Site of Action of 2,5-Dimethoxy-3,6-Dichloro-*p*-Benzoquinone in the Photosynthetic Electron Transport Chain

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Dichlorodimethoxy-*p*-benzoquinone (DCDMQ) was tested for its site of action in the photosynthetic electron transport chain. Hill reaction mediated by DCDMQ was insensitive to DBMIB (1 μm) but sensitive to DCMU, suggesting its site of action before plastoquinone but after Q – the primary electron acceptor of photosystem II. Extraction of freeze-dried chloroplasts with heptane and analyzing their capacity to photo-oxidize water using various Hill oxidants revealed that silicomolybdate (SiMO) and DCDMQ could effectively restore the activity. Diaminodurene (DAD) in the presence of ferricyanide could restore 40% of the activity. But ferricyanide alone failed to restore the ability to photo-oxidize water in heptane extracted chloroplasts. Similarly, Na_2SO_3 which is known to cause a bottleneck in the electron flow at plastoquinone affected the ferricyanide Hill reaction. Hill reactions mediated by SiMO and DCDMQ were insensitive to the addition of Na_2SO_3, suggesting that both these oxidants intercept electrons before plastoquinone. But 50% of the activity was lost when sulfite was added to the Hill reaction mediated by DADox. DNP-INT, melittin and picrylhydrazyl were recently introduced as photosystem II inhibitors inhibiting the electron flow between Q and the PQ pool. While DCBQ and DCDMQ Hill reactions were insensitive to DNP-INT, ferricyanide was highly sensitive. The quinonediamines TMPD and DADox showed 50% decrease in the electron transport rate, similar to heptane extracted or sulfite inhibited chloroplasts. Melittin increased the electron transport rate when ferricyanide or TMPD was the Hill oxidant, while DCBQ and DCDMQ reduction remained unaffected. However, DADox Hill reaction showed 50% inhibition in the presence of melittin. Picrylhydrazyl – which inhibits the electron flow between Q and the PQ pool – inhibited the Hill reaction of all the PS II electron acceptors except that of DCDMQ. It is possible that there is another site of intercepting electrons between Q and plastoquinone before the site where most of the quinonediamines accept electrons.

Introduction

One of the notable recent developments is the introduction of a new group of Hill oxidants which are capable of intercepting electrons from photosystem II, even in unfragmented chloroplasts. These photosystem II electron acceptors, also called class III acceptors, are lipid soluble oxidants with high redox potentials. The use of these acceptors have provided ample information regarding the sequence of energy conserving steps and the topography of the photosynthetic electron transport chain in the chloroplast membrane. Benzoquinones and phenylenediamines reduced by photosystem II are insensitive to DBMIB [1, 2] or KCN [3] and their site of reduction seems to be plastoquinone [4]. Hill reaction mediated by both these compounds are sensitive to DCMU and is coupled to photophosphorylation, although with only half the stoichiometry [5–7]. DCMU insensitive Hill reaction with $HgCl_2$ [8], silicomolybdate [9], silicotungstate [10], phosphomolybdate and phosphotungstate [11] as electron acceptors have been reported. It was shown that by treating the chloroplast with trypsin, the sensitivity of the Hill reaction with ferricyanide for DCMU is lost [12]. Thus, there are two groups of electron acceptors intercepting electrons either from Q in a DCMU insensitive reaction or from the PQ pool in a DBMIB insensitive

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Abbreviations: *p*-BQ, *p*-benzoquinone; DAD, diaminodurene or 2,3,5,6-tetramethyl-*p*-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-1,6-isopropyl-1,4-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCDMQ, 2,5-dimethoxy-3,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCPIP (DPIP), dichlorophenol indophenol; DNP-INT; 2-iodo-6-isopropyl-3-methyl-2′,4,4-trinitrodiphenyl ether; DNPBNT, 2-bromo-6-isopropyl-3-methyl-2′,4,4′-trinitrophenyl ether; KCN, potassium cyanide; MV, methylviologen; PQ, plastoquinone; PS I, II, photosystem I and II; picryl hydrazyl, 2,2-diphenyl-1-picryl hydrazyl; Q, quencher, primary electron acceptor of photosystem II; R, the secondary electron acceptor; SIMO, silicomolybdate; TMPD, N,N,N′N′-tetramethyl-*p*-phenylenediamine.

