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INTERACTION OF OXIDIZED AND REDUCED *N*-METHYLPHENAZONIUM METHOSULFATE (PMS) WITH PHOTOSYSTEM II

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SUMMARY

In 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) poisoned chloroplasts, the restoration of the fluorescence induction is presumed to be due to a back reaction of the reduced primary acceptor (Q^-) and the oxidized primary donor (Z^+) of Photosystem II. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) is known to inhibit this back reaction. The influence of reduced *N*-methylphenazonium methosulfate (PMS) in the absence of CCCP and of oxidized PMS in the presence of CCCP on the back reaction was investigated and the following results were obtained:

(1) Reduced PMS at the concentration of 1 μM inhibits the back reaction as effectively as hydroxylamine, suggesting an electron donating function of reduced PMS for System II.

(2) The inhibition of the back reaction by CCCP is regenerated to a high degree by oxidized PMS which led to assume a cyclic System II electron flow catalysed by PMS.

(3) At concentrations of reduced PMS higher than 1 μM it is shown that both the fast initial emission and more significantly the variable emission are quenched.

INTRODUCTION

The induction of fluorescence in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts was shown to be restored in a dark time of less than 0.5 min [1–3]. The restoration of the fluorescence rise signal has been interpreted as a reaction between the reduced acceptor (Q^-) and the oxidized electron donor (Z^+) of Photosystem II. So far it is not known whether Q^- and Z^+ recombine directly or whether several intermediate steps are involved. The back reaction is inhibited by the phenylhydrazones carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [3] and

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Abbreviations: PMS, *N*-methylphenazonium methosulfate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylendiamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [4]. Renger [5, 6] could show that CCCP also accelerates the deactivation of the S_2 and S_3 states of the watersplitting enzyme (ADRY effect). This led to the speculation that CCCP may act as a reductant of oxidized Photosystem II donors [3, 5]. Such interpretation would be consistent with the finding that electron donors of Photosystem II e.g. hydroxylamine [2–4], ascorbate/*p*-phenylendiamine and ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylendiamine (TMPD) [3] also inhibit the restoration of the fluorescence rise curve. However, more recently Renger [6] excluded a donor function of CCCP for stoichiometric reasons. Thus the mechanisms of the inhibitory action of CCCP should differ from that of electron donors (e.g. NH_2OH).

In this report the influence of oxidized and reduced PMS on the fluorescence induction in the presence of DCMU was measured. The results are interpreted on the basis of the hypothesis of Duysens and Sweers [8] which states that the variable fluorescence is dependent on the redox-state of the primary acceptor Q.

MATERIAL AND METHODS

Pea chloroplasts were prepared as described elsewhere [9]. They were incubated in a Thunberg cuvette for 5 min in darkness in a reaction medium containing 100 mM sucrose, 10 mM NaCl, 20 mM Tricine pH 8.0, 5 mM $MgCl_2$, 10 μM DCMU, 10 mM glucose and 0.8 % methanol (DCMU and CCCP were dissolved in methanol) and further additions which are described in the legends to the figures. Reduced PMS was obtained by adding the oxidized compound and reducing it either by ascorbate or the glucose/glucoseoxidase couple. Complete reduction of PMS was controlled photometrically. The enzymatic PMS reduction was introduced elsewhere [10].

Fluorescence was measured in a noncommercial spectrophotometer. Excitation light which came from a 150 W halogen lamp was filtered through a narrow banded interference filter (Schott) with a peak transmission at 595 nm and a half band width of 10 nm and passed through a photoshutter (Compur) which opened and closed within 3 ms, respectively. Light intensity was 34 kerg $\cdot$ cm^{-1} s. The photomultiplier was situated at a right angle to the actinic light beam and was protected against scattered actinic light by a combination of a broad band interference filter (Balzer K 7) and a narrow-banded interference filter (Schott) with peak transmission at 687 nm and a half band width of 10 nm. The signal was temporarily stored in a Nicolet signal averager having 1024 sampling channels and plotted on a x/y-recorder.

RESULTS

Besides the generally accepted interpretation that the yield of Photosystem II fluorescence is dependent on the redox state of the primary acceptor, Q [8], other parameters can influence the variable fluorescence as well [11–17]. Of these Mg^{2+} was always included in the assay which increased the variable fluorescence by a factor of about 5. In the following, the initial fast rise of fluorescence is designated with F_0 the steady-state level with F_∞.

