Volume 253, number 1,2, 33–37 FEB 07442 August 1989

The protonic shortcircuit by DCCD in photosystem II

A common feature of all redox transitions of water oxidation

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Received 6 June 1989

Photosystem II of green plants oxidizes water at an Mn centre and reduces bound plastoquinone to supply electrons to photosystem I. Owing to the vectorial properties of the electron transfer (water-)protons are released into the lumen of thylakoids, while (quinole-)protons are taken up from the medium. The proton-pumping activity can be shortcircuited by covalent modification of two polypeptides with *N,N'*dicyclohexylcarbodiimide (DCCD). Then (water-)protons are no longer released but, within milliseconds, they are funnelled through the protein to the reduced quinone [(1988) EMBO J. 7,589–594]. When dark-adapted thylakoids are excited by a series of light flashes water oxidation to dioxygen is synchronised to follow a stepped reaction pattern with period four, which is apparent in the pattern of proton release. We asked whether or not the above 'protonic shortcircuit' was restricted to certain reactions out of the four-stepped cycle. This was not observed. Instead, each step was equally affected and the complement of the period four pattern was observed for proton uptake at the site of quinone reduction. This proved that seemingly non-released protons were transferred to the quinone and, for unmodified membranes, it suggested that protons produced during the partial reactions of water oxidation used one and the same DCCD-sensitive outlet to the lumen.

Photosystem II; Proton pump; Proton channel; Water oxidase; Dicyclohexylcarbodiimide, *N,N'*-

1. INTRODUCTION

Photosynthesis of green plants is powered by two photosystems, one of which, photosystem II, oxidizes water and reduces bound plastoquinone [1]. The initial event, vectorial electron transfer from a special chlorophyll *a*, P680, at the lumen side of the thylakoid membrane to bound quinone at the stroma side, is electrogenic and it occurs within the picosecond time scale [2]. The consecutive oxidation of water follows more slowly (100 μs to 1 ms, [3]) together with proton release into the lumen [4]. At the stroma side of the membrane, plastoquinone reduction is followed by uptake of one proton per electron with apparent half-rise time longer than 3 ms [5]. While the primary photochemical reaction is a one quantum/one electron event, water oxidation cycles with a period of four through its redox states [6] and the reduction of plastoquinone with a period of two [7]. Proton release into the lumen reveals the period-of-four oscillation characteristic for water oxidation (e.g. [4]). However, the pattern of proton uptake at the quinone site fails to produce oscillations (period of two) [5]. This is suggestive of a merely electrostatic control of proton uptake at the quinone site, which does not always involve direct protonation of the quinone but transiently also of amino acid side chains (see also [8] for bacterial reaction centres). By analogy it is not certain whether the four-stepped pattern of proton release directly reflects proton release from bound water molecules which are oxidized at the Mn centre (see [1,9] for recent reviews). Instead, the pat-

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; PS II, PS I, photosystem II, photosystem I; DNP-INT, dinitrophenylether of iodonitromythol; DMQ, 2,6-dimethyl-*p*-benzoquinone; cyt, cytochrome

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00145793/89/$3.50

tern of proton release may also be blurred by the more indirect electrostatic response of amino acid side chains in the vicinity of or even away from the centre. In this respect it is worth asking, whether or not the protons liberated during different partial reactions of water oxidation follow one and the same pathway between the Mn centre and the lumen.

A clue was sought in the short circuit of the proton pumping activity of photosystem II which can be induced by covalent modification of the 20 and 24 kDa polypeptides with DCCD. After DCCD treatment of thylakoid membranes, proton release into the lumen by water oxidation is diminished, a rapid electrogenic back reaction (< ms) is apparent (which reveals a kinetic H/D-isotope effect of 1.6) and proton uptake at the quinone site is diminished correspondingly [10]. This has been tentatively interpreted as follows: DCCD blocks the natural outlet to the lumen of protons which are derived from water oxidation. This channels protons from the lumen side of photosystem II across the protein to the pocket with the negatively charged quinone [10] at the stroma side.

In this work we followed the dependence of this effect on the redox state of the water oxidase. The Mn centres were synchronized in the conventional way [6] by dark adaptation of thylakoids prior to excitation with a series of flashes. In DCCD-treated membranes we found that the complement of the period-of-four oscillation of water oxidation became apparent for proton uptake on the other side of the membrane. This implied that protons from all transitions of water oxidation were redirected towards the reduced quinone.

