A CYCLIC PROTOLYTIC REACTION AROUND PHOTOSYSTEM II AT THE INSIDE OF THE THYLAKOID MEMBRANE IN DCMU-POISONED CHLOROPLASTS

Yu-Qun HONG+, Verena FÖRSTER and Wolfgang JUNGE

Lehrstuhl Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, Albrechtstrasse 28, 4500 Osnabrück, FRG

Received 14 July 1981

1. Introduction

The water-oxidizing enzyme system and its interaction with photosystem II is one of the most intensively studied parts of green-plant photosynthesis. More direct indicators of the kinetic behaviour of electron flow from this enzyme system into photosystem II are one EPR signal (IIvf, see [1]) and the release of protons into thylakoid interior as measurable spectrophotometrically with the aid of the dye neutral red [2–8]. In [8] we studied proton release inside thylakoids under conditions where the water-oxidizing enzyme system was switched through its successive oxidation states by exciting photosystem II with a group of 4 short laser flashes starting from the dark equilibrium. The stoichiometric pattern of proton release as function of the flash number was variable depending on the duration of dark adaptation. Under the most stringent conditions, we observed a pattern similar to that in [4,5], which is best explained by attributing a proton-over-electron stoichiometry of 1:0:1:2 to the transitions $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4$, S_0 of the water-oxidizing enzyme system. We also resolved the time course of proton release and found it partially compatible with the 1 ms ($S_3 \rightarrow S_4$, S_0) and 500 μs ($S_2 \rightarrow S_3$) that were to be expected on basis of the EPR work in [1]. However, one very rapid component of proton release (half-rise at 100 μs), which was dominant at the second flash of light, did not quite fit into the expectation. We tentatively attributed this component to a protolytic reaction which is not directly associated with the water-oxidizing enzyme complex, but rather with the secondary donors of P860 (e.g., Z and Y; review [9]). Here we report on protolytic reactions which are associated with photosystem II activity under conditions where water oxidation cannot occur. The data, although not fully interpretable at present, demonstrate clearly that a protolytic reaction is associated with cyclic electron transfer around photosystem II, under DCMU poisoning of electron transport from the primary to the secondary plastoquinone acceptor. The cyclic electron transfer which drives this reaction is apparently non-electrogenic at the time scale of 20 μs an it may require the re-entry of a proton into the membrane before another turnover can occur.

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3 methyl-6-isopropyl-1,4-benzoquinone; EPR, electron paramagnetic resonance spectroscopy; chl, chlorophyll

+ On leave from: the Institute of Plant Physiology, Academia Sinica, Shanghai, China

2. Materials and methods

Spinach chloroplasts were prepared from market spinach by a modification of the method in [10]. They were stored under liquid nitrogen until use. The reaction suspension contained chloroplasts at 10 μM chl or 20 μM chl, 25 μM KCl, 3 mM $MgCl_2$, 0.7 g serum albumin/l, 10 μM benzylviologen as electron acceptor or 2 mM $K_3[Fe(CN)_6]$ and 6 μM DBMIB, respectively; 5 μM or 20 μM DCMU were added when indicated. Absorption changes were measured in a rapid kinetic spectrophotometer with a Biomation transient recorder (6500) and a Tracor averaging computer (TN-1500) interfaced to the former. The sample was excited with flashes from a Q-switched Ruby laser (694 nm, half-width 40 ns, typical energy 5 mJ/cm^2) or Xenon flash lamp (>630 nm, 15 μs, 3 mJ/cm^2). In the exper-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

iments with dark-adapted chloroplasts the chloroplasts were not exposed to light (while thawing and preparing the suspension) until the measuring light was gated open, typically 10 ms before the actual sampling and averaging (to allow for relaxation of the photomultiplier in response to light gating). The total measuring light energy/sample was 3 $\mu J/cm^2$, which excited <5% of the reaction centres [8]. The automatic sampling, DC-offset device and automatic flow system for the exchange of samples after one set of exciting flashes was described in [8].

Far-red background light of an intensity which keeps P700 in its oxidized state (8 mW/cm^2) was provided by a halogen lamp together with a Schott glass filter RG 715. Absorption changes of neutral red which indicate pH-changes inside thylakoids were recorded as in [3]. Signals at 548 nm were recorded in the presence and in the absence of this dye. The subtraction of the latter from the former ones yielded a response solely to pH-changes inside (note the strong buffering of the external phase by bovine serum albumin). This response is practically artefact free, quantitative and kinetically highly resolving [3].

