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Photoacoustic detection of photosynthetic energy storage in Photosystem II submembrane fractions

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Photoacoustic spectroscopy can be used for the measurement of energy storage during photosynthesis. We report here the monitoring of photosynthetic energy storage in a Photosystem II submembrane fraction. The data are treated using an analogy to enzyme kinetics. The semi-reciprocal plot of the energy storage yield (ϕ'_r) against the modulated measuring beam light intensity (I) provided an energy storage yield extrapolated at $i = 0$ (ϕ'_{r0}), and a half-saturation beam intensity (i_{50}). The energy storage reaction was characterized by a ϕ'_{r0} of 42% in all the conditions used. However, the value of i_{50} was affected by electron-transport inhibitors, artificial electron acceptors, and by the measuring beam wavelength. It was inferred that ϕ'_r monitored at any given measuring beam intensity varies with i_{50} in close relation to the electron-transport rate monitored as oxygen evolution. We proposed, from the data presented, that the energy storage monitored in photoacoustic experiments is related to the reduction of the plastoquinone pool.

Introduction

Photoacoustic spectroscopy is used to monitor the thermal deactivation of a solid or semi-solid sample after irradiation with an intensity-modulated light source. There is increasing interest in the application of this technique to the study of photosynthesis. One of the most important aspects in this regard is the fact that with active photosynthetic materials, the portion of absorbed energy which is stored in photochemical intermediates is not released immediately as heat. The comparison of acoustic signals from active and inactive samples thus leads to evaluation of the photosynthetic energy storage yield (also called photochemical loss) [1]. This can be measured independently for photoreactions I and II of photosynthesis. For example, Photosystem I-mediated energy storage was detected in chloroplast (dichlorophenolindophenol as electron donor) and in cyanobacteria (N,N,N',N'-tetramethylphenylenediamine as electron donor) [2,3]. Cyclic Photosystem I was also specifically observed in cyanobacterial heterocysts (NADPH, NADH or H_2 as electron donor) [4].

The energy storage yield has been demonstrated to be strongly dependent on the modulated measuring beam intensity [2,5]. This is due to partial saturation of photosynthesis following progressive closure of the photoreaction centers as the light intensity increases. To alleviate this problem, the reciprocal of the energy storage yield can be plotted against modulated light intensity [5,6]. This treatment produces a linear relationship extrapolating to a yield unaffected by light intensity (zero light intensity) at the ordinate [5]. So far, energy storage yields obtained through the above procedure or by measurements at relatively low light intensity (1–5 $W \cdot m^{-2}$) have consistently been between 10 and 25% of the thermal yield of an inactive sample for most of the materials studied at wavelengths in the region of 680 nm [2–5,7].

Recently, we demonstrated that energy storage can be studied in Photosystem II submembrane fractions [5]. The interest in submembrane fractions comes from their wide range of application in studies using isolated photosystems. In this paper, we present a detailed study of energy storage in such preparations enriched in Photosystem II. The relationship between the modulated beam intensity and the energy storage yield is examined using a procedure similar to enzyme kinetics analysis. The results obtained in the presence of inhibitors and

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCBQ, 2,5-dichlorobenzoquinone; PQ, plastoquinone; Mes, 4-morpholineethanesulfonic acid.

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electron acceptors are compared with measurements of oxygen evolution obtained with the same preparation. The role of the plastoquinone pool in the energy conversion monitored by photoacoustic spectroscopy is discussed.

Materials and Methods

Photosystem-II enriched submembrane fractions were isolated using a modification of the procedure described by Berthold et al. [8]. Spinach leaves were homogenized in 50 mM Tricine-NaOH (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sorbitol, 0.1% ascorbate and 1 mM phenylmethylsulfonyl fluoride. The slurry was filtered through eight layers of cheesecloth and centrifuged for 6 min at $1500 \times g$. The pellet was resuspended in 50 mM Tricine-NaOH (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$ and 0.1% ascorbate and recentrifuged for 6 min at $1500 \times g$. The resulting thylakoid membranes were resuspended at a Chl concentration of 1 $mg \cdot ml^{-1}$ in 20 mM Mes-NaOH (pH 6.2), 15 mM NaCl, 10 mM $MgCl_2$ and 4% Triton X-100. The mixture was incubated on ice and in darkness for 20 min and was then centrifuged for 10 min at $3500 \times g$. The supernatant was collected, and the Photosystem II submembrane fractions were harvested by a 30 min centrifugation at $37000 \times g$. The preparation was kept at a Chl concentration of 2 $mg \cdot ml^{-1}$ in 20 mM Mes-NaOH (pH 6.2), 1 mM NaCl and 0.5 mM $MgCl_2$, and was used the same day.

