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A new way to monitor by-pass restorations of electron transport in inhibited chloroplasts by cyclic electron flow cofactors – A study by modulated fluorimetry

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Inhibition of electron transport in broken chloroplasts by DBMIB, under light-limiting conditions, is shown to be bypassed by PMS in a manner similar to the known effects of the phenylenediamine derivatives TMPD and DAD. These bypasses were demonstrated and further studied by modulated fluorimetry, monitoring DBMIB inhibition by the shift of the steady-state fluorescence towards the F_m level and the release of inhibition by a reverse shift together with establishment of a quenching effect by background far-red light. Comparative studies were also made with electron transport blocked by DCMU or BNT. A weak bypass by TMPD and a weaker one by PMS of the block created by DCMU was observed by modulated fluorimetry. The block created by BNT is similarly shown to be bypassed by TMPD but hardly or not at all by PMS. Bypass effects persisted even in the presence of ascorbate. It appears that, following reduction of the different cofactors by ascorbate in the stroma side, illumination caused the accumulation of a pool of oxidized cofactor molecules in the lumen, which is able to mediate electron transport between reduced plastoquinone and plastocyanin or P-700. The existence and the size of this pool were found to depend largely on the internal pH at the lumen, presenting an artificial system in which electron flow is controlled by the lumenal pH. The bypassing electron transport in the presence of DBMIB presumably avoids the participation of the cytochrome b_6f complex. During its occurrence, there is also a strong imbalance in the activities of the two photosystems for linear electron flow, in favor of PS II. These experiments may thus serve to establish an in vitro model system for a future investigation of effects related to changes in the imbalance between the two photosystems and its regulation. Furthermore, this experimental system may also be utilized to study the role of the internal lumenal pH in control of photosynthesis.

Introduction

Electron transport between Photosystem II (PS II) and PS I of green plant photosynthesis is inhibited by several potent quinone analogue inhibitors, such as dibromothymoquinone-2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which blocks the oxidation of the plastoquinone (PQ) pool by binding to the cytochrome b_6f complex [1–3]. Phenylenediamine compounds such as *N*, *N*, *N'*, *N'*-tetramethylphenylenediamine (TMPD) or 2, 3, 5, 6-tetramethyl-*p*-phenylenediamine (DAD) are able to restore DBMID-inhibited electron transport in broken chloroplasts by a bypass mechanism suggested by Trebst and Reimer [4]. According to this suggestion, the above compounds accept electrons from the reduced PQ pool and transfer them to plastocyanin (PC), bypassing the DBMIB block and creating an electron flow path without the participation of the cytochrome b_6f. Restoration of electron transport by TMPD has been also demonstrated in-vivo in DBMID-inhibited cells of cyanobacterium *Synechococcus* sp. [5], where it was suggested that P-700 is the site of electron donation to PS I by TMPD.

So far, the above conclusions were reached using direct measurements of whole electron transport (i.e., by measurements of oxygen evolution, uptake or NADP

Abbreviations: BNT, bromonitrothymol; DAD, 2, 3, 5, 6-tetramethyl-*p*-phenylenediamine; DBMIB, dibromothymoquinone-2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHC, light-harvesting chlorophyll *a*/*b*-protein complex; MeV, methylviologen; PC, plastocyanin; PMS, *N*-methylphenazonium methosulfate; PS, photosystem; TMPD, *N*, *N*, *N'*, *N'*-tetramethylphenylenediamine.

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reduction). We have further studied this effect using the sensitive technique of modulated chlorophyll *a* fluorimetry, working under light-limiting conditions. In this method, the reduction state of Q_A is followed by the steady-state fluorescence level, F_s, whose shift towards the maximum level, F_m, gives evidence for a block in electron transport. The reverse shift and the ability of far-red light to quench the fluorescence and approach the minimum level, F_0, give evidence for the release of the block. Changes in F_s, relative to F_0 and F_m, may reflect changes in photoactivity distribution between the two photosystems [6] or alternatively the existence of a block in the electron transfer chain.

In a previous work [7] we have studied the effect of *N*-methylphenazonium methosulfate (PMS), TMPD and DAD, under limiting light conditions to increase the imbalance for linear electron flow photoactivities of the two photosystems in light 2 more in favor of PS II. This was attributed to the activation of cyclic electron flow around PS I by the cofactors, causing a competition between cyclic and linear flows. Since PS I uses part of the excitation for cyclic electron flow, its apparent efficiency for linear electron flow decreases, thus causing an apparent shift in the imbalance for linear electron flow activities in favor of PS II. It was also shown that such a shift in the imbalance was released by adding saturating far-red light (mostly absorbed by PS I), which releases the limitation on linear electron transport imposed by the cyclic flow around PS I. It has been suggested that the cofactor, after being reduced by the acceptor side of PS I, penetrates in its reduced form into the lumen of the thylakoid membrane where it is oxidized by donating electrons to either PC or P-700. As is demonstrated below, the distribution of the oxidized and reduced forms of the cofactors between the lumen and the stroma under different conditions (e.g., changing lumenal pH by the introduction of an uncoupler under illumination) determines their effect on fluorescence and on the imbalance, owing to the different ability of each cofactor to accept electrons either in the stroma, or in the lumen side and donate them to PS I.