2. MATERIALS AND METHODS

Stacked thylakoids were prepared from pea seedlings as previously [5]. The resulting stock solution was diluted with 5 mM $MgCl_2$, 10 mM NaCl and 2 mM Tricine-NaOH, pH 7.8, to a Chl concentration of 50 μM and incubated with 150 μM DCCD for 10 min at room temperature under dim light. Following incubation, the membranes were centrifuged at 11000 × g for 10 min and resuspended in 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sorbitol and 100 mM tricine-NaOH, pH 7.8, to yield a final Chl concentration of about 2 mg/ml. Control thylakoids were treated in the same way without adding DCCD to the incubation medium. All samples were stored at −80°C for up to 6 days.

pH transients in the lumen by the indicator dye neutral red [11], pH transients in the medium by phenol red [5] and the transient oxidoreduction of P700 [12] were measured spectrophotometrically in an optical cell with 2 cm path length. Saturating excitation by a xenon flash lamp; duration 5 μs (FWHM), wavelength >610 nm, 1.8 kJ/cm^2. Thylakoids equivalent to 10 μM Chl were suspended in an assay medium containing 5 mM $MgCl_2$, 10 mM NaCl and buffers as indicated in the figure legends. pH-specific absorption changes were obtained by subtracting a signal detected in the absence from a signal detected in the presence of the indicator dye (see also [11]).

Oxygen evolution under single-flash excitation was detected with an electrode as described by Schmidt and Thibault [13]. The measurements were carried out with 50 μM Chl suspended in 10 mM $MgCl_2$, 20 mM NaCl and 30 mM tricine-NaOH.

For all measurements thylakoids were dark-adapted for 15 min and excited with a train of 6 saturating flashes. Unless otherwise indicated the pH was adjusted to 7.5 and hexacyanoferrate III (200 μM) served as terminal electron acceptor which, at this low concentration, was selective for photosystem I [5].

3. RESULTS AND DISCUSSION

Incubation of stacked thylakoids with DCCD affects the electron transport [14] and the proton pumping activity of photosystem II [10]. At higher concentrations of DCCD (K_{150} = 5 mol DCCD/mol Chl) electron transport is inhibited and at lower ones (K_{150} = 2 mol DCCD/mol Chl) the proton pump in photosystem II is short-circuited [10]. While the proton shortcircuit is correlated with covalent binding of DCCD to 24 and/or 20 kDa polypeptides [10], the inhibition of electron transport [14] is reversed by washing the membranes in DCCD-free medium, even when 6 mol DCCD/mol Chl were used during preincubation (data not documented).

We studied the dependence of the protonic shortcircuit on the redox state of the water oxidase. To avoid interference with the reversible effect on electron transport, thylakoids were first incubated with 3 mol DCCD/mol Chl and then centrifuged and resuspended in DCCD-free medium. Fig.1 shows the extent of absorption changes at 820 nM (A) and the oxygen yield (B) induced by a train of 6 flashes given to dark-adapted thylakoids, which had undergone DCCD incubation and one wash. Control thylakoids were treated in the same way, but without addition of DCCD. The above parameters are direct indicators for the activity of PS I and PS II, respectively. According to fig.1, the activity of both photosystems was not altered by treatment with DCCD. In particular, the pattern of oxygen release, both, in con-