Initial rates of oxygen evolution were monitored at 22°C with a Clark electrode as described elsewhere [3]. The reaction medium contained 11 μg Chl $\cdot ml^{-1}$, 0.63 mM 2,6-dichlorobenzoquinone as acceptor (unless otherwise specified), 1 mM NaCl, 0.5 mM $MgCl_2$ and 20 mM Mes-NaOH (pH 6.2).

Photoacoustic measurements (35 Hz, 680 nm) were performed in a home-made apparatus described previously [10]. For these experiments, 1 ml of Photosystem II preparation, diluted at 250 μg Chl per ml in the resuspending buffer, was aspirated through a nitrocellulose filter [9]. The filter was cut into a disk of appropriate dimensions and introduced into the cell. To monitor the photochemical energy storage yield, a non-modulated white background light of saturating intensity (100 $W \cdot m^{-2}$) was directed from a quartz-halogen lamp into the cell through a fiber optic guide.

Results

The acoustic signal from a sample of Photosystem II submembrane fractions at a given modulated light intensity is maximal upon illumination with an actinic non-modulated background light of saturating intensity. This is due to a closing of the photoreaction centers by the saturating light beam, causing a maximal thermal emission yield. Background illumination thus produces little or no effect when using a pre-inhibited sample [2–5].

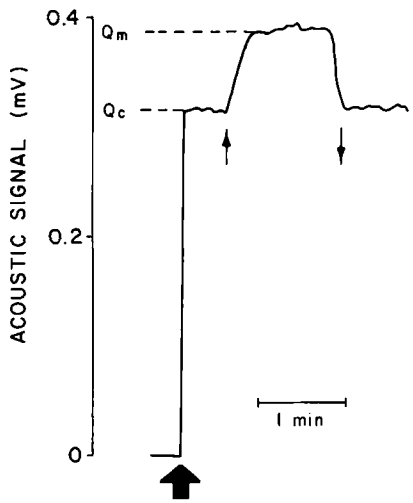

Fig. 1. Photoacoustic signal from Photosystem II submembrane fractions aspirated onto a nitrocellulose filter. ⬆, onset of the measuring light beam (2.5 $W \cdot m^{-2}$). ↑ and ↓, non-modulated actinic beam (100 $W \cdot m^{-2}$) on and off, respectively. The ϕ_r' value calculated as described in the text was 17.8%. Other conditions are described in Materials and Methods.

In Fig. 1, we show a typical trace used for the monitoring of acoustic signals obtained in the presence (Q_m) and absence (Q_c) of the actinic beam. Calculation of the energy storage yield ($\phi_r' = 100\% \times (Q_m - Q_c)/Q_m$) from the trace in Fig. 1 gives a yield of 18%. Due to partial saturation of the photochemistry by the measuring modulated beam, this yield varies with the modulated light intensity. Therefore, to characterize the photoacoustic measurements further, the relationship between the energy storage yield and the measuring beam intensity (I) was studied. Our approach involved the treatment of these data in a fashion similar to enzyme kinetics analysis. Such an analogy has been applied to photosynthetic electron transfer and is discussed in detail by Howell and Vieth [11]. If we consider the primary steps of electron transfer leading to energy storage, the rate constants k_e, k_d and k_D will apply to the following reactions:

$$[P] \xrightarrow{k_e I} [P^*] \tag{1}$$

$$[P^*] \xrightarrow{k_d} [P] + \text{heat} \tag{2}$$

$$[P^*] \xrightarrow{k_D} [P] + \text{energy storage} \tag{3}$$

where [P] and [P*] represent the concentration of photoreaction centers in the ground and in excited

states, respectively. From the above equations, the rate of formation of P* is given by