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reaction. Resolution of the sites of electron acceptance between the DCMU and DBMIB site has been made possible by the introduction of specific inhibitors such as DNP-INT [13], stable radicals [14], substituted *p*-benzoquinones [15] and melittin [16] which inhibit the electron flow between Q and the PQ pool. Recently, substituted benzoquinones with high electron affinity as photosystem II electron acceptors have been introduced [17]. Analysis of various electron acceptors in the presence of different PS II inhibitors revealed that DCDMQ intercepts electrons from R or (B) in the photosynthetic electron transport chain.

Materials and Methods

Chloroplasts were isolated from 10-day old pea plants as described previously [17].

Oxygen evolution or uptake was measured polarographically in a Clark type O_2 electrode at 25 °C, at an irradiance of 100 MW/cm^2.

Heptane extracted chloroplasts were prepared according to the method described by Cox and Bendall [18]. The chloroplasts suspension containing 1 mg chlorophyll/ml was frozen onto the walls of a 500 ml round bottomed flask as a thin layer. Freeze-drying was continued until the flask reached room temperature and the freeze-dried chloroplasts were extracted at room temperature, with heptane at a ratio of 30–40 ml of heptane/mg of chlorophyll in a stoppered conical flask with continuous shaking for 3 h in the dark. The extracted chloroplasts were separated by centrifugation, washed with a small volume of fresh heptane, and evaporated under a stream of N_2. The extracted chloroplasts were re-suspended in the isolation medium and tested for their electron transport capacity. During the process of heptane extraction the chloroplasts were protected from light.

For picryl-treatment chloroplasts were incubated in different concentrations of picrylhydrazyl for 15 min between 0–4 °C in the dark. After washing the incubated plastids in the re-suspension medium once, they were tested for their Hill activity. All other electron acceptors and inhibitors were added directly into the reaction vessel at specified concentrations.

Results and Discussion

In order to probe into the site of action of DCDMQ, the electron transport chain was analyzed using various inhibitors. DBMIB is a plastoquinone antagonist (at 1 μm concentration), and any compound that intercepts electrons before plastoquinone would be insensitive to its addition [19, 20]. Similar rates of O_2 evolution obtained with DCDMQ in the presence and the absence of DBMIB (Table I) suggest that it should intercept electrons at or before plastoquinone. However, it is sensitive to the inhibition of the electron flow by DCMU, suggesting that it is not accepting electrons directly from Q – the primary electron acceptor. Therefore, DCDMQ should intercept electrons between Q and PQ in the photosynthetic electron transport chain. The stimulation of electron flow by NH_4Cl and nigericin in DCDMQ Hill reaction suggestes that the reaction is coupled to photophosphorylation.

It is known that extraction of freeze-dried chloroplasts with non-polar solvents leaves most of the lipids *in situ*, including nearly all the chlorophyll. But the ability to photo-oxidize water is lost and could be recovered by the exogenous addition of plastoquinone [18, 21, 22]. Since plastoquinone is extracted from the chloroplasts, electron acceptors intercepting electrons before PQ (and not at or beyond PQ) alone could restore the rate of Hill reaction in heptane extracted chloroplasts. Silicomolybdate and DCDMQ were able to effectively restore O_2 evolving capacity

Table I. Rates of O_2 evolution in pea chloroplasts mediated by different Hill oxidants. The assay mixture contained 25 mM Hepes–KOH, pH 7.5, 3 mM $MgCl_2$, 50 mM Sorbitol and 15 mM KCl. The final concentrations of the electron acceptors/inhibitors/uncouplers were: DCDMQ 0.5 mM, DCMU 5 μM, ferricyanide 2 mM, DBMIB 0.5 μM, methyl viologen 1 mM, ammonium chloride 1 mM and nigericin 5 μM. Chlorophyll concentration was 30 μg/ml. For further details see Methods.

