THE INHIBITION OF PHOTOSYNTHETIC ELECTRON FLOW BY DCCD

An indication for proton channels

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1. Introduction

Dicyclohexylcarbodiimide (DCCD) is a well known inhibitor of photophosphorylation [1,2] and oxidative phosphorylation [3]. It prevents ATP formation and also suppresses electron flow, the latter rate being restored by the addition of an uncoupler [4]. This shows that DCCD behaves like a classical energy transfer inhibitor. Its mode of action is due to a binding onto an amino acid of the base piece or proton channel (CF_0 or F_0) of the coupling system, which indirectly affects the rate of electron flow [5]. In the photosynthetic electron flow system of chloroplasts however, it is known that DCCD at somewhat higher concentrations has another effect on electron flow in addition to that of an energy transfer inhibitor. The basal (not coupled) rate of electron flow is sensitive to the addition of DCCD but the reversal of the inhibition of the coupled rate by an uncoupler is not complete [1]. This additional effect of DCCD has not been analysed yet in detail, let alone localized at a specific site [6]. By using recent progress in artificial donor and acceptor systems for photosynthetic electron flow we tried to localize an effect of DCCD on the electron flow itself. The data suggest that DCCD affects electron flow at two sites viz the reduction of PQ and its oxidation. It is argued that in vectorial electron flow, proton uptake from outside and release inside the thylakoid membrane in the reduction/oxidation cycle of PQ may involve proton translocation through proton channels and that these may be affected by DCCD.

Abbreviations: chl, chlorophyll; DAD, diaminodurene; DBMIB, 2,5-dibromothymoquinone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea; DCPIP, 2,6-dichlorophenolindophenol; DMMDBQ, dimethylmethylenedioxy-*p*-benzoquinone; DQ, duroquinol; Mv, methyl viologen; PQ, plastoquinone; PS, photosystem; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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2. Methods

Chloroplasts were isolated from market spinach by the procedure in [7] except that ascorbate and BSA were omitted from the grinding medium. The chloroplast pellet was resuspended in a buffer containing: Tricine, 10 mM; sucrose, 0.4 M; NaCl, 10 mM. The electron transport was measured by monitoring the oxygen consumption or evolution polarographically at 20°C with an oxygen electrode (Rank Bros.). The light provided by the projector lamp was passed through a set of two filters (type KG 2 and RG 645 from Jenaer Glaswerk Schott and Gen., Mainz). The incident intensity was 10^5 ergs . cm^{-2} . s^{-1} and was saturating for the reactions studied. Ferricyanide reduction was measured at 420 nm. The conditions of reactions and the concentrations of different compounds used are given under the figures and table.

PQ was isolated from spinach leaves as in [8] and was crystallized from ethanol. It was redissolved in ethanol and its concentration in solution was calculated from the difference spectra at 255 nm using $\epsilon_{mM} = 15$.

3. Results

The effect of different concentrations of DCCD on electron transport from H_2O to methyl viologen in washed spinach thylakoid preparation is shown in fig.1. The experiments were run in the presence of an uncoupler, NH_4Cl, at 10 mM. Under these conditions the energy transfer inhibitor properties of DCCD do not interfere. With increasing concentrations of DCCD there is a progressive inhibition of the rate of electron transport and 50% inhibition is observed at 30 μM DCCD. The experiments were also repeated using another uncoupler, gramicidin, at 10 μM. The DCCD effect is identical irrespective of the uncoupler used (data not shown).

The inhibition by DCCD of the rate of electron flow from H_2O to Mv is also found to be similar in the presence of TMPD and DBMIB (fig.1). The electron flow in the presence of a TMPD bypass does not go through the native plastohydroquinone oxidation site which is blocked by DBMIB [9]. Rather the $TMPD_{ox}$ oxidizes the plastohydroquinone chemically at the inside of the thylakoid [10] and the $TMPD_{red}$ is oxidized through PS I via plastocyanin. The data

