intensity, I, and reached a saturation value, V_0, at $I \approx 0.5$ mJ/cm^2 (Fig. 3). The photovoltage also increased with increasing concentration of chloroplasts (Fig. 3). Within the experimental accuracy, for a given chlorophyll concentration the energy dependence of the photovoltage, V, could be fit by an ex-

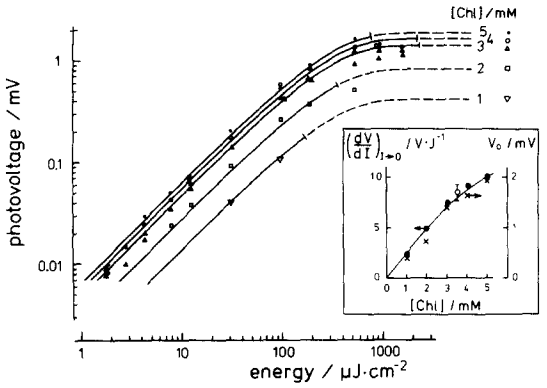

Fig. 3. Double logarithmic plot of the dependence of the photovoltage, V, on the intensity of 12-ns laser flashes at 532 nm for various concentrations of pea chloroplasts. Experimental conditions as in Fig. 2a. The drawn lines represent calculated fits according to Eqn. 1 with $n = 170$. Fit procedure: the maximal photovoltage, V_0, was adjusted to give a good fit at low intensities. The dashed parts of the fit curves indicate a range in which the time-course of the photovoltage deviated from the step-like shape shown in Fig. 2a (see text for discussion). Inset: Dependence of the initial slope of the photovoltage, V, versus energy, I, curve, $(dV/dI)_{I\to 0}$, and the maximal photovoltage, V_0, on the choroplast concentration.

ponential saturation law:

$$V = V_0(1 - e^{-n\varkappa\sigma I}) \tag{1}$$

where n is the mean number of chlorophyll antenna molecules per photosynthetic unit, $\varkappa$ the quantum yield of primary photochemistry, and σ the molar absorption cross section.

The solid and dashed curves in Fig. 3 represent computer fits based on Eqn. 1, using parameters obtained in the following way: V_0 resulted from the plateau photovoltage at high energy (0.5 mJ/cm^2 per pulse) and the exponential term in Eqn. 1 was fit by adjustment in the low-energy range (less than 30 µJ/cm^2). The slopes of the photovoltage vs. energy at low energy, $(dV/dI)_{I\to 0}$, and the maximal photovoltage, V_0, resulting from the fit, depended on the chlorophyll concentration as shown in the inset of Fig. 3. Both quantities were proportional to the chlorophyll concentration, showing the beginning of a saturation at concentrations above 3.5 mM.

Under certain conditions the shape of the photovoltage traces deviated from step functions like shown in Fig. 2a. An overshoot of the signal was observed at high chlorophyll concentration and high excitation energy as well as at low chlorophyll concentration and medium to high excitation energy (traces not shown). The dashed parts of the fit curves in Fig. 3 mark these regimes where the photovoltage signals were shaped. These regimes were excluded in the further analysis. The widest energy range in which no signal shaping was observed was found at a chlorophyll concentration between 3 and 4 mM. For the following experiments we therefore choose a concentration of 3.5 mM.

Since the photovoltage vs. excitation energy could be satisfactorily described by Eqn. 1 (see also Fig. 6), the fit parameter in the exponent, $n\varkappa\sigma = A$, can be used to calculate the antenna size, n:

$$n = \frac{A}{\varkappa \cdot \sigma} \tag{2}$$

Taking a quantum yield for the primary photosynthetic charge separation of $\varkappa = 1$ and an absorption cross section of $\sigma = 1.6 \cdot 10^{-17}$ cm^2, $180 \leqslant n \leqslant 240$ for all pea chloroplasts prepared during 1 year when the peas were grown under comparable light conditions.