Additions	Electron Transport rate [μmol O_2/ mg Chl · h]
+ DCDMQ	74.27 ± 8.5
+ DCDMQ + DCMU	0
+ DCDMQ + DBMIB	72.72 ± 6.6
+ ferricyanide	77.40 ± 5.3
+ ferricyanide + DBMIB	0
+ ferricyanide + DBMIB + DCDMQ	77.89 ± 5.8
+ methyl viologen	35.66 ± 5.6
+ methyl viologen + DBMIB	0
+ methyl viologen + DBMIB + DCDMQ	71.12 ± 4.3
+ DCDMQ	74.63 ± 7.5
+ DCDMQ + ammonium chloride	196.28 ± 8.0
+ DCDMQ + nigericin	335.83 ± 9.3

in heptane extracted chloroplasts (Table II), suggesting that both these acceptors intercept electrons before PQ. When DAD was the Hill oxidant, there was no activity with DAD alone, but about 40% of the control activity was observed when ferricyanide was added, along with DAD. Compounds, which in the oxidized state are acceptors for PS II before DBMIB, are donors for PS I after DBMIB in the reduced form, if they can bypass the DBMIB block by reconnecting photosystems II and I and a good example is DAD [11, 23]. Therefore, the restoration of the ability to photo-oxidize water by DAD in heptane extracted chloroplasts could probably be due to a bypass of the PQ pool. However, this does not explain the decrease in the rate of O_2 evolution observed after heptane extraction. When DCDMQ and silicomolybdate showed 80% of the control rates after heptane extraction, DADox showed only about 40% of the control rates. This could be due to the possibility of DADox accepting electrons both from the PQ pool and from a site before the PQ pool. When most of the PQ pool is extracted, one of the two sites of intercepting electrons could have been damaged. Since ferricyanide accepts electrons after PQ, the rate of O_2 evolution in heptane extracted chloroplasts were very low (Table II).

Recently, it was shown that Na_2SO_3 blocks the electron flow at plastoquinone [24] because of the following reasons: 1) Electron flow from H_2O to silicomolybdate and $DCPIPH_2$ to MV remained mostly unaffected; 2) increase in the *in vivo* Chl a fluorescence, induced by Na_2SO_3, was observed only in the absence of DCMU, suggesting that the site of action of $SO_3^=$ in the photosynthetic electron transport chain was only after the DCMU inhibition site.

Table II. Rates of O_2 evolution in Control and Heptane extracted pea chloroplasts in the presence of different Hill oxidants. For details of assay conditions, see Table I. The final concentrations of the Hill oxidants were: Silicomolybdate 25 µM; Diaminodurene 0.5 mM and 0.5 mM ferricyanide; DCDMQ 0.5 mM; ferricyanide 2 mM. For details of heptane extraction see Methods.

Electron acceptor	Electron transport rate [µmol O_2/mg Chl · h]		% of Control
	Control	Heptane extracted	
Silicomolybdate	58.2 ± 4.5	45.4 ± 5.6	78.0
DAD/Ferricyanide	128.35 ± 5.3	55.65 ± 2.1	43.3
Ferricyanide	70.8 ± 3.3	10.65 ± 1.2	15.0
DCDMQ	81.45 ± 4.3	61.95 ± 2.7	76.1

Table III. Effect of Na_2SO_3 on the rates of O_2 evolution in pea chloroplasts in the presence of different electron acceptors. Na_2SO_3 was added in the light at a final concentration of 5 mM. For details of assay conditions see Tables I and II.

