The Use of O_2-Evolving Subchloroplast Particles to Study Acceptor and Inhibitor Sites on the Reducing Side of Photosystem II

W. S. Cohen and J. R. Barton

School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506/USA

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Photosystem II particles that retain the ability to evolve O_2 have been used to examine acceptor and inhibitor sites in the photosynthetic electron transfer chain between Q and plastoquinone. Employing the water to dichlorobenzoquinone reaction to assay photosystem II activity, we have demonstrated that electron transport in thylakoids and particles is equally sensitive to inhibition by DCMU, dinoseb, metribuzin, HQNO and DBMIB. Based on differential sensitivity to inhibition by DCMU vs. HQNO or DBMIB, we suggest that when synthetic quinones, *e.g.* 2,6-dichlorobenzoquinone operate as Hill reagents in particles they are reduced primarily by the plastoquinone pool. When synthetic quinones, *e.g.* 5,6-methylenedioxy-2,3-dimethyl benzoquinone act as autoxidizable acceptors they accept electron from the Q/B complex at a point that is located between the DCMU and HQNO (DBMIB) inhibition sites.

Introduction

Reports have appeared recently describing the isolation of photosystem II (PS II) particles that appear to be free of contamination with photosystem I and the cytochrome b_6/f complex [1–3]. In addition, the particles retain the ability to produce O_2 at high rates. To date the particles have been used primarily in studies of the oxidizing side of photosystem II [1–5]. The present series of experiments was designed to determine how useful these particles could be in studies of acceptor and inhibitor sites on the reducing side of PS II.

Materials and Methods

Thylakoids were isolated from either field-grown spinach or growth chamber-grown pea plants as described previously [6]. NaBr and Hg^{2+} treatments of thylakoids were performed as previously described [7, 8]. PS II particles were isolated from thylakoid membranes using Triton X-100 (Triton/Chl = 15:1) as described by Berthold *et al.* [1]. In some preparations, the Triton solubilization step was performed at pH 6 and the particles were washed with resuspension medium (at pH 6) containing 1 mM ascorbate [G. Babcock personal communication]. This procedure improved the electron transfer activity of the particles compared to preparations isolated at pH 7.5.

O_2 concentration changes were monitored polarographically [6]. Herbicide binding assays with particles and thylakoids were performed as described elsewhere [9]. Fluorescence induction studies were performed using a lab-built kinetic fluorometer. Membrane polypeptides were separated using SDS-PAGE as described elsewhere [10]. TMBZ staining was carried out as described by [11]. Quinones were recrystallized according to [12].

Results

Treatment of either spinach or pea thylakoids with Triton X-100 as described by Berthold *et al.* [1] yielded PS II particles that retained the ability to evolve oxygen at high rates even after a year of storage at −80 °C. Analysis of the membrane polypeptide composition by SDS-PAGE (Fig. 1) revealed that the particles were missing the apoprotein of CP_1 and the polypeptides associated with CF_1 (when compared to NaBr particles). Staining for peroxidase activity [11] with TMBZ indicated the loss of a polypeptide of ~33 kD in particles

Abbreviations: DAD, 2,3,5,6-tetramethyl phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCDMBQ, 2,5-dimethoxy-3,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-1-(2,4-dinitrophenoxyl)-4-nitrobenzene; FeCy-potassium ferricyanide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; MV, methyl viologen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethyl benzidine.

Reprint requests to Dr. W. S. Cohen.

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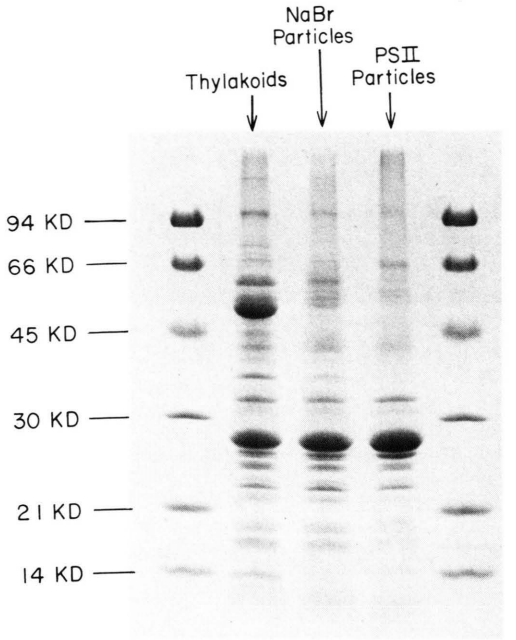

Fig. 1. SDS-PAGE profiles of membrane polypeptides from spinach thylakoids, NaBr-treated spinach thylakoids and spinach PS II particles prepared with Triton X-100. SDS-PAGE was performed as described by Mullett *et al.* [10] using a 7.5–15% linear polyacrylamide gradient. Equivalent amounts of sample (10 µg Chl) were applied to each line. Molec. wt. standards: phosphorylase B, 94 kD; bovine serum albumin, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 21 kD; lysozyme, 14 kD.

compared to thylakoids that may be cytochrome *f* (data not shown).

