Ultraweak photoemission from dark-adapted leaves and isolated chloroplasts

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Received 13 September 1990

Dark-adapted isolated spinach chloroplasts and leaves, unlike sub-chloroplast fractions, are capable of emitting ultraweak light spontaneously (50–125 counts/s per cm²). The emission of leaves is due to two processes with activation energies of 97 and 25 kJ/mol while in isolated chloroplasts, it is the result of a single process (98 kJ/mol), as indicated by the Arrhenius plots of the intensity. Emission spectra demonstrate that the terminal step of these reactions is the excitation of chlorophyll in both samples. We suggest that the additional component in the ultraweak light emission of leaves may be related to mitochondria

Ultraweak light emission; Spinach leaf; Chloroplast; Emission spectrum, Arrhenius plot

1. INTRODUCTION

Extremely weak, spontaneous light is emitted by plant cells previously adapted to darkness for a prolonged period of time as a result of oxidative reactions (for review see [1]). This phenomenon is also known as biophoton emission [2,3], biological chemiluminescence [4,5] or ultraweak photon emission [2]. We have recently reported that dark-adapted spinach chloroplasts emit predominantly red ($\lambda < 650$ nm) ultraweak light even several hours after excitation, as a result of a series of oxygen-dependent reactions possibly related to chlororespiration [6]. Low level photoemission has also been observed in green tissues such as, sprouts [7], *Chlorella* [8] and *Hibiscus* leaves [9].

In order to investigate whether ultraweak light emission from leaves originates in chloroplasts only or whether other sources also contribute, we compared light emission from dark-adapted sub-chloroplast fragments, chloroplasts and green leaves.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Chloroplasts were isolated from market spinach according to Takahashi and Asada [10] and purified by sucrose gradient centrifugation [11]. Purified chloroplasts were washed and diluted (0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM $MnCl_2$, and 50 mM tricine-KOH, pH 7.2) to approximately 2000 μg Chl/ml. Chlorophyll content was determined spectroscopically according to the method of Arnon [12]. This suspension was either diluted further for photon counting experiments or used as starting material for thylakoid membrane and sub-chloroplast fragment preparations. Thylakoid membranes were prepared by washing osmotically shocked chloroplasts [13].

Photosystem II and photosystem I sub-chloroplast fragments were isolated according to [14,15], respectively. After isolation the preparations were resuspended and washed in the above buffer twice. Photosystem II preparations (Chl-*a*/Chl-*b* ≈ 1.8–1.9, P700:Chl ≈ 1:1800–1900) evolved 300–350 μM O_2/mg Chl/h with 250 μM dimethylbenzoquinone. Photosystem I preparations (Chl-*a*/Chl-*b* ≈ 5.1–5.3, P700:Chl ≈ 1:220–240) showed 140–160 μM/mg Chl/h O_2 uptake in the presence of 0.1 mM 2,6-dichlorophenol indophenol and 50 μM methyl viologen.

The rate of oxygen evolution or consumption was measured using samples with 50 μg/ml Chl at 25°C with a Clark-type oxygen electrode (OBH-100, Otsuka Electronics, Japan) as described in [13]. Chlorophyll:P700 ratio was determined from samples containing 25 μg/ml Chl by measuring the oxidized minus reduced difference spectrum of P700 [13] with a spectrophotometer (Hitachi U-3400, Japan).

2.2. *Photon counting measurements*

Light emission was detected with a high sensitivity photon counting system [16] utilizing an R1333 photomultiplier tube with 20 cm² photocathode (Hamamatsu Photonics, Japan), in a cooled housing, protected by a motor-driven shutter. Light emission from suspensions diluted to 100 μg/ml was measured in a metal dish (20 cm²) positioned under the photomultiplier tube in a sample-holder which was kept at the indicated temperature by circulating temperature-controlled water. Leaf disks (8 cm²) were cut from market spinach and placed on a wet, temperature controlled plate. In order to avoid possible artifacts due to tissue wounding, the cut edges were covered with aluminum foil.