Previous studies discussed the role of cytochrome b_6f in controlling either in vivo state transitions [8–11] or the ratio between cyclic and linear electron flows [12], which serve as regulatory mechanisms of the photosystems' imbalance in photoactivities [13–15]. It is therefore important to study the photosystems' imbalance for linear electron flow activities with or without the participation of cytochrome b_6f in order to examine these regulation mechanisms. Indeed, as will be detailed below, we prove here that a significant imbalance for linear electron flow photoactivities is established, when no functional electron flow through the cytochrome b_6f is enabled (e.g. with a bypass of DBMIB block by electron flow cofactors). This establishes an in vitro system to further study imbalance regulation with the sensitive tool of modulated fluorimetry.

Materials and Methods

Materials

TMPD was purchased from BDH Chemicals. Other common biochemicals and buffers were purchased from Sigma. Broken chloroplasts were prepared from market lettuce or from green-house-grown spinach and stored either in liquid nitrogen or in a Revco freezer (−120°C) [16]. The storage buffer contained 20 mM Hepes (pH 7.3), 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 30% (w/v) ethylene glycol. Total chlorophyll concentration was determined spectroscopically. For fluorescence measurements, chloroplasts were diluted in 2 ml of reaction medium within a regular spectrofluorimetric cell, such that the chlorophyll concentration did not exceed 10 μg/ml. Standard reaction mixture was 20 mM Hepes (pH 7.3), 10 mM NaCl, 5 mM $MgCl_2$ and 200 μM methylviologen (MeV). Stock concentrated solutions of PMS (aqueous), DBMIB (in ethanol), oxidized TMPD (aqueous solution of Wurster's blue, $TMPD^+$, pH 7.3), DAD (ethanol/water, 1:1), gramicidin-D (in ethanol) DCMU (in methanol) and sodium ascorbate (aqueous) were prepared and kept at −20°C. The DAD solution was frequently replaced, due to noticeable aging. During the experiments, the stock solutions were kept on ice and were protected from light. The final concentration of added alcohol from the stock solutions did not exceed 0.5% (v/v). 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 with two exciting light sources as described previously [17]. One source provided the modulated light 2 (480 nm; 1 nE cm^{-2} s^{-1}). The second source was not modulated (background light) and served to exert actinic effects. It was either photosynthetically saturating broad-band blue light (about 400–600 nm; 120 nE cm^{-2} s^{-1}) or a narrow band light 1 (720 nm; 30 nE cm^{-2} s^{-1}). The modulated fluorescence signal from the sample was detected at 683 nm and was processed by a lock-in amplifier.

Excitation with the modulated light 2 resulted in a certain steady-state fluorescence signal, F_s, where PS II reaction centers are partially closed. The closure is attributed either to a more favorable light distribution to PS II, or when inhibitors are used, to a block in the electron transfer pathway downstream from PS II. Addition of a photosynthetically saturating background light yielded a momentary maximal fluorescence signal, F_m, corresponding to a closed state of all PS II reaction

centers. Superimposing surplus background light 1 normally caused a fluorescence quenching to a minimum fluorescence level, taken to represent the parameter F_o. However, such quenching reflects the removal of the limitation in PS I excitation rate provided by the modulated light alone and is not expected to take place when electron transfer has a block between the two photosystems.

Whenever it was meaningful, fluorescence parameters were used to calculate the ratio of the light activity distribution coefficients for linear electron flow in PS II (β) and PS I (α). Only when one can assume that $\alpha + \beta = 1$, β (and α) can be calculated using the following equation [6]: $\beta = (F_m - F_o)/(2F_m - F_s - F_o)$. However, the effect of the cofactors in the context of this article is due to a change in α with a presumably unchanged β. Hence, the photosystems' imbalance for linear electron flow activities in favor of PS II is usually expressed by the imbalance term, $[(\beta/\alpha) - 1]$ which can be also calculated directly from the fluorescence parameters [6] without the need for the above assumption: $(\beta/\alpha) - 1 = (F_s - F_o)/(F_m - F_s)$. These expressions are valid only for F_s greater than or equal to F_o. Theoretically, the variation range of the imbalance term is between zero (i.e., $F_s = F_o$) and infinity ($F_s = F_m$), at which all photoactivity is distributed to PS II (i.e., $\alpha = 0$).