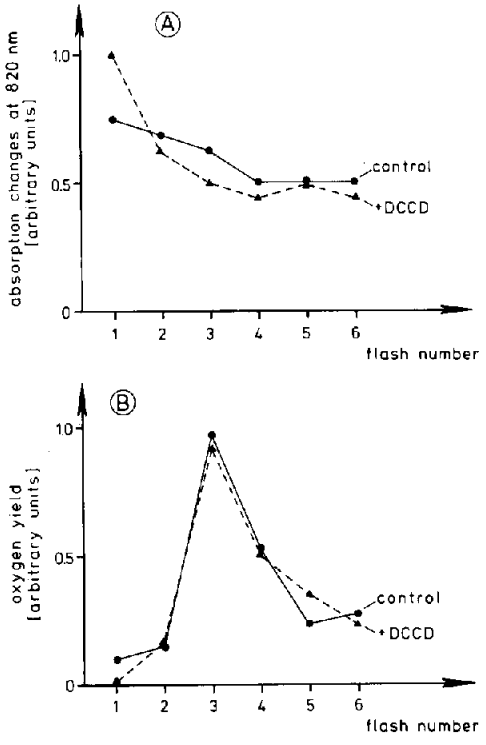

Fig.1. (A) Magnitude of absorption changes at 820 nm in dependence of the flash number. 2 mM Tricine-NaOH was added as buffer. Excitation of dark-adapted thylakoids by a train of short flashes. DCCD preincubation when indicated. (B) Relative yield of oxygen evolution in dependence of the flash number. Assay medium: 10 mM $MgCl_2$, 20 mM NaCl and 30 mM Tricine-NaOH. No electron acceptor added. Other conditions as in A.

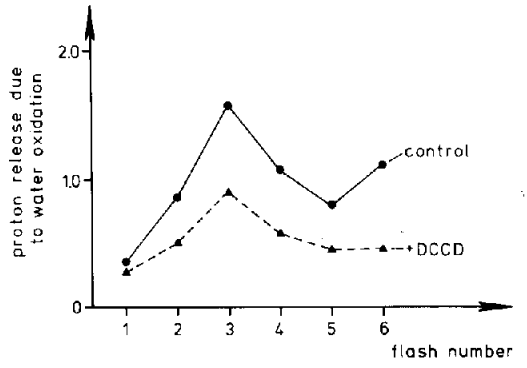

Fig.2. Relative yield of proton release into the lumen as function of flash number. Measurement of absorption changes of neutral red at 548 nm. 5 μM DNP-INT, 10 μM DMQ and 2.6 mg/ml BSA were added to the assay medium. DMQ served to accept electrons from PS II and DNP-INT served to block the oxidation site of plastoquinole at the cyt b_6f-complex. Under these conditions proton release into the lumen was exclusively due to water oxidation. Ordinate scale was normalised to give four protons in the first four flashes in control thylakoids. Other conditions as in fig.1A.

trol thylakoids and in DCCD-treated samples was as usual [6]. The maximum at the third flash indicated that out of the four states, S_0–S_3, S_1 was most stable in the dark. The nearly unchanged values for the oxidation of P700 in controls and in DCCD-treated thylakoids proved the intactness of the linear electron transport chain between PS II and PS I.

Proton release from water oxidation and proton uptake at the site of quinone reduction by PS II were recorded under the same conditions. The extent of flash induced pH transients in the lumen revealed the well-known pattern of proton release due to water oxidation, with a maximum on the third flash [4], both in controls (fig.2, circles) and in DCCD-treated samples (fig.2, triangles). In DCCD-treated samples, however, the magnitude was lowered to about one half. That this holds true for all transitions implied that there was no S-state dependence of the changed routing of protons which were produced at the manganese centre.

The question which way the seemingly non-released protons had taken, was answered by measurements of proton uptake at the plastoquinone reduction site of PS II. Fig.3A shows the original traces of pH indicating absorption changes of phenol red at 559 nm. Fig.3B shows the extent of the flash-induced transients as a function of flash number. In control thylakoids (fig.3B, circles) proton uptake from the medium was nearly the same at every flash, equivalent to one proton per flash [15]. Only in DCCD-treated samples proton uptake from the medium showed a pronounced dependence on the flash number (fig.3B, triangles): no, or very little decrease on the first flash (when there was no proton release by water oxidation in untreated samples) and maximum decrease on the third flash (when proton release was maximum in controls). This corroborated the notion that the seemingly non-produced protons from water oxidation were indeed transferred to the reduced bound quinone.

We normalised the data under the presumption that the sum of pH-indicating absorption changes after the first four flashes reflected release and up-