Excitation with trains of 30-ps flashes

The flashes in the previous experiments did not contain many more photons than necessary to close all traps, and the photons were rather evenly spread over the time interval of 12 ns. Under these conditions annihilation processes are unlikely and the quantum yield for trapping can be assumed to be close to 1. Next we want to look for loss processes and for the trapping efficiency when the same number of photons are delivered in packages of 30 ps duration.

The excitation source for these experiments was a flash-lamp pumped Nd-YAG laser under 'free running' mode-locking conditions, that delivered a train of 30-ps flashes spaced by 7 ns. The energy distribution of the flashes in the train when 20 trains were averaged is shown in Fig. 4b (open circles). It was possible to describe the relative energy of the ith flash, I_i, by a Gauss function:

$$I_i \approx e^{-\left(\frac{i-0.3}{3.4}\right)^2} \tag{3}$$

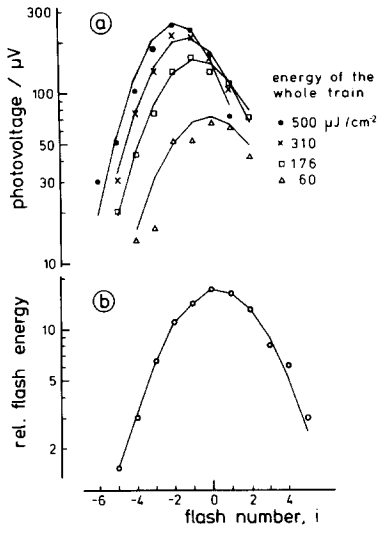

Fig. 4. (a) Dependence of the peak photovoltages elicited by trains of 30-ps flashes (analogous to Fig. 2b) on the total energy which was varied by insertion of neutral density filters. The maximal total energy (= energy of the whole train) was 300 μJ/cm². The drawn lines result from the fit of the whole data set assuming the energy distribution of the individual picosecond flashes (b) and the dependence of the photovoltage on the excitation energy observed with 12-ns flashes (Fig. 2a). (b) Energy distribution of the picosecond flashes in the train, measured with a fast responding photodiode (Motorola, MRD 500) in place of the coaxial cell.

where $i = 0, \pm 1, \pm 2, \ldots$ ($i = 0$ labels the most intense flash). The nominator -0.3 accounts for a jitter in the flash number which resulted from the interaction of small energy fluctuations of individual trains with the setting of the trigger level of the oscilloscope (externally triggered by a photodiode). Eqn. 3 is useful to estimate by extrapolation small photovoltages which were not captured on the oscilloscope screen.

The photovoltage elicited by the train was either measured with high impedance (Fig. 2b) or with 50-Ω impedance (Fig. 2c). Although at high impedance significant 'ringing' was observed, the photovoltage remained essentially constant after the initial fast rise. Following flashes added to the level attained before, thus producing a stair with different step heights. At 50-Ω impedance the major part of the photovoltage decayed quickly. The main part of the decay can be ascribed to the discharge of the capacitance of the cell through the 50 Ω of the preamplifier: $\tau = 50\ \Omega \cdot 33\ \text{pF} =$ 1.65 ns. A smaller fraction decayed more slowly and caused the slight offset of the baseline. We ascribe the latter effect to electrolyte/electrode interfacial relaxations known from electrolyte capacitors [24], since it was sensitive to the type and concentration of the electrolyte used. Despite the mentioned differences between the signals measured with different impedances, we note that the maximal amplitudes were the same in the two cases.

