Biochimica et Biophysica Acta, 462 (1977) 259–272

BBA 47378

GATED PROTON CONDUCTION VIA THE COUPLING FACTOR OF PHOTOPHOSPHORYLATION MODIFIED BY *N,N-ortho*PHENYLDIMALEIMIDE

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(Received March 25th, 1977)

SUMMARY

The membrane bound coupling factor of photophosphorylation is studied after pretreatment of broken chloroplasts with the bifunctional *N,N-ortho*phenyldimaleimide under energization of the thylakoid membrane by mild flashing light. The proton conduction of the membrane is monitored both via the electrochromic absorption changes and via selective pH-indicating dyes. It is found that the coupling factor, after interaction with *N,N-ortho*phenyldimaleimide during the preillumination period, shortcircuits one of the two protons pumped inside after excitation of chloroplasts with one short flash of light. In contrast to the low proton conductivity of the unperturbed thylakoid membrane (relaxation time for a proton gradient >5 s), this extra proton channel leads to a partial relaxation of a proton gradient within a few ms. Although limited to only one proton per electron, this extra proton conducting pathway is not otherwise specific. It operates with protons resulting from both Photosystem I and Photosystem II activity. In addition it operates with protons already present in the internal phase before firing of the exciting light flash. These effects are prevented by the presence of ATP (but not GTP) during the preillumination period. It is suggested that the modified coupling factor is gated open by the light induced electric field across the thylakoid membrane while self closing after passage of one proton per activated coupling factor.

INTRODUCTION

There is increasing interest in markers for conformational changes of the coupling factor for photophosphorylation (CF1). It is conceivable that some of the observed conformational changes are either energy carrying or information carrying (activating) intermediates in the hitherto ill-defined chemistry of photophosphoryla-

Abbreviation: OPDM, *N,N-ortho*phenyldimaleimide; CF1, coupling factor for photophosphorylation

tion. Ryrie and Jagendorf [1, 2] observed that electrochemical energization of the thylakoid membrane induces a special conformation of CF1 which exposes proton exchanging groups to the aqueous environment of the enzyme. It was reported that certain modifying agents interact with CF1 only after electrochemical energization of the membrane. Sulphate and permanganate [3, 4] as well as *N*-ethylmaleimide (5) then inhibit the rate of phosphorylation and the activity of the (Ca^{2+}) ATPase by about 50 %. McCarty and Fagan [6] identified the inhibitory site of *N*-ethylmaleimide as one thiol group on the γ-subunit of CF1. Although the inhibition was found to be only about 50 % there was circumstantial evidence that permanganate and *N*-ethylmaleimide interacted with 100 % of the CF1 population.

Our earlier studies on the ATP production under flashing light, where the electric potential is the dominating part of the electrochemical potential of the proton, revealed a strongly superlinear dependence of the ATP yield on the electric potential [7]. This led us to consider the possibility that the activity of CF1 was electrically gated [8, 9]; that is to say, the magnitude of the electric potential determines the proportion of active and inactive enzyme molecules on the thylakoid membrane. The experimental results [7], although challenged at first [10, 11], were confirmed and extended for spinach [12] and for *chlorella* [13]. More direct evidence for an electrical gating mechanism of the activity resulted from recent experiments on the dependence of nucleotide binding by the membrane bound CF1 on the electric potential across the membrane [14].

In pursuit of the gating mechanism we became interested in the correlation between conformational changes and the electric potential difference across the thylakoid membrane. The extent of the electric potential difference is measurable via electrochromic absorption changes [15] much better under flashing light than under continous light (for reviews see refs. 11 and 16). Therefore we looked for chemical markers for conformational changes which would react with CF1 rapidly, i.e. during the lifetime of the electric potential after excitation of chloroplasts with a short flash of light (about 100 ms). Dr. McCarty suggested the use of the bifunctional reagent *N,N-ortho*phenyldimaleimide (OPDM) because of its rapid reactivity with the membrane bound CF1 after energization of the membrane (Weiss and McCarty, private communication). We observed, as did these authors, that incubation of chloroplasts with OPDM during energization of the thylakoid membrane caused partial inhibition of photophosphorylation. This occurred even after preillumination of chloroplasts with a few groups of only short flashes. However our original aim of defining the critical electric potential difference exposing CF1 to interaction with OPDM was postponed when we realized the strange effect of this modifying agent on the proton permeability of the thylakoid membrane. We observed that if CF1 was modified by OPDM, one out of two protons released into the internal phase after flash excitation was short-circuited rapidly across the membrane. Strangely enough, the other proton was seemingly unaffected. The aim of this communication is to describe the kinetics and the selectivity of the proton channel associated with OPDM-modified CF1.

MATERIALS AND METHODS

Spinach was purchased from the local market. Chloroplasts were prepared according to Siggel et al. [17] and used when fresh. (Longest storage 1.5 hours at 4 °C.)

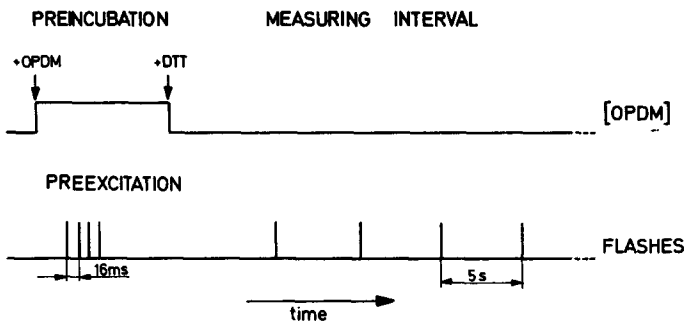

Fig. 1. Illustration of the time course of events during preillumination of chloroplasts in the presence of OPDM and during the subsequent measuring interval (for details see text).

