Modulation of Millisecond Chlorophyll Luminescence by Non-Photochemical Fluorescence Quenching

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By combining a high frequency modulation system for measurement of fluorescence with a phosphoroscope type apparatus for measurement of luminescence, recordings of fluorescence and luminescence induction kinetics under identical conditions were obtained. Both measuring systems tolerated the application of saturating pulses of white light for rapid, transient elimination of photochemical quenching at photosystem II reaction centers, thus allowing determination of the non-photochemical quenching component. The saturation pulse induction curves of luminescence are well correlated with the corresponding curves of fluorescence, suggesting that luminescence yield is lowered by the same type of non-photochemical quenching (mostly "energy dependent quenching") as fluorescence. Hence, in order to evaluate luminescence signals in terms of the rate of charge recombination at photosystem II reaction centers, knowledge of fluorescence quenching is required.

Introduction

Chlorophyll luminescence results from charge recombination at PS II reaction centers (for reviews, see ref. [1–5]). Luminescence intensity is determined by a great number of factors, which make a quantitative interpretation of luminescence data problematic. Different phases of the complex luminescence decay, ranging from about 0.1 μs to several minutes, reflect different precursor states and sources of activation energy for the recombination reaction. "Millisecond-luminescence" (0.2–5 ms decay component) has been preferentially investigated in past work, using the phosphoroscope measuring technique. This component may be considered to originate from reaction centers in a quasi-equilibrium state with respect to the stabilization of the charges separated during a preceding light pulse [3, 4]. Millisecond-luminescence intensity is stimulated by membrane energization, $\Delta\Psi$ and Δ pH (6), the underlying mechanism of stimulation not being fully understood yet. There is also strong influence of the S-states of the oxygen-evolving complex on luminescence yield [1–5]. However, with continuous illumination there is rapid mixing of S-states, such that S-state related changes become negligable some seconds after light-on.

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In contrast to the stimulatory effect of membrane gradients on luminescence (also called delayed fluorescence or delayed light emission) membrane energization is known to cause "quenching" of prompt fluorescence [7, 8]. The question arises whether this "energy-dependent quenching" also affects delayed fluorescence. This may be expected, if, as proposed by Lavorel [9], luminescence is controlled by a fluorescence yield factor. Indeed, there may be not much difference between an exciton arriving at PS II reaction centers after quantum absorption and an exciton originating at PS II reaction centers by recombination. However, as has been discussed by Malkin and Barber [10], there may be a high probability for a luminescence exciton to be trapped by the same reaction center from which it originated. In prompt fluorescence, the initial fluorescence, F_o, is known to be affected in a somewhat different way by energy-quenching than variable fluorescence [11]. As has been pointed out by Lavorel *et al.* [4] it is still not clear to what extent luminescence yield is influenced by fluorescence yield, and whether the initial, variable or maximal fluorescence yield is the main modulating factor.

Recently, methods have been developed to distinguish between photochemical and non-photochemical types of fluorescence quenching [8, 12–15]. We have designed a modulation fluorometer which measures fluorescence yield with a weak measuring beam and which tolerates continuous or pulsed light

of high intensity to induce fluorescence changes and to transiently cause full reduction of PS II acceptors (saturation pulse method) [15]. The fluorescence quenching observed during a saturation pulse with respect to the yield of a dark-adapted sample can be taken as a measure of non-photochemical quenching. In the present contribution, we report on experiments, combining the modulation technique for measuring prompt fluorescence with the phosphoroscope method for measuring ms-luminescence and applying saturation pulses for removing photochemical quenching. It will be shown that there is close similarity between the induction kinetics of saturated fluorescence and luminescence observed upon illumination and repetitive application of saturation pulses, suggesting that fluorescence and luminescence are affected by the same type of non-photochemical quenching.