The particles are not capable of reducing methyl viologen with either water or duroquinol as the electron donor, but they do reduce a wide variety of quinones (Table I). pH profiles for DCBQ (Fig. 2) and MDBQ (Fig. 3) reduction in particles and uncoupled thylakoids are comparable. Kinetic studies of electron transport made with both pea thylakoids and PS II particles in which light intensity and DCBQ concentration [13] were varied indicated little difference between particles and thylakoids (data not shown). The PS II particles also reduce the class III acceptor diaminodurene (assayed in the presence of excess ferricyanide) at rates that are comparable to those observed in uncoupled thylakoids in which the photosystem I component of overall electron transfer has been blocked with DBMIB (Table II).

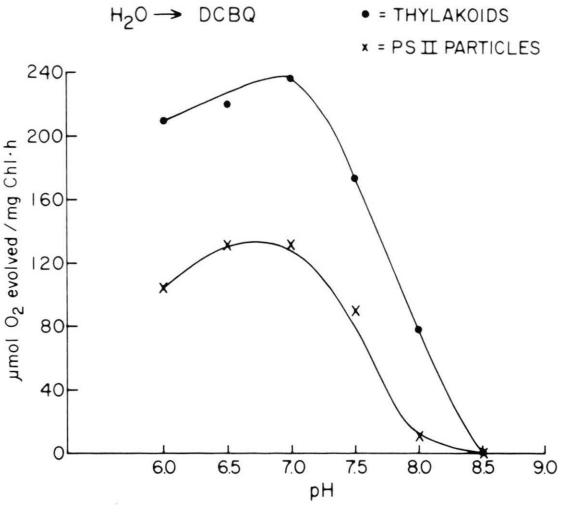

Fig. 2. Effect of pH on H_2O DCBQ activity in thylakoids and PS II particles. The assay mixture contained 50 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.25 mM DCBQ and thylakoids or particles equivalent to 10–15 µg Chl. 5 µg/ml gramicidin D was added to all thylakoid assays. The following buffers were employed at 25 mM: MES, pH 6.0 and 6.5; MOPS, pH 7.0; Tricine, pH 7.5, 8.0, 8.5.

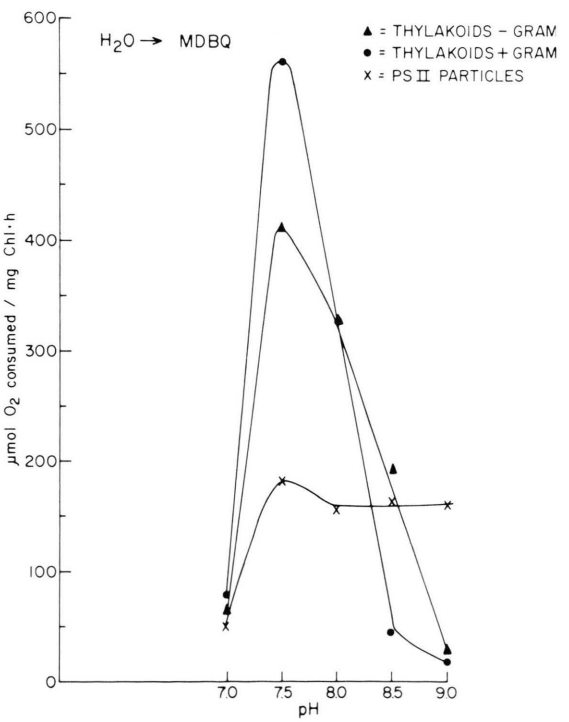

Fig. 3. Effect of pH on $H_2O \rightarrow$ MDBQ activity in thylakoids and PS II particles. The assay mixture was similar to the one described in the legend to Fig. 2 except the DCBQ was replaced by 0.225 mM MDBQ and 0.25 mM $MnCl_2$ was added. The following buffers were employed at 25 mM: MOPS, pH 7.0; Tricine, pH 7.5, 8.0, 8.5; TAPS, pH 9.0.

