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Manipulation of the imbalance for linear electron flow activities between Photosystems I and II of photosynthesis by cyclic electron flow cofactors

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Imbalance for linear electron flow in light 2 (e.g. $\lambda < 690$ nm) in favor of PS II, may be regulated by a mechanism which changes the ratio between competing linear and cyclic electron flows. In order to examine this possibility, artificial PS I-cyclic electron flow cofactors were added to broken chloroplasts, which performed linear whole electron transport in light 2 excitation with methylviologen as an electron acceptor. A significant increase of the apparent photosystems imbalance for linear electron transport activities in favor of PS II, monitored by modulated fluorimetry, occurred upon addition of oxidized or reduced (with excess ascorbate) forms of PMS and TMPD as well as with the reduced form of DAD. It is suggested that these effects arise from a competition with PS II on electron donation to PS I, and establishment of a steady-state where cyclic electron flow competes with the linear one. Most convincing was the observation of a similar increase of this imbalance in favor of PS II, reproduced in leaf discs treated with PMS, performing in this case a more direct measurement of oxygen evolution by photoacoustic spectroscopy. The wavelength dependence of the imbalance for linear electron transport activities was checked in chloroplasts for the case of PMS, and had maxima at wavelengths where absorption of chlorophyll *b* predominates. These observations suggest that the final photoactivity distribution between the two photosystems for the linear electron flow (affected by the cofactor) depends on the basal photosystems imbalance of quanta absorbed in each photosystem due to the pigment distribution pattern between PS I and PS II, as influenced by the cationic level and the abolition of membrane energization. The relevance to a possible regulation mechanism in-vivo, which controls the ratio between cyclic to non-cyclic electron flows, is discussed.

Introduction

It has been demonstrated in whole chloroplasts that the imbalance in the photosystems activities for linear electron transfer in favor of Photosystem (PS) II (as expressed by Emerson enhancement) depends on the type of the final electron acceptor [1–3]. In this dependence, the imbalance increased when the ATP/e requirement for the reduction of the final acceptor was higher. This result could be interpreted by assuming that due to an extra demand for ATP, a cyclic electron path around PS I is activated to produce more ATP.

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DMBIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; F_0, F_s and F_m, minimum, steady-state and maximum fluorescence respectively; G.D., gramicidin-D; Hepes, *N*-2-hydroxyethylpiperazine *N'*-2 ethanelsulfonic acid; LHC, light harvesting chlorophyll *a* / *b*-protein complex; MeV, methyl-iologen; PC, plastocyanin; PMS, *N*-methyl phenazonium methosulfate; PS, Photosystem; PQ, plastoquinone; TEC, tris(ethylenediamine) Cobalt(III) chloride; TMPD, *N,N,N',N'*-tetramethyl-phenylenediamine.

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The competition between cyclic and linear electron flows at a certain site(s) in the linear electron transfer chain between the two photosystems is expected to result in an apparent shift of the imbalance in linear electron flow to strongly favor PS II.

In a previous study [4] we have demonstrated a very strong dependence of the photosystems imbalance for linear electron flow activities in light 2 (e.g. $\lambda > 690$ nm) on cations, uncouplers and ionophores. To observe such an imbalance in favor of PS II the thylakoids must be stacked, i.e suspended in a high enough cation concentration (e.g. 5 mM $MgCl_2$). A particular high level of this imbalance is observed in the presence of uncouplers or ionophores (e.g. gramicidin D or valinomycin and KCl). One of the possibilities, which was considered to account for this effect, was that membrane energization controls the extent of an endogenous cyclic electron flow around PS I [4]; when membrane energization is abolished (i.e. by the addition of an uncoupler), the cyclic path may be accelerated and compete more favorably for more excitation in PS I, thus decreasing the efficiency of PS I towards electrons from PS II and giving rise to an apparent imbalance for the linear electron flow in favor of PS II. This idea, however, seemed unacceptable since the uncoupler's effect was strongly wavelength-dependent [4], indicating more favorably that the photosystems imbalance in photoactivities for linear electron transport is related to excitation distribution via specific pigment composition of each photosystem.

Nevertheless, to obtain more comprehensive knowledge, it is important to study the effect of the change in the ratio between cyclic and non-cyclic flows. This could be manipulated by the addition of different non-physiological cofactors of cyclic electron transport to isolated thylakoids, which also perform linear electron flow with PS I acceptors. We examined particularly the effect of *N*-methyl phenazonium methosulfate (PMS), a classic cofactor for cyclic electron flow around PS I [5], which donates electrons in its reduced form to P-700 [6,7], as well as the effects of other cofactors, which donate electrons to PS I (either through the cytochrome b_6f complex and PC or directly via P-700 such as 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) [8] and *N,N,N',N'*-tetramethylphenylenediamine (TMPD) [7,9]. Under light-limiting conditions, the addition of PMS and TMPD in their oxidized state indeed resulted in a strong shift towards imbalance favoring PS II. A similar phenomenon was observed with PMS, DAD and TMPD in their reduced state (kept by excess ascorbate). Furthermore, a similar increase of the imbalance for linear electron flow activities in favor of PS II was reproduced in O_2 evolution measurements when PMS was introduced to leaf discs. The effect of cyclic electron flow cofactors on the photosystems imbalance in relation to linear electron transport activities in broken chloroplasts was even more dramatic in the presence of gramicidin-D. Resembling the effect of uncouplers per-se, the PMS effect was strongly wavelength-dependent, in contrast to expectations, indicating involvement of the pigment distribution pattern.

Materials and Methods

Materials

HEPES, gramicidin-D, valinomycin, nigericin, methylviologen and PMS were purchased from Sigma. DCPIP was purchased from Fluka. TEC was purchased from Alfa Products. DAD was given as a gift from Prof. Achim Trebst, Ruhr University, Bochum, Germany. TMPD was purchased from BDH Chemicals. Stock solutions of TMPD, DCPIP, PMS and Na-ascorbate (aqueous), DAD (in ethanol : water, 1 : 1), gramicidin-D (in ethanol), DCMU (in methanol) were kept at −20°C. During experiments the stock solutions were kept on ice in the dark. The DAD solution was renewed frequently (about once a week).

