STRUCTURE DEPENDENT CONTROL OF CHLOROPHYLL *a* EXCITATION DENSITY: THE ROLE OF OXYGEN

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1. Introduction

Recent investigations have shown that processes associated with the generation of the ATP forming electrochemical potential X_E exert a control on the density of Chl *a* excitation in photosystem II (for reviews, see [1, 2]). In particular, two processes appear to be involved: i) The light-dependent protonation of the thylakoid interior, which is synchronous with a quenching of Chl *a* fluorescence [3–5]. ii) The light-dependent extrusion of metal cations from the thylakoid to the stroma, which also reduces the yield of Chl *a* fluorescence. The last process is born out by the experiments of Homann [6] and of Murata [7–9], who demonstrated an increase of the Chl *a* fluorescence by adding salts to DCMU-poisoned suspensions of broken chloroplasts, simulating thus a reversal of the cation extrusion event.

Two different mechanisms have been postulated to account for the cation control of the excited population of Chl *a* in DCMU poisoned chloroplasts. First, the quenching which is related to the influx of protons was ascribed to an acceleration of the thermal degradation of the Chl *a* excitation [3, 4]. This implies the participation of quenchers other than the protons, since these cannot function as recipients of electronic excitation. Second, the increase of Chl *a* fluorescence on the addition of salts to suspensions of chloroplasts was assigned to a cation dependent control of the spillover of excess excitation. According to this, cations inhibit the excitation spillover from the fluorescent Chl *a* of photosystem II to the non-fluorescent Chl *a* of photosystem I [7–9].

Starting from the premise that the conformation of the thylakoid membrane may regulate the diffusion of fluorescence quenchers, such as molecular oxygen, to the pigment bed, we designed experiments to examine whether oxygen is required for the expression of the cation dependent fluorescence changes. Our experiments with normal and deoxygenated chloroplasts suspensions suggest that, indeed, the cation control of the Chl *a* excitation density operates through the quenching effect of molecular oxygen.

Abbreviations:
Chl : Chlorophyll.
DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea.
PMS : Phenazine methosulfate.
DAD : *p*-diaminodurence.

2. Methods

Chloroplasts were prepared from fresh market spinach according to Murata [7]. In addition to the chloroplasts, the final suspension contained 50 mM tricine-NaOH pH 7.8, 400 mM sucrose, 10 mM NaCl, and 10 μM DCMU. The absorbance at 678 nm was 0.40 (2.0 μg chlorophyll/ml) in the experiments with $MgCl_2$ and 0.20 (1.1 μg chlorophyll/ml) in those with PMS. In the latter, 0.5 mM ascorbate was also added to the chloroplast suspension.

Degassing of the chloroplast suspensions was carried out by the vacuum freeze–thaw technique in small pyrex glass tubes fitted with 2 mm bore stop-

