The photosynthetic water oxidase: its proton pumping activity is short-circuited within the protein by DCCD

Peter Jahns, Andrea Polle and Wolfgang Junge

Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, Postfach 4469, D-4500 Osnabrück, FRG

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The photosynthetic water oxidase is composed of~15 polypeptides which are grouped around two functional parts: photosystem II and the catalytic manganese centre. Photochemically driven vectorial electron transfer between the manganese centre and bound plastoquinone causes deprotonation–protonation reactions at opposite sides of the thylakoid membrane. Thereby the water oxidase acts as a proton pump. Incubation of stacked thylakoids with N,N'-dicyclohexylcarbodiimide (DCCD) short-circuited its proton pumping activity. Under flashing light, the extent of both proton release into the lumen by water oxidation and of proton uptake from the medium by reduced quinone was diminished. Instead there was a rapid electrogenic backreaction with a strong H/D-isotope effect. Apparently protons which were produced by water oxidation were channelled across the transmembrane protein to the bound quinone. A more rapid protonation of the reduced quinone was evident from a shortening of the time lag for the reduction of photosystem I. These effects were paralleled by the preferential labelling with [14C]DCCD in stacked thylakoids of two polypeptides with 20 and 24 kd apparent molecular mass. These may be capping the oxidizing and the reducing terminus of the water oxidase to control proton extrusion and proton uptake respectively.

Key words: photosynthesis/photosystem II/proton pump/water oxidase/DCCD

Introduction

The photosynthetic electron transport chain in thylakoid membranes is composed of three membrane-spanning enzyme complexes: photosystem II, cytochrome b6f and photosystem I (see Figure 1A). Excitation of the two photochemically active components drives electron flow from water to NADP\(^+\). Plastoquinone mediates electron flow between photosystem II and cytochrome b6f, plastocyanin between cytochrome b6f and photosystem I (Haehnel, 1984). The vectorial electron transport is coupled with proton translocation from the stroma to the lumen side of the thylakoid membrane (Junge, 1982) (illustrated in Figure 1A).

The photosynthetic water oxidase is a complex enzyme which integrates a photochemical reaction centre, photosystem II, and a catalytic centre, a manganese cluster (Barber, 1987). Only two polypeptides, D1 and D2, seem to contain binding sites for all the electron transfer components of the photochemical reaction centre and even some of the coordination sites for the catalytic manganese cluster.

But at least 13 further polypeptides are involved, most of which with still ill-defined function (Murata and Miyao, 1987) (see Figure 1B). In stacked thylakoids the water oxidase is located in the appressed portions of the thylakoid membrane (Andersson and Anderson, 1980). One quantum of light drives a very rapid charge separation from a donor chlorophyll \(a\) at the lumen side to a bound plastoquinone at the stroma side of the thylakoid membrane (Junge, 1982). The donor chlorophyll \(a\) cation is in turn reduced by the manganese centre which, after accumulation of four oxidizing equivalents, oxidizes two molecules of water to yield dioxygen (Kok et al., 1970). Water oxidation is accompanied by proton release into the lumen (see Förster and Junge, 1985). The reduction of bound quinone causes proton uptake.
at the stroma side of the thylakoid membrane (see Polle and Junge, 1986a,b). There is kinetic evidence that both redox cofactors are not in direct contact with the aqueous bulk. Hence there may be transient proton relay with polypeptides capping the respective redox sites. By analogy with cytochrome c oxidase (Wikström, 1987) it is conceivable that the control by certain proteins of the protonic reactions with the bulk phases serves to modify the energetic requirements of the stepped oxidoreduction of the manganese centre and the two stepped one of bound plastoquinones. The rapid electrogenic charge separation together with the two protonic reactions at opposite sides of the membrane make the photosynthetic water oxidase act as an electrogenic proton pump for the purpose of ATP synthesis.

The detailed kinetic analysis of the electrochemical events in thylakoids has been facilitated by flash spectrophotometric techniques for measuring with very high time resolution proton release into the lumen (Junge et al., 1979; Hong and Junge, 1983), proton uptake from the medium (e.g. Polle and Junge, 1986a) and transients of the transmembrane voltage (Junge and Witt, 1968).