In the absence of DCMU, CCCP lowers the steady-state level of variable

fluorescence [18]. This was confirmed by Homann [3] who concluded that CCCP quenches the fluorescence of chloroplasts because it accelerates the System I mediated oxidation of Q^-. Besides this type of action an inhibition of electron transport by CCCP was reported at the oxygen evolving side of system II [19–21]. The former effect should be excluded by the addition of DCMU which blocks electron transport close to Q. Under these conditions Q^- accumulates rapidly upon illumination since it can not be reoxidized by electron transport intermediates. Under moderate light intensities a sigmoidal induction of the emission is observed which was interpreted as an energy transfer between the subunits of Photosystem II [22].

The area above the induction curve is proportional to the number of oxidizing equivalents [23] which are identical with Q in the presence of DCMU. If the ratio F_0/F_∞ is taken as an indicator of the back reaction, qualitatively the same results as from the evaluation of the areas were found.

Under the conditions used here the ratio of F_0/F_∞ is constant for each individual chloroplast preparation but varied between 0.21 and 0.30 for the first excitation of dark adapted chloroplasts in different preparations. After a dark interval in the minute range, a second illumination was given. The resulting F_0/F_∞ values were compared with those of the first excitation assuming that they are indicative for the extent of the back reaction.

Fig. 1 shows typical traces of the time course of the fluorescence induction. In each pair of traces, the lower and the upper one corresponds to the first or second

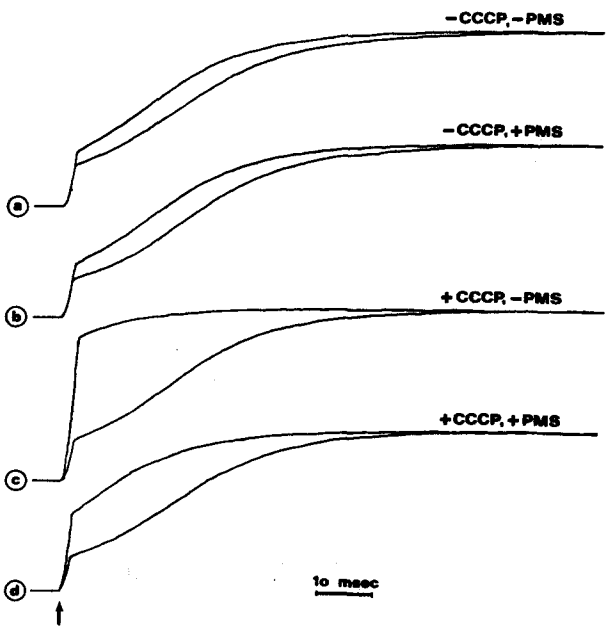

Fig. 1. The effect of CCCP and PMS on the restoration of the fluorescence induction. Reaction mixture as described in Materials and Methods. Where indicated 10 μM CCCP and 20 μM PMS were also present. Chloroplasts were equivalent to 8 μg chlorophyll/ml. Dark incubation 2 min. Duration of the excitations 125 ms. Dark interval between the excitations 2 min. The upward arrow refers to "light on".

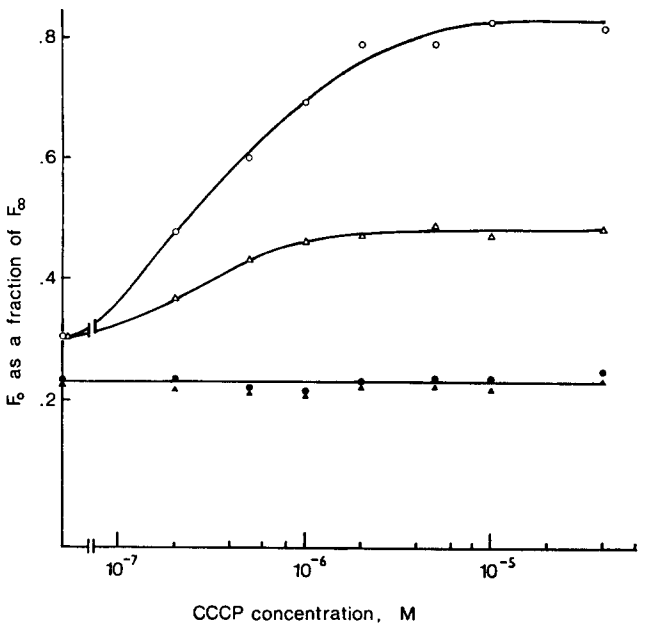

Fig. 2. The restoration of the fluorescence induction as a function of CCCP concentration in the absence and presence of 20 μM PMS. Reaction mixture as described in Materials and Methods. Chloroplasts were equivalent to 8 μg chlorophyll/ml. Closed and open symbols refer to the first and second excitation, respectively. ○, ●, without PMS; △, ▲, with 20 μM PMS. Dark incubation 2 min. Exciting conditions as in Fig. 1. Note, an increase in F_0/F_∞ indicates an inhibition of the dark restoration.