Chloroplasts preparation

Broken chloroplasts from market lettuce were prepared and stored in liquid nitrogen [10]. The storage medium contained 0.3 M sorbitol, 20 mM Hepes, 10 mM NaCl, 5 mM $MgCl_2$ and 30% (v/v) ethyleneglycol, pH 7.3. Total chlorophyll concentration was determined spectroscopically [11].

Reaction mixtures

For fluorescence measurements, chloroplasts were diluted 500–1000-fold in a 2 ml cuvette, so that the concentration of chlorophyll did not exceed 10 μg/ml. The standard reaction mixture contained 20 mM Hepes, pH 7.3, 10 mM NaCl, 5 mM $MgCl_2$ and 200 μM MeV as an electron acceptor. In some experiments, $MgCl_2$ was omitted (except for a residual amount, < 10 μM). Alcohol concentration did not exceed 0.5% (v/v). Before each experiment, the chloroplasts were diluted into the reaction medium and kept in the dark for at least 2 min. All measurements were done at room temperature (22–25°C).

Chlorophyll-a fluorescence measurements

Modulated chlorophyll *a* fluorescence was measured by a home-built fluorimeter as described previously [4], which included several light sources for excitation. One source was used to produce modulated (120 Hz) exciting light 2 (isolated by an appropriate interference filter; 480 nm for most of the experiments) of an intensity within the light-limiting range of electron transport (about 1 nE cm^{-2} s^{-1}). The fluorescence signal from the sample (isolated by a 683 nm interference filter) was detected by a photodiode (EG

and G HUV-4000) and processed by a lock-in amplifier (PAR 128 A). The output corresponded to the amplitude of the modulated fluorescence only and was recorded on a strip chart recorder. Light from a second unmodulated source (background light) served to exert actinic effects. Two sorts of background light were used: (a) a strong blue broad-band light, isolated with a 4-96 corning glass (about 120 nE cm^{-2} s^{-1}), serving to saturate electron transport, thus causing modulated fluorescence to reach the maximum level, F_m; (b) far-red light (light 1), isolated with a sharp-banded interference filter peaked at 720 nm (about 30 nE cm^{-2} s^{-1}), serving to maximally open the reaction centers of PS II, thus resulting in an approach of the modulated fluorescence to the minimum, F_0. Without any background light, the fluorescence level F_s varied between these two limits. An increase in F_s and its approach to F_m indicates an increase in the imbalance of photochemical capacities of the two photosystems in favor of PS II.

Fluorescence induction measurements were made using the same modulated fluorescence set-up, but with the photodiode connected to a storage oscilloscope and illuminating the sample with the non-modulated saturating blue broad-band light only. The onset of illumination was controlled by an electronic shutter (Compur – opening time about 3 ms).

Calculation of photoactivity distribution between the photosystems in the modulated light, from modulated fluorimetry

The distribution of light 2 quanta to PS II, β, can be calculated according to (4), from the fluorescence parameters:

$$\beta = (F_m - F_0)/(2F_m - F_s - F_0)$$

However, this expression is valid provided that $\alpha + \beta = 1$ (where α is the corresponding light activity distribution coefficient of PS I for linear electron flow), namely, that there is no waste of excitation energy except for that utilized for linear electron transport between the two photosystems [12]. In the context of this article, we used a parameter termed imbalance for linear electron flow activities, equal to $(\beta/\alpha - 1)$ (abbreviated simply 'imbalance'), which can be calculated directly, independently of the above assumption:

$$(\beta/\alpha) - 1 = 1/f - 1 = (F_s - F_0)/(F_m - F_s)$$

where f is the fraction of open PS II reaction centers, deduced directly from the fluorescence parameters [4,12]. This expression is valid only as long as $F_s > F_0$. In the limit when F_s tends to equal F_0, this imbalance term tends to zero.

PMS treatment of Hibiscus sp. leaves

Hibiscus sp. leaves were cut from the upper part of a bush growing in the garden and kept in humid conditions. For all measurements, leaf discs (1 cm in diameter) were cut along the leaf vein, using a standard cork borer, and incubated either in water (control) or in an aqueous solutions of PMS at specified concentrations for 15 min at near 0 C in the dark. Incubation was chosen to be at low temperature and in the dark in order to prevent PMS photo-conversion to pyocyanin and to avoid the damage due to oxidized phenolic compounds. Control cold-incubated leaf discs exhibited similar O_2 evolution rates compared to control discs which were not preincubated, indicating that no damage occurred to photosynthetic electron transport during incubation. Following incubation, the leaf discs were rinsed dry, punched gently at several places on the upper surface with a hypodermic needle and placed inside the photoacoustic cell.

Photoacoustic measurements and calculations

Measurements of photosynthetic O_2 evolution and Emerson enhancement of *Hibiscus sp.* leaf discs were done by the photoacoustic method, as described elsewhere [13]. Modulated light was obtained from a 900 W xenon-lamp/monochromator combination (650 nm, about 2 nE cm^{-2} s^{-1}). Non-modulated background light was either a strong, photosynthetically saturating light (white, about 100 nE cm^{-2} s^{-1}) or far-red light (710 nm, about 20 nE cm^{-2} s^{-1}) which was saturating for its effect. By superposing the far-red light with the modulated 650 nm light upon the leaf disc surface for several minutes, a complete transition to state 1, where the photosystems imbalance for linear electron flow activities in light 2 excitation is maximum, was ensured [14]. When the O_2 signal reached a maximum, the 710 nm light was turned off. All measurements of oxygen evolution and Emerson enhancement were executed at a constant low modulation frequency (20 Hz). In the measurements, the phase of the lock-in amplifier was set such that the quadrature channel contained only the modulated O_2 evolution signal, while both modulated photothermal and oxygen signals were present in the in-phase channel. This was set by ensuring that in the presence of photosynthetically saturating light, the quadrature channel had a zero signal. At the same time, the in-phase channel had a maximum photothermal signal (and zero oxygen). Modulated oxygen evolution was calculated from the signal in the quadrature channel when no saturating light was present. As an expression for the yield of modulated O_2 evolution, we used the ratio O/T where O is the O_2 evolution signal in the quadrature channel and T is the maximum photothermal signal in the in-phase channel. Emerson enhancement in O_2 evolution (E) was calculated as the ratio between the signal O in the presence of 710 nm

background light to that when the 710 nm was withdrawn:

$I = (O_1 \ldots \ldots O_1 \ldots \ldots$

Results

Performing modulated fluorescence measurements, we examined the effect of PMS and other cofactors of cyclic electron transport on the imbalance between the two photosystems in favor of PS II, for linear electron flow activities (abbreviated simply as 'imbalance'). During these measurements, linear electron flow through the two photosystems was maintained with MeV as an electron acceptor. Our initial assumption was that such cofactors will introduce a competition between two (cyclic and linear) electron flows, resulting in an apparent increase in the imbalance for light 2 in favor of PS II. Indeed, addition of PMS (5 μM) induced an appreciable increase in F_s and therefore a deeper far-red light-induced quenching of F_s, which indicated an increase of the photosystems 'imbalance'. An example of such an experiment is described in Fig. 1. Notice in

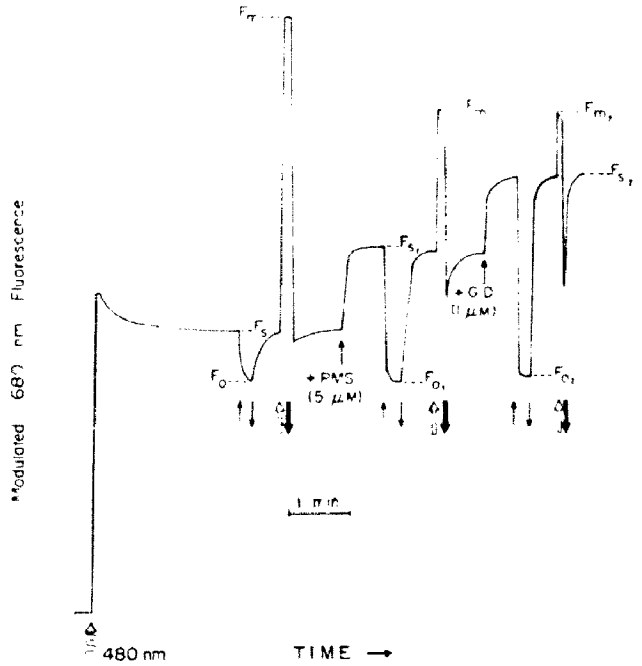

Fig. 1. Time course of the changes in the modulated fluorescence of broken chloroplasts, demonstrating the effect of PMS and the additive effect of gramicidin-D (G.D.) on the photosystems 'imbalance'. The fluorescence parameters are indicated before the addition (F_o, F_s, F_m), after the addition of 5 μM PMS (F_{o_1}, F_{s_1}, F_{m_1}) and after further addition of 1 μM G.D. (F_{o_2}, F_{s_2}, F_{m_2}). The wavy arrow denotes the on-switching of the modulated light 2. Thin arrows upward and downward denote, respectively, the on and off switching of background light 1. Thick arrows, open and closed, denote, respectively, the on and off switching of the background saturating blue light.

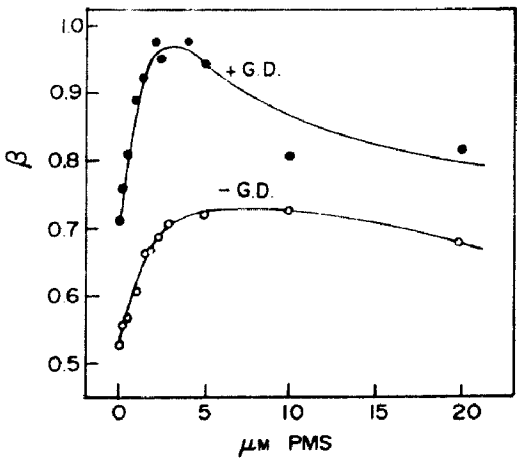

Fig. 2. The apparent distribution of photoactivity to PS II (β) in light 2 as a function of the PMS concentration in the presence (●) or absence (○) of gramicidin-D (G.D.). The experimental protocol, using broken chloroplasts, was made similar to that in Fig. 1.

Fig. 1 that the F_m level is defined by the initial momentary level when the saturating light is switched on. Repetition of this experiment after addition of gramicidin-D caused an additional appreciable increase in the 'imbalance'. In some cases, the combined effect of the presence of both PMS and gramicidin-D was quite dramatic in increasing the steady-state fluorescence level F_s up to almost the F_m level (i.e. reaching maximum 'imbalance' in favor of PS II in the modulated light). A PMS concentration dependence for such a case is shown in Fig. 2 and indicates an optimum for this effect at 3–5 μM PMS, at which point β reached a value of about 0.7 and increased further, upon the addition of gramicidin-D, up to almost the possible maximum value of 1. A similar effect to PMS has been obtained with TMPD but not with DAD.

While the basic effect of PMS (and the other cofactors) was straightforward, as shown in Figs. 1 and 2, it became immediately clear, however, that the effect has further aspects and that additional fluorescence phenomena exist, some of which were also noted before [15–18]. For example, there was a significant decrease of the F_m level relative to the control. (cf. Fig. 1—the maximum fluorescence level without PMS is denoted F_m and in its presence is denoted F_{m_1} or F_{m_2}. Clearly $F_{m_1} < F_m$. Furthermore, the F_m level was further quenched in time with continuation of the strong background light which was used for its assessment. The dependence of the initial F_m level on the PMS concentration is shown in Fig. 3. The initial quenched level of F_{m_1} relative to its value without PMS could be a result of a direct immediate independent interaction of PMS with the chlorophylls in their excited state (i.e. physical quenching) [15]. Thus we assume that the new initial F_m level nonetheless represents the state where all PS II reaction centers are closed and have considered this