$N,N'$-Dicyclohexylcarbodiimide (DCCD) is a hydrophobic carbodiimide with high reactivity towards several organic groups. At room temperature and about neutral pH, DCCD mainly reacts with carboxyl groups [for a review see Azzi et al. (1984)]. In chloroplasts it binds covalently to subunit III of the proton channel, CF$_3$ (McCarty and Racker, 1967), and to subunit $\beta$ of the catalytic part of the ATP synthase, CF$_1$ (Shoshan and Selman, 1980). By covalent modification of a single glutamic acid residue in subunit III it blocks proton conduction through CF$_3$. This parallels its action on the related F$_0$-type proton channels of mitochondria and eubacteria [see Sebald and Hoppe (1981) for a review]. When present at high concentration, DCCD can inhibit electron transport in chloroplasts (McCarty and Racker, 1967; Sane et al., 1979) and in purple bacteria (Pototzky et al., 1981). The inhibition of electron transport, however, could not be related to covalent binding of DCCD (Johanningmeier and Sane, 1981; Zürrer et al., 1983).

Here we describe a new type of action of DCCD on chloroplasts which affects the protonic reactions around photosystem II without the known inhibition of the electron.

Fig. 2. The right column illustrates the events which are represented by the traces at the left. (A) Time course of the absorption changes of cresol red (15 $\mu$M) which indicate pH transients in the medium. With methyl viologen (10 $\mu$M) as electron receptor and in the presence of 2800 units superoxide dismutase/mg Chl (no buffer added). The rapid rise represents proton uptake at photosystem I during the dismutation of formed superoxide (see Polle and Junge, 1986a) and the slow rise proton uptake by photosystem II (Polle and Junge, 1986a,b). (B) Time course of the electrochromic absorption changes, indicating the rapid generation of a transmembrane voltage by both photosystems and its decay (2 mM tricine/NaOH added). (C) Time course of the absorption changes of neutral red (13 $\mu$M), indicating pH transients in the lumen. pH changes in the suspending medium were selectively buffered by added bovine serum albumin (2.6 mg/ml). The rapid rise reflects proton deposition by water oxidation and the slow rise proton deposition resulting from the oxidation of plastoquinol (Ausländer and Junge, 1975; Förster and Junge, 1985). All measurements were performed at pH 7.7 and at room temperature.
transport which requires higher concentrations of DCCD. We found the proton pumping activity of photosystem II short-circuited upon covalent modification by DCCD of two polypeptides with ~20 and 24 kd molecular mass. It will be interesting to identify these polypeptides which decide on the routing of protons, out into the water phase or, alternatively, across the membrane from the manganese cluster to the reduced quinone.

Results

Figure 2 shows the electrochemical events upon excitation of thylakoids with repetitive flashes of light, with and without DCCD (20 μM). The arrows in the schematic drawings at the right illustrate the respective event which is represented by the traces at the left, namely pH transients in the medium, transients of the thylakoid membrane voltage and pH transients in the lumen. Figure 2A shows the transient alkalization of the suspending medium. In the presence of methyl viologen as electron acceptor the extent of the alkalization in controls was due to proton uptake at the reducing side of photosystem I (rapid phase) and at the reducing side of photosystem II (slow phase) (Polle and Junge, 1986a,b). After treatment with DCCD only the slow phase, proton uptake by PS II, was drastically diminished. Figure 2B shows the time course of the electrochromic absorption changes. The extremely rapid generation of a transmembrane voltage by both photosystems is followed by a decay via the ionic conductance of the membrane (see Junge, 1982). In DCCD-treated samples the decay was accelerated ~100-fold (from a half-decay time of ~100 ms to ~1 ms). The extent of the voltage, indicative of the initial photochemical charge separation in both photosystems, however, was hardly affected. Figure 2C shows the transient acidification of the thylakoid lumen. In controls the rise was biphasic with a rapid phase due to proton release during water oxidation and a slow one due to the oxidation of plastoquinol (Ausländer and Junge, 1975; Junge, 1982). DCCD diminished only the rapid phase, proton release by water oxidation. It was noteworthy that the rate of uncoupled oxygen evolution under continuous light was not affected in the presence of 20 μM DCCD. On the same line, the general proton permeability of the membrane was increased very little as is evident from a follow-up of the alkalinization in the medium in the time range of 10 s. Figure 3 revealed that the transmembrane pH difference decayed with a half-decay time >5 s even in DCCD-treated samples. These results showed that treatment of stacked thylakoids with 20 μM DCCD affected mostly the protonic reactions at photosystem II without inhibiting the linear electron transport and the protonic reactions associated with photosystem I activity.