excitation, respectively, which were separated by two minutes of darkness. Curves a and b show the signals in the absence and presence of 20 μM PMS. Under both conditions the induction curves were restored to a high extent within the dark time showing no effect of PMS on the restoration. Curves c and d were measured in the presence of 10 μM CCCP and in the absence and presence of 20 μM PMS. In this case PMS has a marked effect. While the restoration is clearly inhibited by 10 μM CCCP (curve c) it is partly regenerated when PMS is also present (curve d).

Fig. 2 shows the extent of the dark restoration of the fluorescence induction as a function of the CCCP concentration in the presence and absence of 20 μM PMS. Over the whole range of concentration no effect of CCCP could be observed for the first excitation which was 125 ms of duration (closed symbols). For the second excitation, however, F_0 increases with increasing CCCP concentration approaching about 81 % of the maximum fluorescence F_∞ at about 10 μM of CCCP (open circles). This result indicates that at saturating CCCP concentrations the back reaction is markedly but not completely inhibited. When the duration of the first excitation is increased, the restoration of the fluorescence induction at high CCCP concentrations is inhibited to a somewhat higher extent (not shown). When at the given CCCP concentrations 20 μM PMS was also present, no significant changes of the induction curves were obtained for the first excitation (closed triangles). As shown in Fig. 1,

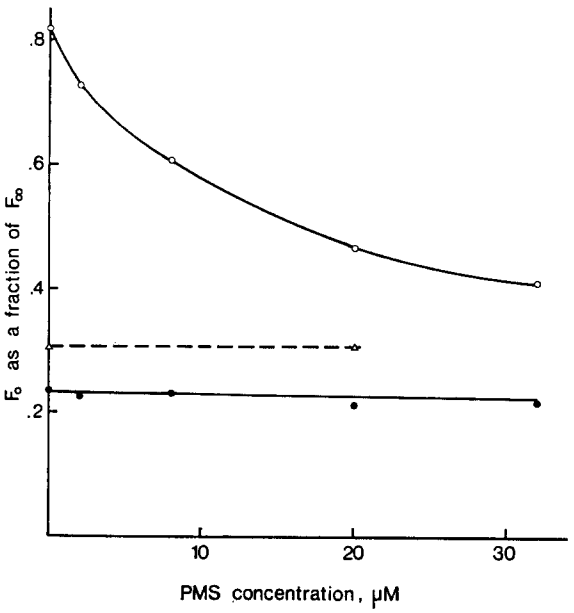

Fig. 3. The restoration of the fluorescence induction in the presence of 10 μM CCCP as a function of the PMS concentration. Reaction mixture as described in Materials and Methods. 10 μM CCCP and PMS were also present at concentrations as indicated. Chloroplasts were equivalent to 8 μg chlorophyll/ml. The dashed line and the triangles show the F_0/F_∞ ratio during the second excitation in the absence of CCCP. Closed and open circles refer to the first and second excitation, respectively. Exciting conditions as in Fig. 1.

PMS facilitates the dark restoration of the induction. Fig. 2 also shows that this PMS catalysed regeneration of the dark restoration and is observed over the whole range of CCCP concentration. Compared with controls which had no CCCP, the degree of regeneration is rather constant at about 65 % with 20 μM PMS.

Fig. 3 shows the re-establishment of the dark restoration at a saturating CCCP concentration as a function of the PMS concentration. The higher the PMS concentration, the lower is F_0 during the second excitation; i.e. the higher the restoration. At the highest PMS concentration used (32 μM) the restoration is almost 80 % compared with controls which had no CCCP. At concentrations of PMS higher than 32 μM, a general fluorescence quenching by oxidized PMS becomes significant. Such an effect was already described by Papageorgiou [24]. Restoration in CCCP poisoned chloroplasts was also obtained when PMS was given after the first excitation.

Thus, in the presence of DCMU, CCCP inhibits the back reaction of Photosystem II which implies that Q^- is conserved under the inhibited conditions. When PMS is also present the inhibition of the back reaction is cancelled. Consequently, PMS seems to catalyze the reoxidation of Q^-. An explanation for this might be that PMS undergoes a redox reaction by accepting electrons from Q^- and donating them to Z^+. This reaction would represent a System II cyclic electron flow catalyzed by PMS.

