Effects of Disalicyldiethylenepropanediamines on Photosynthetic Electron Transport of Isolated Spinach Chloroplasts

Received for publication May 31, 1978 and in revised form November 9, 1978

Norbert Laasch, Werner Kaiser, and Wolfgang Urbach
Institut für Botanik der Universität Würzburg, Germany

ABSTRACT

The effects of disalicyldiethylenepropanediamine (DSPD) and disal-salicyldiethylenepropanediamine (sulfo-DSPD) on the photosynthetic electron transport of isolated chloroplasts have been reexamined.

Our data suggest that DSPD, but not sulfo-DSPD, is an effective inhibitor of electron transport between photosystem II and photosystem I before or at plastoquinin. Furthermore, both DSPD and sulfo-DSPD block electron transport at the site of ferredoxin.

Under certain conditions DSPD and even more so sulfo-DSPD function as autoxidizable electron acceptors.

Finally it is shown that DSPD can cause an inhibition of photophosphorylation.

According to our results the use of DSPD as a specific inhibitor of ferredoxin-dependent reactions has to be questioned.

In 1967, disalicyldiethylenediamines were introduced by Trebst and Burba (23) as inhibitors of photosynthetic electron transport. DSPD and sulfo-DSPD were the main representatives of this new class of inhibitors. The site of inhibition of photosynthetic electron transport by these compounds was localized at ferredoxin by studying the effect of sulfo-DSPD on broken chloroplasts (23). The other compounds were thought to act in the same manner. Inasmuch as only the lipophilic DSPD is able to enter intact chloroplasts or cells, only DSPD was used in vivo experiments. It has been assumed that DSPD acts at the same site as sulfo-DSPD, i.e. as an antagonist of ferredoxin (1, 5-7, 9, 12-16, 20, 21, 23, 26-29). We also tried to study the role of ferredoxin in photosynthetic reactions with the aid of DSPD. We found, however, that DSPD, unlike sulfo-DSPD, inhibits photosynthetic electron transport between PSII and PSI and that it also inhibits photophosphorylation.

MATERIALS AND METHODS

Broken chloroplasts were obtained by the following procedure. Intact chloroplasts were isolated from freshly harvested leaves of Spinacia oleracea var. "Atlanta" according to Cockburn et al. (10) with the following modifications: the grinding medium (solution A) contained 0.33 mM sorbitol, 0.05 mM Mes-NaOH (pH 6.5), 1 mM MgCl₂, 1 mM MnCl₂, and 2 mM EDTA. Instead of muslin, a pad of cotton wool sandwiched between two layers of nylon cloth was used for the filtration of the spinach homogenate and the chloroplasts were washed once in the suspending medium. Both the suspending medium and the basic reaction medium for the experiments with broken chloroplasts (solution B) were identical to solution A, but contained 0.05 mM Hepes-NaOH (pH 7.6) instead of Mes buffer. An aliquot of 0.1 ml of the chloroplast suspension was added to 0.95 ml of water at 0 C to prepare broken chloroplasts. After about 1 min, 0.95 ml of double strength solution B was added and the broken chloroplasts were used at the final concentrations described below.

For O₂ evolution or uptake, which was measured polarographically with a Beckman electrode, broken chloroplasts at a concentration equivalent to 50 μg Chl/ml were used. The test cuvette (total volume 2.0 ml) was fitted with a water jacket and a magnetic stirrer. Illumination was provided by a slide projector. The intensity of white light was about 500 w/m² and the temperature was kept at 18 C.

NADP reduction was carried out with broken chloroplasts (10 μg Chl/ml) in a reaction medium lacking sorbitol but containing 0.05 mM Hepes-NaOH (pH 7.6), 10 mM MgCl₂, 0.25 mM NADP, a saturating amount (0.2 mg) of ferredoxin, 1 mM ascorbate, 0.1 mM DCPIP, and 5 μM DCMU. All other conditions were the same as for the O₂ measurements. Determination of the NADPH formed was carried out by a method of enzymic recycling described by Pinder et al. (18) and modified by Rurainski (personal communication). NADP reduction could not be measured directly at 340 nm because DSPD and sulfo-DSPD also show considerable absorption at this wavelength.

