Energy Conservation in Photoreductions by Photosystem II. Reversal of Dibromothymoquinone Inhibition of Hill-Reactions by Phenylenediamines

Achim Trebst and Susanne Reimer

Abteilung Biologie, Ruhr-Universität Bochum

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The plastoquinone antagonist dibromothymoquinone interrupts the photosynthetic electron transport chain between photosystem II and I by inhibiting the oxidation of plastohydroquinone by photosystem I. The effect of phenylenediamines on NADP and ferricyanide photoreduction inhibited by dibromothymoquinone is studied in isolated chloroplasts. *N*-substituted phenylenediamines fully restore NADPH and ATP formation as well as oxygen evolution in DBMIB inhibited NADP reduction, but are practically inactive in stimulating ferricyanide reduction by photosystem II. On the other hand phenylenediamine and *C*-substituted phenylenediamines are only weakly active in restoring NADP reduction but are very active in stimulating ferricyanide reduction. The P/e_2 ratio in restoration of NADP reduction by phenylenediamines is close to 1, whereas the P/e_2 ratio in ferricyanide reduction is about 0.4 (the ratio is dependent on the DBMIB concentration). The reversal of the DBMIB inhibition in NADP reduction by phenylenediamines is attributed to a bypass of the inhibition site: Reduced phenylenediamines are electron donors for photosystem I after and oxidized phenylenediamines are electron acceptors for photosystem II before the DBMIB inhibition site. To explain the different rates and P/e_2 ratios in the various systems it is assumed that all phenylenediamines are reduced at the expense of water oxidation by photosystem II but via plastoquinone and on the inside of the membrane. Therefore two energy conserving steps (*i. e.* two proton releasing sites on the inside of the membrane) are involved. *N*-substituted phenylenediamines like TMPD reduced by photosystem II inside remain inside the membrane and are reoxidized inside by photosystem I. This way they connect photosystem II back onto photosystem I, electron transport rates are high and the P/e_2 ratio is one in the restored NADP photoreduction. Phenylenediamine and *C*-substituted phenylenediamines on the other hand travel back to the outside through the membrane, and by doing so carry some of the protons released on the inside of the membrane by the energy conserving steps back to the outside. This way they are very active in stimulating the rate of ferricyanide photoreduction but the P/e_2 ratio is only about 0.4. These phenylenediamines being removed from the inside are less active in restoring the rate of NADP reduction.

Introduction

Recently we have described dibromothymoquinone (DBMIB) as potent antagonist of plastoquinone (Trebst *et al.*[1]; Böhme *et al.*[2]). The compound inhibits photosynthetic electron flow in chloroplasts from water to NADP between photosystem II and I at the reducing side of plastoquinone (Trebst[3]). Hill-reactions in the presence of DBMIB are attributed to a photoreduction by photosystem II. We recently reported that such photoreductions are coupled to ATP formation, the P/e_2 ratio depending whether lipophilic or hydrophilic acceptors are used (Trebst and Reimer[4]). Good's group (Saha *et al.*[5]; Ouitrakul and Izawa[6]; Ort *et al.*[7]; Izawa *et al.*[8]) as well as Gimmler[9] also reported on an energy conserving step in photoreductions by photosystem II. Good's group found oxidized *p*-phenylenediamine to be the best electron acceptor for photosystem II (Saha *et al.*[5]) and recently showed that this reduction is not inhibited by DBMIB (Izawa *et al.*[8]).

Reduced phenylenediamines on the other hand are long known to be electron donors for photosystem I (Trebst[10]; Trebst and Pistorius[11]). As already shown photoreductions by photosystem I at the expense of phenylenediamines (kept reduced by ascorbate) are not inhibited by DBMIB (Böhme *et*

Requests for reprints should be sent to Prof. Dr. A. Trebst, Lehrstuhl für Biochemie der Pflanzen, *D-4630 Bochum*, Ruhr-Universität.

