Biochimica et Biophysica Acta, 973 (1989) 105–108
Elsevier

BBA 40219

BBA Report

Oxygen per flash from leaf disks quantifies Photosystem II

W.S. Chow [1], A.B. Hope [2] and Jan M. Anderson [1]

[1] *CSIRO Division of Plant Industry, Canberra and* [2] *School of Biological Sciences, Flinders University, Bedford Park (Australia)*

(Received 1 June 1988)

Key words: Photosynthesis; Thylakoid membrane; Photosystem II; Oxygen evolution; Diuron binding

The quantity of functional Photosystem II (PS II) centres in vivo has been determined by the oxygen yield from leaf disks, exposed to 1% CO_2 and repetitive flashes at 4 Hz (xenon flash lamp, 3 μs), and compared with the diuron-binding capacity of thylakoids isolated from the corresponding tissue of several herbaceous species. With continuous background far-red light present during the repetitive flashing of leaves, a good correlation was obtained between the two assays of PS II, the concentration of diuron-binding sites being only slightly higher. Omitting the background far-red light reduced the estimate of functional PS II centres by only about 10%, even when the PS II/PS I ratio was nearly 2. It is suggested that PS I is able to turn over more than once per flash, thus preventing a backlog of electrons in the plastoquinone pool. The oxygen yield per flash provides a convenient, direct assay of PS II in vivo when conditions are selected so as to avoid limitation by PS I.

Estimation of the functional Photosystem II centres on a chlorophyll basis in isolated plant thylakoid membranes is still controversial, with different values of PS II/PS I stoichiometry being reported. Even when the same species (e.g., spinach) and the same methods were used, the values obtained vary widely [1–7]. Neither is there agreement whether the PS II/PS I ratios may vary or be fixed at unity [2,4,7–9]. Part of these discrepancies may be due to variable degrees of inactivation of PS II function during the isolation of chloroplasts and thylakoids. Our aim was twofold: to develop a method to quantify functional PS II in leaves directly, and to compare the values obtained from leaf discs (in vivo) with those of isolated thylakoids.

The concentration of PS II was measured in leaf pieces by the O_2 evolution induced by repetitive flashes, a method originally used for algae [10,11] or cyanobacteria [12,13]. Leaf pieces were dark-adapted for about 15 min in a Hansatech (King's Lynn, U.K.) O_2 electrode chamber thermostatted at 25 °C; the CO_2 concentration in the electrode chamber was approx. 1%. Background far-red light (approx. 17 μmol quanta $\cdot m^{-2} \cdot s^{-1}$, 700–730 nm) was present during flash illumination to ensure that PS I was not limiting; this level of far-red light did not induce any appreciable oxygen evolution. The flashes, generated by a xenon flash lamp (Strobos-lave type 1539-A) at 4 Hz, were focussed by a lens on the leaf tissue, giving an intensity that was close to saturating. The net rate of O_2 evolution (allowing for dark O_2 consumption and instrumental drift) together with the flash frequency gave the O_2 yield per flash. Typical traces are shown in Fig. 1. The concentration of functional PS II centres was calculated as four times the number of mol O_2 per mol Chl per flash; the atmospheric $[O_2]$ for the local altitude of 800 m was taken as 8.05 mM at 25 °C. To check for any heating artifact due to the flashes, signals were also recorded with a leaf disk pre-infiltrated with 10 μM DCMU for 7 min (trace S′) or heated at 65 °C for 5 min (trace P′). Fig. 1 shows the very small residual responses from leaf disks so treated, compared with fresh disks (traces S and P).