In all fluorescence measurements, the choice for cofactor concentration was to obtain the optimal conditions for their effect on bypass and imbalance. However, we had to consider that these cofactors also act in a secondary way as very effective fluorescence quenchers, particularly of F_m. This limits the range of concentration where the main effect of these compounds can be studied. Similar considerations had to be applied for the use of DBMIB as an inhibitor. A complete blockage of electron transport is achieved with about 10 μM DBMIB. We could not use such a high concentration, as DBMIB at this concentration acts also as a strong physical quencher of chlorophyll fluorescence [18,19]. We relied on a more specific block of electron transport between the reduced PQ (PQH_2) and the Rieske FeS protein of cytochrome b_6f and used a lower concentration (about 0.5 μM) of DBMIB, at which it still inhibits electron transport significantly [20,21], but with a more tolerable extent of fluorescence quenching.

Measurements of electron transport

Auxiliary measurements of electron transport rate were carried out with a regular Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK), using 4 ml of reaction mixture containing 200 μM MeV, 20 mM Hepes (pH 7.3), 10 mM NaCl, 5 mM $MgCl_2$ and 50 μM total chlorophyll. The sample was illuminated through a combination of Corning 4-96 with a 580 nm short-pass (Schott) filters (approx. 500 nE cm^{-2} s^{-1}). Additions were made by carefully injecting small volumes of concentrated solutions using Hamilton syringes to the well-stirred reaction medium.

Results and Discussion

Fig. 1a describes the effect of DBMIB on the modulated fluorescence parameters, excited with modulated 480 nm light (light 2), and the release of its inhibition by TMPD. Prior to DBMIB addition the steady-state fluorescence level, F_s, was quite low relative to F_m, and was further quenched to F_o with the addition of far-red light. Both facts indicate the operation of an unperturbed linear electron transport between the two photosystems with a certain degree of imbalance in favor of PS II. Upon addition of DBMIB there was a significant increase in F_s, approaching F_m, which was also increased somewhat (see below). The increase in F_s is presumably due to accumulation of reducing equivalents in the reducing side of PS II, but by itself still does not necessarily indicate a block in electron transfer. Such an increase could also be due to a limitation by PS I (e.g., by a change in light distribution or quantum yield, or by diversion of electron flow to a cyclic path). A more decisive criterion for the indication of the block was the inability of additional saturating far-red light to quench F_s (Fig. 1). Far-red light even increased F_s slightly in our case, most probably due to its slight actinic effect on the reaction centers of PS II *. Fig. 1 further shows that the inhibition caused by DBMIB was released by addition of TMPD. This is indicated in particular by the restoration and increase of the quenching effect of far-red light to achieve F_o. Such an increase, caused by TMPD addition is in line with the same activity of TMPD, previously demonstrated in uninhibited chloroplasts [7]. It was then interpreted to occur as a result of an apparent shift of photoactivity for linear electron transport in favor of PS II by a competing cyclic electron flow around PS I, which is artificially formed by the cofactor. In this case, such a competition takes place most probably between the by-pass and cyclic flows, both driven by TMPD. At a certain TMPD concentration (e.g., 30 μM) the cyclic flow competes against the by-pass flow most efficiently, yielding an increase in the imbalance for linear electron flow activities in favor of PS II.

* Far-red light (in the intensity range used) did not cause any increase of PS II fluorescence in the absence of the inhibitor (e.g., DBMIB) or in its presence after the addition of cofactors, which bypass the inhibition (see below). The slight increase observed in control DBMIB-inhibited chloroplasts is thus due to irrelevant actinic effects which are mostly pronounced under inhibitory conditions.