Table I. Quinone-mediated electron transport in PS II particles. The reaction mixture contained 50 mM sorbitol, 10 mM KCl, 25 mM MOPS (pH 7.0 buffer), 5 mM $MgCl_2$ and PS II particles equivalent to 10–15 μg/ml Chl. The dimethylenedioxybenzoquinone assays were performed with 25 mM Tricine buffer (pH 8.0) and 0.25 mM $MnCl_2$ present. All quinones were present at 0.1–0.25 mM when used as acceptors. Where indicated methyl viologen (MV) was present at 0.1 mM. The duroquinol assays also contained 0.5 mM duroquinol (DQH_2) and 5 μM DCMU.

Electron transport pathway	Activity [μmol O_2/mg Chl · h]
$H_2O \rightarrow MV/O_2$	0
$DQH_2 \rightarrow MV/O_2$	0
$H_2O \rightarrow$ *p*-benzoquinone	20–40
$H_2O \rightarrow$ duroquinone	50–60
$H_2O \rightarrow$ 2,5-dimethylbenzoquinone	30–70
$H_2O \rightarrow$ 2,6-dichlorobenzoquinone	120–290
$H_2O \rightarrow$ 3,6-dichloro-2,5-dimethoxy-benzoquinone	80–120
$H_2O \rightarrow$ 5,6-methylenedioxy-2,3-dimethyl-benzoquinone	160–250
$H_2O \rightarrow$ phenylbenzoquinone	120–130

Table II. Effect of diaminodurene on ferricyanide reduction in uncoupled thylakoids and PS II particles. The reaction mixture contained 50 mM sorbitol, 10 mM KCl, 25 mM MES (pH 6.5), 5 mM $MgCl_2$, 1.5 mM $K_3Fe[CN]_6$ (FeCy), and thylakoids or particles equivalent to 10–20 μg/ml Chl. Where indicated diaminodurene (DAD) was present at 0.5 mM. Thylakoid assays were performed with 0.5 μM DBMIB and 5 μg/ml gramicidin D present.

Electron transport pathway	Activity [μmol O_2/mg Chl · h]	
	Thylakoids	PS II particles
$H_2O \rightarrow$ FeCy	33	30
$H_2O \rightarrow$ FeCy + DAD	72	61

Analysis of fluorescence induction transients [14] indicated that particles and thylakoids have similar size pools of primary and secondary acceptors (Table III). In particle preparations that had low rates of DCBQ reduction (< 100 μmol O_2/mg Chl · h) the fluorescence rise curves were non-sigmoidal consistent with a partial block in electron transfer between Q and PQ [14].

Electron transfer from water to synthetic quinones is sensitive to inhibition by a wide variety of PS II reducing side inhibitors. The I_{50} values for DCMU, metribuzin, dinoseb, HQNO and DBMIB are com-

Table III. Fluorescence induction in thylakoids and PS II particles. Assay mixtures were similar to those described in the legend to Table II. Estimates of electron pool sizes were carried out as described by Forbush and Kok [14]. F_m = maximal fluorescence; F_0 = initial fluorescence level; Q + A = pool of primary and secondary electron acceptors.

	F_m/F_0	Q + A (−DCMU)	Q + A (+DCMU)	+DCMU/−DCMU
Thylakoids	4.54	11.35	0.56	0.0496
PS II particles	5.00	12.75	0.56	0.0441

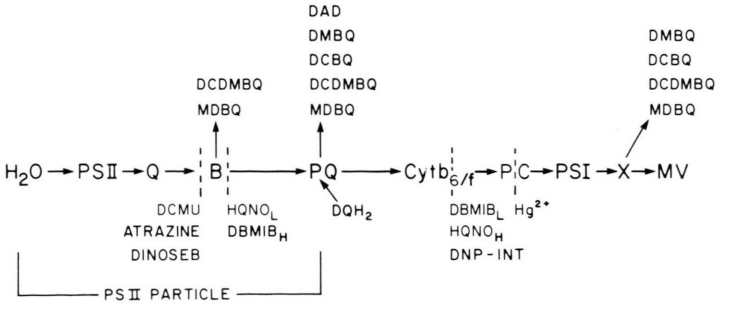

Fig. 4. Inhibitor and acceptor sites in the photosynthetic electron transfer chain. PS II, photosystem II; Q, primary acceptor of photosystem II; B, bound secondary acceptor of photosystem II; PQ, plastoquinone; cyt b_6/f, cytochrome b_6/f complex; PC, plastocyanin; PS I, photosystem I; X, primary acceptor of photosystem I.

Table IV. I_{50} values for inhibition of PS II electron transport. Photosystem II electron transport was assayed as $H_2O \rightarrow DCBQ$ at pH 7.0. I_{50} values were obtained by taking the mean of the half inhibitions from 2–4 separate experiments.