3. Results and discussion

3.1. *Dark-adapted chloroplasts*

Fig.1 shows the absorption changes of neutral red under excitation of chloroplasts which were totally dark adapted (according to [8]) with a series of 4 short laser flashes. Protolytic reactions driven by photosystem I were blocked by addition of DBMIB as in [7,11]. The upper traces show proton release into thylakoids in the absence of DCMU. The middle traces show proton release in the presence of DCMU and the lower traces were obtained under the same conditions as in the middle except that imidazole was added to buffer away pH changes in the internal phase of thylakoids. Comparison of the middle with the lower (buffered) traces shows that the absorption changes of neutral red, which are observable in dark-adapted chloroplasts after their treatment with DCMU, indeed reflect pH changes (for a more thorough discussion of the neutral red technique see [3]). The middle traces reveal two pecularities:

(1) After the first flash, in the presence of DCMU more protons are released inside than in its absence. As DCMU is believed to act on the outside part of photosystem II this is not easily understood.

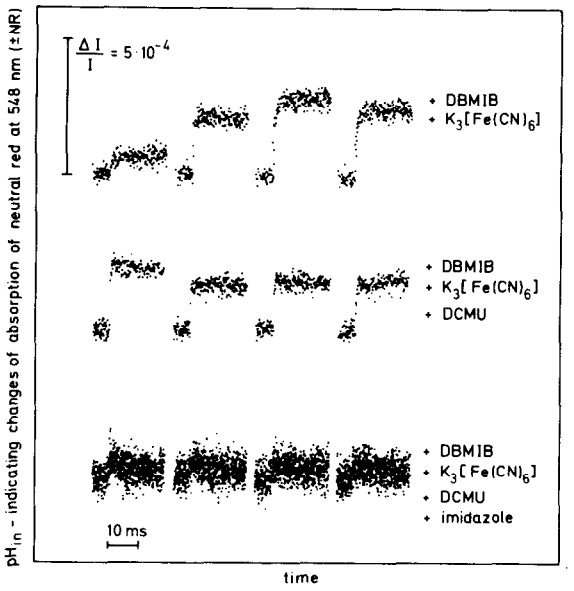

Fig.1. Patterns of proton release inside thylakoids in dark-adapted chloroplasts excited by 4 laser flashes. Proton release coupled with plastohydroquinone reoxidation was inhibited by addition of 2 mM $K_3[Fe(CN)_6]$ and 6 μM DBMIB: (upper) proton release attributed to the water-splitting enzyme system (100 μs/point); (middle) proton release pattern observed after addition of 5 μM DCMU (100 μs/point); (lower) proton release in the presence of 5 μM DCMU is buffered away by addition of the membrane-permeating buffer imidazole (5 mM) (20 μs/point and matched electrical bandwidth caused wider noise band). 50 samples were averaged (for each of ± neutral red): spacing of flashes within a group of four, 3s; dark time between repetitive groups, 20 s.

(2) In the subsequent flashes (where photosystem II can operate cyclically but not linearly, because DCMU blocks electron efflux from the acceptor Q^-) protons are released.

We asked whether the action of DCMU was as expected under our experimental conditions. For this we monitored the electric potential generation via the electrochromic absorption changes at 522 nm [12] and the activity of photosystem I via the absorption changes at 819 nm [13] (at the given time resolution the contribution of photosystem II to absorption changes at this wavelength was virtually absent [14]). The result is shown in fig.2. It is apparent that the first flash induces a charge separation across the membrane in both photosystems, while the subsequent flashes virtually do not produce a charge separation in photosystem II. Such a charge separation is then either absent, or, alternatively it is reversed so rapidly