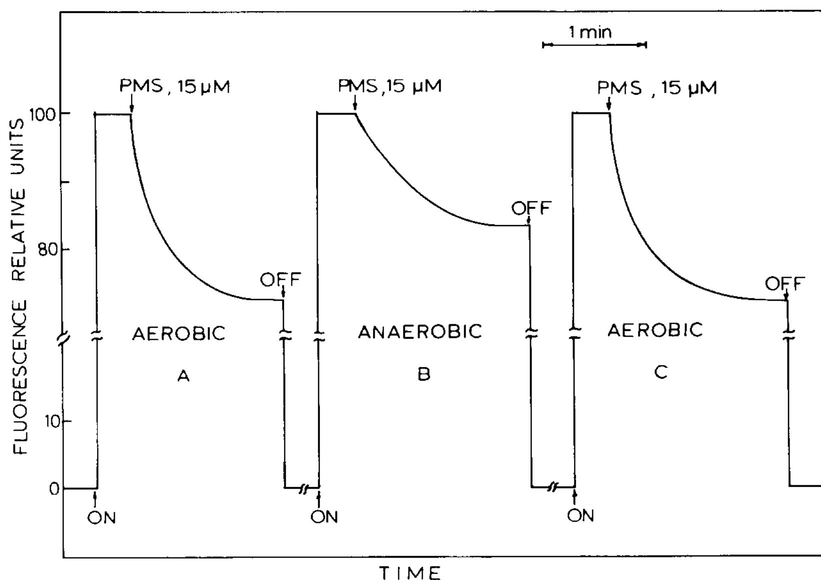

Fig. 1. PMS-induced lowering of the Chl *a* fluorescence in DCMU-poisoned spinach chloroplasts. Arrows indicate excitation light on, and off, and the time of the addition of PMS (15 µM). Noise due to the mixing of PMS is not shown.

cocks. After two freeze–thaw–pump cylces, the suspensions contained less than 10% of the original oxygen, as measured by a Gilson Medical Electronics oxygraph. Samples were designated as anaerobic, only when no more gas bubbles appeared during the pumping stage. Samples designated as aerobic, were subjected to the same freeze–thaw treatment as the anaerobic samples, but the evacuation was omitted. The pyrex glass tubes served as sample holders in the spectrofluorometer.

Fluorescence was excited and detected with an assembled spectrofluorometer described elsewhere [10]. For the experiments with PMS, excitation at 480 nm was isolated from the emission of a 150 W Xe source with a Bausch and Lomb High Intensity monochromator. The excitation intensity was saturating, as judged by the insignificant fluorescence increment produced on adding DCMU to an unpoisoned sample. For the experiments with $MgCl_2$, weak 480 nm excitation was isolated from an incandescent Unitron Lamp by means of a Baird-Atomic interference filter. A Corning C.S. 2-58 cut-off filter protected the analyzing monochromator from stray excitation. Chl *a* fluorescence was observed at 680 nm with a band of 4–6 nm half-width. Additions of PMS and of $MgCl_2$ were made with a microliter syringe and the samples were mixed by shaking.

All samples were at room temp. (ca. 25°) during the fluorescence measurements.

3. Results

Fig. 1 shows typical time courses of the PMS-induced lowering of the Chl *a* fluorescence in normal and deoxygenated samples. Noise due to the mixing of the PMS solution has been omitted from these curves. The final concentration of PMS (15 µM) was sufficient for a maximal lowering of fluorescence. Curves A and B correspond to a normal (aerobic) and to a degassed (anaerobic) sample, respectively, while curve C is from a deoxygenated sample, which was allowed to reequilibrate with air by standing for 2 hr at 0°.

Fig. 2 shows the kinetics of the Mg^{2+}-induced rise of the Chl *a* fluorescence in normal and deoxygenated chloroplasts. The artifact of mixing is not reproduced. Sufficient $MgCl_2$ (final conc. 12 mM) was added for a saturating effect and very weak excitation was used to monitor the fluorescence. The last condition is