Materials and Methods

The experiments were carried out with fully expanded leaves of *Arbutus unedo* L., grown as potted plants in the Würzburg Institute's Botanical Garden. Detached leaves were dark adapted for at least 2 h before the experiments. During measurements leaves were enclosed in a dark cuvette equipped with multibranched fiber optics. If not otherwise stated, they were exposed to a stream of water-saturated air at room temperature (approx. 20 °C).

Fluorescence was measured with a modulation fluorometer (PAM Chlorophyll Fluorometer, H. Walz, Effeltrich, Germany) as previously described [15]. Luminescence was measured with a Becquerel type phosphoroscope (laboratory built), based on a chopper-fiberoptic design. Chopper frequency was 800 Hz and within each cycle the illumination pulse lasted 600 µs and luminescence was integrated from 250 µs to 550 µs following the light pulse. The fiberoptics of fluorometer and luminometer were linked by suitable adaptors for combining the two measuring systems. Fluorescence and luminescence were measured under identical conditions at two equivalent spots of the same leaf, but not simultaneously, as the pulsed fluorescence measuring light caused excessive disturbance of the luminescence signal. Actinic light and saturating light were obtained from the same halogen light source (Xenophot XLX 64634, Osram) which was connected *via* relais switches alternatingly to two different power supplies. The relais switches were operated by the trigger control unit of the PAM Fluorometer (PAM 103 module). The light passed an electromagnetic shutter (Compur electronic-m) and was focused on one branch of the phosphoroscope fiberoptics. Light intensity during a saturation pulse was about 400 W/m^2 at the sample. Pulse duration was about 1 s. Heat filtered white light was used. The intensity of the modulated measuring beam was about 10 mW/m^2 at the sample. The signals were recorded on a digital storage oscilloscope (Nicolet Explorer III) or a chart recorder (Siemens Kompensograph).

Results and Discussion

Fig. 1 shows the induction kinetics of fluorescence and ms-luminescence measured under identical conditions. A dark-adapted leaf of *Arbutus unedo* was illuminated with 20 W/m^2 heat-filtered white light. The induction kinetics of fluorescence and luminescence displayed similarities, particularly during the first two seconds and during the slow decline phase starting at about 20 s following onset of illumination. On the other hand, it may be noticed that the fluo-

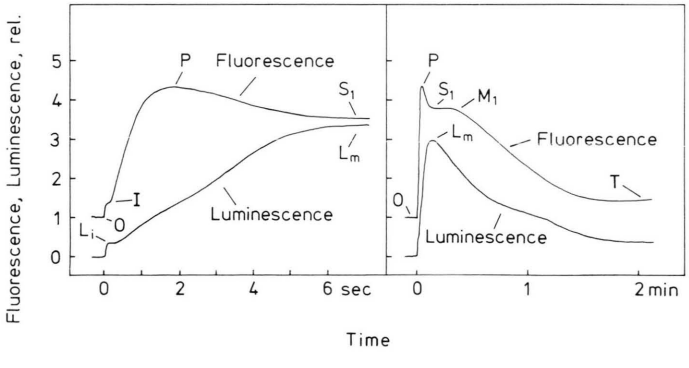

Fig. 1. Simultaneous recordings of chlorophyll fluorescence and luminescence induction kinetics. Rapid and slow transients are shown at two different time scales. Characteristic levels of fluorescence and luminescence are indicated. At time 0 dark-adapted leaves of *Arbutus unedo* were illuminated with 20 W/m^2 actinic light. See Materials and Methods for further details.