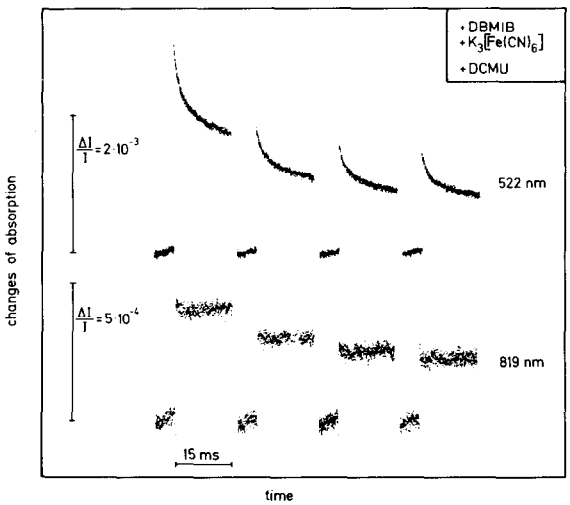

Fig.2. Electrochromic and infrared absorption changes under excitation of dark-adapted chloroplasts by 4 short laser flashes in the presence of DCMU. 2 mM $K_3[Fe(CN)_6]$, 6 μM DBMIB and 5 μM DCMU were present (av. 50 samples, 20 μs/point, 10 kHz bandwidth). Other conditions as in fig.1.

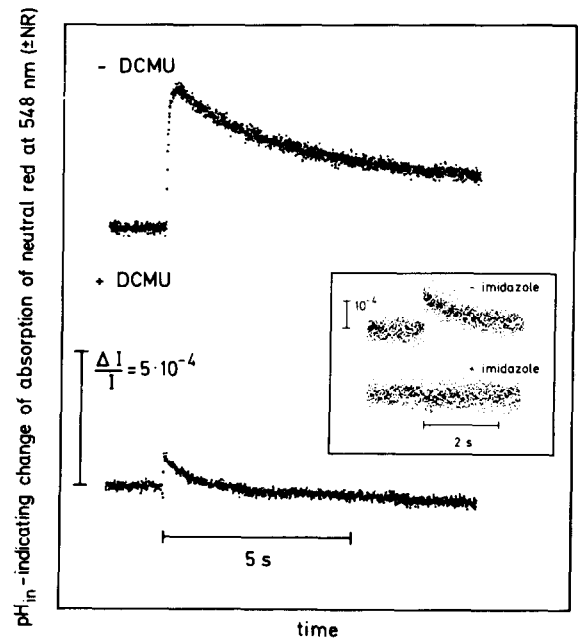

Fig.3. Relaxation of flash-induced acidification inside thylakoids in the absence and in the presence of 5 μM DCMU under repetitive excitation (repetition rate 0.07 Hz). Benzylviologen at 10 μM was used as electron acceptor (av. 20 samples, 5 ms/point). The insert shows that proton release occurring in the presence of DCMU is completely buffered away by 5 mM imidazole.

that it escaped detection (here at 20 μs/address of the transient recorder).

3.2. *Chloroplasts under repetitive excitation*

We studied the release of protons in DCMU-poisoned chloroplasts also under repetitive excitation (period 8 s). Fig.3 shows the release of protons into thylakoids in unpoisoned chloroplasts (above) and in DCMU-treated chloroplasts (below). The insert shows that the neutral red signal in the presence of DCMU is indeed to be buffered away; i.e., it reflects a pH-transient in the internal phase of thylakoids. Again we observed the release of ~0.5 proton/electron under conditions where no oxygen evolution could be detected and where no electrogenic photosystem II activity was detectable at 20 μs resolution (not shown). The most striking feature, however, is the residence time of these protons in the internal phase. The upper traces in fig.3 show that the decay of the flash-induced internal acidification of thylakoids has a half-time of 5 s in the absence of DCMU (and of ADP + P_i). As this half-time is also apparent from measurements on the reversal of the concomitant alkalinization of the external phase (not shown) it is certain that the 5 s characterize the equilibration of a pH-difference across the membrane. In contrast to this the lower trace in fig.3, which was obtained in the presence of DCMU

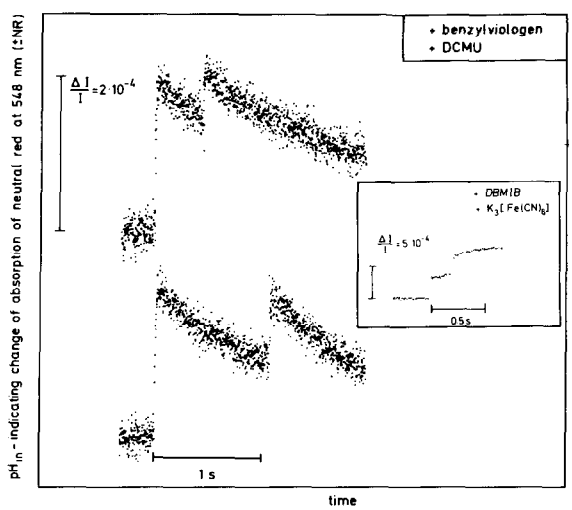

Fig.4. Proton release and relaxation in the presence of 5 μM DCMU studied under repetitive excitation of chloroplasts by a doublet of xenon flashes. The two flashes were spaced 0.4 s and 1 s apart, respectively (repetition rate 0.12 Hz, instrumental time resolution 3 ms). The insert shows proton release of photosystem II in the absence of DCMU for comparison.