At the high time resolution of the electric measurements, a possible contribution from PS II might be present: the reduced state of PS II (DCMU and preillumination) could still be photochemically active [25], according to the reaction:

$$\text{P-680}^* \text{PheoQ}_A^- \rightarrow \text{P-680}^+ \text{Pheo}^- \text{Q}_A^-$$

To separate the contribution of this reaction from that of the PS I photovoltage we delivered a saturating preflash from a ruby laser 5 μs prior to the train. This inactivates PS I by forming quantitatively P-700$^+$ which is known to have a lifetime much longer than tens of microseconds [26]. The result is shown in Fig. 2c, lower trace. Although the chloroplasts used had a fully functioning water-splitting enzyme, as controlled by O_2 measurements in the absence of DCMU, there was no photovoltage observed. The absence of a photovoltage can be rationalized by assuming that the initial charge separation in PS II with reduced Q_A is either not electrogenic or, if it is electrogenic, the reaction does not take place [27,28] (see also accompanying manuscript: Ref. 29).

In Fig. 4a are plotted on a logarithmic scale the photovoltage amplitudes elicited by the 30-ps flashes of the train. The flash of highest energy was labeled with number 0, the preceding ones were counted negative and the subsequent ones positive. As seen in Fig. 4a the highest photovoltage lies at negative flash numbers. This is due to the fact that the flash of highest energy (number 0) is less effective in creating a photovoltage, since at its arrival there may be a large fraction of photosystems already closed by the preceding flashes. Attenuation of the whole train with neutral density filters gave smaller photovoltage and the highest photovoltage moved closer to flash number 0 (Fig. 4a).

To look for possible loss processes (annihilation) being specific to picosecond excitation, the data in Fig. 4 were analysed with respect to three questions: (i) how large is the sum of all photovoltages compared to the photovoltage from 12-ns excitation; (ii) how large is the photovoltage from single 30-ps flashes compared to 12 ns excitation of the same energy; and (iii) does the photovoltage yield from 30 ps-flashes depend on the fraction of already closed traps?

When the sum of all photovoltages, $V_0^{ps} = \Sigma_{i=-\infty}^{\infty} V_i$, elicited by the train of a given total energy, was plotted versus the energy (not shown) the data could be fit by an exponential function (Eqn. 1) with a slope $(dV/dI)_{I\to 0} = 8$ V/J (open circle in the inset of Fig. 3) and with a V_0^{ps} which was only slightly (10–20%) smaller than V_0^{ns} elicited by 12-ns excitation. The conclusions for picosecond excitation are the following. First, if there are losses of excitation energy, they are very small; and second, the traps can be closed by a train of picosecond flashes nearly as effectively as with a 12-ns flash.

Accordingly, the exponential saturation law (Eqn. 1) should be appropriate to fit all data in Fig. 4a with one set of parameters (solid lines). The fit lines result by connection of calculated photovoltages due to flash number i, V_i, by taking the corresponding excitation intensity I_i (from Fig. 4b), the fit parameters $V_0^{ps} = 1.3$ mV and $(dV/dI)_{I\to 0} = 8$ V/J, and taking into account the fraction of traps closed by preceding flashes:

$$V_i = \left(V_0^{ps} - \sum_{j=-\infty}^{i-1} V_j\right) \cdot (1 - e^{-n\times\sigma I_i}) \tag{4}$$

The deviations of the data points from the calculated fit curves are due to energy fluctuations between different trains (approx. 20%) and fluctuations of the half width of the energy distribution (approx. ± 0.2 flash numbers).

However, the data could be analyzed with higher accuracy when the photovoltage from individual 30-ps flashes, V_i^{ps}, was compared with that from 12-ns flashes of the same energy, V^{ns}. This treatment of the train data is shown in Fig. 5, where the photovoltage yield of a given picosecond flash is plotted vs. the fraction of closed traps. The photovoltage yield, Y, is defined as

$$Y = \frac{V_i^{ps}}{V^{ns}} \tag{5}$$

and the fraction of closed traps, f, is calculated by the cumulated photovoltages from the previous flashes in the train divided by the maximum photovoltage, V_0^{ns} obtained for 12-ns flashes:

$$f = \frac{\sum_{j=-\infty}^{i-1} V_j}{V_0^{ns}} \tag{6}$$

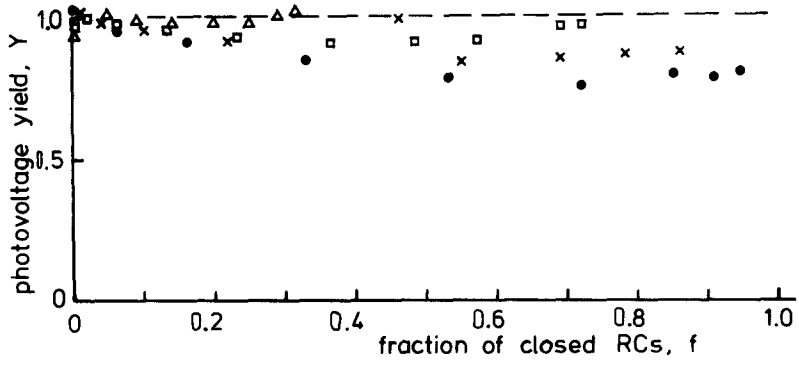

Fig. 5. photovoltage yield evoked by individual picosecond flashes in a train normalized to the photovoltage evoked by 12-ns flashes as a function of the fraction of closed PS I reaction centers.

The data points in Fig. 5 result by dividing the photovoltages from Fig. 4a by the photovoltage calculated from the best fit of Eqn. 1 to the 12-ns data at the same energy (the symbols used in Figs. 5 and 4 correspond to each other). Note, that the sets of equal symbols (i.e., same excitation energy) in Fig. 5 extend differently wide to high energies, because at the end of a train of low energy (△) there are less traps closed than at the end of a train of high energy (●). For the data in Fig. 5 the energy of one single picosecond flash was always less than 80 $\mu J/cm^2$. The photovoltage yield is nearly independent of the number of closed traps (see Discussion).

Excitation with single 30 ps-flashes

To examine loss processes at higher excitation energy of a single 30-ps flash and to test whether all traps can be closed by a single strong picosecond flash, one pulse of the train was selected and amplified by a factor of 10. The energy dependence of the photovoltage elicited by such single 30-ps flashes is shown in Fig. 6 (open circles). For direct comparison the energy dependence was also measured with 12-ns flashes using the same chloroplasts and the same high-impedance amplifier. It can be seen that the data coincide within the experimental acuracy at low energies. However, at higher energies the 30-ps data lie markedly lower, indicating some losses of excitation energy.

In Fig 7 the same data were plotted as the photovoltage yield, i.e, the normalized deviation of the 30-ps data from 12-ns data. As in the previous section, the yield was calculated by dividing the photovoltage from picosecond excitation by the photovoltage at the same energy calculated from the best fit of Eqn. 1 to the 12-ns data of Fig. 6. These data are shown in Fig. 7 (open circles). In addition, we plotted the corresponding data from individual 30-ps flashes of the train experiments (open square symbols; compare Fig. 5). For comparison, Fig. 7 contains a further curve (lower line), which indicates the fraction of still open RCs after a 12-ns flash of a given intensity (best fit of Eqn. 1 to the 12-ns data in Fig. 6).

Trapping kinetics in Photosystem I

The time resolution of the set-up used for the

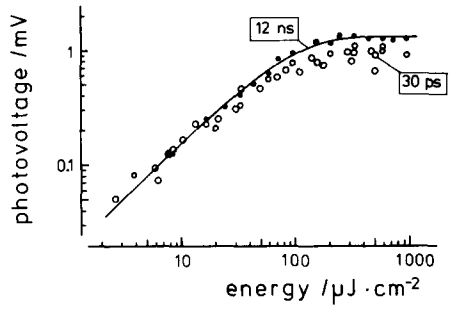

Fig. 6. Double logarithmic plot of the dependence of the photovoltage on the intensity of 12-ns flashed and 30-ps flashes.

above experiments was not sufficient to resolve the kinetics of the primary charge separation in PS I. To achieve a higher time resolution, the slowest component of the above experiments, the 1-GHz oscilloscope, was replaced by a 7-GHz oscilloscope (see Materials and Methods). Even then it is necessary to deduce charge separation kinetics that are close to the time resolution of the apparatus. Therefore, the latter is needed with high precision.