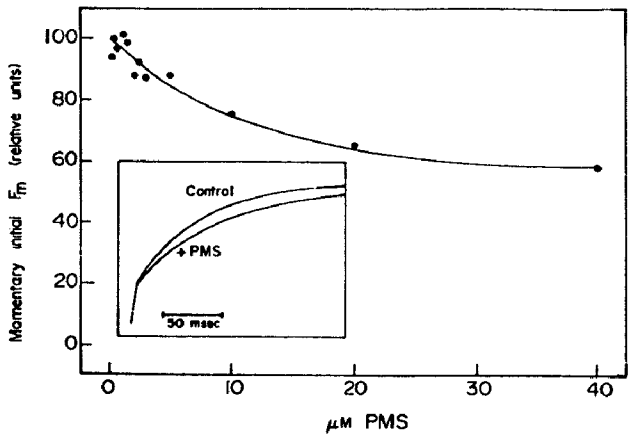

Fig. 3. Effect of the PMS concentration on the initial F_m level (i.e. the level obtained immediately as the saturating blue light is turned on) in broken chloroplasts. The experimental protocol is similar to Fig. 1 except that PMS was pre-added in the dark before illumination (without gramicidin-D). The inset describes a fluorescence induction curve of control and of chloroplasts containing 5 μM PMS. Before the induction measurement the chloroplasts were dark-adapted for 2 min.

level for the calculation of the 'imbalance'. To show the rapidity of this initial quenching we have performed fluorescence induction measurements (inset in Fig. 3) which showed a quenching of about 10% with 5 μM PMS already at initial induction times (less than 50 ms). Because of the rapidity, the accuracy of determining the initial F_m value by the slower modulated fluorimetry (in the chart recorder) was somewhat limited and was estimated to be lower by about 10% of the true value.

Not only F_m, but also F_s and F_0 underwent transient reversible changes under the effect of the strong illumination. These occurred also in control chloroplasts but were very pronounced in the presence of PMS and were reversed when the background light was switched off. Fig. 4 focuses attention on such changes in the absence of PMS (Fig. 4a), its presence (Fig. 4b) and also in the presence of PMS and gramicidin-D (Fig. 4c). In the control experiment, the F_m level was reversibly quenched by the strong background light (to the level denoted F'_m in Fig. 4a) and so also was F_s. The change in F_0 was relatively small. Introduction of PMS yielded immediately a higher F_s level and a lower F_m level, as shown above (Fig. 1). When a strong background light was applied, F_m decreased at a conspicuously faster rate and deeper extent than the control, reaching a new steady-state level F'_m. Relative to the control, big reversible changes were also induced in F_s and in F_0. The change in all these fluorescence parameters, particularly the relative closer approach of the final F_s value to the final F_0 value, actually indicates a transient decrease of the 'imbalance'. Using the time-dependent values of the fluorescence parameters we calculated accordingly a time-dependent 'imbalance' term. It appears that while the initial 'imbalance' in favor of PS II was indeed very high in the presence of PMS, it decreased significantly and rapidly with the strong background light, compared to a slower and milder decrease in the control. For example, after 1 min illumination with the strong light in the presence of 5 μM PMS, the 'imbalance' term decreased from about 0.97 to about 0.28 (70% decrease), compared to a parallel decrease from about 0.36 to about 0.22 (40% decrease) in the control. Some irreversibility in the strong background light-induced effect on F_m was noticed repeating the background light exposure again and again (e.g. the two repetitions in Figs. 4b and c). This partial irreversibility of F_m restoration may be attributed to an artifact due to to the photochemical conversion of PMS to pyocyanin under the strong blue illumination, since pyocyanin by itself did not cause any change in the 'imbalance' (not shown). Indeed, an exposure of a 5 μM PMS solution to the strong blue illumination under the same conditions resulted after 1 min in about 20% irreversible decrease in the absorption peak of PMS at 385 nm (not shown). A further exposure of about 5–10 min resulted in a complete disappearance of PMS absorption peak.

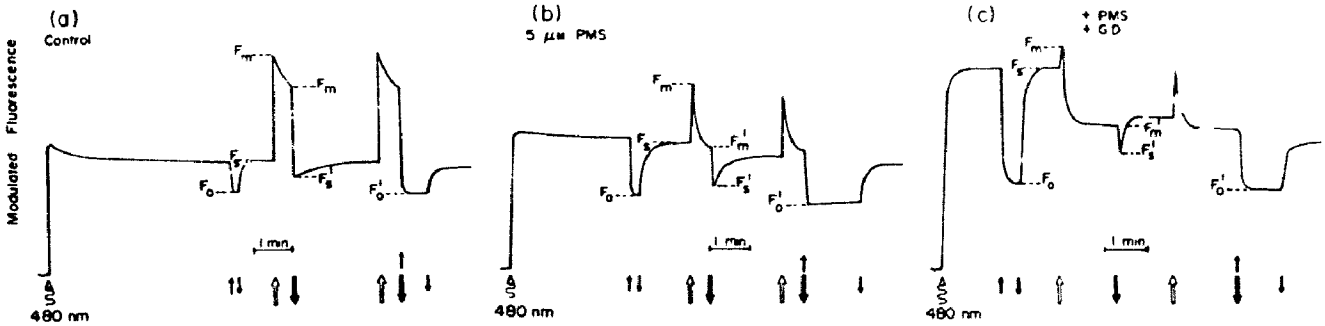

Fig. 4. Time course of light-driven changes in modulated fluorescence parameters of broken chloroplasts in a control sample (a); with pre-added 5 μM PMS (B) or 5 μM PMS with 1 μM gramicidin-D (G.D.) (C). F_0, F_s and F_m denote the fluorescence parameters measured before an extensive application of the background strong light, in each case. F'_0, F'_s and F'_m denote the fluorescence parameters obtained at the end of the saturating light period. Details are as in Fig. 1.

