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PARALLEL INDUCTIVE KINETICS OF FLUORESCENCE AND PHOTOACOUSTIC SIGNAL IN DARK-ADAPTED THALLI OF *BRYOPSIS MAXIMA*

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The inductive kinetics of fluorescence and photoacoustic signal were measured simultaneously in dark-adapted thalli of the green coenocytic alga *Bryopsis maxima*. Under illumination with weak red light modulated at 60 Hz, the fluorescence yield varied, showing three maxima P, M_1 and M_2 almost immediately, 10 s and 6 min after the onset of the illumination, respectively (Yamagishi, A., Satoh, K. and Katoh, S. (1978) Plant Cell Physiol. 19, 17–25). The photoacoustic signal also showed inductive transients which parallel well those of the fluorescence up to the M_2 stage. After M_2, the photoacoustic signal remained at a constant level, while the emission yield gradually decreased. The first peak of the fluorescence induction and a corresponding peak of the photoacoustic transients were selectively eliminated by prior illumination or methyl viologen treatment of the dark-adapted thalli. The second peaks of the two induction curves were abolished by carbonylcyanide-*m*-chlorophenylhydrazone, whereas dicyclohexylcarbodiimide enhanced their peak heights and suppressed the subsequent decreases. The results indicate that the fluorescence yield is mainly determined by the redox state of the Photosystem II reaction center throughout the induction period except the last phase. Mechanisms underlying inductive transients of fluorescence are discussed in the light of the present findings.

Introduction

Illumination of dark-adapted algal cells or plant leaves with strong light induces a series of changes in the yield of chlorophyll *a* fluorescence which is called the fluorescence induction. At physiological temperatures, the fluorescence yield is determined by the redox state of Q, the secondary electron acceptor of Photosystem II, which serves as a quencher in the oxidized state [1]. The emission yield is also modulated by other factors such as the state transition which regulates energy distribution between Photosystems I and II, the influx of proton and efflux of Mg^{2+} across the thylakoid membranes, conformational changes of the membranes and photoinhibition of either or both of the two photosystems [2–4]. Causes of slow transients in the fluorescence induction are still in dispute.

Absorbed light energy that is not utilized by photochemistry is dissipated as fluorescence and heat. The heat production in a sample, which is illuminated with light modulated at acoustic frequencies, can be measured by photoacoustic spectroscopy [5]. Simultaneous measurement of the fluorescence and photoacoustic signal provides important information not only on changes in the efficiency of energy conversion but also on mechanisms underlying fluorescence transients during the dark-light transition of the photosynthetic apparatus. Inoue et al. [6] observed that the photoacoustic signal, after initial small transients, in-

Abbreviations: DCCD, *N*,*N'* dicyclohexylcarbodiimide; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

creases slowly to a high steady state in dark-adapted leaves and suggested that changes in heat conduction in chloroplasts contribute to the slow change in the signal. The photoacoustic signals from photosynthetic materials have been studied in detailes by Malkin and co-workers [7–12]. It was indicated that the high steady state of the photoacoustic signal is due to photosynthetic O_2 evolution, which is modulated at the frequency of the monitoring light [9–12]. The modulated gas-exchange strongly interferes with the measurement of the periodic heat production in leaves by the photoacoustic technique.

We showed recently that the thallus of the green alga *Bryopsis maxima* is a particularly suitable material for photoacoustic spectroscopy, because the contribution of the modulated O_2 evolution to the photoacoustic signal is negligible owing to its unique coenocytic cellular organization [13]. The relative size of the photoacoustic signal is therefore related to the efficiency of photon utilization in photosynthesis. It was shown that, with a weak monitoring light modulated at 60–100 Hz, light energy is mainly stored by electron transport from the electron donor pools to the acceptor pools of the two photosystems with the quantum yield close to unity [13]. The alga also provides very intact chloroplasts, with which kinetics of the fluorescence induction showing three maxima named P, M_1 and M_2 have been studied in detail [14–23]. Early transients are related to the inductive changes of cytochrome *f* [15,16,19], the electrochromic shift of carotenoids [16,19] and the delayed fluorescence [23].

In the present work, we measured simultaneously time-courses of fluorescence and photoacoustic changes in dark-adapted *Bryopsis* thalli to investigate the relationship between the emission yield and rate of electron transport during the induction of photosynthesis. Effects of several treatments of the thalli on the two induction kinetics were also studied.

Materials and Methods

Dark-adapted thalli of *B. maxima* were employed throughout the present work [13,24]. The dark adaptation was carried out by incubating the thalli in aerated sea-water for at least 2 h in the dark. Where indicated, additions were made at least 30 min prior to measurements to ensure their penetration into the cells.