Abbreviations: DAD, diaminodurene; DAT, diaminotoluene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone-dibromothymoquinone; DCIP, 2,6-dichlorophenolindophenol; MMPD, 2-methyl-5-methoxy-*p*-phenylenediamine; PD, *p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

al.[2]; Trebst[3]). Because then neither the oxidation of reduced phenylenediamines by photosystem I nor the reduction of the oxidized forms by photosystem II is inhibited by DBMIB, phenylenediamines should bypass the inhibition site of DBMIB.

We wish to report here on the effect of various substituted *p*-phenylenediamines on the restoration of the NADP Hill-reaction inhibited by DBMIB and on the stimulation of ferricyanide photoreduction in the presence of DBMIB. The energy conserving sites in these systems and the side of the membrane involved are discussed.

Results

As shown already dibromothymoquinone inhibits photosynthetic NADP reduction by isolated chloroplasts (Trebst *et al.*[1]; Böhme *et al.*[2]; Trebst[3]). Total inhibition is obtained at a concentration of about 10^{-6} M DBMIB. The addition of plastoquinone reverses electron flow from water to NADP, restoring NADPH and ATP formation as well as oxygen evolution (Böhme *et al.*[2]; Trebst[3]). Electron donor systems for photosystem I like DAD + ascorbate or DCIP + ascorbate also restore NADPH formation in DBMIB inhibited chloroplasts (Böhme *et al.*[2]; Trebst[3]), but they do not restore oxygen evolution, of course (this is again indicated in Table III, where the effect of DAD + ascorbate or TMPD + ascorbate is shown to restore NADPH formation in DBMIB and DCMU inhibited chloroplasts). Certain *p*-phenylenediamines in catalytic amounts and in the absence of ascorbate also restore NADPH and ATP formation in the presence of DBMIB, but in addition they restore also oxygen evolution (Table I). We had demonstrated such an effect already with DCIP, though this system yields very low rates of restoration (Böhme *et al*[2]). Whereas a *p*-phenylenediamine + ascorbate is a substitute electron donor system after the site of inhibition of DBMIB, catalytic amounts of a *p*-phenylenediamine in the absence of ascorbate are bypassing the inhibition site. According to Table I TMPD yields the highest percentage of restoration in the NADP system, whereas *p*-phenylenediamine and the *C*-alkyl-substituted *p*-phenylenediamines like DAT or MMPD are less active. We interpret these results in the NADP system as a bypassing of the inhibition site of DBMIB by phenylenediamines. This requires that phenylenediamines in the reduced form are electron donors for photosystem I and in the oxidized form are acceptores of photosystem II. The first has been amply demonstrated in the literature (Trebst[10]; Wessels[12]; Trebst and Pistorius[11]; Arnon *et al.*[13]; Vernon *et al.*[14]; Izawa *et al.*[15]). The later has recently been proposed by Saha *et al.*[5] and has recently been substantiated by experiments with KCN (Ouitrakul and Izawa[6]) or poly-lysine (Ort *et al.*[7]) inhibited chloroplasts.

Table I. Reversal by a *p*-phenylenediamine (10^{-4} M) of NADP-photoreduction inhibited by a low concentration (10^{-6} M) of dibromothymoquinone. Conditions: Photosynthetic activity of chloroplasts, prepared according to Nelson *et al.*[29] were assayed at 15 °C under N_2 and illumination for 10 min with 30,000 lux. The medium contained in μmoles in a volume of 3 ml: Tris buffer pH 8 80, $MgCl_2$ 10, ADP 10, inorganic phosphate, labelled by P^{32} 10, chloroplasts with 0.2 mg chlorophyll and ferricyanide 20 or NADP 6 and ferredoxin 0.01 respectively. Oxygen evolution was followed manometrically, NADPH was measured at 340 nm and ATP was measured by the incorporation of P^{32} according to the modification of Conover *et al.*[30].