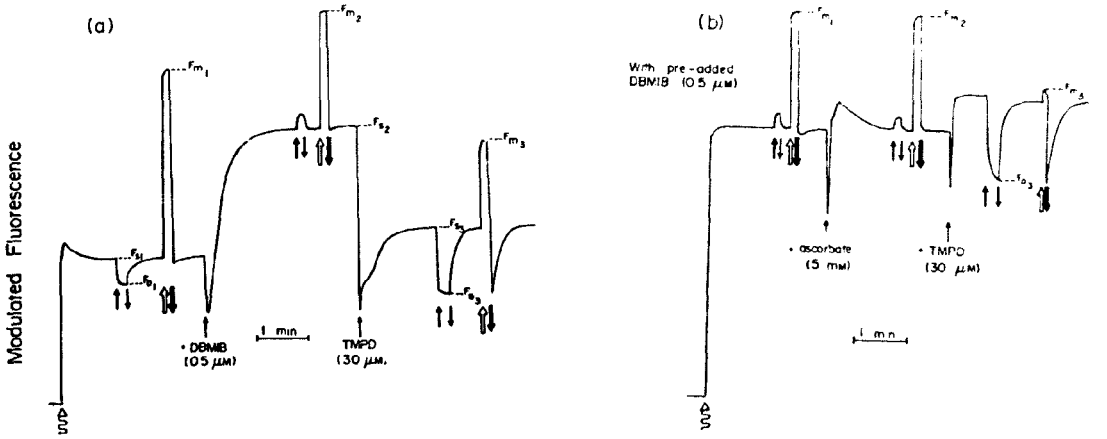

Fig. 1. Effect of DBMIB inhibition and its release by TMPD reflected by modulated fluorimetry of broken chloroplasts. Time-course of typical experiments. (a) Time-course in control chloroplasts followed by sequential additions of DBMIB (0.5 μM) and TMPD (30 μM). The levels of fluorescence parameters are indicated before (F_{o1}, F_{s1}, F_{m1}) and after DBMIB addition (F_{s2}, F_{m2}) and after the further addition of TMPD (F_{o3}, F_{s3}, F_{m3}). The wavy arrow denotes the switch-on of 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 [illegible] light. (b) Time-course when DBMIB (0.5 μM) was pre-added followed by the sequential addition of ascorbate (5 mM) and TMPD (30 μM). The definitions of fluorescence parameters are as in (a) except for F_{m1} and F_{m2} which denote, in this case, the F_m levels obtained after additions of DBMIB and ascorbate, respectively. It is important to note that the transient and further oscillation observed with the addition of ascorbate is only due to mixing as was verified by a control experiment in which only buffer or water was added (not shown). Reaction mixtures and other experimental details are as described in Materials and Methods.

Restoration of the quenching imposed by far-red light in DBMIB-inhibited chloroplasts was affected not only by the oxidized form of TMPD (Fig. 1a) but also when it was kept in its reduced form in the presence of ascorbate (Fig. 1b). Fig. 2 shows that PMS can also release the inhibitory effect of DBMIB, both in the

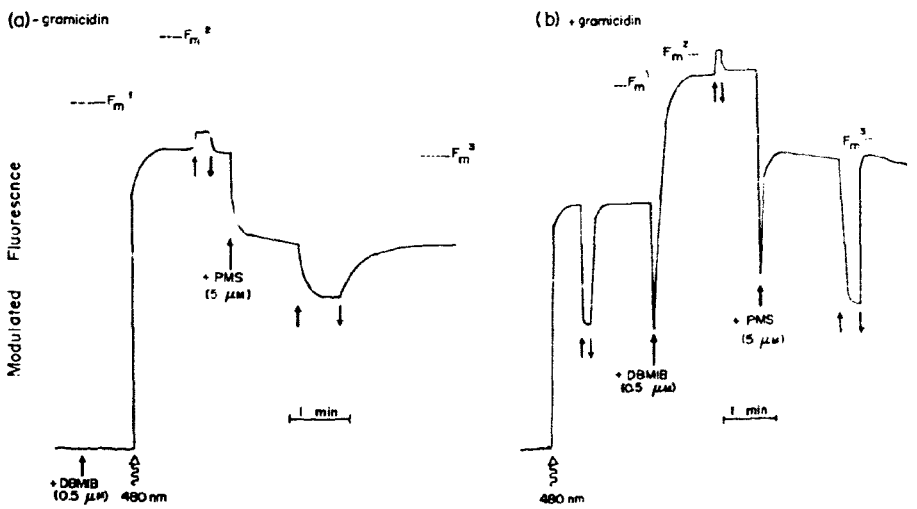

Fig. 2. Effect of DBMIB inhibition and its release by PMS in the absence (a) and presence (b) of the uncoupler gramicidin-D. Time-courses of the experiment. In (a) DBMIB (0.5 μM) was preadded to the chloroplasts. The F_m levels are marked by dashed horizontal lines and were measured before and after DBMIB addition (F_{m1} and F_{m2} resp.), and after the addition of 5 μM PMS (F_{m3}). For simplicity, the effects of the saturating background light to reach F_m in each case are not shown. Upward and downward arrows represent turning on and off the non-modulated light 1. In (b) gramicidin-D (1 μM) was pre-added to the chloroplasts. DBMIB (0.5 μM) and PMS (5 μM) were added sequentially as indicated. The F_m levels obtained before (F_{m1}), after (F_{m2}) DBMIB addition and after PMS addition (F_{m3}), are marked. Other details are as in Fig. 1.

TABLE I

The effect of ascorbate and gramicidin-D on the restoration of far-red light-induced F_s quenching by cyclic electron flow cofactors in DBMIB-inhibited chloroplasts

The quenching effect of Fs by far-red light is expressed as percent of the steady-state fluorescence obtained in each particular experiment. All measurements were performed in the presence of 0.5 μM DBMIB. Other experimental details are as described in Fig. 1. The slight increase (instead of quenching) observed upon addition of far-red light in control samples is presented as being a zero quenching effect because it occurred only in control samples (in the presence of DBMIB) and is considered to be a result of non-relevant actinic effects which slightly affect fluorescence (cf. text).