The photoacoustic apparatus, which had been described previously [13], was modified so as to measure the photoacoustic and fluorescence kinetics simultaneously. The bottom surface of the cuvette (15 × 20 mm) was covered with pieces of the thalli and the red monitoring light (620–700 nm) modulated at 60 Hz was provided through the upper glass window. The photoacoustic singal was monitored by a microphone (Matsushita-Tushin WM-034 AY), amplified by a AC amplifier, fed into a lock-in amplifier (Ithaco Dynatrac 391A) and recorded with a Hitachi recorder. After measurements were done, a strong continuous light (585 W/m^2, 430–750 nm) was added by means of a half mirror to obtain the maximum amplitude of the signal [7]. Chlorophyll *a* fluorescence from the upper surface of thalli was guided to the photomultiplier by a glass-fiber cable. To avoid interference from the exciting light, fluorescence was measured at 735 nm by placing a Corning 7-69 filter and a 735 nm interference filter in front of the photomultiplier.

All measurements were carried out at room temperature (20–25 °C).

Results

Inductive transients of fluorescence and photoacoustic signals

Fig. 1 shows time-courses of changes in the fluorescence and photoacoustic signal measured simultaneously during the illumination of the dark-adapted *Bryopsis* thalli for 60 s. Red light modulated at 60 Hz served as the excitation light for fluorescence and as the monitoring light for photoacoustic signal. On illumination with the modulated light of 35 W/m^2, the fluorescence intensity increased rapidly to a maximum P and then decreased to a minimum S_1 (trace a). A second maximum M_1 appeared about 10 s after the onset of illumination, followed by a gradual decrease to a quasi-steady-state level S_2. The transient features of the fluorescence induction at 735 nm were essentially the same as those observed at 685 nm with the continuous excitation light [14,17]. This and the following observations indicate that

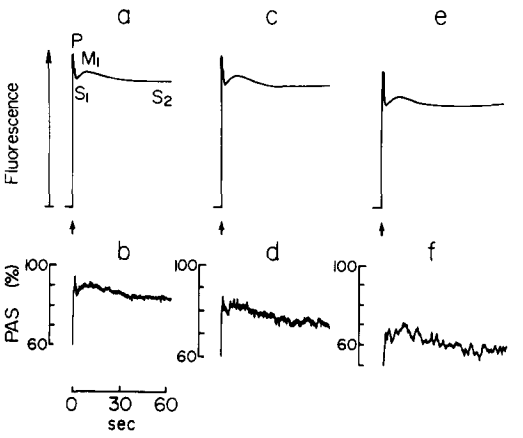

Fig. 1. Inductive transients of fluorescence and photoacoustic signal (PAS) in dark-adapted *Bryopsis* thalli. Upper and lower traces are time-courses of fluorescence and photoacoustic changes, respectively. Intensity of the monitoring light was 35 W/m^2 for a and b, 17.5 W/m^2 for c and d and 7 W/m^2 for e and f. Modulation frequency, 60 Hz. Arrows indicate times when the monitoring light was on. For scales on the left side of the photoacoustic signals, see text.

the emission intensity is modulated by a common factor(s) at the two wavelengths in a similar way.

The photoacoustic signal was found to show inductive transients, which parallel well those of the fluorescence yield (trace b). Two maxima, which correspond to P and M_1 of the fluorescence induction, appeared almost immediately and 10 s after the modulated light was turned on, respectively, and a quasi-steady-state level was attained at the end of the illumination period.

When the intensity of the monitoring light was reduced, the photoacoustic transients became less clear due to increased noise levels (traces d and f). It is to be mentioned that the sample was renewed in each measurement because, once illuminated, the fluorescence and photoacoustic transients were restored only slowly in the dark. Thus, the absolute intensity of the fluorescence and photoacoustic signal cannot be compared between different sets of traces. The background illumination method of Lasser-Ross et al. [8] was, therefore, employed to compare the photoacoustic signals from different samples. After 60 s of illumination, strong continuous light was added to the modulated monitoring light to obtain a reference, in which absorbed modulated light is totally converted to heat because the photochemical system is saturated. The maximum signal size thus obtained was taken as 100% in scales on the left side of traces. The percent signal size is proportional to the fraction of absorbed light energy that is dissipated as heat. Physiologically more meaningfull is the energy yield, which is defined as the fraction of absorbed light energy that is stored in intermediates relative to the total absorbed light energy [13]. The energy yield is obtained from the following equation:

$$1 - \frac{\text{signal without the background illumination}}{\text{signal with the background illumination}}$$

Fig. 1 shows that higher energy yields were obtained with weaker monitoring light and that the efficiency of photon utilization increased gradually during illumination. The energy yield varied between 0.05 and 0.15 at 35 W/m^2 and between 0.3 and 0.4 at 7 W/m^2. Obviously, the monitoring light itself serves as the actinic light even at 7 W/m^2 [8,12]. The use of weaker monitoring light is limited by increased noise level.