The time resolution of the complete apparatus was determined in two ways. First, we made use of the primary charge separation in purple membranes from *Halobacterium halobium,* which is most likely faster than 5 ps [30,31]. In addition, these photobiological membranes possess no antenna pigments. The purple membranes were electrically preoriented in the coaxial cell with a d.c. voltage of 2 V, where they deposit as a thin oriented layer on one electrode. This way of determining the rise time of the apparatus has the advantage that it tests the same instrumental configuration as used for the light-gradient experiments, i.e., it accounts for the flash duration as well as for the rise time of the coaxial cell itself, the preamplifiers and the oscilloscope. Computer analysis of such photovoltage traces revealed that the rising phase could be described by an error function having an exponential time constant of $\tau = 70 \pm 5$ ps [31]. Second, we calculated the total rise time by geometrical addition of the 10–90% rise times of the single components the apparatus was composed of. This also yielded a $\tau = 70$ ps.

To determine the mean trapping time in PS I and to examine whether the distance between an

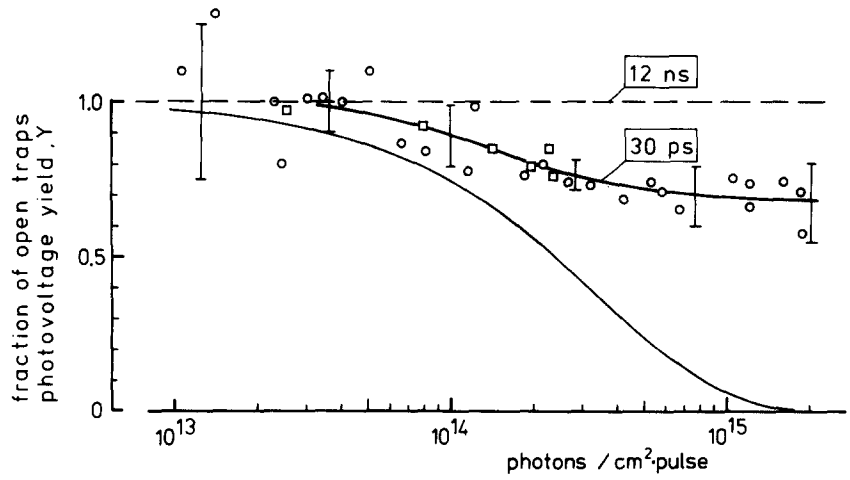

Fig. 7. Comparison of the picosecond data (O) and the 12-ns data (dashed horizontal line), expressed as normalized deviation from the exponential fit of the 12-ns data. The lower solid line indicates the fraction of still open RCs after a 12-ns flash of a given energy.

antenna pigment that absorbs a photon and the trap (P-700) has an influence on the trapping kinetics, the photovoltage was measured at high and low excitation energy, 500 $\mu J/cm^2$ and 50 $\mu J/cm^2$, respectively (Fig. 8a and b). The photovoltage at high energy developed with a time constant close to the instrumental rise time, as can be seen by comparison with a computed error function with $\tau = 80$ ps (Fig. 8a). The photovoltage at low energy developed significantly slower, as seen by the deviations when a fit was tried with the instrumental rise time as the only one (Fig. 8b). However, a good fit of this trace was obtained when a mono-exponential kinetics of the charge separation of $\tau_t = 90$ ps was convoluted with the instrumental rise time (Fig. 8b).