Electron acceptor	Electron transport rate [µmol O_2/mg Chl · h]		% of Control
	Control	+ Na_2SO_3	
Silicomolybdate	51.00 ± 8.6	48.00 ± 7.9	94.1
DAD/Ferricyanide	130.37 ± 9.7	65.17 ± 5.3	49.9
Ferricyanide	78.75 ± 4.5	0	0
DCDMQ	79.12 ± 3.9	74.48 ± 5.6	94.2

It is clear from Table III that Na_2SO_3 inhibits the electron flow when ferricyanide is the Hill oxidant, but does not affect the Hill reactions mediated by silicomolybdate and DCDMQ. DAD showed 50% of the activity in $SO_3^=$ inhibited chloroplasts in the presence of ferricyanide, indicating again that it could bypass the electrons and probably accept electrons from two sites in the electron transport chain. The possibility of direct action of $SO_3^=$ on ferricyanide was excluded from the observation that further addition of ferricyanide in excess could not relieve $SO_3^=$ inhibition of O_2 evolution. (The direct chemical reduction of ferricyanide by Na_2SO_3 was also checked by recording absorption spectra.)

Studies using the recently introduced PS II inhibitors between Q and the PQ pool provided additional insight into the site of action of these electron acceptors. It was shown by Trebst *et al.* [13] that DNP-INT inhibits the electron flow between R and PQ as do higher concentration of DBMIB [23]. Recent findings of Sarojini *et al.* [27] (Table IV) show that while DCBQ and DCDMQ Hill reactions are insensitive to DNP-INT, ferricyanide is highly sensitive. The other quinonediamines TMPD and DADox showed 50% decrease in the electron transport rate, similar to the heptane extracted or sulfite inhibited chloroplasts. Barr and Crane [14] showed that 2,2-diphenyl-1-picrylhydrazyl inhibited PS II reactions and all PS II reaction except that of silicomolybdate could be restored by α-tocopherol, suggesting that one of the picrylhydrazyl affected sites of the electron transport chain in PS II is between the DCMU and DBMIB inhibition site. In order to avoid the possible direct action of picrylhydrazyl on the electron acceptors, the chloroplasts were incubated in the presence of various concentrations of picrylhydrazyl for 15 min to remove

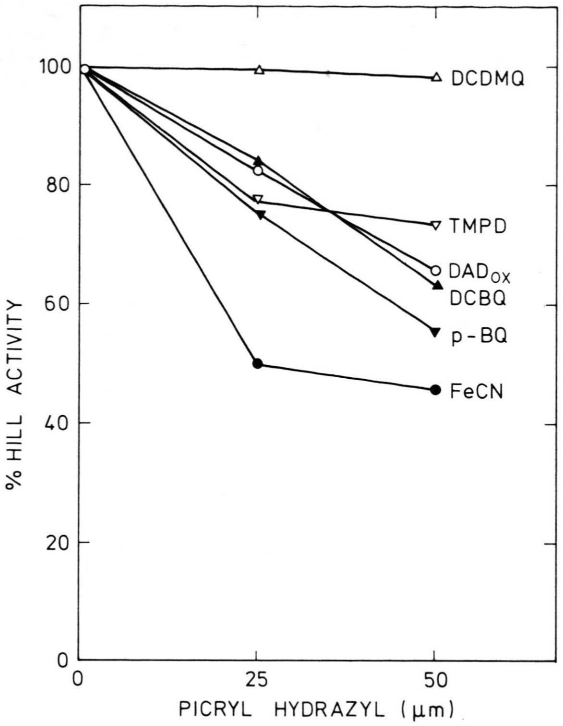

Fig. 1. Effect of picrylhydrazyl treatment on the Hill reactions of different electron acceptors. Chloroplasts were incubated in different concentrations of picrylhydrazyl for 15 min between 0°–4 °C in the dark, washed in the resuspension medium and assayed for their Hill activity. Control chloroplasts were also given identical treatments in the absence of picrylhydrazyl. The assay conditions and final concentration of the electron acceptors are given in Tables I and IV. Control (100%) rates of the indicated reactions were (in µmol O_2/mg Chl · h): △–△ DCDMQ, 86; ▽–▽ TMPD, 140; ▼–▼ *p*-benzoquinone, 56; ○–○ DADox, 138; ●–● ferricyanide 82; and ▲–▲ DCBQ, 84.