Additions to 10^{-6} M DBMIB	μequiv. O_2 evolved	μmole NADPH formed	μmole ATP formed	P/e_2
control without DBMIB	3.9	4.3	4.3	1.0
—	0.3	0.2	0.1	
TMPD	3.1	3.6	2.6	0.72
N,N-dimethyl-*p*-phenylenediamine	3.1	3.6	2.5	0.7
N,N-diethyl-*p*-toluidine	2.8	3.1	2.0	0.65
p-phenylenediamine	1.2	1.6	1.2	0.75
DAT	1.1	1.5	1.2	0.8
MMPD	1.1	0.8	2.9	cyclic
DAD	0.6	0.4	2.9	cyclic

Of particular interest are the P/e_2 ratios in the restored NADP reduction by phenylenediamines (Table I). The P/e_2 ratio is about 0.75, *i.e.* approaching the stoichiometry of ATP formation to electron transport of 1 in the control (Table I). This seems surprising at first because it has been shown that the photoreduction of NADP by photosystem I by TMPD + ascorbate is not coupled to ATP formation (Trebst[10]; Wessels[12]; Izawa *et al.*[15]) (whereas the DAD/ascorbate system is coupled (Trebst and Pistorius[11])). In Table I the P/e_2 ratio also in the *N*-substituted phenylenediamine systems is close to one as in the control. The phenylenediamines which are coupled, when used for photoreductions by photosystem I in the presence of ascorbate like DAD, yield very high ATP values in the experiments of Table I indicating a superimposed cyclic electron flow.

Table II shows the effect of *p*-phenylenediamines on the photoreduction of ferricyanide in the presence of DBMIB. Because certain phenylenediamines stimulate the rate of oxygen evolution this indicates

Table II. Reversal by a *p*-phenylenediamine (10^{-4} M) of ferricyanide-photoreduction inhibited by a low concentration (10^{-6} M) dibromothymoquinone. Conditions as in Table I.

Additions to 10^{-6} M DBMIB	μequiv. O_2 evolved	μmole ATP formed	P/e_2
—	1.4	0.7	0.5
TMPD	1.4	0.5	0.35
N,*N*-dimethyl-*p*-phenylenediamine	1.7	0.5	0.29
N,*N*-diethyl-*p*-toluidine	1.4	0.5	0.35
p-phenylenediamine	3.7	1.8	0.49
DAT	3.8	1.6	0.42
MMPD	5.4	2.8	0.52
control without DBMIB	3.8	3.6	0.95
control without DBMIB + PD	5.8	3.6	0.66

that these phenylenediamines in the oxidized form are the better electron acceptors for photosystem II and are shuttling the electrons to ferricyanide. As Table II indicates the *N*-substituted *p*-phenylenediamines like TMPD are inactive in stimulating ferricyanide reduction, whereas phenylenediamine and *C*-substituted phenylenediamines like DAT or MMPD are very active. By comparing Tables I and II it becomes apparent, that those phenylenediamines particularly active in restoring NADP reduction have small effects in the ferricyanide system and vice versa. The rate of ferricyanide reduction in the presence of DBMIB is also increased by quinones as already reported (Trebst and Reimer [4]). Saha *et al.* [5] have first shown that phenylenediamine is stimulating ferricyanide photoreduction by photosystem II. The ratio of ATP formation to electron transport (P/e_2) in the experiments of Table II is about 0.5 in the presence of 10^{-6} M DBMIB, *i.e.* about half the ratio of the control (Table II). This agrees well with the values reported earlier (Saha *et al.* [5]; Trebst and Reimer [4]; Ouitrakul and Izawa [6]; Ort *et al.* [7]). Phenylenediamines then couple ferricyanide reduction with half the stoichiometry of the one in restored NADP reduction.