To discriminate this effect of DCCD against previously published ones, especially the inhibition of the electron transport (Sane et al., 1979), we investigated its concentration dependence. The results are given in Figure 4. Five observables were monitored as a function of the DCCD concentration. They fell into two classes: three of them were only affected at higher relative concentrations of DCCD, K₁₅₀ ~5 mol DCCD/mol Chl; the two others were more sensitive, K₁₅₀ ~2 mol DCCD/mol Chl. The parameters in the first subset—the initial extent of the transmembrane voltage (full circles), proton release by plastoquinol oxidation (open triangles) and the rate of uncoupled oxygen evolution under continuous light (full squares)—were indicative of the linear electron transport involving both photosystems. The parameters in the second subset—rapid proton release from water oxidation (open circles) and proton uptake at the plastoquinone reduction site (full triangles)—were indicative of protonic reactions around photosystem II. A comparison of the five parameters at a ratio of 2 mol DCCD/mol Chl showed that proton release by water oxidation and proton uptake by plastoquinone reduction were apparently half inhibited, while the integrity of the electron transport chain was not significantly affected. At higher concentrations (DCCD:Chl = 5) proton release due to plastoquinol oxidation driven by photosystem I was still 50% of the control, while proton uptake from the medium at the site of plastoquinone reduction at photosystem II was already down to 10%. Did this indicate that DCCD induced a transfer of protons, which were produced during water oxidation at the
lumenal side of the thylakoid membrane, across the membrane to serve the just reduced bound quinone at the other side? If so, we expected an accelerated decay of the flash-light-generated transmembrane voltage, which was indeed observed as documented in Figure 2B.

If the accelerated decay of the transmembrane voltage reflected passage of protons across the photosynthetic water oxidase, we expected a primary kinetic isotope effect if protons were replaced by deuterons. We measured the decay of the electrochromic absorption changes in H₂O and D₂O. The result is shown in Figure 5. Analysis of the accelerated decay in the presence of DCCD revealed two phases. Both were ~1.6 times slower in D₂O than in H₂O. Did this reflect the primary kinetic isotope effect of a moving proton or a higher order isotope effect, e.g. through a changed protein conformation in D₂O? The latter is characterized by a strong pH dependence in H₂O. Since this was not observed in our experiments in the pH range 7.0–7.8 the decay of the transmembrane voltage was probably accelerated by proton movements.

As a critical test for the proposed short-circuiting of the proton pump it remained to be established that the seemingly non-released protons from water oxidation were really supplied to the reduced quinone which is located at the other side of the membrane. As the protonation of the reduced quinones cannot be spectroscopically resolved, until now we had to rely on an indirect test. After oxidation of the plastochinone pool and photosystem I by far-red pre-illumination, a short flash of light which turns over photosystem II reduces a bound quinone which, after protonation to yield the quinol, is liberated from its binding site, diffuses in the membrane, and transiently reduces the oxidized primary electron donor of photosystem I (see Figure 4). The reduction of P700 can be followed via an absorption increase at 700 nm. It proceeds in 10–20 ms (half-rise-time) starting with a time lag of some milliseconds duration which, according to Haehnel (1976) reflects mainly the necessity for the protonation of bound plastochinone, after its reduction at the acceptor side of photosystem II. We measured the duration of this time lag via the transient absorption changes of the primary electron donor of photosystem I, P700, in the absence and in the presence of DCCD (20 μM). As revealed in Figure 6, this lag was shortened from ~4 ms in controls to ~2 ms in DCCD-treated samples. We approximated the reduction kinetic of P700 by first-order consecutive reactions:

\[ \text{PQ}^2^- \xrightarrow{k_1} \text{PQH}_2 \xrightarrow{k_2} \text{P700} \]

A numerical fit of the data revealed rate constants \( k_1 = 100 \text{ s}^{-1} \), \( k_2 = 35 \text{ s}^{-1} \) for the control and \( k_1 = 250 \text{ s}^{-1} \), \( k_2 = 35 \text{ s}^{-1} \) in the presence of DCCD respectively. The influence of DCCD on the rate constant \( k_1 \) (and the unchanged \( k_2 \)) indicated that only the protonation of the reduced plastochinone was accelerated in the presence of DCCD. This corroborated the interpretation that protons from water oxidation were channelled across the membrane to serve the reduced bound quinone.