Cofactor	Ascorbate (mM)	Far-red quenching effect (% of F_s) −gramicidin-D	Far-red quenching effect (% of F_s) +1 μM gramicidin-D
Control	0	0	0
	5	0	0
DAD (100 μM)	0	18.8	0
	5	24.8	2.4
TMPD (30 μM)	0	33.4	51.4
	5	29.5	40.6
PMS (5 μM)	0	26.8	52.2
	5	28.7	0

absence (Fig. 2a) and presence (Fig. 2b) of gramicidin-D. Similar effects were exhibited by DAD. Table I summarizes the bypass effects of the three cofactors TMPD, PMS and DAD, in the absence and presence of ascorbate and gramicidin-D, on the block created by DBMIB. In the absence of the uncoupler, all the above cofactors act to release the inhibition, whether added in the oxidized form or kept reduced with excess ascorbate. There is no doubt that the release of the DBMIB inhibition is due to the ability of these cofactors to accept electrons upstream and donate them downstream from the inhibition site. This idea was raised previously for the effect of TMPD in restoring oxygen evolution in the presence of DBMIB [4].

The majority of the increase in F_m, which occurred as a result of DBMIB addition (Fig. 1a) did not occur in the presence of gramicidin-D (Fig. 2b) and thus it is attributed to the prevention of an electrochemical gradient build-up (e.g., of 'energy-dependent' quenching) as a result of the block of electron transport created by DBMIB. The small increase in F_m that still persisted in the presence of gramicidin-D (Fig. 2b) cannot be attributed to energization-dependent processes but rather to a possible effect of low DBMIB concentrations on PS II reaction centers or antenna. It thus cannot be excluded that while high concentrations of DBMIB impose a physical quenching of PS II fluorescence [18,19], low DBMIB concentrations may induce the opposite effect due to a certain interaction with PS II reaction centers or antennae.

The persistence of the bypass effect in the presence of ascorbate may seem inconsistent conceptually at first sight as apparently the cofactor becomes totally reduced and cannot accept electrons. This must be therefore explained in terms of the existence of a second phase to which ascorbate cannot penetrate, i.e., the lumen side of the thylakoid membrane, implying transport effects between the stroma and lumen side. Such an explanation was invoked in our previous work on the effect of these cofactors on the imbalance between the photosystems [7]. Without ascorbate, the cofactors accept electrons at the stroma, initially from the PS II side (e.g., PQH_2) but also from PS I. Being reduced, the cofactors penetrate into the lumen and become oxidized by donating electrons to PS I via PC or P-700, bypassing the DBMIB block. As they become oxidized in the lumen, the cofactors may accumulate in the lumen, being relatively trapped in their oxidized form and serving as electron acceptors in the lumen side (e.g., from PQH_2). In the presence of ascorbate, all bypasses must operate via the lumen side, as no oxidized form of the cofactor in any significant concentration exists outside to accept electrons from the stroma side of PS II or PS I. There must be a build-up of entrapped oxidized pool of the cofactor in the lumen side as a result of its penetration in its reduced form through the membrane into the lumen and the donation of electrons to PC or P-700 [7]. With the existence of an electron acceptance site (presumably from PQH_2) also in the lumen side, besides one at the stroma side [4], a bypass path is formed, mediated by the lumenal oxidized pool of the cofactor. With the inner relatively lower pH (i.e., in the absence of an uncoupler), a protonation equilibrium is established in the lumen where the oxidized forms of PMS and DAD are charged positively and are indeed able to be entrapped and accumulate in the lumen. TMPD in its oxidized form is always positively charged at any pH.

A significant quenching of steady-state and maximum fluorescence levels occurred with the addition of PMS or TMPD in the presence of DBMIB either with or without gramicidin-D (Fig. 2b and Table I), so that it cannot be related to 'energy-dependent quenching'. Also, other processes which drive a decrease in PS II fluorescence such as state 1 to state 2 transitions or photoinhibition are ruled out, since the experiments were performed in the complete absence of ATP and the quenching occurred only after the addition of the cofactors. Light-driven accumulation of a large pool of oxidized cofactor, which can accept electrons from PQH_2 in the luman may be responsible for the observed cofactor-induced quenching of F_m under illumination, as been suggested previously [7]. The observed cofactor-induced quenching of F_s is simply due to the

release of DBMIB inhibition by the cofactors, which decreases the number of reducing equivalents at the PS II side of the electron transport chain.

In the presence of the uncoupler but in the absence of ascorbate, only TMPD and PMS were effective, and even more strongly so compared to their effect in the absence of the uncoupler (Table I). In the presence of both gramicidin-D and ascorbate, only TMPD was significantly effective. DAD was not effective at all in the presence of gramicidin-D, with or without ascorbate. The effect of the uncoupler may be accounted for by its influence on setting a different internal pH. The inability of both ascorbate-reduced DAD and PMS to bypass in the presence of the uncoupler, when the inner pH remained relatively high, may be due to the fact that their half-oxidized forms, produced by one electron donation to PC or P-700 in the lumen, are uncharged and therefore can easily leak out where they are reduced by ascorbate. In such a case, no significant concentration build-up of their oxidized forms occurs in the lumen, to be effective in electron acceptance from the PS II side (i.e., from PQ). This is not the case with TMPD, which is always charged in its oxidized forms. The lack of DAD effect at pH 7.3 in the presence of gramicidin-D also in the absence of ascorbate, may be explained by a low affinity for electron acceptance or donation in the thylakoid system of the existing mostly uncharged forms of DAD (pK nearly 8). PMS, on the other hand, has still a high affinity in the absence of ascorbate even at the high lumenal pH, perhaps due to the fact that it is charged in its fully oxidized state, as in the case with TMPD. The positive charge itself is thus another possible factor in changing the affinity of the cofactor at the site for electron acceptance or donation.