The concentration of DBMIB employed is of importance. The low concentration (10^{-6} M) of DBMIB used in Tables I and II already inhibit NADP completely and ferricyanide reduction to the maximal obtainable value. Higher concentrations of DBMIB do have an effect in the restoration of these reactions by phenylenediamines. Increasing the concentration of DBMIB above 10^{-6} M changes proportionally the rate and the stoichiometry of some of the reactions investigated here, whereas other reactions are not effected. In Tables III and IV the

Table III. Reversal by a *p*-phenylenediamine (10^{-4} M) of NADP-photoreduction inhibited by a high concentration ($5 \cdot 10^{-6}$ M) dibromothymoquinone. Conditions as in Table I.

Additions to $5 \cdot 10^{-6}$ M DBMIB	μequiv. O_2 evolved	μmole NADPH formed	μmole ATP formed	P/e_2
control without DBMIB	3.9	4.3	4.3	1.0
—	0.2	0.1	0.1	
TMPD	2.3	2.5	2.1	0.84
N,*N*-dimethyl-*p*-phenylenediamine	2.5	2.7	2.4	0.89
N,*N*-diethyl-*p*-toluidine	1.7	2.4	1.7	0.71
p-phenylenediamine	0.7	0.7	0.3	0.43
DAT	0.7	0.9	0.5	0.55
Additions to $5 \cdot 10^{-6}$ M DBMIB, $2 \cdot 10^{-5}$ M DCMU and 10 μmoles ascorbate				
TMPD	0.3	1.8	0.2	0.11
N,*N*-dimethyl-*p*-phenylenediamine	0.4	2.4	0.1	0.05
p-phenylenediamine	0.2	1.2	0.1	0.08
DAD	0.3	3.7	3.7	1.00
MMPD	<0.1	3.1	3.1	1.00

Table IV. Reversal by a *p*-phenylenediamine (10^{-4} M) of ferricyanide-photoreduction inhibited by a high concentration ($5 \cdot 10^{-6}$ M) dibromothymoquinone. Conditions as in Table I.

Additions to $5 \cdot 10^{-6}$ M DBMIB	μequiv. O_2 evolved	μmole ATP formed	P/e_2
control without DBMIB	3.8	3.6	0.95
—	1.3	0.2	0.15
TMPD	1.6	0.3	0.2
N,*N*-dimethyl-*p*-phenylenediamine	1.2	0.1	0.1
p-phenylenediamine	2.2	0.7	0.32
DAT	2.3	0.6	0.26
MMPD	2.3	0.6	0.3
DAD	1.7	0.3	0.18

values obtained in reversing NADP and ferricyanide photoreduction in the presence of $5 \cdot 10^{-6}$ M DBMIB are summarized. At higher concentrations of DBMIB the rate of NADP reduction (compare Tables I and III), is somewhat lower but no influence or actually an increase of the P/e_2 value in

the TMPD system is observed. On the other hand at the higher concentration of DBMIB the P/e_2 values in the PD and DAT system are reduced to about half. In the ferricyanide system higher concentrations of DBMIB reduce the rates as well as the P/e_2 values (compare Tables II and IV). In addition Table III shows, as already reported in the literature (Trebst [10]; Trebst and Pistorius [11]; Izawa *et al.* [15]), that the electron donor systems for photosystem I (*i.e.* a phenylenediamine + ascorbate) are coupled to ATP formation only in the case of DAD and MMPD, whereas the system *p*-phenylenediamine and *N*-substituted *p*-phenylenediamines + ascorbate are not coupled.