Emission spectra of the ultraweak light were detected by an R1333 photomultiplier (Hamamatsu Photonics, Japan) based filter spectral analyzer utilizing colored filters (Toshiba Co., Japan) described by Inaba et al. [17]. Leaf disks and isolated chloroplasts (100 μg/ml) were measured in a 1.6 cm diamter glass tube at 20°C. All samples were kept in the dark in the measuring chamber for 1 h before measurement in order to avoid any contribution of delayed fluorescence. Photon counting data were collected with a personal

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

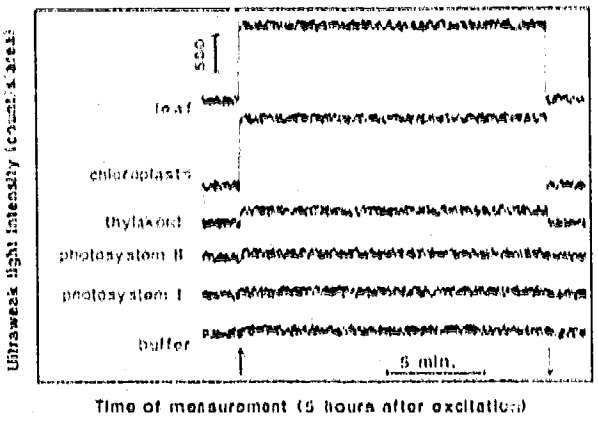


Fig. 1. Ultraweak light emission detected at 21°C from spinach leaf, isolated chloroplasts, thylakoid membrane, photosystem II and photosystem I sub-chloroplast fragments, stored in the dark for one hour before measurement. All suspensions contained 100 μg/ml chlorophyll. The lowest trace represents light emission from the suspending buffer. The area of observation was 8 cm^2 for leaves and 20 cm^2 for all other samples. The arrows indicate the opening and closing of the shutter located between the sample and the photomultiplier tube. Dark counts (shutter closed) were 72 ± 5 counts/s per 20 cm^2.

computer (NEC PC-9801, Japan) which was also used for calculations. The spectral analyses were carried out by computing the count rate for each spectral region defined by the subtraction of the two transmission curves of the corresponding colored glass filters as described earlier [17]. Spectra were corrected to the spectral response of the photomultiplier tube. The spectral resolution of the filter spectral analyzer system was about 25–35 nm.

3. RESULTS

As shown in Fig. 1, leaves and isolated chloroplasts are capable of emitting ultraweak light after one hour of dark adaptation, even by the time long lived fluorescence components (data not shown) have decreased. This dark photoemission is stable for several hours when it starts decreasing, possibly due to sample degradation. Photosystem I- or photosystem II-enriched subchloroplast fragments, although still having 60–70% of their initial electron transport activity (data not shown), feature no light emission after one hour in the dark. The remarkable lower intensity of light from broken thylakoid membranes, as compared to chloroplasts, indicates that this process requires not only the presence of both photosystems but also chloroplast intactness.

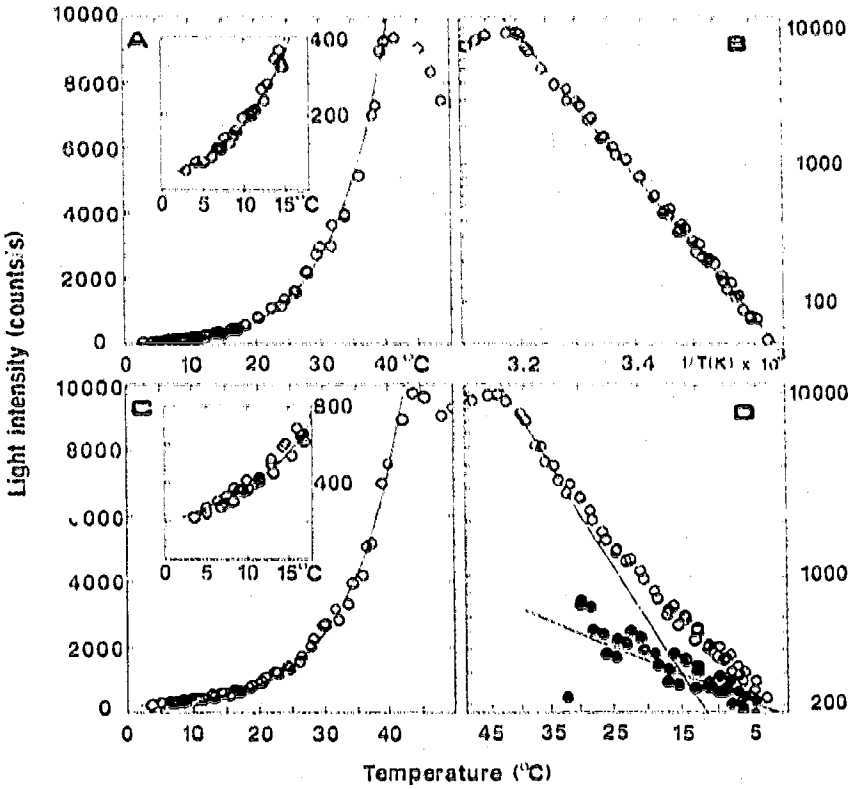
For an energy comparison of the processes leading to ultraweak light emission in leaves and in isolated chloroplasts we measured the temperature dependence fo the emission intensity in both samples. As shown in Fig. 2A, the emission intensity from chloroplasts increases with increasing temperature and starts decreasing around 40°C, possibly due to degradation. An Arrhenius plot of this temperature dependence (Fig. 2B), indicates the presence of one component with an activation energy about 98 kJ/mol. Leaves feature a similar increasing temperature dependence function (Fig. 2C), but, as shown in the Arrhenius plot (Fig. 2D), this is a result of two components. Besides a process with the same activation energy as in the case of chloroplasts (97 kJ/mol) there is another reaction with approximately 25 kJ/mol.