A comparison between Figs. 1a and 2a indicates another interesting difference between the bypass effects of TMPD and PMS. With TMPD in the presence of DBMIB, the initial quenching of the fluorescence upon TMPD addition was transiently stronger than in the steady-state, reversing slowly and partially during illumination (Figs. 1a and 3a). At TMPD concentration above about 30 μM, the reversal after the initial quenching was inhibited; the initial quenching increased and predominated. The immediate fluorescence quenching can be interpreted as reflecting the establishment of electron transfer to TMPD at the stroma side, upstream from the inhibition site. The second phase of the gradual increase of F_s (Fig. 3a), is attributed to light-induced accumulation of the reduced form of the cofactor, a consequent depletion of the oxidized form and also the establishment of a cyclic electron flow around PS I in addition to the bypass electron flow [7]. Consistently, the time for this transient increased as the concentration of the cofactor increased (Fig. 3a). The competition between the by-

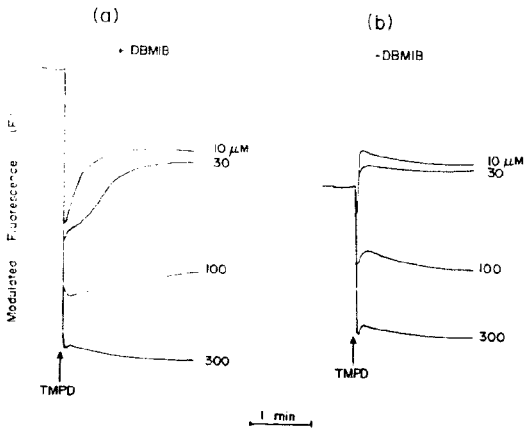

Fig. 3. Transient changes in modulated fluorescence following TMPD addition. Changes in steady-state fluorescence were monitored with modulated light 2 alone. Different concentrations of TMPD (as indicated) were added to the chloroplasts in the presence (a) or absence (b) of 0.5 μM DBMIB. Other details are as in Fig. 1.

pass electrons and electrons arriving from PS I on the donation side to the oxidizing side of PS I leads to a partial accumulation of reducing equivalents in PS II and hence an increase of fluorescence, which can be quenched by excess far-red light. This last effect is DBMIB-independent, as shown in our previous work [7]. In the present case TMPD plays a double role, providing both bypass and cyclic flows. In a control experiment without DBMIB (Fig. 3b), there was rather an immediate small increase in F from an initial low value (close to F_o) upon the addition of TMPD. This increase has been interpreted to reflect an apparent increase in the imbalance in favor of PS II for linear electron flow [7], resulting from the limitation at PS I due to the operation of a cyclic electron flow around PS I. The case of PMS in the presence of DBMIB is similar, except that no transient in F occurred after the initial quenching effect (Fig. 2). There was rather a small additional slow phase of decline. It seems that in the case of PMS the cyclic flow operates almost immediately, which indicates a very high affinity of PMS for electron donation to PS I already at a very low concentration of the reduced form. The slow phase of fluorescence decline can be explained as a reflection of the build-up of an oxidized PMS pool in the lumen, which accepts electrons from PS II in a higher lumenal affinity site.

As was shown previously for uninhibited chloroplasts [7], TMPD and PMS by themselves or TMPD, PMS and DAD in presence of ascorbate increase the imbalance in favor of PS II. However, above a certain optimal concentration the imbalance was reduced. This reduction in the extent of the imbalance was attributed to the increase of a bypass flow from a lumenal site at

higher concentration of the oxidized form, which accumulates in the lumen. Fig. 4 demonstrates this phenomenon in control and in DBMIB-treated chloroplasts with increasing concentration of TMPD. As the concentration of TMPD increases above the optimal point for the imbalance, there was a deeper quenching of all fluorescence parameters, but still, the stronger effect was that F_s approached F_o more closely, so that the imbalance decreased. With DBMIB there is a considerable shift to a lower value of the optimal TMPD concentration for maximum imbalance. This may be explained as follows: Without DBMIB, there is a competition between TMPD and cytochrome b_6f for electrons from reduced PQ. With DBMIB, TMPD becomes more favorable for electron acceptance, resulting in a requirement for less TMPD to have the maximum effect on the imbalance. Fig. 4 also shows that the presence of gramicidin-D enhances the effect of TMPD on the imbalance in DBMIB-inhibited chloroplasts. This is similar to the effect of the uncoupler in uninhibited chloroplasts [7] and is due to a synergistic effect, as the uncoupler by itself shifts the imbalance in favor of PS II towards higher values [17]. Approximately the same imbalance in favor of PS II is obtained in the presence of TMPD whether with or without DBMIB (Table II), implying a restoration of the photosystems' imbalance by TMPD (but also by PMS and DAD).