To compare the concentrations of PS II in leaves with those of isolated thylakoids, chloroplasts were extracted from leaves strictly comparable with those used in the O_2 electrode as previously [7] and kept at 77 K until needed. Chl was determined by the method of Arnon [14]. DCMU-binding analyses followed the method of Tischer and Strotmann [15]; other details were as for atrazine [7]. ^{14}C-DCMU (Amersham International, Amersham, U.K.) was dissolved in ethanol and the stock concentration was checked by: (1) the specific activity of the stock solution, and (2) the absorbance at 250 nm compared with a nonlabelled gravi-

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; FR, far-red (light); PS I, II, Photosystem I, II; Q_A, primary electron acceptor in PS II; q_Q, Chl fluorescence parameters related to reduction of variable fluorescence by oxidised Q_A.

Correspondence: W.S. Chow, Division of Plant Industry, CSIRO, GPO Box 1600, Canberra City, ACT 2601, Australia.

metrically standardised solution of DCMU (Serva, Heidelberg, F.R.G.) for which the difference absorption coefficient at 250 nm relative to 280 nm was 23.5 $mM^{-1} \cdot cm^{-1}$. DCMU was added to give a final concentration of 60–400 nM, with a maximal ethanol content < 0.7% (v/v). The maximum DCMU-binding capacity was obtained from the extrapolation of a linear regression fitted to a double-reciprocal plot. Our estimated DCMU-binding capacity was typically 20–30% higher than the corresponding atrazine-binding capacity. For spinach grown in a glasshouse in spring/summer, we obtained a DCMU-binding capacity (approx. 3.3 mmol per mol Chl) which agrees with the value for commercial spinach reported by Graan [16].

A comparison of [PS II] in leaves of a large number of plants with the concentration of DCMU-binding sites in thylakoids isolated from corresponding tissues reveals four important points (Fig. 2). First, the concentration of PS II centres in leaves correlates well with the number of DCMU-binding sites found in their corresponding isolated thylakoids. The linear regression through the origin fitted to these points has a slope of 1.14 (cf. Fig. 1 in Ref. 7); a slope of greater than unity is partly attributable to a miss factor [7]. Thus the method of repetitive flashing of leaf disks provides a convenient, direct measure of the functional PS II centres in leaves, without artifacts due to inactivation of PS II during thylakoid isolation. This leaf method, which to our knowledge has not been published by others, should prove useful in studies of plant development or environmental stress to which PS II is particularly vulnerable.

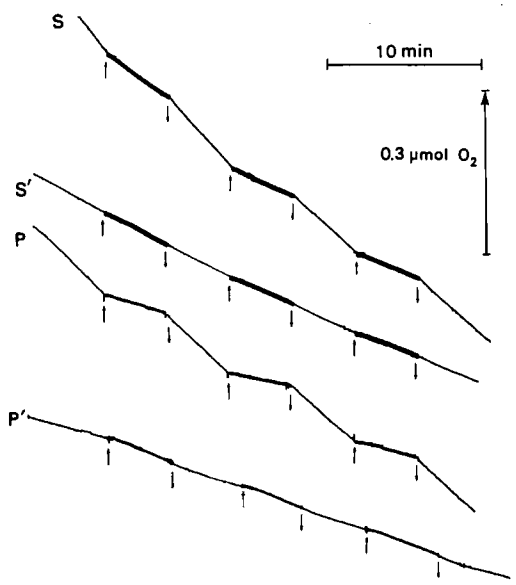

Fig. 1. Amplified O_2 exchange signals during and after repetitive flash illumination at 4 Hz of leaf disks of spinach control (S), spinach disk pre-incubated with 10 µM DCMU for 7 min (S'), pea control (P) and pea disk pretreated at 65 °C for 5 min (P'). The Chl contents of the leaf disks were 80.4, 48.9, 120 and 124 nmol, respectively. The plants were grown in a glasshouse. Upward or downward arrows indicate the beginning or end of a train of flashes, respectively.