The time-dependent quenching of F_m in strong light in the presence of PMS has been observed previously. While initially thought to reflect the build-up of an electrochemical gradient of protons (the 'high-energy state'), it has been shown later to be rather affected by other factors [15,17]. Indeed, the reversible time-dependent quenching of F_m with PMS was obtained even in the presence of an uncoupler (Fig. 4c), which eliminates the 'high-energy state' as a sole quenching factor in this case. Blocking electron transfer from PS II to the plastoquinone pool by DCMU still did not prevent the saturating light-driven quenching of F_m in the presence of PMS, which still persisted in the presence of an uncoupler (Fig. 5). This is in contrast to the control without cofactor, where the transients in F_m, F_s and F_0 are strongly inhibited by uncouplers [4]. Similar effects have been obtained with 30 μM TMPD.

It is clear that in the presence of the above cyclic electron flow cofactors there is a range of time-dependent phenomena, which are not obviously understood a-priori. Most probably, an explanation of these phenomena will be found considering the presence of oxidized and reduced forms of the cofactors, their ratios and distribution in the stromal and lumenal sides. Changes in these parameters will change the ratio of cyclic to linear electron flows and also the extent of F_m quenching. These are affected reversibly by the strong background light (cf. Discussion). Nevertheless, it is quite evident that PMS affects the 'imbalance' of photoactivities in the light-limiting range, manifested particularly by the clear increase of F_s and the resulting stronger far-red light-induced quenching of the fluorescence from F_s to the (almost) unaffected F_0 level. This effect was most clearly seen with a minimum exposure to the strong background light, just to probe the F_m level. Most of the 'imbalance' data refers

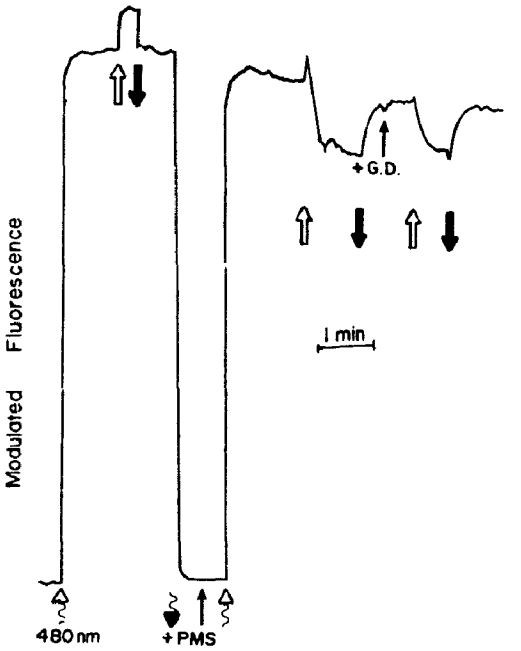

Fig. 5. Saturating light and PMS-driven quenching of modulated fluorescence of broken chloroplasts in the presence of DCMU. PMS (5 μM) and DCMU (10 μM) were added in the dark prior to the experiment. Gramicidin-D (G.D.) (1 μM) was added under illumination at the second stage of the experiment, as indicated. Other details are as in Fig. 1.

therefore to the light-limiting conditions, using the initial value of F_m.

One expects a similar effect of the above cofactors when they are brought to and kept in their reduced form by excess ascorbate. This is because the electron donation to PS I, by itself, is the main cause for the competition with PS II and thus would cause electron accumulation at the reducing side of PS II. The ques-

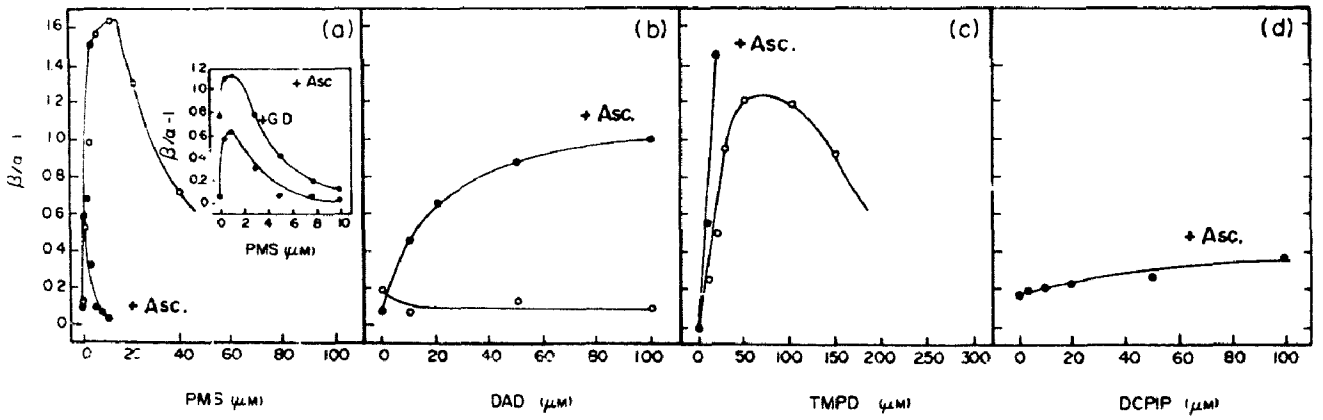

Fig. 6. Changes in the 'imbalance' term, $(\beta/\alpha - 1)$, as a function of the concentration of different electron flow cofactors. Different concentrations of PMS (a), DAD (b), TMPD (c) and DCPIP (d) were introduced under steady state modulated light 2 illumination in their native (mostly oxidized) form either to a standard chloroplast reaction mixture without (○) or with (●) 5 mM ascorbate. The inset in (a) presents the data corresponding to PMS in the presence of ascorbate in more detail and also compares them to the 'imbalance' values obtained after a a further addition of gramicidin D (G.D.) (1 μM).

tion in this case would be whether PS I, when over-excited with far-red light, would be able to over-balance both the cofactor and PS II electron donation to cause fluorescence quenching down to F_0. It was found that in the presence of ascorbate, PMS, as well as DAD and TMPD indeed shifted the fluorescence level away from F_0 and closer to F_m, while far-red light caused significant opposing quenching effects. One may visualize this again as an apparent shift in the 'imbalance' between the two photosystems regarding whole linear electron transport between them.