In Fig. 3 emission spectra of ultraweak light emission from leaves (Fig. 3A) and isolated chloroplasts (Fig. 3B), are compared. In order to avoid the effect of absorbance differences between the two samples, chloroplasts were measured at 100 μg/ml chlorophyll concentration, which is higher than the saturating concentration for ultraweak light emission (É. Hideg, unpublished observations). We found that, within the resolution of the measurement, the two spectra closely resemble each other. The emission predominates in the red region with an important contribution of a band around 720–730 nm. There is practically no emission below 650 nm and emission features above 800 nm are also questionable because of the low sensitivity of the photomultiplier tube.

4. DISCUSSION

Leaves and isolated spinach chloroplasts show a spontaneous low level light emission which is not due to delayed fluorescence since the samples were kept in darkness long before the experimental period. Light emission from both chloroplasts and leaves is highly dependent on temperature. Temperature dependence functions of leaf fluorescence reportedly show a characteristic maximum in the 8–18°C region, corresponding to a discontinuity in the Arrhenius plot, which identifies changes in the membrane fluidity [18,19]. The lack of equivalent features in the temperature dependence of ultraweak light emission indicates that the slope of these Arrhenius plots refers to activation energies of two different processes rather than to the effect of phase transition. With this concept, the Arrhenius plot of the intensity identifies two reactions in leaves with activation energies of 25 and 97 kJ/mol, the latter corresponding to the activation energy of the emission process in chloroplasts (98 kJ/mol). This suggests that one component is due to chloroplasts in the leaf tissue. We have suggested earlier [6], that the reactions resulting in ultraweak light emission of chloroplasts are, at some level, associated with a hypothesized slow back flow of electrons from NADPH to oxygen via, the plastoquinone pool: chlororespiration [20,21]. Supporting this assumption, the estimated 98 kJ/mol activation energy of ultraweak light emission is similar to the previously reported activation energies of other thylakoid membrane related electron-transport backreactions (80–90 kJ/mol, in [22]).

Many authors attribute low level chemiluminescence of plant material to an oxidative damage of unsaturated



. 2. Temperature dependence and Arrhenius plots of ultraweak light emission from dark adapted spinach chloroplasts (A and B, respectively) 0 μg/ml Chl) and from leaves (C and D, respectively). The area of observation was 20 cm^2 for chloroplasts and 8 cm^2 for leaves. Circles = data ints; solid lines = least squares fits of the data as one (B) and two (D) exponential components, and the calculated temperature dependence (A) l (C). Full circles in (D) = data points after the subtraction of one component. The insets of (A) and (C) show the 0–15°C range on a magnified *y*-scale.