Discussion

Phenylenediamines kept reduced by ascorbate have been used extensively as electron donors for photosystem I, when electron flow from water through photosystem II is blocked by DCMU (Trebst [10]; Wessels [12]; Trebst and Pistorius [11]; Vernon *et al.* [14]; Izawa *et al.* [15]). These photoreductions by photosystem I are also insensitive to DBMIB (Trebst *et al.* [1]; Böhme *et al.* [2]). An important feature of photoreductions by photosystem I at the expense of phenylenediamines + ascorbate is that some systems are coupled to ATP formation (DAD (Trebst and Pistorius [11]) also DCIP (Losada *et al.* [16])), whereas others are not (TMPD, phenylenediamine, *N*-dimethylphenylenediamine (Trebst [10]; Wessels [12]; Izawa [15])). The difference between DAD (coupled electron donor) and TMPD (not coupled electron donor) has been related to the fact that DAD can carry hydrogens across the membrane whereas TMPD forms a radical ion instead of a chinoid diimine upon oxidation [17]. The oxidation is to occur by plastocyanin on the inside of the membrane because plastocyanin is located inside (Hauska *et al.* [17]). Only at rather high concentrations (10^{-3} M) of *p*-phenylenediamine (Arnon *et al.* [13]) or TMPD (Schwartz [18]) an ATP formation has been observed in photoreductions by photosystem I with these compounds.

These results were interpreted to indicate that electron flow through photosystem I and the electron transport chain from plastocyanin to NADP is not coupled to ATP formation. The energy conserving step or steps in non cyclic electron flow were thought to be closer to photosystem II. As has been documented by other methods one energy conserving step seems to be coupled to the oxidation of plastohydroquinone (Reinwald *et al.* [19]; Böhme and Cramer [20]). With the introduction of the plastoquinone antagonist, dibromothymoquinone, it was possible to study the properties of photoreductions by photosystem II. We have recently reported on the energy conservation in such photoreductions (Trebst and Reimer [4]). The results indicate that the reduction by photosystem II of the polar ferricyanide on the outer surface of the membrane yields a lower P/e_2 ratio than those of lipophilic *p*-benzoquinones reduced in or inside the membrane.

According to the results of Saha *et al.* [5] oxidized phenylenediamine may also be reduced by photosystem II. This reduction is neither inhibited by high concentration of KCN (an inhibitor of plastocyanin (Ouitrakul and Izawa [6])) nor by poly-lysine (Ort *et al.* [7]) nor by lower concentrations of DBMIB (Izawa *et al.* [8] and results in this paper) and is coupled to ATP formation with a P/e_2 value somewhat less than half the stoichiometry in Hill-reactions comprising both photosystems (like reduction of NADP or methylviologen).

Because these results in the literature had shown that *p*-phenylenediamines in the reduced form are electron donors for photosystem I (not inhibited by DBMIB) and that oxidized *p*-phenylenediamines are acceptors for photosystem II (also not inhibited by DBMIB) it was to be expected that phenylenediamines (in the absence of ascorbate!) are able to bypass the inhibition site of DBMIB. Such a restoration has already been observed by catalytic amounts of DCIP, though with rather low rates (Böhme *et al.* [2]). The results presented here substantiate this prediction. Certain *p*-phenylenediamines are reversing the DBMIB inhibition of NADP reduction. Because NADPH and ATP formation as well as oxygen evolution are restored, it is concluded that the inhibition site of DBMIB is bypassed and photosystem II is coupled again to photosystem I via the phenylenediamine. The properties of the phenylenediamines used are of important consequence as to the rate of restored electron flow and also to the ratio of coupled ATP formation. *N*-substituted *p*-phenylenediamines are highly active in restoring the rate of electron flow from water to NADP via photosystem I whereas *p*-phenylenediamine and *C*-substituetd phenylenediamines are less active. The P/e_2 ratios for NADP

reduction restored by *N*-substituted phenylenediamines (like TMPD) are approaching 1.0, the value for the control, and are independent of the DBMIB concentration. These high P/e_2 ratios seem surprising at first because – as discussed above – the photoreduction of NADP by photosystem I at the expense of TMPD + ascorbate is not coupled to ATP formation (Trebst[10]; Wessels[12]; Izawa *et al.*[15]). *C*-substituted *p*-phenylenediamines like DAT also show a P/e_2 ratio of 1.0 at low concentrations of DBMIB, but the ratio is decreasing proportional to increasing the DBMIB concentration with about 0.5 at the high concentration of $5 \cdot 10^{-6}$ M DBMIB. DAD and MMPD are cofactors of cyclic photophosphorylation, therefore the ATP values in these systems cannot be considered in the context.