The above-described effects of intermediate concentrations of DCCD were observed with stacked thylakoids, but they were not induced if unstacked thylakoids, suspended under low-salt conditions (no MgCl₂), were incubated with DCCD. This is shown in Figure 7. If thylakoids were incubated with DCCD in the absence of Mg²⁺ (i.e. in the unstacked configuration, see upper traces in Figure 7) the drastic acceleration of the decay of the transmembrane voltage was not observed. (The decay was the same as the one in controls which is not shown in Figure 7.) This was dependent of whether the flash spectrophotometric experiment was carried out in a medium with or without added magnesium, i.e. with restacked or with unstacked thylakoids (upper traces in Figure 7). On the other hand, once induced by incubation of stacked thylakoid membranes with DCCD, the acceleration was not abolished upon unstacking (Figure 7, lower traces). In conclusion, the described effects were probably caused by a covalent modification of thylakoid
membranes with DCCD, which occurred only if the membranes were in the stacked configuration during incubation.

The differential effect of DCCD on stacked and on unstacked thylakoid membranes offered a clue for the identification of the responsive polypeptides in discrimination against other polypeptides which were also covalently labelled by DCCD (e.g. subunit III of CF\text{0}). Figure 8 shows densitometric scans of autoradiographed SDS gels of thylakoid membranes which were treated with \textsuperscript{14}C\textsubscript{DCCD}. Comparison of the traces S (incubation with \textsuperscript{14}C\textsubscript{DCCD} of stacked thylakoids) and U (...unstacked...) revealed a similar pattern for peak locations, but great differences for the peak intensities. The largest difference was observed for two particular polypeptides migrating in the gel according to an apparent molecular mass of 20 and 24 kd.

**Discussion**

Incubation of stacked thylakoids with DCCD short-circuited the proton pumping activity of the photosynthetic water oxidase. Under single turnover of photosystem II by flashing light fewer protons from water oxidation were released into the lumen and proton uptake from the medium was also diminished. Instead, a rapid electrogenic backreaction was observed as if protons from water oxidation were transferred across the protein from the inner to the outer side of the thylakoid membrane. That protons were responsible for the accelerated decay of the transmembrane voltage was evident from the primary kinetic H/D-isotope effect. The confinement of the conducting pathway in the protein was evident from the absence of an appreciably higher proton conduction between the lumen and the outer medium. That the reduced quinone was the recipient of the conducted protons was suggested by the shortening of the time lag of the reduction of P700.

The above effect of DCCD was clearly distinct from other previously reported ones. It was elicited at chlorophyll-related DCCD concentrations which were intermediate (typically 2 mol DCCD/mol Chl) between the lower ones which blocked the proton channel CF\text{0} by covalent modification of subunit III (0.5/1) (McCarty and Racker, 1967) and the higher ones (5/1) inactivating the linear electron transport chain (Sane et al., 1979). As to the specificity of the effect for stacked thylakoids it was conceivable that stacking of the membranes provided the required hydrophobic environment for DCCD binding to the respective polypeptide. Except for the short-circuiting the effect of DCCD on the water oxidase somehow resembles the one of DCCD on subunit III of cytochrome c oxidase where a partial inhibition of proton pumping was reported without a proportional inhibition of electron transport (Casey et al., 1980; Prochaska and Fink, 1987).

It was puzzling that an intraproteinaceous proton channel seemed to be induced by DCCD, an agent which, on the contrary, is notorious as a blocker of proton channels (Sebal and Hoppe, 1981; Azzi et al., 1984). This was to be understood, however, if DCCD blocked the outlet of protons from the site of water oxidation into the thylakoid lumen and perhaps also the inlet of protons from the medium to the site of the bound quinones.

We found that the short circuit of the proton pump was paralleled by the selective labelling with \textsuperscript{14}C\textsubscript{DCCD} of two polypeptides of ~20 and 24 kd apparent molecular mass. At least four different polypeptides in this range have been associated with the water oxidase (Murata and Miyao, 1987, Åkerlund and Jansson, 1981; Dunahay and Staehelin, 1986; Liveanu et al., 1986; Murata et al., 1987). The identity of the polypeptides which were labelled by \textsuperscript{14}C\textsubscript{DCCD} is currently under investigation.