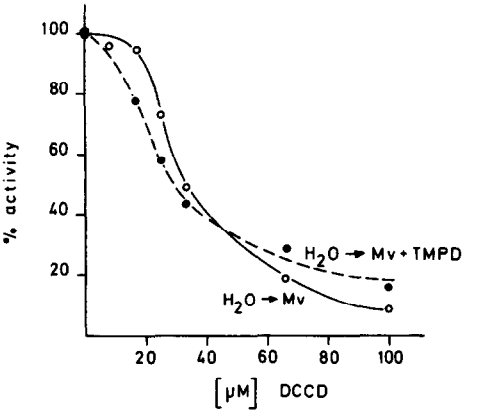

Fig.1. Effect of DCCD on the reduction of Mv with H_2O as the electron donor in the presence of NH_4Cl. The reaction mixture in 3 ml total vol. contained Tricine–NaOH, (pH 8.0) 50 mM; NaCl, 50 mM; $MgCl_2$, 5 mM; Mv, 0.1 mM; NaN_3, 0.3 mM; NH_4Cl, 10 mM; and chloroplasts equivalent to 50 μg chl. In the case of TMPD bypass, TMPD (30 μM) and DBMIB (1 μM) were added. The DCCD solution in methanol was added prior to the addition of chloroplasts. The control rates without DCCD from water to Mv and in the presence of TMPD bypass were 857 and 675 μequiv . μg chl^{-1} . h^{-1}, respectively.

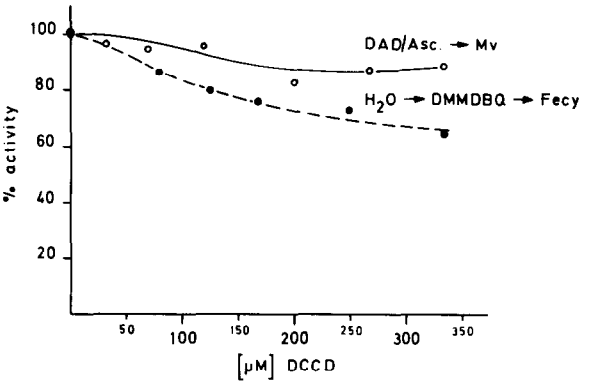

Fig.2. Effect of DCCD on the photoreduction of ferricyanide with H_2O as electron donor and Mv with DAD/ascorbate as electron donor. The reaction mixture as in fig.1 contained either: ferricyanide, 3 mM; DMMDBQ, 0.4 mM; DBMIB, 1 μM; or Mv, 0.1 mM; NaN_3, 0.3 mM; DAD, 1 mM; ascorbate, 3 mM; DCMU, 1 μM. The control rates in the absence of DCCD were 390 μequiv. and 1740 μ equiv. for ferricyanide and Mv reduction, respectively. NH_4Cl did not improve the rates of either reaction.

in fig.1 then indicate that the electron flow from H_2O to Mv is primarily inhibited by DCCD at a stage prior to the oxidation of plastohydroquinone as the TMPD bypass does not overcome the DCCD inhibition.

The inhibition by DCCD is not beyond the TMPD donor site to PSI via plastocyanin as the electron transport from DAD/ascorbate to Mv is virtually insensitive to DCCD (fig.2). Even at 300 μM DCCD this PS I reaction is inhibited by 10% only. The electron flow sequence from H_2O to ferricyanide through PS II in the presence of DBMIB and the lipophilic mediator DMMDBQ [11] is also only slightly inhibited by DCCD (fig.2). We also checked the photoreduction of DCPIP in the presence of DBMIB and in the presence or absence of DMMDBQ. These reactions showed the same insensitivity to DCCD (data not shown). These results indicate that the section of electron transport chain which is affected by DCCD lies beyond the site at which PS II acceptors such as DMMDBQ withdraw electrons but before the oxidation of DAD or TMPD by PS I.

The introduction of an electron donor to the PQ permits study of electron transport from plastohydroquinone to Mv. The donor DQ [12,13] has been shown to reduce PQ in the presence of DCMU.

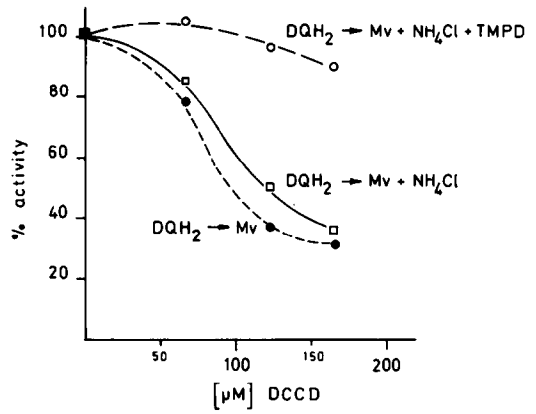

Fig.3. Effect of DCCD on the reduction of Mv with Dq as the donor. The reaction mixture for Mv reduction as in fig.1 additionally contained DCMU, 1 μM and DQ 0.5 mM (freshly prepared in methanol). NH_4Cl was added after observed the basal rates. For TMPD bypass TMPD (30 μM) and DBMIB (1 μM) were added. In the absence of DCCD the basal rate was 342, the uncoupled rate 737 and the TMPD bypass rate 1440 μequiv. mg chl^{-1} . h^{-1}.