An error consideration of the measured rise times allows one to calculate (or to give limits to) the molecular processes involved: in the case of high energy excitation ($\tau = 80 \pm 10$ ps), trapping and charge stabilization occurs in less than 50 ps. Picosecond absorbance change measurements on isolated PS I particles under conditions of exciton annihilation have previously shown that Chl* disappears with a time constant of 40 ps in the presence of P-700 as well as in the presence of $P\text{-}700^+$ [45]. In the case of low-energy excitation, the $\tau_t = 90 \pm 15$ ps can be interpreted as the mean trapping time, since the charge stabilization step is much faster.

The experiments with trains of picosecond flashes have shown that the yield of the primary photochemistry was nearly independent of the fraction of closed traps (Fig. 5). The following experiment aims at the question whether or not the presence of closed traps effects the kinetics of trapping. Fig. 8c shows the photovoltage when half of the traps were in the closed state. This was achieved by applying white background light of appropriate intensity as to halve the photovoltage. The trace could also be fit by $\tau_t = 90 \pm 15$ ps. This demonstrates that the trapping kinetics does not depend on the fraction of closed traps.

Discussion

Origin of the photovoltage

Recently doubts have been raised whether or not the photovoltage from chloroplasts due to the light-gradient effect reflects directly the primary photosynthetic charge separation [32]. In this reference the photovoltage was ascribed to the Dember effect which arises when inhomogeneous light absorption gives rise to a gradient of positive and negative charges with different mobilities. We want to reject this explanation, since the fastest rise times of the photovoltage of less than 50 ps correspond to the known kinetics [35] of the primary charge separation without an indication of a delay phase that could be expected for consecutive movements of mobile ions to be in the nanosecond range.

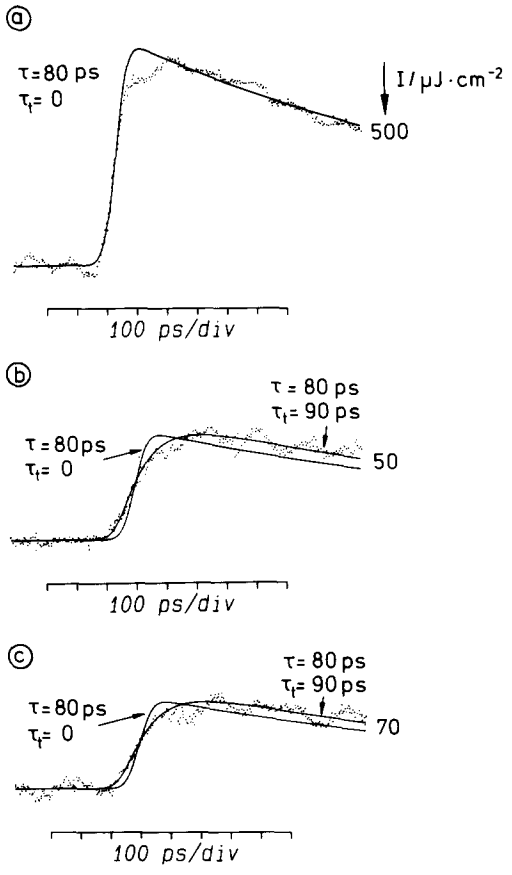

Fig. 8. Time-courses of the photovoltage excited by single 30-ps flashes of high energy (a), low energy (b), and when half of the traps were closed by white background illumination (c). The drawn lines represent computer curves resulting by convolution of charge displacements in a capacitor (exponential kinetics, τ_t) with the frequency characteristics of the apparatus (error function, τ).

Early charge separation events in the reaction center of Photosystem I

Several acceptors, A_0, A_1, F_X, F_A and F_B, are known to be involved in the primary photochemistry of PS I (for a review, see Ref. 33). There is no general agreement on the sequential or parallel connection of these acceptors, and little is known on the kinetics of the fastest forward electron-transfer reactions at room temperature. The initial charge separation between P-700 and the A_0 acceptor (a chlorophyll molecule absorbing around 690 nm) is thought to occur in about 15 ps [34], whereas the electron transfer from A_0^- to A_1 (vitamin K_1) takes place with a time constant of about 30 ps [35].