$$\frac{d[P^*]}{dt} = k_e I[P] - k_d[P^*] - k_D[P^*] \quad (4)$$

During a measurement at a given light intensity, a stationary concentration of P* is obtained. In this condition,

$$[P^*] = \frac{k_e I[P]}{k_d + k_D} \quad (5)$$

The rate of electron transfer can be described as

$$R = k_D[P^*] \quad (6)$$

If Eqns. 5 and 6 are combined and if $[P_0]$ is considered as the total concentration of photoreaction centers P* + P,

$$R = \frac{k_D I[P_0]}{(k_d + k_D)/k_e + I} \quad (7)$$

The maximal rate of electron transfer is

$$R_m = k_D[P_0] \quad (8)$$

Thus, Eqn. 7 can be expressed as

$$R = \frac{R_m I}{i_{50} + I} \quad (9)$$

where, in analogy with K_m, i_{50} represents the constant ratio

$$i_{50} = \frac{k_d + K_D}{k_e} \quad (9a)$$

and coincides with the half-saturating light intensity. Eqn. 9 indicates that the rate of electron transfer increases with light intensity. However, in photoacoustic experiments, the rate of electron transfer is probably measured as energy storage in an intermediate acceptor of the electron-transfer chain, presumably in the PQ pool [5]. The energy storage yield can be expressed as:

$$\phi'_r = \phi'_{r0} R_m - R/R_m \quad (0 \le R \le R_m) \quad (10)$$

where ϕ'_{r0} represents the maximal energy-storage yield. Accumulation of the reduced form of intermediates causes saturation at a rate proportional to light intensity. To apply Eqn. 9 in photoacoustic measurements, one has to consider $1/I$ instead of I as the concentration of substrate. From Eqn. 9 and 10 we obtain:

$$\phi'_r = \frac{\phi'_{r0}}{I/i_{50} + 1} \quad (11)$$

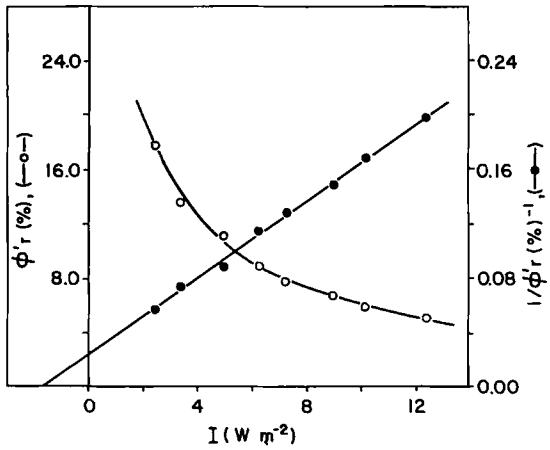

Fig. 2. Dependency of the energy storage yield (open symbols) and the reciprocal of the energy storage yield (closed symbols) on the modulated light intensity for Photosystem II submembrane fractions. The value of ϕ'_{r0} determined as described in the text is 42%. Conditions are as in Fig. 1.

The general equation of the Lineweaver-Burk type of plot applied to the photoacoustic measurements becomes:

$$\frac{1}{\phi'_r} = \frac{1}{\phi'_{r0}}\left(\frac{I}{i_{50}} + 1\right) \quad (12)$$

The semi-reciprocal plot of $1/\phi'_r$ vs. I thus produces a linear relationship with the intercept at the ordinate giving a ϕ'_r value for $I = 0$ (ϕ'_{r0}).

Eqns. 11 and 12 are illustrated by the results presented in Fig. 2, which were obtained from traces similar to the results in Fig. 1. For these experiments, the material used was prepared the same day and only the first measurement of energy storage (background light on and off) is used, since there is a rapid drop of ϕ'_r as a result of photoinhibition by the intense actinic beam [9]. The energy storage yield decreases with I, showing partial saturation of the photochemistry by the measuring beam as discussed above. Energy storage yields at $I = 0$ were obtained for several experiments similar to those in Fig. 2 using different Photosystem II preparations, and a yield of $42 \pm 2\%$ was repeatedly obtained. The value of half-saturating modulated light-intensity (i_{50}) was about 1.7 $W \cdot m^{-2}$. Both ϕ'_{r0} and i_{50} are higher values than those we have presented in a previous report [5]. These reflect the greater integrity of the preparation when it used on the same day that it has been isolated.