First peaks

Fig. 2 shows that the early transients of the fluorescence and photoacoustic signal are similar

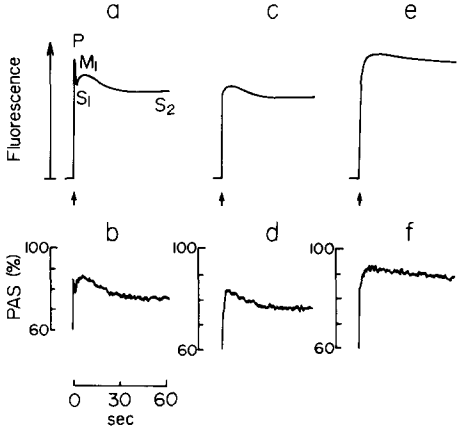

Fig. 2. Effects of prior illumination and methyl viologen treatment of the dark-adapted thalli on fluorescence and photoacoustic transients. Intensity and modulation frequency of the monitoring light were 35 W/m^2 and 60 Hz, respectively. Other conditions were as in Fig. 1. a and b, no treatment; c and d, thalli were illuminated for 5 s with the monitoring light 30 s prior to measurement; e and f, thalli were incubated with 10 mM methyl viologen 30 min prior to measurement.

not only in kinetics but also in response to treatments of the thalli. Illumination of the dark-adapted thalli for 5 s prior to measurement eliminated the first peaks of the fluorescence and photoacoustic induction curves (traces c and d). The thalli, which had been long incubated with methyl viologen, showed the inductions without the first peaks (traces e and f). The results strongly suggest that the first peaks of the two induction curves are different manifestations of the same event(s) in the photosynthetic apparatus.

It is also seen that the two treatments affected the energy yield differently. The high energy yield observed after the prior illumination is ascribed to a partial photoactivation of the photosynthetic machinery. On the contrary, the methyl viologen treatment resulted in a significant lowering of the energy yield. This reflects retardation of electron transport by the proton gradient set up across the thylakoid membranes, because the effect of methyl viologen can be largely reversed on addition of an uncoupler [13].

Second peaks

A close relationship exists between the second fluorescence maximum M_1 and the proton gradient formed across the membrane [17]. The peak height of M_1 is elevated and the subsequent decrease is suppressed by energy transfer inhibitors, which inhibit proton efflux coupled with the ATP formation, whereas uncouplers, which dissipate the proton gradient, specifically reduce or eliminate the second fluorescence peak. These effects were reproduced with intact thalli which had been incubated with DCCD and CCCP (Fig. 3).

It was found that DCCD and CCCP affect the second peak of the photoacoustic induction curve similarly. The energy transfer inhibitor slightly increased the peak height and strongly suppressed the subsequent decrease. The peak disappeared completely in the CCCP-treated thalli. We conclude therefore that the second photoacoustic peak also depends upon the proton gradient. DCCD also affected the first peak with unknown reasons.

Slower transients

Fig. 4 illustrates kinetics of the fluorescence and photoacoustic changes measured during a longer illumination period. After S_2, the fluorescence rose to the third maximum M_2. About 6 min of illumination was needed to attain M_2 with monitoring light of 35 W/m^2. The emission intensity then decreased gradually to the terminal level T.

The photoacoustic signal varied in parallel with the fluorescence changes up to M_2. Then, its kinetics deviate from those of the fluorescence, remaining at a constant level during a prolonged illumination. The results suggest that the slower fluorescence rise to M_2 is also determined by the redox state of Q, whereas the final M_2-T decline is independent of electron transport.

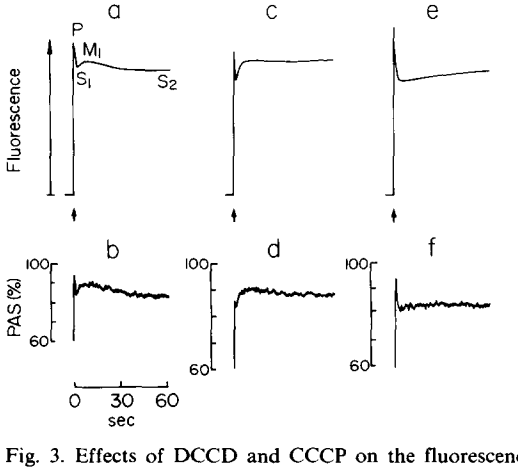

Fig. 3. Effects of DCCD and CCCP on the fluorescence and photoacoustic transients. Experimental conditions were as in Fig. 1. a and b, no addition; c and d, 50 μM DCCD; e and f, 5 μM CCCP.