For a more direct demonstration, we also measured the restoration of the oxygen uptake reaction with the

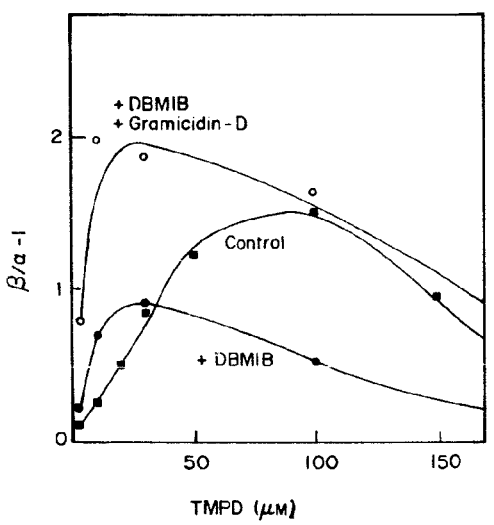

Fig. 4. Effect of TMPD concentration on the imbalance term. The imbalance term, $\beta/\alpha - 1$, was calculated from the fluorescence parameters measured at different TMPD concentrations for the following cases, as indicated: Control chloroplasts (■) and with 0.5 μM DBMIB in the presence (○) or absence (●) of 1 μM gramicidin-D. Other details are as in Fig. 2.

TABLE II

Restoration of the imbalance in photoactivities between PS I and PS II by cyclic electron flow cofactors in DBMIB-inhibited chloroplasts

The imbalance term, $\beta/\alpha - 1$, was calculated from fluorescence parameters before and after addition of cyclic electron flow cofactors in the presence or absence of 0.5 μM DBMIB. The experimental conditions are as described in Fig. 1.

Cofactor	Imbalance (−DBMIB)	Imbalance (+DBMIB)
Control	0.14	-
TMPD (30 μM)	0.8	0.83
PMS (5 μM)	0.95	0.4
DAD (100 μM)	0.19	0.19

addition of PMS and TMPD in DBMIB partially inhibited chloroplasts without ascorbate. In these experiments we had to use light intensities closer to saturation, since at lower light intensity it was difficult to obtain measurable rates. Fig. 5 presents an example which again proves that the DBMIB block was bypassed by not only TMPD (Fig. 5a), as was observed previously in a similar experiment [4], but also by PMS (Fig. 5b).

The site of electron acceptance by TMPD at the PS II region has been suggested previously to be the reduced PQ pool [22], since DCMU addition abolished the ability of TMPD to restore oxygen evolution in DBMIB-inhibited chloroplasts. In the present case, using modulated fluorimetry, it was surprisingly noticed that PMS and TMPD had the ability to bypass the block exerted by DCMU. This is deduced from the restoration of the far-red light-induced quenching of fluorescence (Figs. 6a and b). Further addition of DBMIB to DCMU-inhibited chloroplasts did not change the restoring effect of TMPD (Fig. 6a). One may suspect that the decrease observed in fluorescence upon addition of the cofactor in the presence of DCMU occurs as a result of 'energy-dependent quenching' (e.g., Ref. 23). Addition of gramicidin-D, however, affected neither the fluorescence decrease nor the bypass effects by the cofactors (not shown), in line with previously-presented data (Ref. 7, Fig. 5). The decrease in fluorescence by addition of the cofactors in the presence of DCMU is comparable to the quenching obtained in F_m upon addition of the cofactors in the presence of DBMIB (e.g., Fig. 1). However, note that, even in the presence of DCMU, where the steady-state fluorescence approaches F_m, the quenching of fluorescence by far-red light in the presence of the cofactors still persists, indicating the partial restoration of electron transport between PS II and PS I. It appears from these experiments that TMPD and PMS may accept electrons from a site closer to PS II reaction center than the Q_B site (e.g., from Q_A or even from pheophytine). Such a site may be close to the

outer surface of the thylakoid membrane or within the membrane itself. It is possible that under the saturating light conditions used in previous studies (where oxygen evolution and uptake or NADP reduction were monitored), the dynamics of electron transport is such that the Q_A bypass is limited and the cyclic flow around PS I takes over: thus no electron flow can easily be detected.