The effect of substitution of the phenylenediamine in restoring NADP reduction in the presence of DBMIB is the opposite to the one in stimulation of the rate of ferricyanide reduction by photosystem II (*i. e.* in the presence of DBMIB). *N*-substituted *p*-phenylenediamines are practically inactive in stimulating, whereas *p*-phenylenediamine and the *C*-substituted derivatives are very active. In the latter cases the P/e_2 ratios in the presence of a low concentration of DBMIB are about 0.5 as Good's group has already reported (Ouitrakul and Izawa[6]; Ort *et al.*[7]). However, by increasing concentrations of DBMIB this ratios drops continuously below 0.5 and is 0.25 at $5 \cdot 10^{-6}$ M DBMIB (Table IV).

It is not sure, whether the high or low concentration of DBMIB is the more appropriate concentration to be considered. At lower concentrations of DBMIB the inhibition of electron flow through plastoquinone into photosystem I might not be complete. At higher concentrations DBMIB might exert additional effects. In addition to inhibiting the reoxidation of plastohydroquinone by the next carrier in the electron transport chain (possibly cytochrome f) (Böhme and Cramer[21]; Lozier and Buter[22]; Haehnel[23]), we would like to suggest that higher concentrations of DBMIB also inhibit passage and orientation of plastoquinone/plastohydroquinone through the membrane, required for hydrogen transport from the outside to the inside (see below). Also at higher concentration DBMIB, being reduced by photosystem II is autoxidized by oxygen or reacting with ferricyanide thus acting as lipophilic electron carrier. This is particularly obvious in experiments by Kimimura and Katoh[24], who introduced $HgCl_2$ treated chloroplasts to study photoreductions by photosystem II.

As basis for the explanation for the principal observation in this paper: "*N*-substituted phenylenediamines are active in restoring NADP reduction but are inactive in stimulating ferricyanide reduction, whereas just the opposite effect is observed with phenylenediamine and *C*-substituted phenylenediamines which are very active in ferricyanide reduction but only weakly active in NADP reduction" the side of the chloroplast thylakoid membrane involved in reduction and oxidation of a phenylenediamine is of deciding importance. The argumentation is as follows:

According to the hypothesis of Mitchell[25] the electron transport chain is traversing the membrane in loops. Two proton releasing sites (*i. e.* two energy conserving steps) are operating in photosynthetic electron transport: the water splitting reaction inside the membrane (Witt *et al.*[26, 27]) and the transport of hydrogens across the membrane by plastohydroquinone (Reinwald *et al.*[19]). Accordingly plastoquinone is reduced by photosystem II outside or close to the outside of the membrane picking up protons from the outside. Plastohydroquinone is oxidized inside the membrane, releasing protons inside (Reinwald *et al.*[19]). Photosystem I oxidizes its primary electron donor, possibly plastocyanin on the inside of the membrane (Schliephake *et al.*[27]; Hauska *et al.*[28]). The polar NADP and ferricyanide are reduced on the outside. DBMIB inhibits the reoxidation of plastohydroquinone by photosystem I (via cytochrome f) as discussed above but not the reduction of plastoquinone by photosystem II. DBMIB at higher concentrations might also interfere with the orientation of plastoquinone/plastohydroquinone in the lipid phase, needed for transporting hydrogens across the membrane.

Oxidized phenylenediamines are reduced on the inside by plastohydroquinone in a reaction which is not inhibited by DBMIB. Reduced phenylenediamines may be oxidized by ferricyanide outside the membrane or inside by plastocyanin. In the later case the phenylenediamine is connecting photosystem I back onto photosystem II past the inhibition site of DBMIB.