Table IV. Effect of DNP-INT on the Hill reactions of different electron acceptors. DNP-INT at a final concentration of 3.5 µM was added directly to the reaction vessel. The final concentrations of the Hill oxidants were: DCBQ 0.5 mM; DCDMQ 0.5 mM; TMPD 0.5 mM; DAD 0.5 mM and 0.5 mM ferricyanide; *p*-benzoquinone 0.5 mM; ferricyanide 2 mM. For details of the conditions see Table I. After G. Sarojini, H. Daniell, W. Vermaas, and Govindjee [27].

Electron acceptor	Electron transport rate [µmol O_2/mg Chl · h]		% of Control
	Control	+ DNP-INT	
ferricyanide	80.66 ± 8.7	13.44 ± 3.5	16.6
DAD_{ox}	137.77 ± 20.1	68.89 ± 11.2	50.0
TMPD	136.19 ± 8.6	70.37 ± 3.8	51.7
p-benzoquinone	48.39 ± 6.5	22.43 ± 5.5	48.2
DCBQ	84.37 ± 8.3	80.20 ± 9.7	95.1
DCDMQ	86.43 ± 11.0	85.89 ± 11.2	99.3

Table V. Effect of melittin on the Hill reactions of different electron acceptors. Melittin was added at a final concentration of 5 µM directly into reaction vessel. Details of assay conditions and final concentrations of the Hill oxidants are given in Tables I and IV.

Electron acceptor	Electron transport rate [µmol O_2/mg Chl · h]		% of Control
	Control	+ 5 µM Melittin	
ferricyanide	81.86 ± 5.5	135.90 ± 4.9	166
DAD_{ox}	130.10 ± 8.3	70.25 ± 5.2	54
TMPD	125.32 ± 9.8	181.72 ± 6.3	145
p-benzoquinone	52.50 ± 4.9	78.06 ± 5.7	149
DCBQ	84.10 ± 6.5	83.51 ± 8.2	99
DCDMQ	87.50 ± 4.3	85.75 ± 5.1	98

tocopherol and after washing them once with resuspension medium, were tested for their Hill activity. Picryl-treatment affected DCBQ, ferricyanide and DADox severely, while TMPD was less affected. But DCDMQ remains insensitive to picryl-treatment (Fig. 1). More recently, melittin-a lytic peptide was introduced as an inhibitor of PS II capable of resolving the two sites of electron acceptance prior to DBMIB block by quinones and quinonediamines [16]. In our experiments with melittin, there was increase in the electron transport rate when ferricyanide and TMPD were the Hill oxidants, while the DCDMQ and DCBQ reaction remained unaffected. However, DADox Hill reactions showed slight inhibition. The interpretation of the results on melittin inhibition becomes more complicated with its direct action on the membranes increasing the bilayer permeability [16]. The partial inhibition of some of the quinonediamine reactions by melittin has been interpreted as a clue to resolve the two sites of electron acceptance prior to DBMIB block [16]. Similar partial inhibition of quinonediamine reactions were observed earlier in the presence of DNP-INT [13], higher concentrations of DBMIB [23] or in heptane extracted or sulfite inhibited chloroplasts (see Tables II, III). It is probable that these quinonediamines may accept electrons from two sites at and before PQ. When the reduction of PQ is affected, the electron transport rate is diminished due to hampering of one of the two sites of electron acceptance. Therefore, melittin probably inhibits the electron flow at the same site as DNP-INT or 10 µm DBMIB. Since DCDMQ remains insensitive to picryl-treatment, it is possible that there is another site of intercepting electrons between Q and PQ before the site where most of the quinonediamines accept