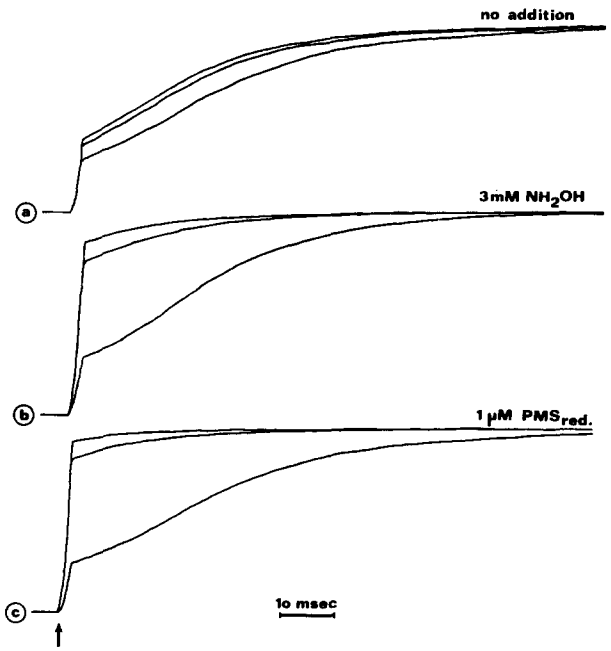

Fig. 4. The influence of NH_2OH and of reduced PMS on the dark restoration of the fluorescence induction. Reaction mixture as described in Materials and Methods. Where indicated 3 mM NH_2OH or 1 μM PMS plus glucose oxidase (about 1 unit/ml) were also present. Chloroplasts were equivalent to 7.8 μg chlorophyll/ml. Dark incubation 5 min. Duration of the excitations 250 ms. Dark interval between the excitations 1 min. The upward arrow refers to "light on".

TABLE I

INHIBITION OF THE DARK RESTORATION OF THE FLUORESCENCE INDUCTION BY REDUCED PMS

Reaction mixture and conditions as described in Materials and Methods. Where indicated the assays also contained about 1 unit/ml glucose oxidase, 5 mM ascorbate, and 1 μM PMS. Chloroplasts were equivalent to 7.1 μg chlorophyll/ml. The duration of each excitation was 250 ms. The dark time between the excitations was 1 min.

Additions	F_0/F_∞		
	First excitation	Second excitation	Third excitation
None	0.28	0.35	0.37
Enzyme	0.29	0.40	0.42
Ascorbate	0.27	0.47	0.51
PMS	0.28	0.38	0.42
PMS plus enzyme	0.28	0.82	0.91
PMS plus ascorbate	0.27	0.83	0.88

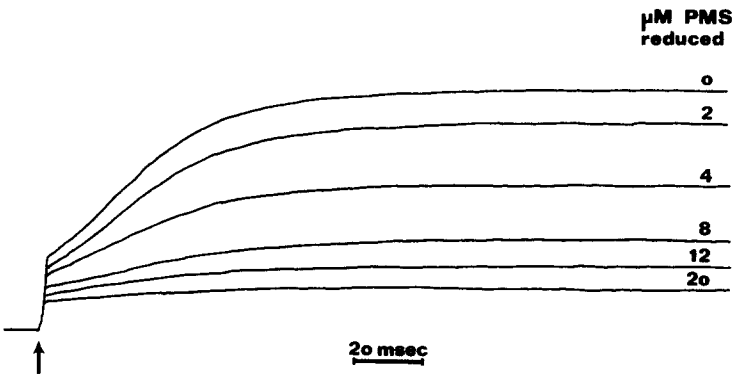

Fig. 5. Quenching of fluorescence by reduced PMS. Reaction mixture as described in Materials and Methods. PMS concentration as indicated. Chloroplasts were equivalent to 7.1 µg chlorophyll/ml. PMS was reduced by glucose oxidase (about 1 unit/ml) within a dark incubation of 5 min. The fluorescence induction signals at the given PMS concentrations are superimposed. The upward arrow refers to "light on".

From the following results a donating function of reduced PMS to Z^+ is actually indicated. As already mentioned typical electron donors to Photosystem II also inhibit the dark restoration of the fluorescence induction [2–4]. This is shown for NH_2OH in Fig. 4 (curves b). In these experiments the fluorescence induction curves of three light periods which were separated by dark intervals of 1 min were measured. Without any addition, F_0 is only slightly increased during the second and third excitation (curve a) indicating an almost complete back reaction. When, however, NH_2OH or 1 µM reduced PMS were present, F_0 for the second and third excitation has markedly increased (curves b and c).