We examined the relationship between ϕ'_r and I at several wavelengths. In Fig. 3, the results are summarized for 670, 680 and 700 nm. A ϕ'_{r0} of 41 percent was obtained in all of the above cases, since the same reaction in Photosystem II was studied. However, the value of i_{50} was dependent on the absorbency of the sample at the selected wavelength. It increased from 1.7

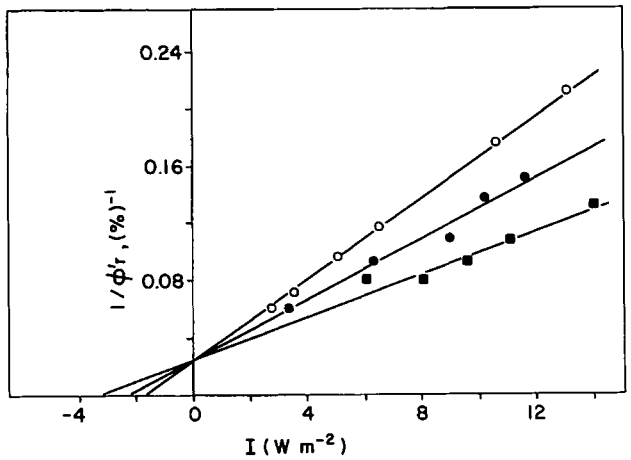

Fig. 3. Effect of the measuring beam wavelength on the semi-reciprocal plot. The i_{50} values (W·m^{-2}) are given in brackets: ○, 680 nm [1.7]; ●, 670 nm [2.2]; ■, 700 nm [3.3]. ϕ'_{r0} was 41% in all cases.

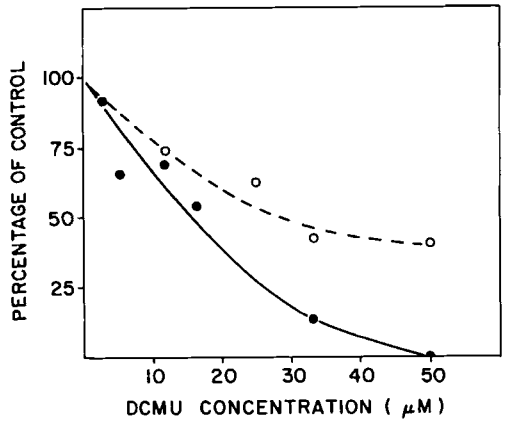

Fig. 5. Effect of DCMU on the initial rate of oxygen evolution (●) and on the yield of energy storage (○). The control rates were 12.2% for ϕ'_r and 425 μmol O_2 per mg Chl per h for oxygen evolution. The measuring beam intensity for photoacoustic measurements was 5 W·m^{-2}.

to 3.3 W · m^{-2} with decreasing absorbancies (from 680 to 670 and 700 nm, respectively, 678 nm being the absorption maximum in these preparations), in agreement with the requirement for a higher light intensity to saturate the photochemistry at wavelengths of lower absorbencies.

In Fig. 4, we present the data obtained from a control sample in comparison to Photosystem II submembrane fractions inhibited with 25 μM 3-(3,4-dichlorophenol)-1,1-dimethylurea or 5 μM ruthenium red. DCMU affects electron transfer on both the donor and acceptor sides of P-680 in these preparations [12], and ruthenium red is a potent inhibitor of water splitting [13]. In the semi-reciprocal plots of Fig. 4, the inhibition was seen as a decreased value of i_{50} in comparison to the control experiment. However, ϕ'_{r0} remained unchanged following inhibition, since maximal energy storage yields can be obtained when the light intensity is low enough to prevent saturation of the weak electron-transfer capacity of the partially inhibited samples.