From these points it follows that DBMIB interrupts electron flow on the inside of the membrane. In NADP reduction two carriers on the inside of the membrane have to be reconnected by a phenylene-

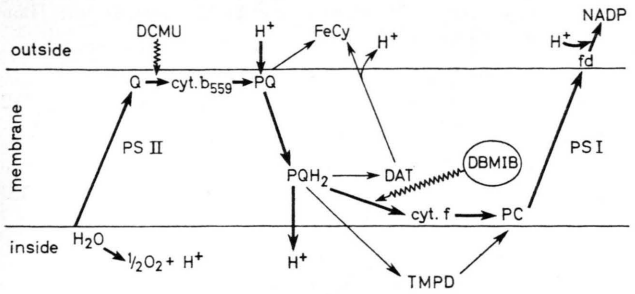

Fig. 1. Scheme of photosynthetic electron flow with the inhibition site of DBMIB being bypassed by TMPD on the inside of the membrane in NADP reduction and the stimulation of ferricyanide photoreduction by DAT which is reduced in or inside the membrane and oxidized outside.

Heavy arrow = native electron flow.
Two proton releasing sites inside are indicated, but no H^+/e stoichiometry.

diamine, *i. e.* a phenylenediamine is oxidized and reduced inside. At the start of the reaction reduced phenylenediamine has to permeate through the membrane. In ferricyanide reduction by photosystem II the phenylenediamine is also reduced inside, but oxidized outside. It will have to traverse the membrane in the reduced state from the inside to the outside and in the oxidized state from the outside to the inside. In addition of a reduction of ferricyanide by photosystem II a restoration of reduction by photosystem I is feasible. (In photoreductions of NADP by photosystem I by a phenylenediamine plus ascorbate or in cyclic photophosphorylation catalysed by certain phenylenediamines the reduced form has to travel from the outside to the inside and the oxidized form from the inside to the outside.)

The chemistry of the phenylenediamine is of importance as to the coupling of the reaction. *N*-substituted phenylenediamines are oxidized to the positively charged stable radical. There is no hydrogen transfer in their redoxreactions, whereas in redoxreactions with phenylenediamine and *C*-substituted phenylenediamines hydrogens are involved. Depending on the pH of the compartment (inside or outside) and on the pK of the oxidized and reduced forms the phenylenediamines are protonated. By travelling from the inside to the outside (in ferricyanide reduction) the reduced phenylenediamine or *C*-substituted phenylenediamines take hydrogens back to the outside. This is an uncoupling effect which is stoichiometric to electron transport. Therefore the P/e_2 ratio is lowered in the ferricyanide system, but not in the NADP system, where the phenylenediamines are oxidized and reduced inside.

The pH gradient built up in electron transport across the membrane lowers the pH inside and increases the redoxpotential of the phenylenediamine closer to the redoxpotential of plastocyanin, limiting electron transport with some of the compounds.

In the presence of ferricyanide the phenylenediamines are kept oxidized outside. Positively charged phenylenediamine radicals like TMPD will not penetrate the membrane against the membrane potential, which is positive inside. Therefore TMPD is inactive in stimulating ferricyanide reduction, because it can neither connect photosystem II to ferricyanide nor can it connect photosystem II to photosystem I past the DBMIB inhibition site because it remains outside. The oxidized form of phenylenediamine and *C*-substituted phenylenediamines would still travel through the membrane as neutral forms and would actually be pulled by protonisation due to the lower pH inside.

The scheme in Fig. 1 summarizes some of the points as to the pathway of electron flow in NADP and ferricyanide reduction, when the two groups of phenylenediamines represented by TMPD and DAT reverse the DBMIB inhibition. The results of this paper interpreted in the view of the Mitchell-hypothesis suggest that the energy conserving sites in non cyclic electron flow are connected with photosystem II. Photoreductions by photosystem II are coupled to ATP formation with P/e_2 ratios lower than those of photoreductions by photosystem II plus I. However, the conclusion of Izawa *et al.* [8] that therefore an energy conserving site is operating after the DBMIB inhibition site is not quite valid and has to be reevaluated by considering the sides of the membrane involved and the hydrogen carrying properties of the carriers used.

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