The fact that the fast photovoltage in Fig. 8a could be described (within the time resolution of 35 ps) by a step function and its convolution with the response characteristics of the apparatus, thus demonstrates that the PS I electrogenicity is developed at least at the stage $P\text{-}700^+A_0A_1^-$. The slight difference between the rise time in the case of purple membranes (70 ps) and PS I at high energy (80 ps) might be an indication of the existence of two electrogenic steps in the forward electron transfer, connected with the formation of the states $P\text{-}700^+A_0^-A_1$ and $P\text{-}700^+A_0A_1^-$, respectively. Presently our time resolution or signal-to-noise ratio is not sufficient for a clear-cut statement.

The present electric measurements, however, do not allow a distinction between the other acceptors, since they do not manifest themselves by electrogenic contributions. At longer times (up to 50 ns) the photovoltage continues like a step function (Fig. 2) without indication of further electrogenic charge displacements. This result means that all those acceptors that are reduced in the time range between 35 ps and 50 ns lie in one plane of the thylakoid membrane, which is most likely the membrane/water interface.

Competition between trapping and exciton annihilation

The quenching curve in Fig. 7 is not comparable to similar ones known from fluorescence quenching experiments [18,19,23,36,40]. Fluorescence quenching refers to losses with respect to the total number of absorbed photons, whereas the drop in the photovoltage yield (Fig. 7) refers by definition of Eqn. 5 to losses in antenna systems with open traps only.

In the range of excitation energies applied in this study one does not expect exciton annihilation to take place with long (12 ns) flashes [19]. On the other hand, annihilation must be considered with shorter flashes (30 ps). The decreased amplitude of the photovoltage elicited by 30-ps flashes compared to that generated by 12-ns flashes (Fig. 6) demonstrates that such annihilation processes occur in PS I antenna. The extent of annihilation did not exceed 25% as seen in Fig. 7, where the photovoltage yield is plotted vs. the flash energy. At low energies there is no indication of a difference between the two flash lengths.

Annihilation becomes significant above approx. $2 \cdot 10^{14}$ photons/cm^2 per pulse. This value corresponds to a mean value of 1.7 excitations per antenna system assuming 200 chlorophyll molecules per RC. According to the Poisson statistics, at this excitation density 50% of the units have absorbed two or more photons. This means that the onset of annihilation occurs at an energy that is one order of magnitude higher for PS I than for PS II [18,40].

When the photovoltage from individual picosecond pulses of the train are treated the same way as for the single picosecond flashes (correcting for the fraction of traps already closed by preceding flashes), a comparable decrease of the photovoltage yield is found (Fig. 7, squares).

The decreased yield of the photovoltage at high energies could be due to different annihilation processes such as singlet-singlet or singlet-triplet (including the Fission mechanism). However, the present experimental data do not allow a distinction between them.

Models for the organization of Photosystem I antenna units

The dependence of the photovoltage on the energy of a 12-ns flash could be fit with an exponential saturation law (Fig. 6) in the regime discussed before. Such a law is expected from a statistical consideration, in terms of hits per photosystem [41]. Under the reasonable assumption that only one charge separation occurs in a unit, whether it is hit by one or several photons, the cumulative one-hit Poisson distribution applies [42,43]. The fraction, Y, of traps closed by a flash of a given energy, assuming a quantum yield of 1, is then:

$$Y = \phi(1 - e^{-x}) \qquad (7)$$

where x is the number of average hits. This is the typical behaviour expected for isolated photosynthetic units [44], or for equal efficiency of quenching of closed or open RCs.