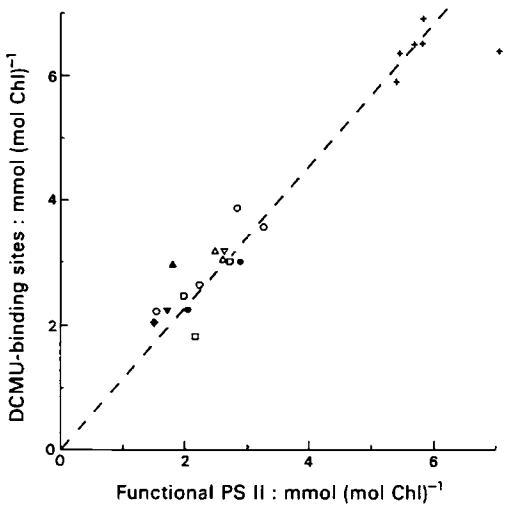

Fig. 2. Correlation between functional [PS II] in vivo, determined in leaf disks via the O_2 yield per flash in the presence of far-red light, with the DCMU-binding sites of thylakoids isolated from corresponding tissues. The herbaceous species were spinach (○), pea (□), lettuce (▽), wild-type barley (△), Chl *b*-less barley (+), *Alocasia macrorrhiza* (●), *Dianella revoluta* (▲), *Colysis ampla* (◆) and *Helmholtzia* glaberima (▼). Varying functional PS II values were obtained for some species grown at different irradiances.

Secondly, our results show that the amount of functional PS II per unit Chl in a number of species is not constant. Shade leaves and sun plants grown at low irradiance have lower [PS II] than plants grown at high irradiance. Thus, in the case of spinach, *Alocasia* and pea plants, the lower values for [PS II] correspond to lower irradiance in the growing period, as found from comparable studies with chloroplasts [7,17]. Values of about 3 mmol per mol Chl were typical for peas and spinach grown in high light. In chloroplasts isolated from high-light-grown peas or spinach we reported values for functional PS II around 2 [7,17] but now routinely obtain 2.8–3.0 when using appropriately low concentrations of PS II acceptors, such as 100 µM DMQ. Higher concentrations of quinone acceptors depress the yield of O_2 (unpublished data, and cf. Ref. 18).

Thirdly, since it is well known that [PS I] is relatively invariant at about 1.6 mmol per mol Chl [4,7,8,17], the variability in [PS II] determined by the leaf method here demonstrates a variable PS II/PS I stoichiometry, a conclusion first reached by Melis and Brown [2] who assayed PS II by a light-induced absorbance change at 325 nm. These two independent demonstrations argue against the view that the PS II/PS I stoichiometry is fixed at unity [4,8].

Fourthly, the close correlation between the functional [PS II] in leaves with the DCMU-binding sites in isolated thylakoids clearly demonstrates that practically all PS II centres which bind DCMU are connected to

the plastoquinone pool; we find no evidence for a substantial pool of non-functional PS II in any non-stressed leaves. This result contrasts with the conclusion that up to 40% of PS II centres are unable to deliver electrons to the plastoquinone pool during repetitive flashing [5].

In assaying PS II in leaves, we have routinely used background far-red light during repetitive flashing. The presence of background far-red light (preferentially absorbed by PS I) was intended to prevent a backlog of electrons in the plastoquinone pool, if, as expected, [PS II]/[PS I] > 1 [7,17]. In the absence of far-red background light, when [PS II]/[PS I] > 1, there may be more electrons delivered by PS II into the plastoquinone pool than are removed from the pool by PS I on each flash, leading to an over-reduction of the pool and hence the reduction of some Q_A. Indeed, in the absence of far-red background light, the method might merely give a value corresponding to the number of PS I centres (approx. 1.6 mmol/mol Chl). However, to our surprise, the effect of far-red light was to increase the O_2 per flash by a factor of only 1.11 ± 0.04 (S.E. of 11 experiments with pea, spinach or lettuce leaf disks). With Myers and Graham [11], who observed no effect of background 703 nm light on O_2 per flash in *Chlorella*, we do not have a complete explanation of why PS II continues to evolve O_2 at the observed rate during repetitive flashing, but it is possibly explained by double turnovers in PS I.