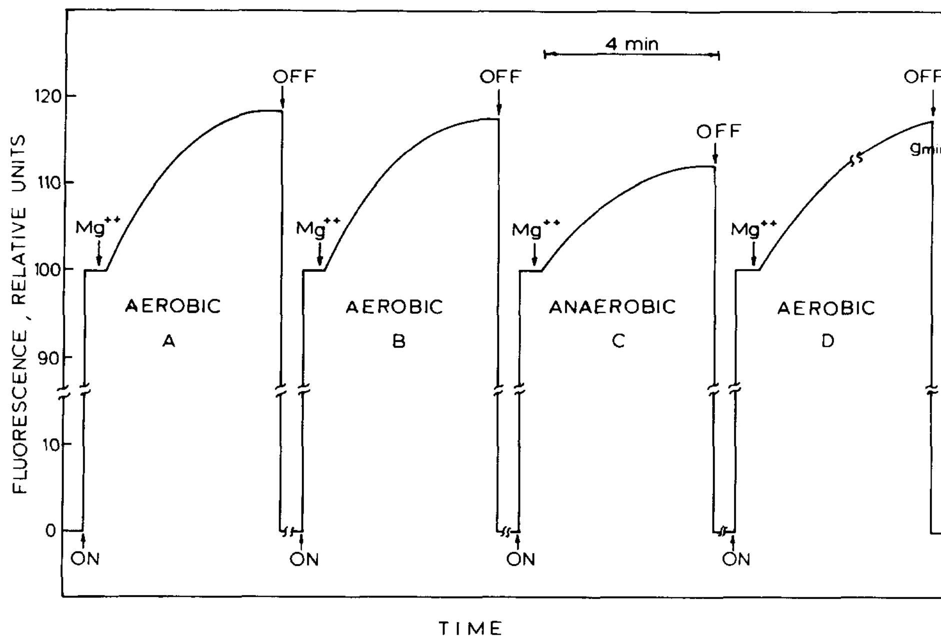

Fig. 2. $MgCl_2$-induced increase of the Chl *a* fluorescence in DCMU-poisoned spinach chloroplasts. Arrows indicate excitation light on, and off, and the time of the addition of $MgCl_2$ (12 mM). Noise due to the mixing of $MgCl_2$ is not shown.

necessary for a maximal fluorescence increment, since the light-induced high energy state has the tendency to expel metal cations from the interior of the thylakoid [11]. Contrary to the PMS-induced lowering, the Mg^{2+}-induced fluorescence rise is not a photochemical process, as it develops also in the darkness. Curves A and B of fig. 2 correspond to aerobic samples, with the latter having experienced the freeze–thaw treatment of the anearobic sample (curve C), but without the air pumping. Curve D has been derived from an anaerobic sample reequilibrated with the air by standing at 0° for 2 hr.

These results illustrate characteristically that:
i) The freeze–thaw treatment does not impair the Chl *a* fluorescence response to PMS and $MgCl_2$.
ii) The PMS and the Mg^{2+}-induced fluorescence differentials are significantly smaller in the anaerobic chloroplasts. iii) The effect of anaerobiosis is reversible. On reequilibration with the atmosphere the full response to these additives is reinstated.

4. Discussion

Murata and Sugahara [3] attributed the Chl *a* fluorescence lowering induced by PMS, a cofactor of the cyclic electron transport, to an X_E-linked enhancement of the thermal degradation of the Chl *a* excitation. Wraight and Crofts [5], driving the cyclic electron transport by means of the DCMU–DAD system, demonstrated the dependence of the Chl *a* fluorescence quenching on the increased proton concentration inside the thylakoid. Sherman and Cohen [5] confirmed this result. On the other hand, Homann [6] ascribed the Chl *a* fluorescence rise, induced by metal cations to changes in the fine structure (conformation) of the lamella, and Murata [7–9] suggested a structure dependent control of the excess excitation spillover from the fluorescent photosystem II to the non-fluorescent photosystem I.

Oxygen can quench dynamically low energy singlet excitation in a non-destructive manner [12, 13]. Our results suggest that cation dependent changes in the conformation of the thylakoid membrane modify the accessibility of the pigment bed to this

quencher. At the high energy state (excess of protons inside the thylakoid), the conformation of the membrane elements is such as to facilitate access of molecular oxygen to the pigment bed. The reverse situation (excess of metal cations inside the thylakoid), on the other hand, favors a structural protection of the pigment bed from molecular oxygen, leading to a rise of the fluorescence yield. We have shown previously [10], that cations regulate the accessibility of the chlorophylls *in situ* to the fluorescence quencher nitrobenzene. Fluorescence quenching by molecular oxygen has been proposed as a tertiary structure probing technique for fluorochrome labeled proteins [14].

Structurally controlled oxygen quenching of Chl *a* excitation may operate in parallel with other control mechanisms, such as the regulation of the spillover rate. This may explain our inability to suppress completely the PMS-induced quenching and the Mg^{2+}-induced rise. We cannot, however, discount the possibility that the partial suppression of these effects is due to the incomplete deoxygenation of the samples.

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