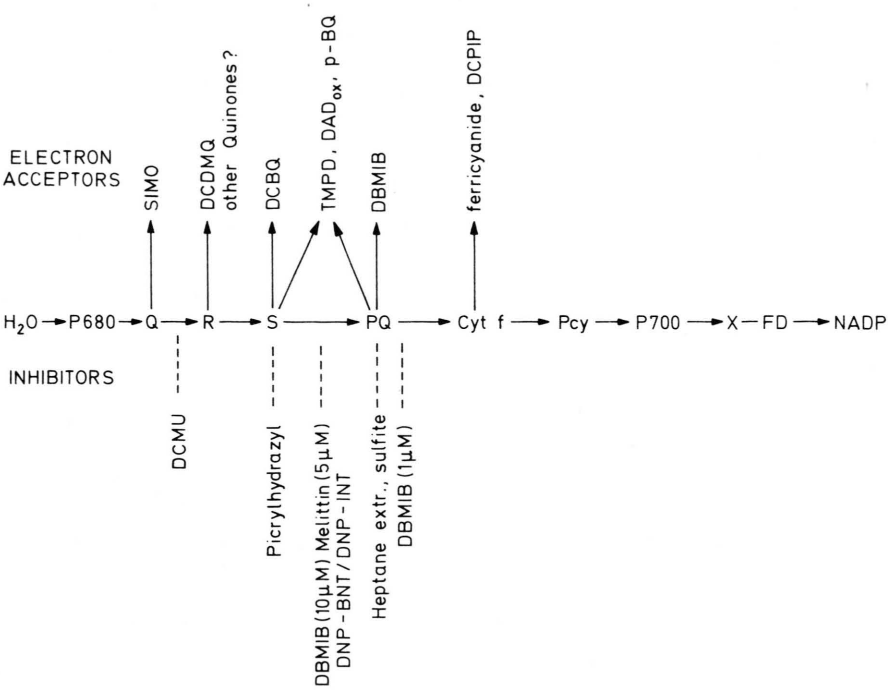

Fig. 2. Proposed sites of inhibition and acceptance by different photosystem II inhibitors (- - -) and electron acceptors (→). Scheme suggests that there are two sites of electron acceptance between Q and the PQ pool. Acceptors intercepting electrons at the first site (R) are insensitive to picrylhydrazyl treatment. Those accepting electrons from the second site (S) are insensitive to DNP-INT, 10 µM DBMIB and Melittin but sensitive to picrylhydrazyl treatment. The quinonediamines may accept electrons from two sites *i.e.,* from PQ and S because they are affected only partially by the inhibitors that inhibit the reduction of PQ. P680, photosystem II reaction center; Q, primary electron acceptor of PS II; R and S, the proposed two sites of acceptance of electrons by different electron acceptors; PQ, plastoquinone pool; cyt. f, cytochrome f; Pcy, plastocyanin; P700, PS I reaction center; x, primary electron acceptor of PSI; FD ferredoxin; NADP, Nicotinamide-adenine dinucleotide phosphate oxidised.

electrons. The quinonediamines also differ from the quinones in their redox potential and response to uncouplers [11]. Electron transport between Q and PQ involves an additional component situated between Q and PQ which is reduced by Q^- in two one-electron transfer reactions and oxidized by PQ in a two-electron transfer reaction [25]. Plastoquinone is a two-electron acceptor, and R must accumulate two negative charges

$$[Q \cdot R \xrightarrow{h\nu} Q^- \cdot R \leftrightarrow Q \cdot R^- \xrightarrow{h\nu} Q^- \cdot R^- \leftrightarrow Q \cdot R^{2-}]$$

before it is reoxidized by the PQ pool [26]. Therefore, we suggest that there could be two sites of electron acceptance between Q and the PQ pool, the first site (R) where DCDMQ (probably other substituted benzoquinones) accepts electrons and the second site (S) where most of the quinonediamines intercept electrons (Fig. 2).

Since bicarbonate depletion is known to affect the electron flow between Q and the PQ pool in isolated chloroplasts, it would be an ideal system to resolve the precise site of action of these PS II electron acceptors. The results on the depletion and reconstitution with bicarbonate in the presence of various Hill oxidants in isolated thylakoids done in collaboration with Prof. Govindjee will be published elsewhere [27].

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