ACCEPTOR SPECIFIC INHIBITION OF PHOTOSYSTEM II ELECTRON TRANSPORT BY UNCOUPLING AGENTS

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1. Introduction

A number of groups have recently demonstrated that lipophilic oxidants such as quinones and oxidized phenylenediamines will support photosystem II-associated electron transport [1–6]. This photosystem II electron transport is sensitive to DCMU, but only partially sensitive to low concentrations of EDAC [6], DBMIB [7] or KCN [8].

The present study deals with the effect of uncouplers on photosystem II electron transport catalyzed either by phenylenediamines or quinones. Photosystem II-sensitized quinone reduction (using either high DBMIB concentrations or 2,5-dimethyl-*p*-benzoquinone as acceptor) in EDAC-treated chloroplasts was neither inhibited nor stimulated by uncouplers. In contrast, reduction of oxidized phenylenediamines (diaminodurene or *p*-phenylenediamine) in DBMIB-poisoned or EDAC-treated chloroplasts was inhibited 35–75% by uncoupling agents at normal uncoupling concentrations. Concomitant measurements of electron transport and msec delayed light emission (DLE) in the uncoupler-treated chloroplasts suggest that the inhibition of phenylenediamine reduction is related to the effect of the uncoupler on intrathylakoid pH. In addition, the msec DLE patterns observed in the presence of uncoupling agents do not support the suggestion of Gould and Ort [9] that the inhibition of phenylenediamine reduction involves an effect of the uncoupling agent on the oxidizing side of photoreaction II.

Abbreviations: CF_1 – chloroplast coupling factor$_1$; DBDCTFB – 4,7-dibromo-5,6-dichloro-2-trifluoromethylbenzimidazole; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU – 3(3,4-dichlorophenyl)-1,1-dimethylurea; Diaminodurene – 2,3,5,6-tetramethyl-*p*-phenylenediamine; DLE – delayed light emission; EDAC – 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; S-13 – 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

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2. Materials and methods

Chloroplasts were isolated from market spinach as described elsewhere [10] EDTA washing and EDAC treatment of the isolated chloroplasts were performed as described previously [6,10]. Chlorophyll was determined by the method of Arnon [11].

Electron transport was measured either by following changes in oxygen concentration with an oxygen electrode [12] or by following ferricyanide reduction spectrophotometrically [13]. Light-induced proton uptake was monitored as described elsewhere [10]. Msec delayed emission was measured as previously described [10,14].

DAD and DBMIB were generously supplied by Dr S. Izawa. 2,5-Dimethyl-*p*-benzoquinone was purchased from K & K Laboratories. DBDCTFB, DCMU, EDAC, S-13 and X-464 were gifts from Dr A. Jagendorf, P. Heytler, R. McCarty, P. Hamm and W. E. Scott respectively. Crude coupling factor, prepared from spinach chloroplasts according to Vambutas and Bertsch [15], was generously supplied by Dr V. Vambutas.

3. Results

Table 1 shows that in the absence of DBMIB, gramicidin uncoupled electron transport catalyzed by either ferricyanide or methyl viologen, but had relatively small effects on electron transport catalyzed by oxidized diaminodurene (DADox) or oxidized *p*-phenylenediamine (PDox). In the presence of a low concentration of DBMIB (5×10^{-7} M), electron transport catalyzed by the phenylenediamines was generally inhibited 35–50% by gramicidin; the maximum inhibition ever observed was 75%. In contrast, ferricyanide plus DBMIB was unaffected by gramicidin. DADox reduction in the presence of DBMIB was also inhibited by the uncouplers nigericin (X-464), NH_4Cl, S-13, DBDCTFB and after EDTA treatment. Valinomycin (plus 10 mM KCl) and the phosphorylation reagents ADP/P_i had little or no effect on DADox (plus DBMIB) reduction.