Table 1
Effect of externally added plastoquinone on the rate of electron transport from H_2O to methyl viologen in the presence and absence of DCCD

Addition	Rate of electron transport (μequiv. mg chl^{-1} . h^{-1})	
	−PQ	+ PQ
1. Control	155	170
2. + NH_4Cl	565	565
3. + NH_4Cl + 30 μM DCCD	297	424
4. + NH_4Cl + 60 μM DCCD	170	254
5. + NH_4Cl + 75 μM DCCD	113	226

Basic conditions as in fig.1 for Mv reduction except NH_4Cl (10 mM) and PQ (30 μg) were added only in some reactions

The PQH_2 is oxidized through the native plastohydroquinone oxidation site as it is practically completely inhibited by DBMIB [13]. The inhibition of Mv reduction by DBMIB from DQ can be overcome by TMPD as it bypasses the DBMIB inhibition site (unpublished observations). The curves in fig.3 show that the electron flow from DQ to Mv in the absence (basal rate) as also in the presence of NH_4Cl (the extent of stimulation of the electron transport by the uncoupler is 2–3-fold) is indeed inhibited by DCCD but at higher DCCD concentration than the one needed for the inhibition of H_2O to Mv electron flow. To observe a 50% inhibition of the rate of electron flow from DQ to Mv 120 μM DCCD is required. In the presence of DBMIB, the TMPD bypass completely restores the electron flow and this DBMIB-insensitive TMPD-catalyzed photoreduction to Mv from DQ is virtually insensitive to DCCD. These data suggest that DCCD also interferes with the oxidation of PQH_2 through the native oxidation site. If this site is excluded by DBMIB and a TMPD bypass the sensitivity is lost. It thus appears that there are two sites at which DCCD interferes with the photosynthetic electron flow, one in the reduction of PQ and another in the oxidation of PQH_2.

Since the experiments so far indicate that the reduction and oxidation of PQ is affected by DCCD we tried to find out if PQ added externally can overcome this inhibition. The data presented in table 1 show that addition of 30 μg PQ (this is ≃ 10 fold the amount of PQ present in the chloroplasts) to the DCCD-inhibited non-cyclic electron flow from H_2O to Mv restores the electron flow to a considerable extent. It has very little effect on the control basal rate or the uncoupled rate.

It may be pointed out that the sequence of addition to the reaction mixture of uncoupler and DCCD is not important indicating that the preincubation with DCCD is not necessary. Different chl:DCCD ratios change the concentration of DCCD needed for 50% inhibition. At higher chlorophyll concentrations the amount of DCCD required for 50% inhibition is high as is expected.

4. Discussion

DCCD at low concentrations (depending on chlorophyll concentration) acts as an energy transfer inhibitor and in this way indirectly affects the electron flow rate in addition to blocking ATP formation. A direct effect of DCCD on the electron flow system itself (already suspected by earlier observations [1]) was therefore studied in uncoupled systems to dissociate its direct effect on electron flow from the indirect one by its energy transfer inhibitor property. The effect of 30 μM, and above, DCCD on

partial reactions (see fig.2,3) of the electron flow system chloroplasts was used to localize the inhibition site(s). The results are:

1. Electron flow from water to an acceptor of PS I like Mv, is considerably sensitive to DCCD.
2. Neither the reduction of a PS II electron acceptor (like ferricyanide + lipophilic mediator in the presence of DBMIB) nor the photoreduction of Mv at the expense of an artificial donor for PS I via plastocyanin-like DAD/ascorbate is sensitive to DCCD.
3. The DQ donor system for PS I photoreductions, which is DBMIB-sensitive and passes through PQ, is inhibited by DCCD, though at concentrations higher than those for non-cyclic electron flow from water.
4. The TMPD system, which bypasses the DBMIB inhibition site (see fig.1) and the native plastohydroquinone oxidation system in non-cyclic electron flow from water to Mv, is still sensitive to DCCD, though a little less so than the control system.
5. A TMPD bypass in the DQ system is no longer DCCD-sensitive (fig.3).

These results suggest that DCCD interferes with the electron transport system at two places, one at the PQ reduction site, and another at plastohydroquinone oxidation. The PQ reduction site is more sensitive to DCCD (half-maximal inhibition at 30 μM) than the PQ oxidation site (half-maximal inhibition at 120 μM). It should be noted again that the concentration of DCCD needed for the inhibition of electron flow is higher than that concentration of DCCD needed for energy transfer inhibition.

The results showing that the reduction of the PS II acceptors in the presence of DBMIB is not much affected by DCCD seems surprising at first, if it is concluded above that DCCD inhibits PQ reduction. Though it is possible that a carrier before PQ is responsible for the reduction of PS II acceptors another explanation for the DCCD insensitivity of PQ function in a PS II reduction system is offered below.