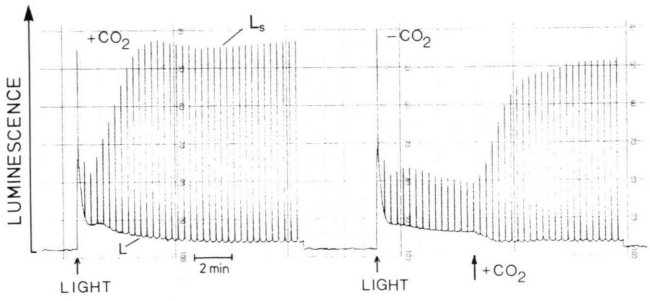

Fig. 2. Saturation pulse induction curves of chlorophyll luminescence in *Arbutus unedo*. Illumination by 50 W/m^2 continuous light was superimposed by 1 s pulses of 400 W/m^2 applied every 20 s. Induction curves in presence and absence of CO_2 are shown. Where indicated, a stream of CO_2-free air was exchanged against a stream of air containing CO_2. Luminescence intensity observed during saturation pulses (L_s) follows a time course which differs considerably from the luminescence curve in continuous light (L).

rescence peak (P) did not coincide with the main peak of luminescence. L_m, which occurred considerably later, when fluorescence had already decayed to the S_1-level. Detailed interpretation of the various parallel and antiparallel transients of fluorescence and luminescence is complicated by the large number of parameters determining fluorescence and, even more so, luminescence yield. However, it is well known that uncouplers eliminate the main luminescence rise starting at the L_i-level [6]. This was confirmed for intact leaves, using NH_4Cl as uncoupler ([16], Bilger and Schreiber, in preparation). Therefore in first approximation, the rise to the peak level (L_m) may be considered to reflect establishment of the proton gradient by light driven electron transport. Interpretation of the following decay is less clear. In fluorescence, the occurrence of the M_1-peak points to a retardation of electron flow, before Calvin cycle is activated, and with a delay of about 10–15 s to the start of illumination, energy dependent fluorescence quenching develops [15]. In the following experiments we will show that also luminescence is affected by this type of quenching.

We have previously reported on so-called "saturation pulse induction curves" of fluorescence [15] and demonstrated that these curves reflect the development of energy-dependent fluorescence quenching upon illumination and its relaxation when Calvin cycle sets in, ATP is consumed and membrane energization is lowered. In analogy, in Fig. 2 "saturation pulse induction curves" of luminescence are shown, which were measured under conditions similar to those of the fluorescence experiment in Fig. 6 of ref. [15]. During actinic illumination, the light intensity was raised every 20 s to a near saturating level of 400 W/m^2 in pulses of 1 s duration. As with fluorescence, the saturation level of luminescence was first lowered and then, after about 1 min, it slowly relaxed again and this relaxation required Calvin cycle activity, as it only occurred when CO_2 was present. There were substantial differences between the conventional luminescence induction curves (L-curves) and the induction curve following the envelope of the saturation levels (L_s-curves). With the onset of CO_2-fixation L decreased while L_s increased. In Fig. 3 L_s-curves are depicted for different intensities of the continuous actinic light. As with fluorescence [15], relaxation of L_s was most pronounced at moderate light intensities, *i.e.* when ATP-consumption and photophosphorylation are most effective in decreasing the light-driven gradients.

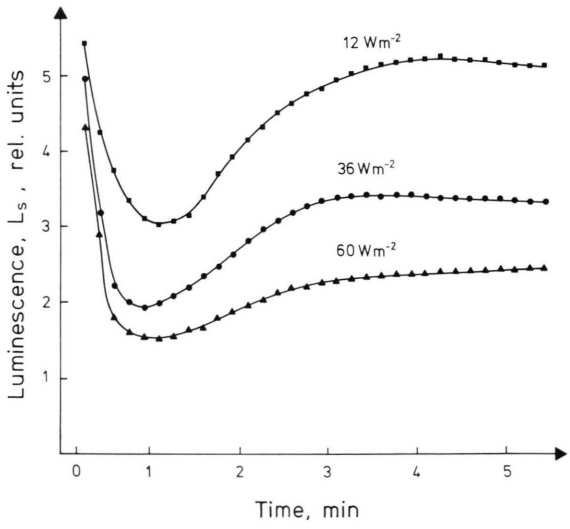

Fig. 3. Saturation pulse luminescence induction curves at different intensities of continuous illumination. Dark adapted leaves of *Arbutus unedo* were illuminated at the indicated intensities and luminescence during repetitively applied 400 W/m^2 saturation pulses (L_s) was recorded.