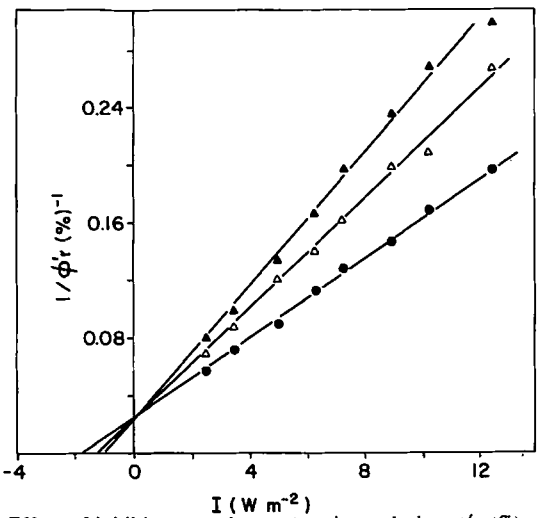

Fig. 4. Effect of inhibitors on the semi-reciprocal plot. ϕ'_{r0} (%) and i_{50} (W·m^{-2}) are given in brackets: ●, control sample [42;1.8]; △, sample inhibited with 25 μM DCMU [42,1.3]; ▲, samples inhibited with 5 μM ruthenium red [42,1.1].

It was demonstrated before in this laboratory that i_{50} is closely related to the electron-transfer rate [14]. The decrease of i_{50} from 1.7 to 1.3 or 1.0 W · m^{-2} after the addition of the above compounds indicates an inhibition of 24 and 41% of energy storage with DCMU and ruthenium red, respectively, under the present experiment conditions. Because ϕ'_{r0} remained constant even in the presence of inhibitors, the relative change in i_{50} could be indirectly monitored by following the value of ϕ'_r at a given measuring beam intensity. The relationship between the electron-transfer rate and ϕ'_r was studied at several DCMU concentrations in Fig. 5. The initial rate of oxygen evolution decreased with increasing DCMU concentrations, and full inhibition in the preparation used was obtained with 50 μM DCMU. On the other hand, energy storage was less affected by DCMU. The saturation of the effect of DCMU on ϕ'_r, which is obtained above 35 μM, indicates that part of the energy storage cannot be inhibited by DCMU even at the concentration of 50 μM.

The presence of Photosystem II electron acceptors did not increase the value of ϕ'_r or i_{50}. As seen in Fig. 6, the reverse effect was indeed obtained, since submembrane fractions in the presence of 5 mM potassium ferricyanide or 300 μM DCBQ behaved as inhibited samples (see Figs. 4 and 6). Again, a ϕ'_{r0} of 42% was calculated in each experiment. The lower i_{50} obtained in the presence of acceptors indicated that the energy storage measured at these light intensities is decreased by the electron sink created by the electron acceptors.

We have studied the effect of DCBQ in greater detail. In Fig. 7, we compare the initial rates of oxygen evolution with ϕ'_r at variable DCBQ concentrations. In the conditions used, the maximal rate of oxygen evolu-

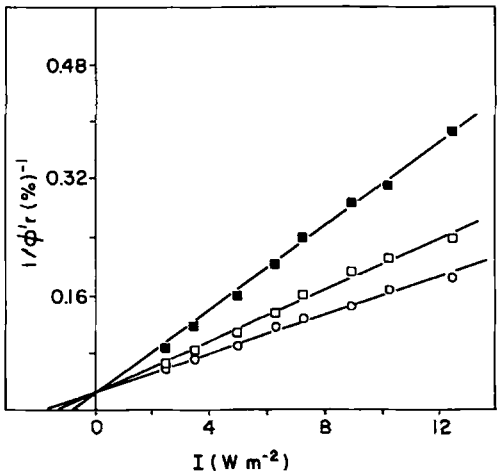

Fig. 6. Effect of electron acceptors on the semi-reciprocal plot. ϕ'_{r0} (%) and i_{50} (W·m^{-2}) are given in brackets; ○, control [42,1.7]; ■, 5 mM potassium ferricyanide (42,0.7]; □, 300 μM 2,6-dichlorobenzoquinone [42,1.3].

tion was obtained with DCBQ concentrations above 1.2 mM. The maximal effect on ϕ'_r was reached at a similar DCBQ concentration; however, part of the energy storage (25%) remained unaffected. This situation is similar to the case reported in Fig. 5 for the inhibition by DCMU, where about 35% of the energy storage remained, even in the presence of high DCMU concentrations. Both the reciprocal of ϕ'_r and the ratio of DCBQ concentration to the initial rate of oxygen evolution gave a linear relationship with DCBQ concentration (not shown). The concentration causing half the maximal effect of DCBQ on the above parameters, which was obtained by extrapolation of the traces calculated by linear regression, was 0.35 and 0.31 mM for ϕ'_r and oxygen evolution, respectively. The similar values indicate that a common process, presumably the oxidation of reduced intermediates implicated in electron transport on the acceptor side of Photosystem II, is responsible for the effect of DCBQ on oxygen evolution and on the energy storage monitored in the photoacoustic experiments.