Inhibition of DADox reduction by gramicidin was also observed with EDAC-treated [6] chloroplasts (table 2); EDAC is believed to inhibit between the sites of inhibition of DBMIB [7] and KCN [8]. Using EDAC-treated chloroplasts we observed that electron transport catalyzed by quinones (either DBMIB or 2,5-dimethyl-*p*-benzoquinone) was relatively insensitive to either gramicidin or EDTA treatment (data not shown).

The effects of EDTA treatment on energy-linked reactions in chloroplasts can be partially reversed on the addition of coupling factor protein (CF_1) [16]. As shown in table 3, addition of a crude coupling factor preparation to EDTA-stripped thylakoids resulted in a partial restoration of light-induced proton uptake. Concomitantly, electron transport to methyl viologen was slowed while electron transport to DADox (plus

Table 1
Effect of gramicidin on electron transport using various acceptors

	Acceptor System	μmol O_2 evolved or consumed/mg chl·hr	
		– gram	+ gram
Exp't. 1	$H_2O \rightarrow$ FeCy	44	132
(pH 7.5)	$H_2O \rightarrow$ MV-O_2	20	66
	$H_2O \rightarrow$ DADox	180	166
	$H_2O \rightarrow$ FeCy + DBMIB	22	26
	$H_2O \rightarrow$ MV-O_2 + DBMIB	0	0
	$H_2O \rightarrow$ DADox + DBMIB	148	67
Exp't. 2	$H_2O \rightarrow$ FeCy	74	154
(pH. 8.3)	$H_2O \rightarrow$ MV-O_2	31	93
	$H_2O \rightarrow$ PDox	237	267
	$H_2O \rightarrow$ PDox + DBMIB	172	110

The reaction mixture (in 5 ml) contained 100 mM sucrose, 40 mM Tricine–NaOH (pH 7.5 or 8.3), 10 mM KCl, 2 mM $MgCl_2$, chloroplasts equivalent to 100 μg chlorophyll and the indicated acceptor system. The acceptor systems were: FeCy (0.6 mM potassium ferricyanide); MV-O_2 (0.1 mM methyl viologen and 0.5 mM NaN_3); DADox (0.4 mM diaminodurene plus 1.2 mM ferricyanide); PDox (0.4 mM *p*-phenylenediamine plus 1.2 mM ferricyanide). Where indicated, DBMIB was present at 5×10^{-7} M and gramicidin (GRAM) at 10^{-6} M. The assays were performed at 25°C.

Table 2
Effect of gramicidin on electron transport in EDAC-treated chloroplasts

	Condition	μmol O_2 evolved or consumed/mg chl·hr	
		– gram	+ gram
	Untreated		
Exp't 1	$H_2O \rightarrow$ MV-O_2	27.6	88.5
	$H_2O \rightarrow$ DADox	132.5	104.0
	EDAC-treated		
	$H_2O \rightarrow$ MV-O_2	0	7.4
	$H_2O \rightarrow$ DADox	102.5	56.0
	Untreated		
Exp't 2	$H_2O \rightarrow$ MV-O_2	27.3	–
	EDAC-treated		
	$H_2O \rightarrow$ MV-O_2	4.4	–
	$H_2O \rightarrow$ DADox	78.0	37.4
	$H_2O \rightarrow$ DMQox	78.0	76.5
	$H_2O \rightarrow$ DBMIB + FeCy	43.6	37.4

Reaction conditions as in table 1, the pH was 7.5. EDAC-treated chloroplasts were prepared in the following way: Spinach chloroplasts (100 μg/ml) were illuminated for 5 min in 20 mM Tricine–NaOH (pH 8.0), 25 mM NaCl, 0.5 mM PMS, and 2 mM EDAC. The treated chloroplasts were then washed and resuspended in 0.4 M sucrose, 0.5 M Tricine–NaOH (pH 7.8) and 0.1 M NaCl at 1 mg/ml. Control chloroplasts were treated in a similar fashion, but were not exposed to EDAC. The acceptor systems were: MV-O_2 (0.2 mM methyl viologen plus 0.5 mM NaN_3); DADox (0.4 mM + 1.2 mM ferricyanide); DMQox (2,5-dimethyl-*p*-benzoquinone plus 1.2 mM ferricyanide); DBMIB plus FeCy (0.1 mM DBMIB plus 0.5 mM ferricyanide). Gramicidin (GRAM) was used at 1×10^{-6} M.