For measuring photophosphorylation, 1 ml of a suspension of broken chloroplasts (50 μg Chl/ml) in addition to the basic reaction medium, 1 mM KH₂PO₄ labeled with 10 μCi ³²P and 1 mM ADP. For PMS-catalyzed cyclic photophosphorylation the reaction mixture was changed as follows: 5 mM ADP, 5 mM KH₂PO₄ containing 10 μCi ³²P, 5 mM MgCl₂, 1 mM Na-ascorbate, 0.1 mM PMS, and 5 μM DCMU. Photophosphorylation was carried out in 25-ml centrifuge tubes in a modified Warburg apparatus. Illumination (about 500 w/m²) was provided by 60-w Krypton lamps from the bottom of the water bath. Incorporation of ³²P into organic compounds was measured after isobutanol-benzene extraction (4) in a liquid scintillation counter.

Chl was measured according to Arnon (2).
Pretreatment of chloroplasts with high concentrations of KCN (50 mM) blocks noncyclic electron transport at plastoeyacin (17). After this treatment, endogenous pseudocyclic electron flow from water to O₂ is inhibited (Table II), indicating that a site of O₂ reduction is indeed after plastoeyacin. DSPD restored O₂ reduction, whereas addition of sulfo-DSPD causes no reversal of the inhibition of electron flow by KCN (Table II). The O₂ reduction occurring in the presence of DSPD is inhibited by DCMU or DBMB in a similar way to the endogenous O₂ uptake (Fig. 2 and Table III). The restoration of KCN-inhibited O₂ uptake is fully reversible on a trace level. The basic reaction medium (solution B) described under "Materials and Methods" contained additions of 1 mM ferricyanide and 10 mM NH₄Cl. The light was switched on or off where indicated. Numbers beside the traces give rates of O₂ production in μmol O₂/mg Chl-h.

Table I. Effect of DSPD (7.5 x 10⁻⁵M) and sulfo-DSPD (7.5 x 10⁻⁵M) or different types of electron transport in broken chloroplasts.

<table>
<thead>
<tr>
<th>Systems</th>
<th>control</th>
<th>DSPD</th>
<th>sulfo-DSPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) H₂O→FeCy</td>
<td>333</td>
<td>47</td>
<td>14</td>
</tr>
<tr>
<td>b) H₂O→DCPIP</td>
<td>124</td>
<td>41</td>
<td>52</td>
</tr>
<tr>
<td>c) H₂O→MV→O₂</td>
<td>407</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>d) H₂O→DBMB→O₂</td>
<td>43</td>
<td>45</td>
<td>105</td>
</tr>
<tr>
<td>e) asc/DCPIP→NADP</td>
<td>56</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>f) asc/DCPIP→MV→O₂</td>
<td>840</td>
<td>1886</td>
<td>225</td>
</tr>
<tr>
<td>g) H₂O→O₂</td>
<td>27</td>
<td>25</td>
<td>93</td>
</tr>
</tbody>
</table>

FIG. 2. Comparison of effects of 5 μM DCMU, 5 μM DBMB, and 0.75 mM DSPD on endogenous O₂ uptake (pseudocyclic electron flow) of isolated chloroplast lamellae. The basic reaction medium contained additions of 0.2 mM KCN and 10 mM NH₄Cl. The light was switched on or off where indicated. The inhibitors were added in a volume of 20 μl in ethanol to give the final concentrations mentioned above. Numbers beside the traces give rates of O₂ uptake in μmol O₂/mg Chl-h.
reduction by DSPD and the DCMU and DBMIB sensitivity of this restored process can be explained only if DSPD acts as an autooxidizable electron acceptor between plastoquinone and plastocyanin. Besides acting as an autooxidizable electron carrier, DSPD ought to have some additional inhibitory effect on electron transport, as indicated by the strong inhibition of some high rate photoreductions (Table I). The reduction of sulfo-DSPD and its reoxidation by O₂ must take place in the electron transport chain at a site after plastocyanin, presumably after PSI, which was already concluded from the results in Table I.

The experiments described thus far were done in the presence of an uncoupler (NH₄Cl) to avoid any interference by photophosphorylation. In order to study possible direct interactions of DSPD and sulfo-DSPD with photophosphorylation the effects of DSPD and sulfo-DSPD on ³²P incorporation in broken chloroplasts in the presence of different electron acceptors were tested. DSPD (0.75 mM) inhibits PMS-catalyzed photophosphorylation even more than NH₄Cl does at 10 mM (Table IV). Sulfo-DSPD at the same concentration has no effect at all. Assuming that the inhibition site of DSPD is localized before or at plastocyanin, the inhibition of PMS-catalyzed photophosphorylation by DSPD cannot be due to an inhibition of the electron transport. Thus, DSPD must inhibit photophosphorylation.