It is known that O_2 itself can oxidise reduced species near PS II [18–20]. It is possible to envisage occasional double turnovers of PS I even in the time of the relatively brief flash used (approx. 3 μs at half height) during which O_2^-, formed for example from the oxidation of plastoquinol, might reduce P-700$^+$. Evidence for double turnovers of PS I has come from experiments with isolated thylakoids where durohydroquinone, in the presence of DCMU, NaN_3 and superoxide dismutase, was able to donate about 3.2 mmol e^- per flash per mol Chl to methyl viologen (Table I), a value approximately twice the concentration of PS I centres. The hypothesis of double turnovers of PS I is further consistent with our finding with thylakoids that the number of electrons transferred from H_2O per flash was similar for the PS II acceptor DMQ as for the PS I acceptor methyl viologen (NaN_3 and superoxide dismutase also present).

If double turnovers of PS I occur, repetitive flash illumination should not readily lead to an over-reduction of the plastoquinone pool that eventually results in a steady-state population of reduced Q_A. To test this idea, we monitored the average redox state of Q_A during repetitive flashing (at 4 Hz) by measuring the relative Chl fluorescence yield with a PAM fluorometer. As illustrated in Fig. 3, the weak modulated light (m) alone gave a lower Chl fluorescence yield corresponding to practically all PS II reaction centres being open (i.e., Q_A oxidised). On the other hand, a one-second saturating test flash (s) gave a high fluorescence yield corresponding to closed reaction centres. During repetitive flashing, the steady-state fluorescence yield was low; in the terminology of [21], q_Q was 0.97 (−FR) or 0.99 (+FR). It is apparent that during 4 Hz flashing, Q_A was mostly oxidised in-between flashes, even in the absence of far-red light. This finding is consistent with our observation that the effect of background far-red light on the O_2 per flash was only slight.

In conclusion, repetitive flashing of leaf discs in the presence of far-red light provides a convenient way to determine functional PS II without the possibility of

TABLE I

Electrons transferred through PS I per flash in the presence of DCMU

The assay medium contained 330 mM sorbitol, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 5 μM gramicidin D, 0.1 mM methyl viologen, 1 mM NaN_3, 500 units/ml superoxide dismutase, 10 μM DCMU, thylakoids equivalent to 30 μM Chl, and, where present, 0.2 mM durohydroquinone, 0.2 mM diaminodurene, 0.05 mM $DCPIPH_2$ (2,6-dichlorophenolindophenol) and 0.2 mM sodium ascorbate. The concentration of PS I in the thylakoids was 1.53 ± 0.03 (n = 3) mmol per mol Chl. It was assumed that 1 O_2 molecule is equivalent to two electrons transferred.

Electron donor system	mmol e^- per flash per mol Chl
Durohydroquinone	3.22 ± 0.03 (3)
Diaminodurene/ascorbate	2.97 ± 0.03 (3)
$DCPIPH_2$/ascorbate	2.23 ± 0.01 (3)