It was interesting to check whether an inhibition at a similar site obtained by another inhibitor, bromonitrothymol (BNT), would be also bypassed by these cofactors. BNT is a phenol-type inhibitor which binds to the Q_B site [24] in a non-competitive manner but probably not to the exact same region as DCMU [25],

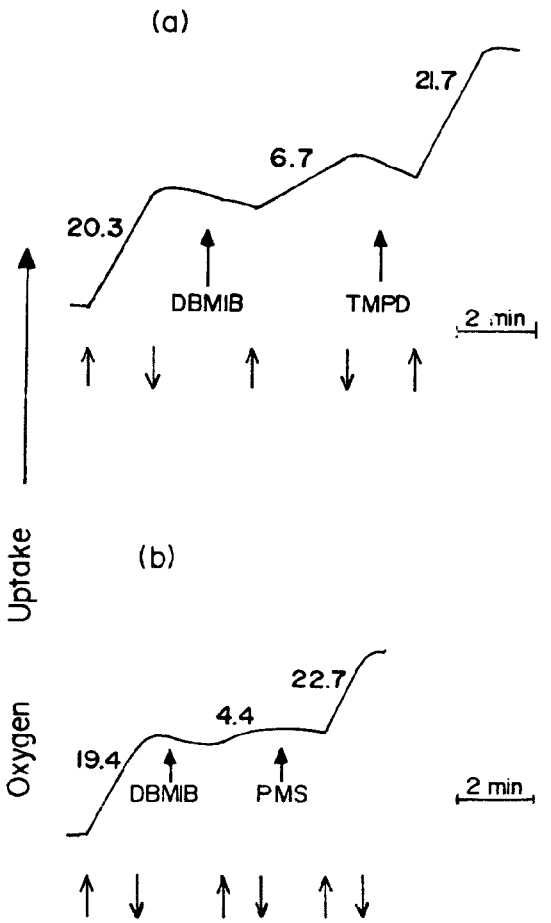

Fig. 5. By-pass restoration of oxygen uptake by TMPD and PMS. Changes in oxygen uptake were measured as described in Materials and Methods. DBMIB (0.5 μM) was added to chloroplasts suspended in a reaction mixture containing 200 μM MeV, 20 mM Hepes, 10 mM NaCl, 5 mM $MgCl_2$ and 50 μM chlorophyll. (a) 30 μM TMPD or (b) 10 μM PMS was added after DBMIB addition. Rates of oxygen uptake in units of μM O_2 per min are given next to each uptake curve.

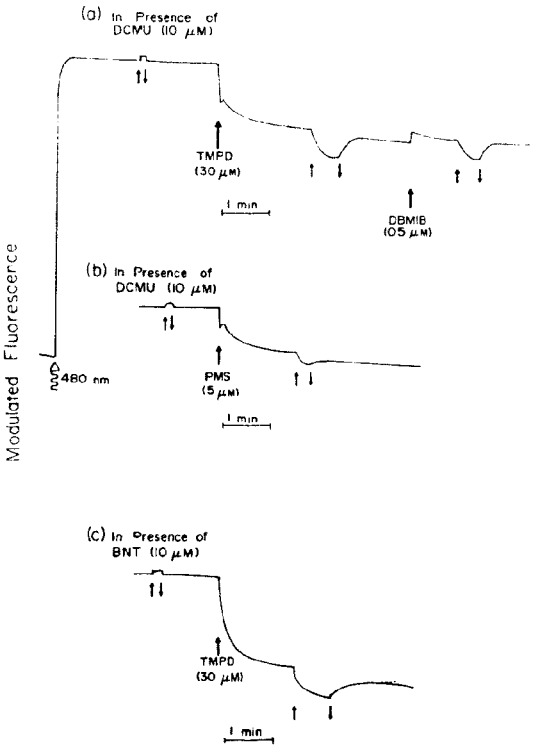

Fig. 6. By-pass of DCMU and BNT blocks. Time-courses of modulated fluorimetry experiments. Restoration of the modulated steady-state fluorescence quenching induced by light 1 and the photosystems' imbalance were monitored in chloroplasts inhibited by either 10 μM DCMU or 10 μM BNT. (a) Bypass effect of TMPD (30 μM) in DCMU-inhibited chloroplasts. Notice the persistence of the by-pass with further addition of DBMIB (0.5 μM). (b) Bypass effect of PMS (5 μM) in DCMU-inhibited chloroplasts. (c) Bypass effect of TMPD (30 μM) in BNT-inhibited chloroplasts. The baseline of all measurements was similar. Other experimental details are as in Fig. 1.

which binds in a competitive manner. It was indeed found that the inhibition at the Q_B site by BNT could be bypassed by TMPD, following the restoration of the far-red light quenching effect on the fluorescence (Fig. 6c). In this case however, PMS was ineffective (not shown).

In conclusion, we used modulated fluorimetry as a sensitive monitor for the recovery of electron transport by certain electron flow cofactors in inhibited chloroplasts. The results presented here show that modulated fluorimetry can give clues to the sites and the dynamics of the interaction of the cofactors with the thylakoid membrane. This should be utilized in a more quantitative analysis of the effect of lumenal pH on photosynthesis control. Furthermore, the ability to demonstrate an imbalance in photoactivities between PS II and PS I under conditions where the cytochrome b_6f does not

participate in electron transport (e.g., Table II) opens the possibility for studying the role of the cytochrome b_6f in mediating and controlling the state transitions and changes in the ratio between linear and cyclic electron flows.

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