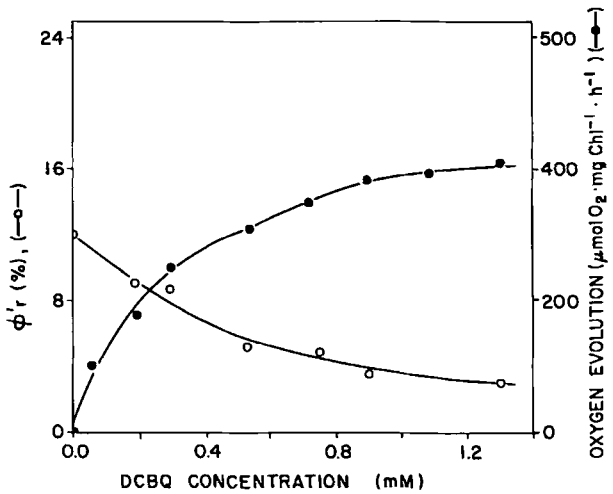

Fig. 7. Effect of DCBQ on the energy storage yield (○) and on the initial rate of oxygen evolution (●). The measuring beam intensity for photoacoustic measurements was 5 W·m^{-2}.

Discussion

Photoacoustic spectroscopy provides a unique way of detecting the amount of absorbed energy that is stored in reduced intermediates during photosynthetic electron transport. Since the light reactions of photosynthesis can be simplified to a kinetic model similar to a second-order enzymatic reaction [10], we have applied this general treatment to energy storage. The use of this analogy allows for a quantitative examination of the semi-reciprocal plot of $1/\phi'_r$ vs. the modulated light intensity. Two parameters were defined. The energy storage at a measuring beam intensity intrapolated to zero provides a value of energy storage in the absence of light saturation (ϕ'_{r0}), and the light intensity extrapolated at $1/\phi'_r = 0$ represents the intensity of half saturation (i_{50}) for the photoreaction studied.

The value of ϕ'_{r0} is related to the photoreaction quantum yield (ϕ) through the following equation [1]:

$$\phi'_{r0}(\lambda) = \phi(\lambda)\frac{\Delta E_p}{Nhc/\lambda} \qquad (13)$$

where $\Delta E_p/(Nhc/\lambda)$ represents the ratio of stored energy over absorbed energy. N is the Avogadro's number; h, the Planck's constant; c, the light velocity; and λ, the measuring beam wavelength. ΔE_p is the amount of thermal energy per mol stored by the photochemical reaction.

The quantum yield of photochemistry at the photoreaction centers is generally accepted to be greater than 0.8 [15,17]. However, it is not unity because some of the absorbed energy is lost during energy (exciton) migration from the absorbing holochrome to the reaction center, and because some of the reaction centers are closed [18]. In order to obtain a ϕ'_{r0} of 100%, ΔE_p would have to be greater than Nhc/λ. This requirement can be met if the concerned reaction is accompanied by a positive change of entropy. In the present case, we have obtained values of about 40%, which indicates that more than half of the absorbed energy was released immediately as heat.