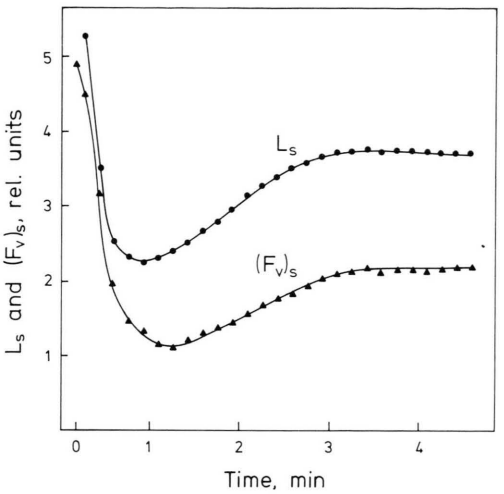

Fig. 4. Comparison of saturation pulse induction kinetics of fluorescence and luminescence. Variable fluorescence yield, $(F_v)_s$, and luminescence, L_s, during repetitively applied saturation pulses were recorded. Conditions as for Fig. 3 at an actinic light intensity of 36 W/m^2.

In Fig. 4 saturation pulse luminescence, L_s, and saturation pulse fluorescence, $(F_v)_s$, are directly compared for induction at 36 W/m^2. Luminescence and fluorescence were measured under identical conditions by the system described in Materials and Methods. There was close similarity between the two types of saturation pulse induction curves, suggesting that luminescence and fluorescence yield become lowered with respect to their maximal level by the same quenching mechanism. Detailed information from fluorescence work [7, 8, 15, 17] suggests that in the experiments of Fig. 2–4 the lowering of maximum fluorescence yield during a saturation pulse is related primarily to intrathylakoid proton accumulation.

In principle, other types of non-photochemical fluorescence quenching do exist, as *e.g.* caused by a state I → state II transition or by photoinhibition (see *e.g.* ref. [17]). However, the contribution of these quenching mechanisms appears negligable under the given experimental conditions. Therefore, it may be suggested that ms-luminescence and fluorescence are equally affected by "energy-dependent quenching".

Hence, the same parameter, *i.e.* membrane energization, which stimulates ms-luminescence yield by lowering the activation energy for recombination, also suppresses luminescence yield by non-photochemical quenching. The reason, why there is still a pronounced energy-dependent ms-luminescence peak upon onset of illumination, may be seen in the fact that energy-dependent quenching is delayed by about 10–15 s (depending on species and growth conditions) with respect to the initiation of membrane potential formation and proton pumping [15, 16, 18].

These results suggest that the variation of fluorescence yield has to be taken into consideration, when luminescence signals are to be evaluated in terms of the rate of charge-recombination at photosystem II reaction centers, as proposed earlier by Malkin and Barber [10]. The question arises whether in addition to non-photochemical quenching also photochemical quenching has to be considered. This depends on the extent of exciton delocalization between PS II units. Although a recombination exciton originates in combination with an open reaction center, at room temperature there is a certain probability of transfer to neighboring units [19, 20]. In this way, the overall reduction state of PS II acceptors could influence luminescence yield after exciton formation, with a higher reduction level favoring luminescence yield. On the other hand, it should be noted that only centers with oxidized acceptors can experience charge separation and recombination yielding exciton formation. Also the aspect of PS II heterogeneity has to be taken into consideration: There is a population of PS II centers with separate pigment units (PS II β) [21–23] which may contribute significantly to luminescence emission, as has been pointed out by van Gorkom [24]. Further work will be required to establish the role of photochemical quenching in modulating luminescence yield.

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