It is known that one of the two energy conserving sites in chloroplasts is associated with the translocation of protons across the thylakoid membrane via the reduction/oxidation cycle of PQ. Considering the effect of DCCD on the proton channel of ATP synthetase in photosynthesis (CF_0) and respiration (F_0) as well as on the protein conducting channel of cytochrome oxidase [14] it is tempting to suggest that the inhibition by DCCD of the electron flow system of chloroplasts exactly before and after PQ function may also be due to an interference with the uptake of protons needed for the reduction of PQ and with the release of protons during PQH_2 oxidation. The chemical reduction of PQ by DQ and also the chemical oxidation of PQH_2 by a TMPD bypass completely overcomes the DCCD effect. This suggests that neither the reduction of PQ by an artificial donor nor its oxidation by an artificial acceptor as such is affected by DCCD. Rather the reduction of PQ via PS II which requires proton uptake and its oxidation via PS I which results in the deposition of protons in the inner space [15] are affected by DCCD. It was shown [16] that proton uptake needed for the reduction of PQ is delayed relative to electron transport probably because of a diffusion barrier in an uncharacterized protein shield. We postulate that this protein shield is a proton conducting channel needed for the reduction of PQ by PS II. DCCD blocks this proton channel and thus inhibits the reduction of PQ. A similar proton channel is postulated to exist where PQH_2 is oxidized in the membrane. The protons released on the oxidation of PQH_2 are translocated to the inner space via this proton channel. DCCD blocks this proton channel and inhibits PQH_2 oxidation. The observation that artificial donor and acceptor systems for PQ are not sensitive to DCCD show that chemical reduction and oxidation of PQ is not limited by these two proton channels from either side of the membrane.

On the basis of this proposal the reduction of PS II acceptors in the presence of DCCD is easier to explain. If PS II acceptors are reduced after PQ the proton translocation may not impose a limitation because a plastosemiquinone could function as an electron donor to PS II acceptors. Alternately even if the PQH_2 is the electron donor to PS II acceptors, the oxidation of PQH_2 will release protons into the membrane towards the external side (as against the internal oxidation in the intact system) which could again be used for the reduction of PQ. This obviates the necessity for continuous proton uptake in an artificial acceptor system and a catalytic amount of protons can serve the purpose.

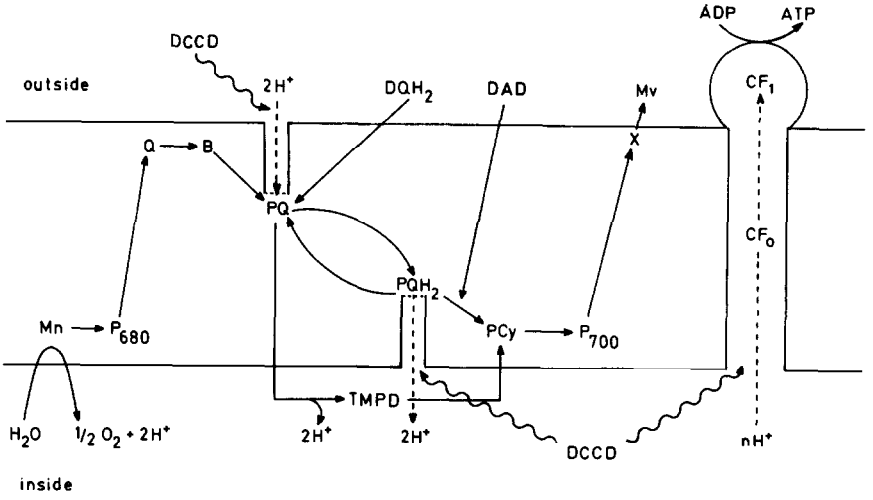

Fig.4. A schematic diagram showing the presence of postulated proton conducting wells towards PQ reduction and oxidation sites as the sites of DCCD inhibition. The donation of electron by Dq and DAD and the TMPD bypass are also indicated.

The significance of the reversal of DCCD inhibition by externally added PQ is not quite clear. A possibility is that the added PQ may act as an artificial chemical quinoid shuttle transporting protons and electrons from PS II to PS I, bypassing the proton channels.

The results presented in this communication could be summarized in a schematic way as shown in fig.4. The reduction and oxidation of PQ is associated with proton uptake and release which involves two proton conducting channels or wells. The DCCD is postulated to block the proton conduction through them and thus inhibit electron flow.

It is obvious that the inhibition by DCCD of electron flow is mediated through its effect on proton conduction and is therefore, a 'proton translocating inhibitor' rather than an 'electron transfer inhibitor'.

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