Table 3
Proton uptake and electron transport in EDTA-uncoupled and reconstituted chloroplasts

Condition	H^+ uptake	Electron Transport	
	(neq/mg chl)	(μmol of O_2 consumed or evolved/mg chl·hr	
		$H_2O \rightarrow$ MV-O_2	$H_2O \rightarrow$ DADox + DBMIB
Untreated	308	42	206
EDTA-treated	5	170	54
EDTA-treated plus CF_1	100	107	99

The reaction mixture for proton uptake (in 5 ml) contained: 40 mM NaCl, 1 mM Tricine–NaOH (pH 7.1), 1 mM $MgCl_2$, 0.1 mM methyl viologen, and chloroplasts equivalent to 100 μg of chlorophyll. Electron transport was measured by following changes in oxygen concentration in a reaction mixture (in 5 ml) containing: 20 mM NaCl; 20 mM Tricine–NaOH (pH 7.8); 1 mM $MgCl_2$; either 0.1 mM methyl viologen plus 0.5 mM NaN_3 or 0.4 mM diaminodurene, 1.2 mM ferricyanide and 0.5 μM DBMIB; and chloroplast equivalent to 100 μg of chlorophyll. Reconstitution was carried out according to McCarty [26] in a 1 ml reaction mixture containing: 40 mM sucrose, 5 mM Tricine–NaOH (pH 7.8), 1 mM NaCl, 5 mM $MgCl_2$, EDTA-treated chloroplasts equivalent to 100 μg chlorophyll and crude CF_1 equivalent to 285 μg of protein.

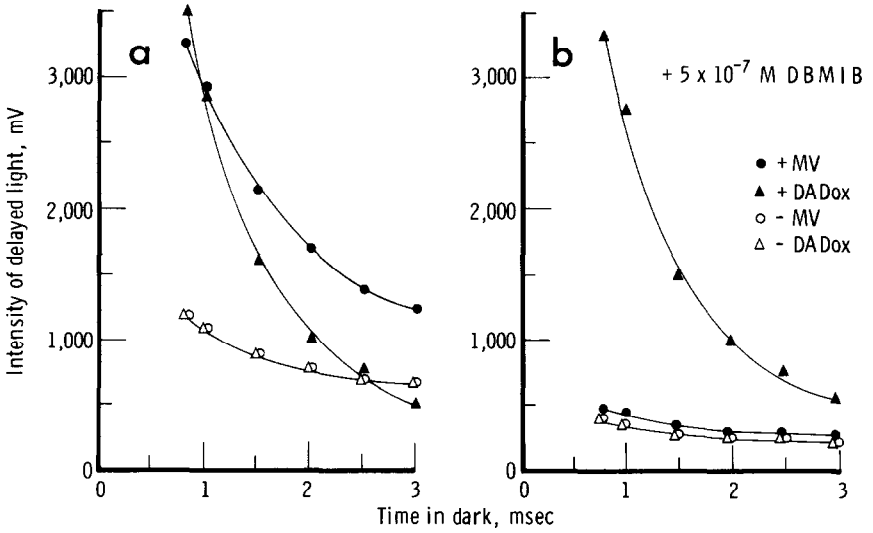

Fig. 1. Effects of methyl viologen and oxidized diaminodurene on msec DLE in the absence and presence of DBMIB. The reaction mixture was similar to one described in table 1, the pH was 7.5 and the chlorophyll concentration was 10 μg/ml. The final concentration of methyl viologen was 0.5 mM and the final concentrations for the DAD system were 0.2 mM DAD and 0.6 mM ferricyanide. DBMIB was present at 5×10^{-7} M. Rates of reduction (μequivalents/mg chl·hr) were: unpoisoned – $H_2O \rightarrow$ MV, 80; $H_2O \rightarrow$ DADox, 725. DBMIB poisoned – $H_2O \rightarrow$ MV, 0; $H_2O \rightarrow$ DADox, 592.