A comparison between the different effects of adding PMS, DAD, TMPD and DCPIP in the absence or presence of excess ascorbate is presented in Fig. 6. All these compounds yielded different concentration dependencies. In the absence of ascorbate, TMPD and PMS are similar in yielding curves with low values at low and high concentrations and an optimum point of the 'imbalance' in between. DAD in this case had, however, a very small effect at all concentrations. When kept reduced by excess ascorbate, PMS and TMPD gave different responses: with reduced TMPD, the 'imbalance' term tended to infinity (β tends to 1) as the concentration increased, while with reduced PMS, there was an optimal point and a decrease of the 'imbalance' at higher concentrations. DAD, when kept reduced by ascorbate caused an increase in the 'imbalance' as the concentration increased, inclining towards an asymptotic value. Reduced DCPIP, which donates electrons to P-700 [7,19,20] yielded only a small effect of increase in the imbalance (Fig. 6d). Neither reduced nor oxidized DCPIP caused any strong light-induced quenching of any of the modulated fluorescence parameters. As already noted above, at relatively high cofactor concentrations (e.g. above 40 μM for oxidized PMS, 70 μM for TMPD, 10 μM for reduced PMS with ascorbate and 20 μM for reduced TMPD with ascorbate), all fluorescence parameters and particularly the initial F_m were very strongly quenched, such that the whole span of change became small until the estimation of the 'imbalance' became doubtful, thus reaching the limit of the method of the imbalance assessment by modulated fluorimetry. With the reduced cofactors, the effect of F_m quenching was even stronger, in a perfect agreement with the results on PMS obtained by Papageorgiou [15].

To complement the fluorescence measurements and avoid the complications due to fluorescence quenching phenomena, we also obtained preliminary results of the rate of oxygen uptake by an ordinary Clark oxygen electrode which indicated an increase of Emerson enhancement in broken chloroplasts, from about 2.3 without PMS to about 3.4 with 10 μM PMS. These measurements were carried out similarly to that described in Ref. 4. More extensive experiments were carried out on the effect of PMS on oxygen evolution from leaf

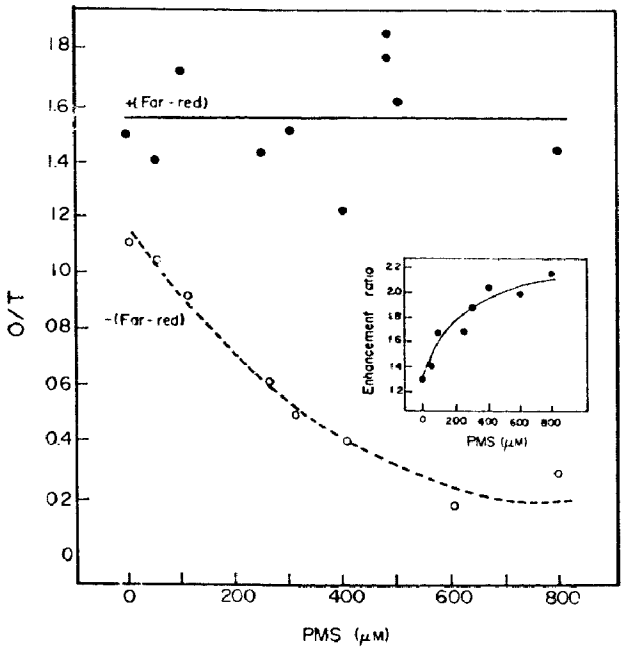

Fig. 7. The effect of PMS on leaf photosynthesis-oxygen evolution in light 2 and its enhancement by background light 1 as a function of PMS. *Hibiscus sp.* leaf discs were treated with PMS and the ratio (O/T) of the photobaric and photothermal photoacoustic signals with (●) or without (○) additional far-red illumination was determined (cf. Materials and Methods). The inset describes the resulting calculated enhancement ratio.

discs of *Hibiscus sp.*, which yielded very clear results. Leaf discs were immersed in PMS solutions at various concentrations and incubated in the dark, hoping that some of the PMS would penetrate through the interior of the leaf and to the chloroplasts. Photoacoustic measurements were performed, in which the ratio between the modulated oxygen and the thermal signals was determined (Fig. 7). This ratio decreased with PMS concentration, implying a decrease in the quantum yield of modulated oxygen evolution for light 2 alone. However, upon addition of saturating far-red background light (i.e. under conditions where the reaction centers of PS II are presumably all open), this ratio increased and reached again the same maximum of the control value at all PMS concentrations. This implies that PMS did not induce any inactivation per-se in PS II or any decrease in the maximum quantum yield of the overall electron transfer chain, but competed with PS II and thus increased the extent of closure of PS II reaction centers. This competition could be balanced by excess far-red light. The parallel increase in the Emerson enhancement to the decrease in O/T without the far-red light is shown in the inset to Fig. 7.

All the above results demonstrate that an increase in the photosystems 'imbalance' for light 2 in favor of PS II can be achieved artificially by the addition of different cyclic electron transport cofactors under different conditions, presumably by creating an artificial

cyclic electron flow pathway around PS I. In a previous paper [4] we have argued that such a cause for an apparent 'imbalance' should be wavelength-independent, since the fraction of PS I activity utilized for the cyclic path should depend only on competition between two dark rates of electron feeding, either from the cofactor or from the reduced endogenous acceptors of PS II. We used this argument for the effect of uncouplers [4] which was wavelength-dependent and thus excluded the possibility that their effect is due to the opening of an endogenous cyclic path. We therefore examined the wavelength dependence of the PMS effect on the 'imbalance'. For this, we checked the ratio f_0/f_+ vs. wavelength, where f_0 and f_+ are the fraction of open centers of PS II in the modulated light as defined by the fluorescence parameters in the absence and the presence of PMS, respectively. Contrary to expectation, it was found that this ratio depends on the wavelength in a similar manner to the imbalance induced by gramicidin-D [4] (Fig. 8). This raises some doubts about our previous arguments on the effect of