Inhibitor	Thylakoids	PS II particles
	I_{50} [M]	
DCMU	1.7×10^{-8}	2.8×10^{-8}
Metribuzin	1.0×10^{-7}	2.9×10^{-7}
Dinoseb	1.0×10^{-6}	1.4×10^{-6}
HQNO	2.5×10^{-6}	1.8×10^{-6}
DBMIB	2.0×10^{-6}	2.7×10^{-6}

Table V. Metribuzin binding properties in thylakoids and PS II particles. Binding assays were performed as described by Barton *et al.* [9] at a chlorophyll concentration of 50–70 μg/ml.

Membrane preparation	I_{50} [M]	K_B [M]	X_G [nmol/mg Chl]
Thylakoids	0.10×10^{-6}	0.07×10^{-6}	2.33
PS II particles	0.29×10^{-6}	0.58×10^{-6}	1.33

parable to those observed with uncoupled thylakoids (Table IV). [^{14}C]metribuzin binding studies made with both particles and thylakoids indicated that the particles bind approximately half the amount of metribuzin compared to thylakoids (Table IV) with a lower binding affinity ($k_B = 0.074 \times 10^{-6}$ M for thylakoids vs. 0.59×10^{-6} M for particles). Bound [^{14}C]metribuzin can be displayed from both PS II particles and thylakoids by DCMU and dinoseb, and not by HQNO (data not shown).

To gain further information concerning acceptor sites in the photosynthetic electron transfer chain, we assayed electron transport in both particles and thylakoids in the presence of inhibitors that block either between Q and PQ or between PQ and P_{700}. The data in Table VI obtained with uncoupled pea thylakoids, indicate that DMBQ and MDBQ accept electrons from both PS II and PS I, whereas DCBQ and DCDMBQ accept electrons primarily from PS II. With particles electron transfer to synthetic quinones was completely sensitive to inhibition by DCMU and triazine-type inhibitors. To further explore quinone interaction sites on the acceptor

Table VI. Effect of inhibitors on quinone-mediated electron transport in pea thylakoids. Assay conditions were similar to those described in the legend to Figs. 2 and 3.

Electron transport pathway	0.5 μM DBMIB	10 μM DNP-INT	100 μM Nitrofluoren	Hg^{2+} Treatment
	% control			
$H_2O \rightarrow MV/O_2$	0	0	15	0
$H_2O \rightarrow DMBQ$	40	48	47	37
$H_2O \rightarrow DCBQ$	76	86	83	88
$H_2O \rightarrow DCDMBQ$	90	88	–	–
$H_2O \rightarrow MDBQ/O_2$	50	57	36	54

Table VII. Effect of pH and inhibitors on electron transport in PS II particles. The reaction mixture was similar to the one described in the legend to Table I. When present, the final concentrations of DBMIB and HQNO were 10 and 20 μM respectively.

Electron transport pathway	Electron transport activity [μmol O_2/mg Ch · h]					
	pH 6			pH 8		
	–	+HQNO	+DBMIB	–	+HQNO	+DBMIB
$H_2O \rightarrow DCBQ$	238	24	41	0	–	–
$H_2O \rightarrow DCDMBQ$	109	14	–	119	81	–
$H_2O \rightarrow MDBQ$	88	7	38	183	124	112
$H_2O \rightarrow DMBQ$	51	6	–	0	–	–
$H_2O \rightarrow DAD/FeCy$	58	22	28			

side of photosystem II, we resorted to the use of inhibitors that act on the reducing side of plastoquinone but whose site of action is not well characterized. With particles at pH 6 we observed that the Hill reaction mediated by all quinones tested was sensitive to inhibition by 20 μM HQNO (Table VII). In contrast at pH 8 electron transfer to MDBQ and DCDMBQ was largely insensitive to HQNO. DCBQ reduction at pH 6 was inhibited 83% by 10 μM DBMIB, whereas MDBQ reduction at pH 8 was inhibited only 39%. 10 μM DNP-INT inhibited both DCBQ reduction at pH 6 and MDBQ reduction at pH 8 to a very small extent ~ 15% (data not shown).