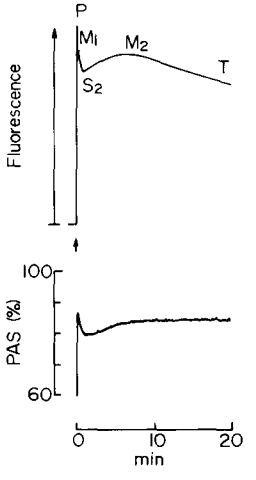

Fig. 4. Slow inductive transients of fluorescence and photoacoustic signal. Experimental conditions were as in Fig. 1, except that a slower recording time was used.

Discussion

Competitive relationship between fluorescence and electron transport

Since chlorophyll *a* fluorescence is a loss of light energy absorbed by plants, the emission yield is expected to vary competitively with the efficiency of photosynthesis during the induction period. Antiparallel kinetics of the fluorescence and photosynthetic O_2 evolution do not hold, however, in slow phases of the induction [25,26]. This is generally explained in terms of the modulation of the fluorescence yield by various additional factors as stated in Introduction. It is also to be stressed that O_2 evolution does not always serve as a good measure of photosynthesis during the induction period, because O_2 is not only evolved but also consumed as an acceptor of photosynthetic electron transport in dark-adapted algae with the inactive CO_2 fixation system [27].

We showed previously that, with the monitoring light modulated at 60 Hz, the energy yield is directly related to rate of electron transport in *Bryopsis* thalli, where the heat production can be measured by the photoacoustic technique without interference from the modulated O_2 evolution [13]. Inductive variations of noncyclic electron transport can be, therefore, monitored by the photoacoustic technique in the dark-adapted thalli. Contribution of cyclic electron transport to the energy yield is considered to be of minor significance, because electron transport from the donor pool of Photosystem I to, for instance, the plastoquinone pool stores less energy as compared with the charge separation between the donor and acceptor pools of Photosystem I. The kinetics of fluorescence changes are parallel to the photoacoustic transients and therefore are antiparallel to the rate of electron transport up to the M_2 stage. Thus, the present work clearly demonstrates that a competitive relationship exists between the fluorescence yield and photosynthetic electron transport over the entire induction period except the last M_2-T phase. In the following, we shall discuss each inductive transient of the fluorescence yield in the light of the present findings.

First peaks

The first wave of the fluorescence induction is attributed to redox changes of Q in dark-adapted intact *Bryopsis* chloroplasts, because the rise to P and the subsequent decline from P to S_1 are accompanied by a parallel transient reduction and reoxidation of cytochrome *f*, respectively [15]. On illumination, Q is rapidly reduced because electron transport is blocked on the reducing side of Photosystem I in dark-adapted chloroplasts [15,16,18]. The block is removed during illumination and consequently Q is rapidly reoxidized by electron transport to Photosystem I. Thus, the first peak is absent from the briefly illuminated thalli (Fig. 2c). Methyl viologen eliminates the first peak by accepting electrons directly from the reducing site of Photosystem I and hence by bypassing the inactivated site of electron transport (Fig. 2e).

The present work provides a strong support for the Q-dependence of the early fluorescence transients. The kinetics of the photoacoustic changes indicate that noncyclic electron transport is indeed strongly suppressed shortly after the onset of illumination and then restarts with increasing rate in parallel to the fluorescence rise to P and the subsequent decrease from P to S_1, respectively.

The appearance of P is often related to the inactive state of the CO_2 assimilation. It should be emphasized, however, that the rapid initiation of electron transport at the P-S_1 phase cannot be explained in terms of the photoactivation of CO_2 reducing cycle, which requires at least a few minutes of illumination. In addition, the P-S_1 decline is totally insensitive to KCN at concentrations, where the CO_2 fixation is completely inhibited [15].

The results are incompatible with an explanation that the flourescence decrease is caused by the energization of the thylakoid membranes, because the formation of the proton gradient across the membranes is expected to slow down electron transport and consequently increase the photoacoustic signal. A previous work has demonstrated that the P-S_1 decline depends on the stroma pH but not on the energized state of the thylakoid membranes [24].