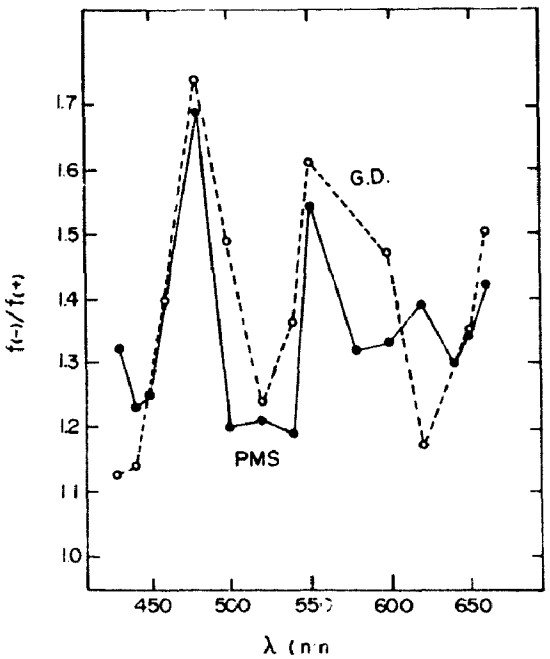

Fig. 8. Wavelength-dependence of the calculated f_0/f_+ ratio obtained, respectively, in broken chloroplasts with 5 μM PMS alone (●, continuous curve) or with 1 μM gramicidin-D (G.D.) alone (○, dashed curve). Data for the gramicidin-D effect were taken from Ref. 4. Fluorescence parameters were measured in the steady-state before (f_0) and after (f_+) the addition of these compounds to the standard reaction mixture. The wavelength of the modulated light was varied using interference filters (bandwidth 5–10 nm). The incident light intensity was adjusted for equal absorbed light intensity at all wavelengths (using light absorption measurements with an integrating sphere). In both cases, the f_0/f_+ ratio was maximum at 480 nm. The 580 nm and 650 nm points were exceptional in that the interference filters did not pass enough light intensity to reach the same absorbed intensity as for the other points.

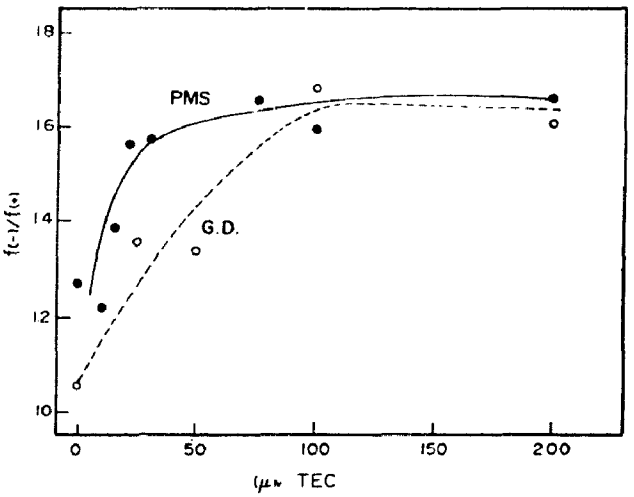

Fig. 9. Dependence of f_0/f_+ ratio in broken chloroplasts on the cation (TEC) concentration with PMS alone (●, continuous curve) or with gramicidin-D alone (○, dashed line). The ratio f_0/f_+ was calculated from fluorescence parameters measured in steady-state before (f_0) and after (f_+) the addition of either 5 μM PMS or 1 μM gramicidin-D (G.D.). Reaction mixtures were standard except that $MgCl_2$ was not included and with varying amounts of TEC. These results were obtained with chloroplasts, isolated as described in Materials and Methods but in the complete absence of $MgCl_2$ in order to avoid a possible effect of residual Mg^{2+}. Other details are as in Fig. 1.

uncouplers [4] and its possible interpretation. Although still preliminary, it seems that the PMS effect varies with wavelength in a similar way to the uncouplers effect and to Emerson enhancement in-vivo, in that its effect is maximum at about 480 nm and about 640 nm [4,14].

Another comparison between the uncoupler and the PMS effects on the 'imbalance' is brought in Fig. 9. It seems that even in the absence of cations (i.e. with unstacked membranes) there is already a significant PMS effect, while the gramicidin-D effect strictly follows the degree of stacking [4]. A similar observation was that the additional effect of gramicidin-D when PMS was present (see Fig. 2) did not show up in the absence of cations (not shown). It follows that the PMS and the uncoupler effects represent independent phenomena, which may act in synergism.

Discussion

Compounds like PMS, DAD and TMPD are capable of donating electrons to PS I when reduced. This causes competition to electron transfer by PS II to PS I, resulting in reducing equivalents accumulation in PS II and thereby, an increase in steady-state fluorescence. This competition is largely removed by increasing PS I activity with additional far-red light, indicating enough capacity of PS I to over-balance the combined PS II and the cofactor electron donation. The presence

of such cofactors apparently results in a shift of the photosystems 'imbalance' for light 2 excitation in favor of PS II.

In a more detailed explanation we have to introduce the sideness of the membrane, in view of the different membranal locations where the cofactor can be reduced or oxidized [21]. With this, we have to consider the different affinities to the sites of reduction and oxidation and to the permeabilities through the membrane. To explain the PMS concentration dependence of the 'imbalance' (Fig. 6a), for example, one needs to assume that PMS at relatively low concentrations accepts electrons mainly from PS I at the stroma side and returns them to PS I at the lumen side. The competition to the normal electron transport increases with the concentration of the cofactor. This explains the rising part of the curve. At higher concentrations, however, PMS accepts electrons from a PS II site either at the stroma or the lumen sides. This creates a bypass mechanism similar to that which was shown for TMPD [18], and independently shown recently also for PMS (Braun et al., submitted). The bypass serves as an additional route of the electron transfer from PS II to PS I and hence decreases the 'imbalance'. One could raise another possibility that when PMS is added at a high concentration, electron transfer may proceed via reduced PMS to oxygen and thus linear electron flow would compete better with the cyclic one, causing a decrease in the photosystems 'imbalance'. However, this idea requires that a high concentration of reduced PMS is accumulated, which is untenable with the light limiting conditions and the short time of measurement. This idea was anyway checked experimentally. It was found that indeed PMS does not act in this fashion: addition of high PMS concentrations (e.g. 300 μM) to illuminated chloroplasts, in the presence of MeV, decreased the rate of oxygen uptake significantly (not shown). Without MeV, addition of PMS resulted rather in a small decrease of the small endogenous rate of oxygen uptake. These effects occurred both under low-intensity or strong illuminations. This indicates a competition between PMS and MeV on electrons, while reduced PMS has no affinity for electron transfer to oxygen.