³²P incorporation coupled to electron transport from water to ferricyanide is more sensitive to DSPD than is electron transport, leading to a continuous decrease of the P/2e ratio from an initial value of 0.85 to zero (Fig. 3). Sulfo-DSPD affected neither electron transport nor ³²P incorporation (not shown). DSPD seems to uncouple photophosphorylation or is an energy transfer inhibitor. It is difficult to distinguish between these two alternatives. A stimulation by DSPD of a basal electron transport would indicate a function of DSPD as an uncoupler. Since DSPD inhibits all open chain electron transport reactions which are coupled to photophosphorylation, this kind of experiment cannot be done successfully.

**DISCUSSION**

Both DSPD and sulfo-DSPD have often been used (1, 5-7, 9, 12-16, 20, 21, 23, 26-29) to study the role and participation of ferredoxin in reactions of isolated chloroplasts as well as algal cells. Although the extremely different water solubility of both compounds might have given rise to the idea that the lipophilic DSPD might reach—and perhaps act at—different sites of the thylakoid membranes than the water-soluble sulfo-DSPD, both were thought to act in the same way only as antagonists of ferredoxin. Our experiments demonstrate clearly that this is not the case. In contrast to sulfo-DSPD, DSPD additionally inhibits photosynthetic electron flow between the photosystems and it also blocks photophosphorylation. Thus, using DSPD for an evaluation of the role of ferredoxin in photosynthetic organisms can be highly inconclusive and misleading. Earlier experiments and results on this subject should be carefully reexamined.

On the other side the interactions of DSPD and sulfo-DSPD with the photosynthetic membrane seem very interesting. As already mentioned by Trebst and Burba (23), sulfo-DSPD might be reduced by the photosynthetic electron transport chain, and the same might be true for DSPD. Since DSPD is able to restore O₂,

---

**Table IV. Effect of DSPD (7.5 x 10⁻⁴M), sulfo-DSPD (7.5 x 10⁻⁴M) and NH₄Cl (10 mM) on PMS cyclic photophosphorylation. Conditions; see Materials and Methods.**

<table>
<thead>
<tr>
<th>additions</th>
<th>µmol /mg Chlorophyll x h</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>----</td>
<td>746</td>
<td>100</td>
</tr>
<tr>
<td>DSPD</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>sulfo-DSPD</td>
<td>799</td>
<td>107</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>15</td>
<td>1.8</td>
</tr>
</tbody>
</table>

---

**Fig. 3. a: Comparison of effects of various concentrations of DSPD on O₂ evolution (O—O) and photophosphorylation (▲▲▲) in presence of ferricyanide. b: Decrease of the P/2e ratio of these reactions with increasing concentrations of DSPD. The basic reaction medium contained an addition of 10 mM ferricyanide.**
reduction after inhibition by KCN treatment, we assume that it can be reoxidized by molecular O₂, thus acting as an electron carrier connecting the electron transport chain between plastoquinone and plastocyanin with O₂. Sulfo-DSPD even increases the normal rate of endogenous O₂ reduction, but not in KCN-pre-treated chloroplasts. Apparently sulfo-DSPD is also autooxidizable, but can accept electrons only after PSI. Considering the high water solubility of sulfo-DSPD, this finding is not surprising. It is very well known that the primary acceptors of PSI are located in or at the outer surface of the thylakoid membranes (24).

As already mentioned above (compare “Results”), we cannot decide from our present data whether the inhibition of phosphorylation by DSPD is due to uncoupling or to an inhibition of energy transfer. Theoretically, it could be expected that the inhibition of phosphorylation by DSPD is due to uncoupling, because DSPD is a lipophilic amine. Amine uncoupling is relatively well understood (11). It requires permeation of the amine base through the membrane, a process which would readily occur with the lipophilic DSPD, but not with the hydrophilic sulfo-DSPD. Thus it seems reasonable that sulfo-DSPD cannot affect phosphorylation at all.

Acknowledgments. We thank Prof. Dr. A. Trebst for advice and gifts of sulfo-DSPD and DMB and Dr. H. J. Rumanksi for discussion and experimental support. The skilful technical assistance of Mrs. Spidol-Niemann is gratefully acknowledged.

LITERATURE CITED