ological lipids [5,9,23]. Boveris et al. [23] have ported two reactions with 20 kJ/mol and 68 kJ/mol tivation energies, corresponding to the enzymatic poxygenase) and non-enzymatic oxidation, respec-ely, of unsaturated fatty acids in seeds. Comparing e energies of activation, it seems to be possible that the lower energy (25 kJ/mol) process we observed in leaves, also corresponds to enzymatic lipid peroxidation yielding singlet oxygen or carbonyl radicals [5]. However, the emission spectra do not confirm this assumption. The dimol emission of singlet oxygen occurs with main bands of equal intensity at 634 and 702

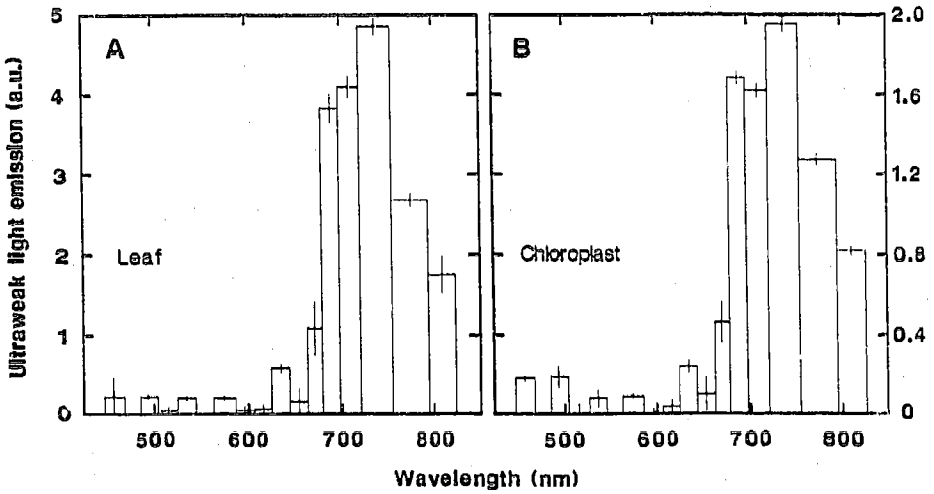


Fig. 3. Emission spectra of ultraweak light from leaves (A) and isolated chloroplasts (B), 100 μg/ml.

nm with little, 1–2% of the total, light at 668 nm [24,25]. Triplet carbonyls can be identified by their characteristic emission around 420 nm [26]. These bands are broadened and may be shifted by about 10 nm in a complex medium [27]. However, we found that ultraweak emission spectra of both leaves and chloroplasts are dominated by a broad emission feature around 720 nm, with practically no emission below 650 nm. Although, within the resolution of our spectra, the possibility of a small (approximately 5% of the total emission) contribution of singlet oxygen can not be excluded, the bulk of the emission is due to chlorophyll which has also been suggested as the emitter of low level luminescence in *Chlorella* [8] and in germinating plants [7]. The close similarity between the emission spectra of leaves and chloroplasts indicates that even the additional (25 kJ/mol) emission process of leaves involves energy transfer to chlorophyll.

Bean mitochondria have also been shown to produce low level chemiluminescence [28]. In our experiment, mitochondria in the spinach leaf might contribute to ultraweak light emission as a result of an enzymatic or electron-transport process. Excited states generated in this process might then transfer energy to chlorophyll molecules, which have been found to be efficient energy harvesters, even as externally added labels in chemiluminescent systems [29]. Supporrting this assumption, the activation energies of some mitochondrial enzymes associated with the oxidation of NAD(P)H and succinate have been reported to be 23–35 kJ/mol in mammalian and plant mitochondria [30].

In summary, we suggest that the ultraweak light emission of dark-adapted leaves is mainly (at least 95%) emitted by chlorophyll molecules, whose excitation is the result of two different reactions. One (with an activation energy of 98 kJ/mol) originates in chloroplasts and the other (25 kJ/mol) process might be relevant to enzymatic or electron transport activity of mitochondria. A further study of the effect of inhibitors and activators of the two reactions could determine, in detail, the contribution of each process to ultraweak light emission from leaves.

Acknowledgement: The Research Development Corporation of Japan (JRDC) is a statutory entity of the Japanese Government administered by the Science and Technology Agency. The Biophoton Project is a research activity of the JRDC's Exploratory Research for Advanced Technology Program (ERATO).

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