Discussion

Employing data from this laboratory and from other laboratories [9, 15–19], we have designated a number of inhibitor sites in the interphotosystem electron transfer chain as indicated in Fig. 4. In addition, Fig. 4 also contains information concerning acceptor sites for synthetic quinones. Based on kinetic fluorescence data, electron transport studies and inhibitor studies, the PS II particles isolated from either spinach or pea thylakoid membranes with Triton X-100 contain the electron transfer chain from water to the plastoquinone pool. This conclusion is in agreement with the data of Lam and Malkin [20, 21]. Based on sensitivity to inhibition by DCMU, HQNO and DBMIB, it appears that all of the quinones tested accept electrons from the plastoquinone pool at pH 6. At pH 8, MDBQ and DCDMBQ appear to be capable of accepting electrons from a point in the chain that is located between the DCMU and HQNO inhibition sites. It has been suggested [22, 23] that DCDMBQ is capable of accepting electrons from a site that is at or near the Q/B complex when it is operating as a Hill oxidant. Our data (Table VI) seem to be consistent with another hypothesis namely that DCDMBQ can only interact with the Q/B complex when it is acting as an autoxidizable acceptor.

Ogilvie *et al.* [24] have suggested that PS II particles, prepared according to Berthold *et al.*, lack the site on the acceptor side of PS II for the reduction of quinonediimides. Results from this study (Table II) indicate that the particles do possess the capacity to reduce quinonediimides albeit at very low rates. The rates of diaminodurene reduction observed with PS II particles are comparable to those observed with uncoupled thylakoids inhibited with DBMIB. Since the particles are totally uncoupled, such low rates of quinonediimide reduction are not surprising [25–27].

In addition to being useful in acceptor studies, the oxygen-evolving particles employed in this study should also prove valuable in PS II inhibitor studies, since their sensitivity to various inhibitors is comparable to that observed with native thylakoid membranes. PS II particles isolated using digitonin and octyl glucoside [28] do not evolve oxygen and thus electron transport studies can only be performed with a limited number of artificial electron donor-acceptor combinations. The digitonin/octyl glucoside PS II particles also have greatly altered inhibitor sensitivities when compared to untreated thylakoids.

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[1] D. Berthold, G. Babcock, and C. Yocum, FEBS Lett. **134,** 231 (1981).
[2] Y. Yamamoto, M. Doi, N. Tamura, and M. Nishimura, FEBS Lett. **133,** 265 (1981).
[3] T. Kuwabara and N. Murata, Plant Cell Physiol. **23,** 533 (1982).
[4] J. Lavorel and M. Seibert, FEBS Lett. **144,** 101 (1982).
[5] M. Boska, K. Sauer, W. Buttner, and G. Babcock, Biochim. Biophys. Acta **722,** 327 (1983).
[6] W. Cohen, Plant Sci. Lett. **11,** 191 (1978).
[7] A. Kamienietzky and N. Nelson, Plant Physiol. **55,** 282 (1975).
[8] M. Kimimura and S. Katoh, Biochim. Biophys. Acta **283,** 279 (1972).
[9] J. Barton, W. MacPeek, and W. Cohen, J. Bioenerg. Biomemb. **15,** 93 (1983).
[10] J. Mullett, J. Burke, and C. Arntzen, Plant Physiol. **65,** 814 (1980).
[11] J. Guikema and L. Sherman, Biochim. Biophys. Acta **637,** 189 (1981).
[12] C. Yocum, Meth. in Enzym. **69 c,** 576 (1980).
[13] J. Guikema and C. Yocum, Biochim. Biophys. Acta **547,** 241 (1979).

[14] B. Forbush and B. Kok, Biochim. Biophys. Acta **162,** 243 (1968).
[15] W. Bugg, J. Whitmarsh, C. Rieck, and W. Cohen, Plant Physiol. **65,** 17 (1980).
[16] J. Barton and W. Cohen, Plant Sci. Lett., in press (1983).
[17] A. Trebst, Meth. in Enzym. **69c,** 675 (1980).
[18] J. Guikema and C. Yocum, Arch. Biochem. Biophys. **189,** 508 (1978).
[19] J. Bowes and A. Crofts, Arch. Biochem. Biophys. **209,** 682 (1981).
[20] E. Lam and R. Malkin, FEBS Lett. **144,** 190 (1982).
[21] E. Lam and R. Malkin, FEBS Lett. **152,** 89 (1983).
[22] G. Sarojini and H. Daniell, Z. Naturforsch. **36c,** 656 (1981).
[23] G. Sarojini, H. Daniell, and W. Vermaas, Biochem. Biophys. Res. Comm. **102,** 944 (1981).
[24] P. Ogilvie, L. Reschl, and S. Berg, Arch. Biochem. Biophys. **220,** 451 (1983).
[25] W. Cohen, D. Cohn, and W. Bertsch, FEBS Lett. **49,** 355 (1975).
[26] A. Trebst and S. Reimer, Biochim. Biophys. Acta **325,** 546 (1973).
[27] J. Guikema and C. Yocum, Biochemistry **15,** 362 (1976).
[28] J. Mullett and C. Arntzen, Biochim. Biophys. Acta **635,** 236 (1981).