Tentatively, the observed short-circuiting by DCCD of the proton pumping activity of the water oxidase was interpreted as follows: DCCD blocked proton conduction in two polypeptides which cap the sites of water oxidation and of quinone reduction. This stopped the extrusion into the lumen of

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**Fig. 7.** Time course of electrochromic absorption changes. All samples—either unstacked (no Mg\textsuperscript{2+} present) or stacked (with 5 mM MgCl\textsubscript{2}) thylakoids—were incubated for 10 min with 20 \textmu M DCCD. Following centrifugation (10 min at 10 000 g) the membranes of both incubation conditions were resuspended in a medium without or with 5 mM MgCl\textsubscript{2} and incubated for 10 min at room temperature to induce stacking or unstacking respectively. To avoid confusion by overlapping data points, two of the four traces were presented by smoothed curves. The control, chloroplasts which were pretreated as before but without addition of DCCD, yielded traces which were identical to those obtained with unstacked thylakoids.

**Fig. 8.** Densitometric scans of autoradiograms of \textsuperscript{14}C\textsubscript{DCCD}-labelled polypeptides which were separated by SDS–PAGE. S = stacked thylakoids; U = unstacked thylakoids. Molecular masses in kilodaltons are indicated.
protons which were produced during water oxidation and it also stopped the uptake of protons from the medium at the site of plastoquinone reduction. This forced protons, which were produced during water oxidation, through the protein and across the membrane to the reduced quinones. The intra-protein proton channel, which was activated by DCDD, was apparently not used in untreated membranes.

Materials and methods

Stacked thylakoids were prepared from laboratory-grown pea seedlings as previously described (Polle and Junge, 1986a). By flash spectrophotometric techniques we measured the transient oxidoreduction of photosystem I via P700 (Haehnel, 1984), transients of the transmembrane voltage via electrochromism (Junge and Witt, 1968), pH transients in the lumen via neutral red (Junge et al., 1979) and pH transients in the medium via cresol red (Polle and Junge, 1986a). Thylakoids equivalent to 10 μM Chl were suspended in an assay medium containing 5 mM MgCl₂, 10 mM NaCl and buffers as indicated in the legends. Unless otherwise indicated, the pH was adjusted to 7.8 and hexacyanoferrate III (200 μM) served as terminal electron acceptor for photosystem I. pH-specific absorption changes were obtained by subtracting a transient signal detected in the absence from another one detected in the presence of the respective indicator dye [for details see Junge et al. (1979); Junge (1982)].

The rate of oxygen evolution under continuous illumination was determined by a Clark-type electrode. Samples containing 30 μM Chl, 5 mM MgCl₂, 10 mM NaCl, 20 mM tricine/NaOH, pH 7.8, 2 mM hexacyanoferrate III and 0.5 μM nigericin were illuminated with saturating white light. Thylakoids were incubated with DCDD for 10 min at room temperature under dim lights at pH 7.8. For the measurements in heavy water (D₂O) the apparent pH value obtained with a standard glass electrode was converted into the equivalent pD value by adding 0.45 units (Covington et al., 1968). The medium for the measurements in D₂O contained <0.1% H₂O₂.

The polypeptide composition was analyzed by SDS–PAGE according to Laemmli (1970) with some modifications as described by Engelbrecht et al. (1986); 1 M urea was present in the sample buffer and a 12.5% acrylamide gel was used. Samples contained ~35 μg chlorophyll and staining was performed with Coomassie brilliant blue R-250.

Labelling of thylakoids with [³⁵S]DCCD was carried out at room temperature by incubation of thylakoids equivalent to 1 μM Chl/same medium as used for flash spectrophotometric measurements with 2 μM [³⁵S]DCCD (Amersham, Braunschweig). Following incubation, samples were centrifuged (15 min, 20 000 g) and the pellet used for SDS–PAGE. The stained gel was treated with Amplify (Amersham, Braunschweig) prior to drying. Autoradiograms were obtained by using a Kodak X-AR5 X-ray film, exposed for 72 h at −70°C and densitometrically scanned (Biorad 1650).

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