The State II-I transition would induce a parallel decrease in the fluorescence and photoacoustic signal by adjusting the energy distribution between the two photosystems favorably for Photosystem I and also for electron transport. The state transi-

tion, which may involve phosphorylation or dephosphorylation, or intramembrane redistribution of light-harvesting chlorophyll proteins [29] should be, however, a much slower process than the P-S_1 decline, which completes within the first 1 or 2 s of illumination.

Bradbury and Baker [30] and Krause et al. [31], who titrated fluorometrically the redox state of Q during the induction, showed that oxidation of Q is the main cause of the fluorescence decline from P in bean leaves and *Chlorella*, respectively. The energy-dependent quenching is predominant in a slow decline from P in intact chloroplasts isolated from higher plants [31]. The P-S_1 decline accompanied by the transient oxidation of cytochrome *f* precedes, however, the slow energy-dependent quenching in intact spinach chloroplasts [32].

A question arises as to what serves as electron acceptor at this stage of the induction, where the CO_2 reducing system is still largely in the inactive state. In this respect, of special interest is the finding of Radmer and Kok [27] that photosynthetic electron transport to O_2 takes place in dark-adapted algae in complement to that with CO_2 as electron acceptor. Satoh [22] also showed that the P-S_1 decline is selectively suppressed under anaerobic conditions in intact *Bryopsis* chloroplasts. We suggest, therefore, that the P-S_1 decline is caused by oxidation of Q through electron transport to O_2. The kinetics of the fluorescence and photoacoustic transients indicate that electron transport to O_2 is limited until Q is substantially reduced. This implys that the accumulation of electrons on the reducing side of Photosystem I is important for the initiation of a rapid electron flow to O_2. The mechanism of the light-dependent switch in electron transport on the reducing side of Photosystem I remains to be studied in future.

Second peaks

Two mechanisms have been proposed to explain the occurrence of M_1, which is related to the formation of the proton gradient across the thylakoid membranes [17]. The first mechanism assumes that M_1 reflects changes in the structure of the thylakoid membranes caused by protonation of the membranes [17]. The structural changes would alter the distance between chlorophyll molecules or their mutual orientation, upon which the yield of fluorescence strongly depends. The fluorescence yield is, however, at most only a few percent of the absorbed photons and a small change in the fluorescence yield should not appreciably affect the heat production by itself. The parallel appearance of the second peak in the photoacoustic transients favors the second mechanism, which relates the fluorescence rise to M_1 to a partial reduction of Q [23]. Regulation of electron transport by the proton gradient in the plastoquinone region would result in the accumulation of the reduced Q, which in turn increases the light emission and the heat production.

The fluorescence decrease from M_1 to S_2 was inhibited by DCCD, which blocks proton efflux coupled with the ATP synthesis. Methyl viologen, which suppresses ATP consumption in the CO_2 reducing system, also inhibited the M_1-S_2 decline. We suggest that the M_1-S_2 transition reflects the initiation of CO_2 assimilation. Recently, Andreeva and Tikhonova [28] compared inductive transients of fluorescence and P-700 in bean leaves as a function of the dark-incubation time and suggested that a fluorescence decrease from the second peak is due to the photoactivation of the CO_2 reducing cycle.

Slow transients

Much less is known about fluorescence changes after S_2. The photoacoustic measurements provided, however, important information on mechanisms of slow fluorescence transients. The S_2-M_2 rise is accompanied by a parallel increase in the photoacoustic signal and may be related to a change in electron transport. The slow fluorescence increase cannot be attributed to the State I-II transition, because the state transition always changes the energy distribution in a direction to reduce the heat production.

Exceptionally, the M_2-T transition was found to be independent of the redox state of Q. The constant level of the photoacoustic signal indicates a steady state of electron transport at the last phase of the induction. This rules out a possibility that the fluorescence decrease is caused by a photoinactivation of Photosystem II [33]. The M_2-T decline cannot be ascribed to the energy-dependent quenching, either, because it was not reversed on addition of uncouplers (data not presented).

In concluding, the present work emphasizes the importance of electron transport in regulating the fluorescence yield throughout the induction period except the last phase in dark-adapted *Bryopsis* thalli. Any other single mechanism cannot provide a better explanation for the fluorescence transients which parallel the photoacoustic signal changes. We cannot, however, totally exclude the superposition of additional fluorescence modulations, especially, at the slow phases of the induction. Much more has to be learned to understand mechanisms underlying the slow fluorescence transients. Finally, the present work shows that photoacoustic spectroscopy serves as a powerful tool for the investigation of the inductive transitions of fluorescence, provided that a plant material suitable to the technique is chosen.

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