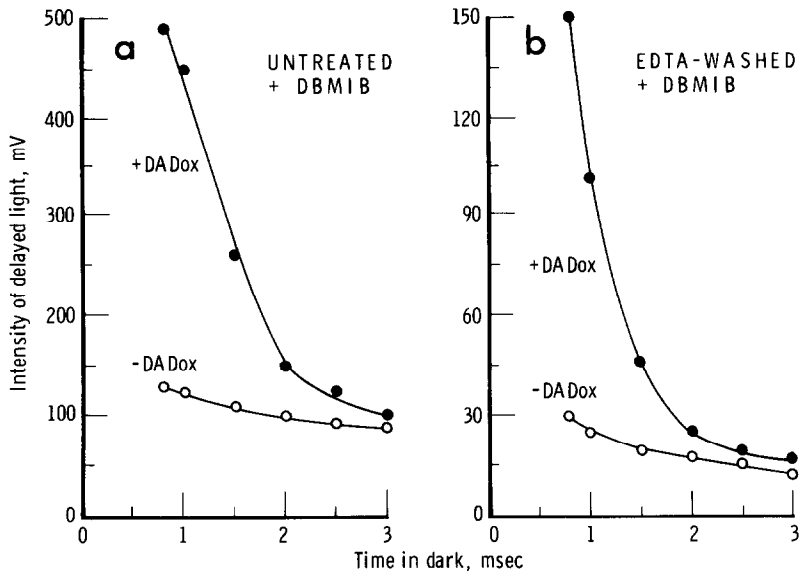

Fig.2. msec DLE from untreated and EDTA-washed chloroplasts. The reaction conditions as in fig.1. Rates of DADox reduction were 908 and 332 equivalents/mg chl·hr in untreated and EDTA-washed chloroplasts respectively.

DBMIB) was speeded, by addition of CF_1. Gramicidin stimulated electron transport to methyl viologen by 30% in the reconstituted chloroplasts, while electron transport to DADox (plus DBMIB) was inhibited 38% (data not shown):

Fig. 1a shows the effects of adding either methyl viologen or DADox on the intensity and dark decay kinetics of msec DLE. The dark decay in the presence of DADox is significantly faster than with methyl viologen or other Hill reagents [17]. As shown in fig.1b, poisoning chloroplasts with 5×10^{-7} M DBMIB completely prevented the effect of methyl viologen on msec DLE concomitantly with complete inhibition of methyl viologen photoreduction. In contrast, DBMIB

Table 4
Effect of semicarbazide on electron transport in heat-treated and uncoupler-inhibited chloroplasts

Condition	Electron Transport (μequivalents/mg chl·hr)	
	−Semicarbazide	+ Semicarbazide
Untreated ($H_2O \rightarrow$ FeCy)	112	118
Heat-treated ($H_2O \rightarrow$ FeCy)	31	64
Untreated ($H_2O \rightarrow$ DADox plus DBMIB)	238	230
Heat-treated ($H_2O \rightarrow$ DADox plus DBMIB)	25	64
Gramicidin-treated ($H_2O \rightarrow$ DADox plus DBMIB)	112	100

The reaction mixture as in table 1, the pH was 7.8. The following acceptors were used: $H_2O \rightarrow$ FeCy, 0.3 mM FeCy; $H_2O \rightarrow$ DADox, 0.2 mM DAD plus 0.6 mM FeCy. When added, DBMIB was present at 5×10^{-7} M, gramicidin at 1.5×10^{-6} M, and semicarbazide at 3×10^{-3} M. Heat-treated chloroplasts were prepared by heating 1.5 ml (1 mg/ml) of spinach chloroplasts at 50°C for 2.5 min.

poisoning had very little effect on the changes induced by DADox, though the intensity of DLE in the absence of an acceptor was reduced to about 30% of the value of unpoisoned chloroplasts. The changes in msec DLE induced by DADox were completely abolished by 2×10^{-6} M DCMU.