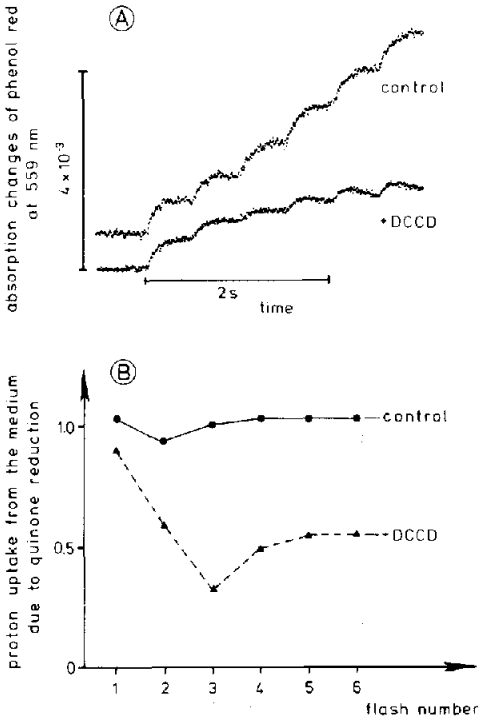

Fig.3. (A) Absorption changes of phenol red at 559 nm, indicating proton uptake from the medium at the reducing site of photosystem II. No buffer added. (B) Relative yield of proton uptake as function of flash number, calculated from the traces in A. Normalisation as in fig.2. Other conditions as in fig.1A.

take, respectively, of four protons. The diminution of proton release into the lumen as a function of flash number was compared with the diminution of proton uptake from the medium. As shown in fig.4 the respective differences (uptake/release) were very similar. This indicated that all protons which, due to DCCD modification, were seemingly not released into the lumen were used to protonate the site of the reduced quinones.

These results supply new information about the outlet of protons from the manganese centre. There must be a common pathway for protons which are produced during water oxidation. DCCD may block this path and force protons to follow the electrostatic attraction of the reduced quinone towards the other side of the transmembrane protein. How protons are conducted by the protein remains unknown. The pathway has certainly not been designed by nature for this wasteful

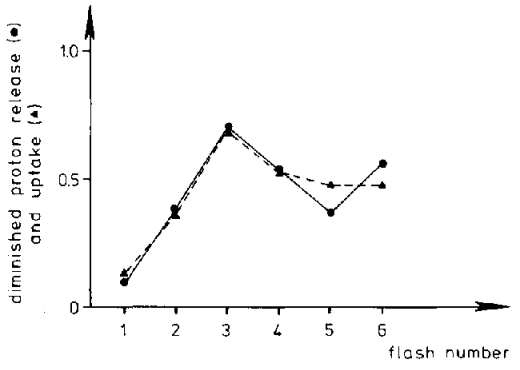

Fig.4. Normalised values of diminished proton release into the lumen (circles) and diminished proton uptake from the medium (triangles) after modification by DCCD. The extent was calculated by subtracting the results in fig.2 and fig.3B, respectively.

purpose. The results also suggest, that none of the proton release steps of water oxidation is peripheral and therefore unrelated to the production of a proton in the Mn centre. Instead, protons seemed to be produced with the known stoichiometric pattern (1:0:1:2 in $S_0/S_1/S_2/S_3/S_0$). It will be interesting to identify the polypeptides which were covalently modified by DCCD under conditions producing the above effects. J. Hoppe determined the sequence of a CNBr-fragment (23 residues) of the selectively labelled 24 kDa polypeptide. This fragment contained the DCCD-binding amino acid, probably the only Asp which was present (unpublished). From sequence comparisons between this fragment and known protein and gene structures associated with photosystem II the fragment was neither identical with polypeptides from the reaction centre including D1/D2/Cyt *b*-559, the extrinsic polypeptides (16, 23 and 33 kDa), nor with other known proteins in the 20–24 kDa range [16,17]. Immunoblots with antibodies against different photosystem II polypeptides revealed some crossreactivity of the labelled 24 kDa polypeptide with antibodies against CP II [18] and CP 24 [19] (Camm and Green, personal communication).

4. CONCLUSION

This work corroborated that the covalent modification(s) of certain proteins by DCCD

redirected the path of protons which were produced by water oxidation. The appearance of a period four oscillation for proton uptake at the reducing site of photosystem II showed in a direct way that protons produced by water oxidation were transferred to the quinone reduction site. This occurred to an equal extent for the transitions S_0–S_1, S_2–S_3 and S_3–S_4 of the water oxidase. It strongly suggested that these protons originated from the very neighbourhood of the Mn centre and it argued against a peripheral protolytic response of polypeptides.

Acknowledgements: We wish to thank Dr Jürgen Hoppe for sequence analysis, Drs George Schmidt and Klaus Bader for the opportunity for oxygen yield measurements and Hella Kenneweg for drawings and photographs. DNP-INT was a gift from Dr A. Trebst. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 171, Teilprojekt A2).

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