Table 1
Amplitudes of infrared absorption changes and proton release inside thylakoids in DCMU-poisoned chloroplasts with and without background illumination by continuous far-red light

Redox agents	DCMU	Far red light	Extent of absorption changes	
			819 nm	548 nm (±neutral red)
Benzylviologen (10 μM)	5 μM	No	100%	100%
Benzylviologen (10 μM)	5 μM	Yes	19%	65%
DBMIB (6 μM) + $K_3[Fe(CN_6]$ (2 mM)	20 μM	Yes	10%	71%

shows a 10-fold faster decay (half-decay time 500 ms). This could indicate that protons released inside are taken up back into the membrane rather before they can leak out into the external phase. (The possibility that the 'DCMU-protons' are released into another subcompartment than those which are ejected into the internal aqueous bulk can be rejected on the ground that they are accessible to the same set of buffers (see insert in fig.3 and non-documented further experiments analogous to fig.3 in [8]).

We asked whether proton release under cyclic electron transfer around photosystem II requires the reentry of protons into the membrane for reactivation of the cycle. From fig.4 it is obvious that proton release under these conditions requires the return of a proton before a next turnover can be initiated. The insert in fig.4 shows that this is not so if photosystem II operates linearly in the absence of DCMU.

We checked the attribution of proton release under DCMU poisoning to photosystem II. For this we compared the activity of photosystem I as apparent from the absorption changes at 819 nm with the neutral red signal under far-red background light. The result is documented in table 1. It clearly shows that proton release is due to photosystem II.

In the absence of DCMU the rise time of the internal acidification due to photosystem II activity is fast and multiphasic [7,8]. The most rapid component rises at 100 μs, the second most rapid at 250 μs in dark-adapted chloroplasts [8]. We compared the rise of proton release in the absence and in the presence of DCMU under repetitive excitation (see fig.5). Proton release in the presence of DCMU (lower trace) rises biphasically at apparent half-rise times of ≤10 μs and 100 μs, while the rapid phase in the absence of DCMU rises with 180 μs (upper trace).

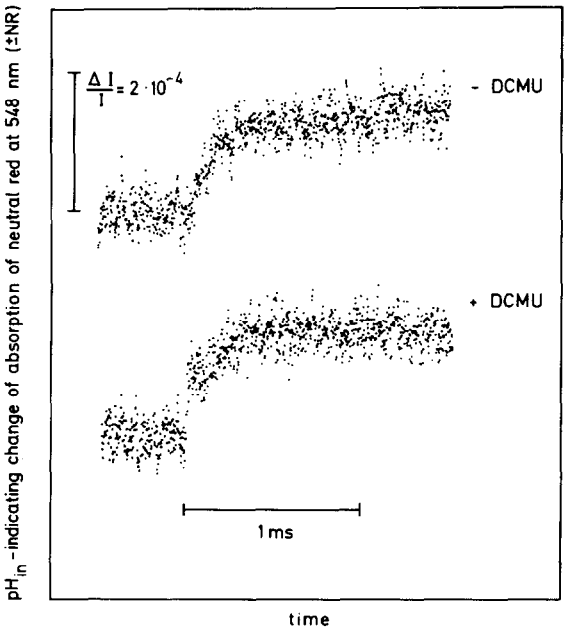

Fig.5. High time resolution of flash-induced proton release inside thylakoids under repetitive excitation in the absence and in the presence of 5 μM DCMU; 10 μM benzylviologen was used as electron acceptor (av. 200 samples; the traces were obtained at 300 kHz bandwidth at the amplifier and an address setting of 2 μs of the transient recorder).