In the experiments carried out with trains of 30-ps flashes most of the photons in a flash are absorbed by chloroplasts in which a variable fraction of closed reaction centers is present. This feature allowed the determination of the yield of charge separation as a function of the fraction of closed traps according to Eqn. 6. Neglecting small losses occurring in the picosecond flashes of higher energy, the photovoltage yield was nearly independent of the fraction of closed traps (Fig. 5), demonstrating that the trapping efficiency is independent of the fraction of closed PS I reaction centers. Although this observation seems at a first sight compelling evidence in favour of a model of organization of PS I units in separate units, it can also be reconciled with the opposite view of fully connected units [16]. However, in this last case, there is a requirement for the quenching efficiency of P-700 and P-700$^+$ to be equal. An identical conclusion has been derived by measuring the PS I antenna absorption cross section in various mutants [45]. On the other hand, Delepelaire and Bennoun [46] have presented conclusive evidence in favour of a connection between at least three PS I photosynthetic units.

Excitation trapping in Photosystem I and comparison with fluorescence decay analysis

Under natural light conditions there is never more than one exciton at the same time in one antenna system. Since the mean trapping times usually refer to this case, an exciting picosecond flash should contain as few photons as possible to avoid non-linear effects. The photoelectric determination of the trapping time in PS I was done with flashes of 50 μJ/cm^2. This energy was at the lower limit as to give a photovoltage with sufficient signal-to-noise ratio for the kinetic analysis. Taking an antenna size of 200 chlorophylls per P-700, an absorption cross section of $1.6 \cdot 10^{-17}$ cm^2, and assuming Poisson statistics, one can calculate that 27% of the photosystems have absorbed one photon and 67% none; only 6% have absorbed two or more photons. Hence, the trapping time of 90 ± 15 ps found by the photoelectric assay (Fig. 8b) can be considered to be close to the one that is valid for infinitely low excitation energy.

The values reported for the trapping time in PS I as determined by fluorescence decay analysis range from 50 ps to 130 ps [11–13,36–38]. The value of 90 ps reported here, however, is in close agreement with the most recent fluorescence analysis by Holzwarth et al. [12], that accounts for

four exponentials and fits simultaneously at different wavelengths with one set of parameters.

With increasing excitation energy two phenomena could decrease the trapping time. On the one hand the probability increases that a photon is absorbed by an antenna pigment lying closer to the trap. Consequently, both the diffusion length and the diffusion time for the first visit of the trap are shorter. If the exciton is trapped at this first encounter (diffusion-limited case [39]), one would expect shorter trapping times at higher photon densities. In this case the trapping times would be equivalent to the first passage times. On the other hand, in the trap-limited case, when the excitation can visit the reaction center several times before being trapped, the probability of annihilation processes increases with increasing pulse energy. This reduces the exciton lifetime which in turn decreases the trapping time.

Conclusions

The present electrical measurements make possible to draw a rather detailed picture of the trapping processes and the charge stabilization in PS I. The concept of the electrical detection of the primary photosynthetic charge separation is that if the charge separation is a fast step, say less than 10 ps, then it can be used to monitor the arrival of excitons from the antenna pigments at the trap.

In agreement with current literature [34,35,45] we found that the charge stabilization is faster than 50 ps (Fig. 8a). Therefore, the slower kinetics observed in Fig. 8b can be interpreted as the kinetics of excitation energy transfer. The transfer time of 90 ± 15 ps determined photoelectrically agrees with fluorescence decay kinetics [11–13,36–38]. Further, we found that the transfer time was not affected by the presence of closed traps (Fig. 8c).

The latter finding would suggest rather a 'separate unit' organization of the PS-I antenna pigments than a 'lake' model, since in the latter one would expect a lengthening of the transfer time due to the longer distance the exciton has to travel. An antenna organization in 'separate units' is also supported by the exponential closure of traps with increasing energy (Fig. 3) as well as by the independence of the photovoltage yield on the fraction of closed traps (Fig. 5). However, if the quenching efficiencies of P-700 and $P\text{-}700^-$ are equal, our data are also compatible with a lake model.

Excitation with single 30 ps-flashes showed that more than 75% of the traps can be closed by one intense picosecond flash (Figs. 6 and 7) and that losses of excitation energy by annihilation are small (less than 25%) compared to the trapping path (Fig. 7).

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