As Table I shows, the glucose/glucose oxidase couple in the absence of PMS has only little effect on the F_0/F_∞ ratios. Therefore, it can be excluded that the anaerobiosis or the H_2O_2 which are produced by the enzymatic reduction of oxygen cause the rise of F_0. Ascorbate, however, induces a somewhat higher increase of F_0 for the second and third excitation than the control indicating a certain degree of electron donation of the compound to Photosystem II which has already been described [25, 26]. But clearly the most dramatic rise of F_0 is observed when reduced PMS is present, independently of whether ascorbate or the glucose/glucose oxidase couple is the reductant.

It has been shown that reduced PMS is a highly effective quencher of chlorophyll *a* fluorescence [10]. This quenching becomes significant at concentrations of reduced PMS higher than 1 µM. As Fig. 5 shows both the variable fluorescence and F_0 are quenched with a more pronounced quenching, however, for the variable fluorescence. For this reason the influence of reduced PMS on the dark restoration of the fluorescence induction was only investigated at a PMS concentration at which this quenching effect is marginal.

DISCUSSION

PMS is generally used as a highly effective catalyst of cyclic photophosphorylation of Photosystem I. The results shown here indicate that PMS in its oxidized

and reduced state can also interact with Photosystem II.

The light reaction of Photosystem II may be expressed as

$$Z\ P\ Q \overset{h\nu}{\rightleftharpoons} Z^+\ P\ Q^-$$

where P is the photoactive pigment and Z and Q are the primary donor and acceptor, respectively.

In the presence of DCMU the oxidation of Q^- by the electron transport chain is inhibited. In spite of this inhibiton a restoration of the fluorescence induction is observed [1–3] indicating a reoxidation of Q^- by other reactions.

It was assumed that a back reaction of Z^+ and Q^- regenerates Z P Q. Hydroxylamine which is known as an electron donor of Photosystem II inhibits the back reaction [2, 3]. Bennoun interpreted the inhibitory effect of NH_2OH by a reduction of Z^+ [2]

$$Z^+\ P\ Q^- + D \rightarrow Z\ P\ Q^- + D^+$$

were D represents an artificial electron donor. Thus Z^+ is no longer available for the back reaction with Q^-. Following this interpretation reduced PMS can be also ascribed an electron donating function to Photosystem II since the restoration of the fluorescence induction is effectively inhibited by the reduced compound whereas oxidized PMS and the PMS reducing agents have only a small effect.

For the inhibition of the back reaction by CCCP two possible explanations may be discussed. (1) Both Q^- and Z^+ are conserved since CCCP inhibits the reaction(s) which lead to a recombination. (2) Z^+ is reduced by an internal reductant facilitated by the action of CCCP while Q^- is conserved.

Thus the second possibility would have the same consequence as artificial donors (e.g. hydroxylamine or reduced PMS).

In either case PMS seems to catalyse the reoxidation of Q^-. Since the low fluorescent reaction center Z P Q is regenerated under the inhibited conditions, Z^+ has also to be reduced. For this reaction either reduced PMS or an internal compound may be the reductant. As reduced PMS presents itself as an effective donor to Z^+, the most simple explanation may be a cyclic electron flow from Q^- to Z^+ via PMS. In Fig. 6 a simplified model is presented which shows the possible sites of action of CCCP, reduced and oxidized PMS and hydroxylamine in the presence of DCMU.

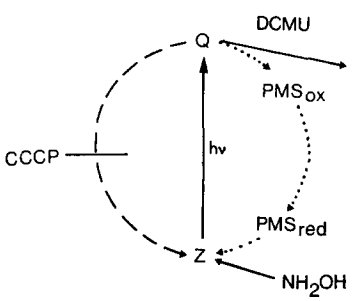

Fig. 6. Simplified model for the sites of action of CCCP, hydroxylamine and oxidized and reduced PMS. The dashed and the dotted lines symbolize the back reaction in the absence and presence of CCCP and PMS, respectively.

Whether the same reaction of PMS accounts for the back reaction in the absence of CCCP is uncertain.

Cramer and Böhme [4] discussed cytochrome b_{559} high-potential to be involved in a System II cyclic electron flow. They also found [27] that FCCP transforms cytochrome b_{559} high-potential into the low-potential form thus inhibiting the back reaction. Within this model PMS would replace cytochrome b_{559} high-potential for the back reaction in the presence of the phenylhydrazons FCCP or CCCP.

Further results [30] also indicate that the known light dependent quenching of fluorescence in the presence of PMS [24, 28, 29] is at least in part due to an interaction of PMS with Photosystem II.

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