When electron transport is inhibited by treatments (e.g. Tris-ageing [13], heating [18], etc.) which block water oxidation, msec DLE in the absence of an acceptor is characterized by extremely rapid dark decay kinetics. Addition of the acceptor did not change the decay characteristics of this rapid DLE. As shown in fig.2, inhibition of DADox reduction (plus DBMIB) by EDTA washing does not result in more rapidly decaying DLE in the absence of an acceptor. Similar results were also obtained with inhibition by gramicidin and NH_4Cl.

In addition, fig.2 also shows that the uncoupling treatment lowers the overall intensity of DLE in the presence of DADox (plus DBMIB); this effect of uncouplers has been ascribed to a reduction in the magnitude of the proton motive force [10,19,20]. This result suggests that msec DLE can be used to monitor the interaction between the high energy state and photoreaction II.

We also tested the ability of an artificial electron donor to photosystem II to restore electron transport in the inhibited chloroplasts. Table 4 shows that although the electron donor semicarbazide was capable of partially restoring electron transport in heat-treated chloroplasts, it was without effect on DADox (plus DBMIB) reduction in gramicidin-inhibited chloroplasts.

4. Discussion

Izawa et al. [2,7,8,21] have shown that in well coupled chloroplasts phenylenediamines can accept at two sites in the photosynthetic electron chain; one site is located close to photosystem I and the other site appears to be at the reducing end of photosystem II. In the presence of DBMIB or EDAC, only the photosystem II site appears to be functional [2,6,7,8,21] (table 1 and 2). Electron transport to phenylenediamines in the presence of either one of these inhibitors is inhibited up to 35–75% by uncoupling agents. The small effect of uncouplers on phenylenediamine reduction in the absence of DBMIB (or EDAC) may reflect a balancing of inhibition at the photosystem II site and stimulation due to uncoupling at the photosystem I site.

The above data suggest that the rate of reduction of oxidized phenylenediamines in the presence of DBMIB (or EDAC) is directly related to the establishment of a proton motive force (pmf). The fact that amines and nigericin (in the absence of valinomycin) inhibit, and the observation that the inhibition in EDTA-treated chloroplasts can be reversed by the subsequent addition of coupling factor (table 3) suggests that the transmembrane proton concentration gradient (ΔpH) may be the form of the pmf which is necessary for high rates of phenylenediamine reduction. The failure of ADP/P_i to lower the rates of phenylenediamine reduction (in the presence of DBMIB) may be related to the fact that ADP/P_i reduces Δ pH far less than uncouplers [22].

Gould and Ort [9] have suggested that the uncoupler-induced inhibition of phenylenediamine reduction is related to secondary effects of the uncoupler on the oxidizing side of photosystem II. From our experimental results (fig.2; table 4), this seems unlikely since, 1) all uncouplers tested induce the inhibition at neutral pH and normal uncoupling concentrations, whereas it has been shown that uncouplers inhibit on the oxidizing side of photosystem II only at pH's greater than 8.5 [11,23], or at high concentration at neutral pH [11,24], 2) a block between the photosystems is required, which is not true of any of the other known treatments which inhibit on the oxidizing side of photosystem II [12,24,25], 3) the uncoupler-inhibited chloroplasts do not exhibit msec DLE patterns similar to chloroplasts which have been inhibited on the oxidizing side [11,12,17], 4) artificial donors do not restore electron transport, and 5) the inhibition is specific for oxidized phenylenediamines, and is not observed with other Hill acceptors (ferricyanide or quinones).

The observation that photosystem II electron transport is differentially affected depending on the nature of the acceptor may reflect the fact that, 1) the sites of reduction of quinones and phenylenediamines are different as suggested by Gould and Izawa [2] or 2) the site of reduction may be the same for the two acceptors but due to the chemical nature [3] of the acceptors they may be affected differently (midpoint potential, chemical form, etc.) by the more alkaline intrathylakoid pH associated with uncoupled chloroplasts. Experiments are in progress to decide between these two alternate hypotheses.

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