4. Conclusions

These experiments corroborate our suggestion that proton release into thylakoids by photosystem II is not entirely due to the water-oxidizing complex, but that at least one protolytic reaction occurs at the level of the secondary electron donors to photosystem II. In addition, the experiments demonstrated the exis-

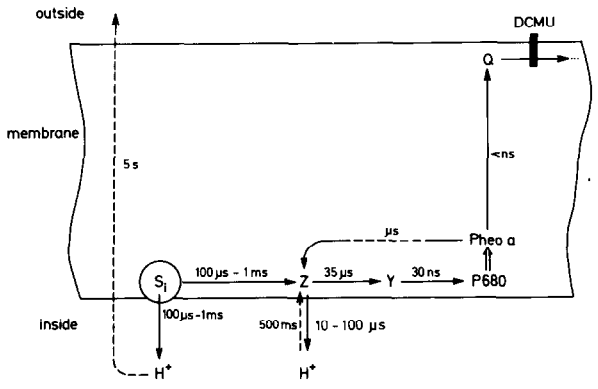

Fig.6. Tentative scheme of electron transport and protolytic reactions at the donor side of photosystem II.

tence of a cyclic (electron) transport reaction around photosystem II which is accompanied with proton extrusion into thylakoids but, more interestingly, which requires the reprotonation of one carrier, possibly Z, before a second turnover can be initiated. The cycle is not electrogenic at a time resolution of 20 µs. Hence, all its chemical components are either located close to the inner surface of the membrane or the reactions are more rapid than detected at the given time resolution.

Fig.6 tentatively illustrates the arrangement and the reaction sequence of the involved electron carriers. Only P680 (chlorophyll *a*) and pheo *a* (pheophytin *a*) are chemically defined until now, while the other components were inferred from kinetic studies (reviews [9,18]). Our experiments suggest that <1 proton/P680 is released under cyclic activity. Fig.4 may be misinterpreted to indicate that operation of the cyclic electron transfer-required protonation of Z. This conclusion, however, is unwarrented, since protons were the only observable in our studies. Instead, the report [16] of a non-electrogenic electron transfer around photosystem II (under DCMU poisoning) suggests (see fig.4 in [16]) that the cycle may be operative even if a doublet of flashes is fired, closer than reprotonation can occur. It is premature to conclude as to the necessity for protons in this cyclic electron transfer. Protonation of an electron donor (DI in [17]) to P680 had been postulated on indirect evidence from the reduction kinetics of $P680^+$ [17]. Our data corroborate that postulation.

Acknowledgements

We are very grateful to Margret Offermann for technical help and for the graphs. Gitta Moehrke did the typing. This work was financially supported by the Deutsche Forschungsgemeinschaft, the Solar Energy Program of the European Commission and by a scholarship of the Chinese Government to Y.-Q. H.

References

[1] Babcock, G. T., Blankenship, R. E. and Sauer, K. (1976) FEBS Lett. 61, 286–289.
[2] Ausländer, W. and Junge, W. (1975) FEBS Lett. 59, 310–315.
[3] Junge, W., Ausländer, W., McGeer, A. T. and Runge, Th. (1979) Biochim. Biophys. Acta 546, 121–141.
[4] Saphon, S. and Crofts, A. R. (1977) Z. Natuforsch. 32e, 810–816.
[5] Velthuys, B. R., Crowther, D. and Hind, G. (1979) Biochim. Biophys. Acta 547, 138–148.
[6] Junge, W., Renger, G. and Ausländer, W. (1977) FEBS Lett. 79, 155–159.
[7] Junge, W. and Ausländer, W. (1978) in: Photosynthetic Water Oxidation (Metzner, H. ed) pp. 213–228, Academic Press, London.
[8] Förster, V., Hong, Y.-Q. and Junge, W. (1981) submitted.
[9] Velthuys, B. R. (1980) Annu. Rev. Plant Physiol. 31, 545–567.
[10] Reeves, S. G. and Hall, D. O. (1973) Biochim. Biophys. Acta 314, 66–78.
[11] Ausländer, W. and Heathcote, P. and Junge, W. (1974) FEBS Lett. 47, 229–235.
[12] Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b. 244–254.
[13] Ke, B. (1973) Biochim. Biophys. Acta 301, 1–33.
[14] Van Best, J. A. and Mathis, P. (1978) Biochim. Biophys. Acta 503, 178–188.
[15] Junge, W. (1977) Annu. Rev. Plant. Physiol. 28, 503–53
[16] Eckert, H. J. and Renger, G. (1980) Photochem. Photobiol. 31, 501–511.
[17] Conjeaud, H. and Mathis, P. (1980) Biochim. Biophys. Acta 590, 353–359.
[18] Bouges-Bocquet, B. (1980) Biochim. Biophys. Acta 594, 85–103.