The effect of PMS persists even in the presence of ascorbate: PMS is reduced at the stroma side by ascorbate, the reduced form easily penetrates to the lumen side where it donates electrons to PS I. Thus, a pool of the oxidized form is formed at the lumen even in presence of ascorbate, as the hydrophylic dissociated ascorbate anions are hardly penetrable and the charged oxidized PMS diffuses only slowly out. The decrease of the 'imbalance' at high PMS concentrations in the presence of ascorbate can be explained, as before, by a bypass mechanism through the lumenal site of electron acceptance from PS II. The existence of such a site has recently been shown more directly by PMS alleviation of DBMIB inhibition even in the presence of ascorbate (Braun et al., submitted).

The strong F_m quenching and the decrease in the imbalance induced by the saturating light must also be related to the distribution ratios of the cofactor between the stroma and the lumen. The rapid stromal photoreduction in strong light results in a massive transport of the cofactor (in its reduced form) from the stroma to the lumen. Thereafter, the cofactor, being oxidized by the oxidizing side of PS I, accumulates in the lumen. As the concentration of oxidized PMS becomes low outside and high inside, the extent of the cyclic pathway diminishes largely and that of a bypass mechanism increases. This explains the decrease of the 'imbalance' in high light. To explain the strong F_m quenching, one must assume that the physical F_m quenching by the cofactor is exerted most strongly from the lumen side [15]. F_m and the 'imbalance' are slowly regained, after the strong light has been switched off, as a steady-state of concentrations is re-established in the weak light with the oxidized form of the cofactor slowly diffusing from the lumen to the stroma.

The different concentration saturation curves for the other cofactors (Fig. 6) must be assigned to different affinities of electron donation or acceptance. TMPD behaved in a similar way to PMS but with a lower affinity (Fig. 6b). It was much more active when kept reduced with ascorbate, probably indicating a high affinity for donation of electrons to PS I. DAD was effective when kept reduced by ascorbate but not when added by itself (Fig. 6c). It is possible that oxidized DAD has a very high affinity to accept electrons from PS II in a bypass mechanism which then return to PS I. In this way, electrons from PS II compete better with electrons originating from the reduced side of PS II on the donation to PS I.

It is interesting to note that DCPIP had almost no effect whether in the oxidized or the reduced form despite the fact that it has been used in its reduced form as an efficient electron donor in the presence of DCMU. Our ability to detect the effect of oxidized DCPIP was quite limited because of its strong blue color but nevertheless it was still feasible to see no effect in a low concentration range. One may conclude that in the reduced form it had a rather low efficiency in donating electrons to PS I compared with the efficiency of the other compounds under the experimental conditions employed. This point, however, needs further investigation.

While the fluorescence phenomena become complex and the determination of the 'imbalance' may be uncertain at high cofactor concentrations, there is no doubt regarding the validity of the effect when measured by O_2 evolution. The effect became particularly clean in intact leaves where it is clearly shown that

PMS introduces competition between PS II donation of electrons and (artificial) cyclic electron flow around PS I.

The wavelength dependence of the oxidized PMS effect on the 'imbalance' (Fig. 8) was not as expected. In a model where there is competition between linear electron flow through MeV and a cyclic flow through PMS, such that the ratio between them, ρ, depends only on the PMS concentration and is particularly independent of the excitation rate in PS I, one may write an equation expressing equal linear fluxes through PS II and PS I:

$$f_{\cdot}\beta = [\rho/(1+\rho)]a_{\cdot}$$

Without PMS ($\rho \to \infty$) the equation takes the form:

$$f_{\cdot} = \beta = a$$

and hence:

$$f_{\cdot}/f_{\cdot} = (1+\rho)/\rho$$

i.e., one expects that the $f_{\cdot}/f_{\cdot}$ ratio is constant and hence wavelength-independent. Since this result was not obtained (Fig. 8) and the $f_{\cdot}/f_{\cdot}$ ratio was variable, higher when β was larger, the model had to be modified. One possibility is to assume a fixed rate of the cyclic flow, r_c, (with the condition $a > r_c$). In this case a resulting equation is:

$$f_{\cdot}\beta = a - r_c$$

from which it follows that

$$f_{\cdot} = f_{\cdot} = a/(a - r_c)$$

The left-hand side of the last equation becomes indeed higher as β increases (i.e. a decreases). This expression may hold only within certain ranges of the parameters. It is more difficult to interpret the meaning behind the last two mathematical equations in terms of a concrete model. Indeed, a constant low rate of the cyclic flow indicates a limitation which is not observable in experiments where the cofactor is added alone. This may be resolved by a two site competition between MeV and PMS on electrons from PS I. In such a model, there is a main branch, potentially a high-rate one, where both MeV and PMS are efficiently reduced, but where the reduction of MeV competes much more strongly. In addition there is a side branch where PMS is mostly reduced, but which has a limited rate, r_c.

In conclusion, one may consider that regulation of the apparent 'imbalance' of photoactivities in-vivo could also occur through the operation of cyclic electron flows. The in-vitro 'imbalance' has been shown to depend largely on the inter salt levels, on the amount of membrane energization and on pigment distribution between PS I and PS II [4,12]. Here, it is demonstrated in broken chloroplasts that these parameters, particularly pigment distribution, play an important role in the effect of cyclic electron flow cofactors, which affect the 'imbalance' by changing the ratio between cyclic and linear electron flows.

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