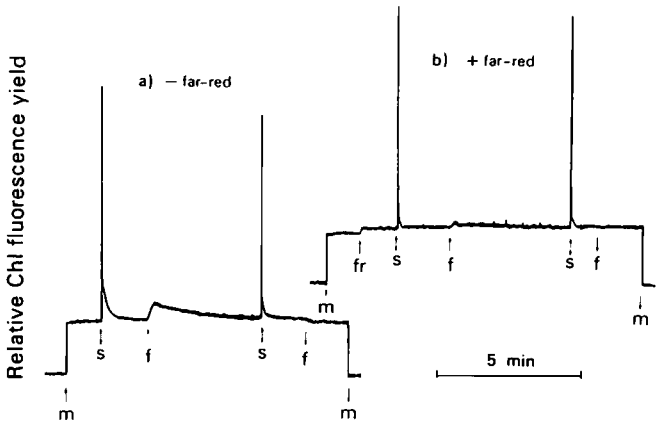

Fig. 3. Relative Chl fluorescence yield before, during and after repetitive flash illumination of a spinach leaf attached to the rest of the glasshouse-grown plant. Relative fluorescence yield was measured with a PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, F.R.G.). Upward arrows indicate light on, downward arrows light off, with the symbols, m for weak modulated light (less than 0.01 $\mu mol \cdot m^{-2} \cdot s^{-1}$), fr for continuous background far-red illumination, s for a 1 s saturating light pulse (to fully reduce Q_A), and f for repetitive flash illumination at 4 Hz. Repetitive flashes (4 Hz) were delivered to one side of the leaf through a heat filter, whereas the other lights were conducted by a multifurcated light guide positioned on the other side. Reversing the leaf surfaces gave the same results.

inactivation during thylakoid isolation. We conclude that much variation in the ratio of PS II to PS I centres is possible amongst plants growing in various light environments [22], that the functional [PS II] in plants such as pea and spinach grown in high light is close to 3 mmol/mol Chl, and that there are not an appreciable number of PS II centres which have herbicide-binding sites but are unconnected to the plastoquinone pool in vivo.

A joint CSIRO/Flinders University grant promoted collaboration between Canberra and Adelaide, for which we are grateful. We thank Ms. Stephanie Hossack-Smith for excellent technical assistance, Mr. Lyn Meredith of the Austrian National Botanic Gardens for some plant materials, and Prof. C.B. Osmond for the use of the PAM chlorophyll fluorometer.

References

1 Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219–239.
2 Melis, A. and Brown, J.S. (1980) Proc. Natl. Acad. Sci. USA 77, 4712–4716.
3 Eckert, H.-J., Renger, G. and Witt, H.T. (1984) FEBS Lett. 167, 316–320.
4 Whitmarsh, J. and Ort, D.R. (1984) Arch. Biochem. Biophys. 231, 378–389.
5 Graan, T. and Ort, D.R. (1986) Biochim. Biophys. Acta 852, 320–330.
6 Jursinic, P.A. and Denneberg, R.J. (1988) Biochim. Biophys. Acta 934, 177–185.
7 Chow, W.S. and Hope, A.B. (1987) Aust. J. Plant Physiol. 14, 21–28.
8 Graan, T. and Ort, D.R. (1984) J. Biol. Chem. 259, 14003–14010.
9 Jursinic, P.A. and Dennenberg, R.J. (1985) Arch. Biochem. Biophys. 241, 540–549.
10 Emerson, R. and Arnold, W. (1932) J. Gen. Physiol. 16, 191–205.
11 Myers, J. and Graham, J.-R. (1983) Plant Physiol. 71, 440–442.
12 Kawamura, M., Mimuro, M. and Fujita, Y. (1979) Plant Cell Physiol. 20, 697–705.
13 Myers, J., Graham, J.-R. and Wang, R.T. (1982) Biochim. Biophys. Acta 722, 281–290.
14 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
15 Tischer, W. and Strotmann, H. (1977) Biochim. Biophys. Acta 460, 113–125.
16 Graan, T. (1986) FEBS Lett. 206, 9–14.
17 Chow, W.S. and Anderson, J.M. (1987) Aust. J. Plant Physiol. 14, 9–19.
18 McCauley, S. and Melis, A. (1987) Photochem. Photobiol. 46, 543–550.
19 McCauley, S.W., Taylor, S.E., Dennenberg, R.J. and Melis, A. (1984) Biochim. Biophys. Acta 765, 186–195.
20 McCauley, S.W. and Melis, A. (1986) Photosyn. Res. 8, 3–16.
21 Schreiber, U., Schliwa, U. and Bilger, W. (1986) Photosyn. Res. 10, 51–62.
22 Chow, W.S., Anderson, J.M. and Hope, A.B. (1988) Photosyn. Res. 17, 277–281.