The significance of the energy storage monitored during photoacoustic experiments is certainly a matter of interest. Endothermic reactions from conformational modifications have been detected in photoacoustic experiments using *Halobacterium halobium* [19,20]. The close relationship observed between the effect of DCBQ and DCMU on the rate of oxygen evolution and on the energy storage yield (Figs. 5 and 7) indicates the involvement of linear electron transfer in the process

leading to energy storage in the Photosystem II submembrane fractions. Because plastoquinone is the last acceptor present in these preparations, and the decay rate of the other electron-transport intermediates present between P-680 and plastoquinone is faster than the time resolution of photoacoustic measurements [21], we have previously postulated that energy storage should involve the reduction of plastoquinone [5]. Competitive inhibition of energy storage was obtained with the addition of inhibitors that block electron transfer, and by electron acceptors that prevent the reduction of plastoquinone (Figs. 4 and 6). We therefore assumed that energy storage due to reduction of the plastoquinone pool is an important factor to explain the above results. The plastoquinone pool will be reoxidized only at a slow rate by dissolved oxygen [25,26]. Thus, energy storage saturates at relatively low light intensities (up to 2 $W \cdot m^{-2}$). Another form of energy storage could result from the formation of a pH gradient during plastoquinone reduction. However, we do not expect the preparation used to form a proton gradient because it probably consists of open membrane fractions.

The fact that DCBQ concentrations which are optimal for oxygen evolution did not produce a complete loss of energy storage (Fig. 7) could indicate that some plastoquinone reduction still occurs under these conditions. Another interpretation resides in the detection of energy storage due to the reduction of added artificial electron acceptors. In that case, the decreased rate of energy storage (decreased i_{50}) obtained in presence of DCBQ or potassium ferricyanide could be explained if we assume that the acceptor molecules spend a longer time at their reduction site than endogenous plastoquinone. Energy storage due to artificial acceptors should produce a different value of ΔE_p than when plastoquinone is involved. However, it seems that this variation in ΔE_p was too small to be detected in Fig. 6 as a change in ϕ'_{r0} under the precision allowed by the present experiments. Conversely, the incomplete inhibition of energy storage encountered in the presence of DCMU at a concentration where oxygen evolution is fully inhibited reflects fundamental differences between the two types of measurement. It is conceivable that the actual DCMU (or DCBQ) concentration in the samples used for photoacoustic measurements is lower than expected, owing to the increased membrane concentration after their aspiration onto the nitrocellulose filter, and to the filtration of most of the liquid medium through the filter, including solubilized inhibitors or electron acceptors. However, this situation would increase the apparent saturating concentrations of additives whereas in our case, these concentrations were similar in photoacoustic and oxygen evolution measurements (Fig. 5 and 7). Another interpretation of the uncomplete inhibition by DCMU is the occurrence of energy storage involving primary acceptors of Photosystem II in presence of the inhibition. Energy storage due to pheophytin has been reported in Photosystem II core complexes [22] and storage due to primary acceptors of both photosystems was detected by time-resolved photoacoustic spectroscopy of submembrane fractions [23]. In that case, in presence of DCMU we could expect a change in ϕ'_{r0} owing to a different ΔE_p for the energy storage due to an earlier acceptor than plastoquinone. Such variation was not seen in Fig. 4 were a DCMU concentration that nearly produces the maximal inhibitory effect was used. As discussed earlier for the experiments with electron acceptors, the variation of ΔE_p may be too small to be detected.

Regarding the above experiments, we could also speculate that an increase of gas pressure due to oxygen evolution in the photoacoustic cell could cause an apparent decrease in ϕ'_{r0}. Because oxygen evolution requires multiple excitations of the reaction center, its effect will increase in importance with increasing light intensity. However, in the type of preparation used, the participation of oxygen to the pressure wave at the phase angle of the thermal signal probably represents less than 10% of the total acoustic signal [3,5,24].

Because the exact nature of the components responsible for the energy storage monitored during photoacoustic experiments was not known, the use of a submembrane fraction containing only one of the two higher-plant photosystems has greatly simplified the situation. The type of preparation used contains an active water-splitting system and electron transport intermediates from water to plastoquinone [9,27,28]. The data presented sustain the hypothesis that energy storage is mostly attributed to the reduction of the plastoquinone pool when no additive is present. In studies using heterocyst cells from blue green algae, we have shown that energy storage could be induced by cyclic Photosystem I [4]. This electron transfer pathway also involved the plastoquinone pool. However, energy storage was lost when reduced equivalents were exhausted following oxydation by dissolved oxygen or when the electrons were diverted towards the respiratory chain through cytochrome oxidase [4]. The above supports the present idea that photoacoustic spectroscopy represents an appropriate methodology to assess the plastoquinone redox state.

Acknowledgements

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