Unless otherwise indicated, the chloroplasts were suspended at an average chlorophyll concentration of 10 μM in the following standard reaction medium: KCl, 20 mM; $MgCl_2$, 3 mM; benzylviologen, 6.7 μM. The pH was adjusted to either 7 or 8 (depending on the pH-indicating dye used) by addition of HCl and NaOH, respectively. The pH-indicating dyes were added to the following final concentration: cresolred, 33 μM (pH 8) and neutralred, 6.6 μM (pH 7) together with bovine serum albumin, 1.3 mg/ml.

Preillumination of chloroplasts in the presence of OPDM

Chloroplasts were suspended in the standard medium while kept in the dark. The time course of the subsequent events is illustrated in Fig. 1. OPDM was added to yield a final concentration of 6.7 μM in the reaction medium. (OPDM was originally a gift from Dr. McCarty. Later it was purchased from EG A-Chemie, recrystallized from acetone and finally dissolved in Me_2SO. Care was taken to keep the Me_2SO concn. in the reaction medium below 0.5 %.) The sample was preilluminated with ten groups of short flashes spaced 10 s apart. Each group consisted of six flashes at 16 ms intervals. About 30 s after preillumination ceased dithiothreitol was added (final concentration 10 μM) in order to remove the unreacted OPDM from the reaction mixture. The addition of dithiothreitol alone did not cause any of the effects which are ascribed below to the action of OPDM. The OPDM concentration used was sufficient to saturate the observed effects. When half saturation occurred at between 2 and 4 μM under the given preillumination conditions freshly prepared chloroplasts were used.

Photometric measurement of the membrane's electric conductivity

After preillumination of the sample in the presence of OPDM the optical absorption cell (thickness 2 cm) was mounted into a rapid kinetic flash spectrophotometer (for reviews on instrumentation, see refs. 18 and 19). The chloroplast suspension was excited with a short flash of light (wavelength greater than 600 nm, half-time of duration 15 μs, saturating energy: 0.5 mJ/cm^2). This caused each photosystem to translocate one electron. Changes of absorption were monitored. To avoid the build-up of a significant pH gradient under the influence of the measuring light, its intensity was kept low (less than 10 μW/cm^2). In addition, it was open only during the sampling interval. To improve the signal-to-noise ratio of the absorption changes, signals were

induced by repetitive flashes (period 10 s) and averaged on a Nicolet 1072 computer.

It is known that absorption changes around 520 nm which are observed on flash excitation of chloroplasts are mainly due to the electrochromic response of carotenoids and of chlorophyll-*b* to the light-induced electric field across the thylakoid membrane (for recent reviews, see refs. 11 and 16). The extent of the flash-induced absorption changes at this wavelength depends pseudolinearly on the voltage. The decay of absorption is indicative of the breakdown of the pulse-induced voltage by ionic currents across the membrane. Complications inherent to the interpretation of the decay in terms of the electric current density across an average thylakoid membrane have been discussed elsewhere [20].

Photometric measurement of pH changes in both aqueous phases of thylakoids

We had previously observed that sulphonic pH-indicating dyes, such as cresolred (e.g. ref. 21) do not penetrate into the internal phase of thylakoids during reasonably long periods of time (say 1 h). These non-permeating dyes can therefore be used as true indicators of pH changes in the external phase of thylakoids [21–24]. If a chloroplast suspension is excited twice: first in the absence and second in the presence of a non-permeating buffer, then the difference between the respective absorption changes observed at a wavelength characteristic for the non-permeating pH indicator represents accurately the response of the indicator to pH changes in the outer phase. The subtraction of the absorption changes from a buffered suspension eliminates possible response of the dye to events other than pH changes in the external phase. The calibration of this method [23] and problems related to its kinetic resolution when applied to chloroplasts were discussed elsewhere [24].

The pH transients in the internal phase of thylakoids were measured by recording absorption changes of a permeating pH-indicating dye (neutralred) in the presence of a non-permeating buffer (e.g. bovine serum albumin) under two sets of conditions as previously reported [21], first in the absence and secondly in the presence of the permeating buffer imidazole. Subtraction of the latter absorption changes from the former yields the response of the dye to pH transients in the internal phase of thylakoids. This method was introduced for studies under flashing light [21], where the pH changes in the internal phase are in the order of 0.1 unit only. Although also operative in a qualitative way under continuous light, its extension for quantitative studies under these conditions proved difficult [25]. The major reason for this difficulty was the redistribution of the permeating dye between the two aqueous phases and the membrane under the influence of a larger pH gradient. This redistribution of weak acids is difficult to handle quantitatively [25, 26]. It must be pointed out, however, that these complications are not relevant to studies under flashing light. This is due mainly to the fact that neutralred accumulates at the inner side of the thylakoid membrane in the dark (its virtual concentration in the internal volume is about ten times higher than outside [25, 42]. This makes the inwardly directed transport of neutralred under the influence of a small pH difference negligible [42]. Under flashing light neutralred then acts as a rapid and linear indicator of pH changes in the internal phase. Transient absorption changes which are labelled in the figures as "pH_{out}-indicating absorption changes of cresolred at 574 nm" and "pH_{in}-indicating absorption changes of neutralred at 552 nm," respectively, were obtained by the above described subtraction of signals measured in the absence and in the presence of appropriate buffers. (Whether

the absorption changes of neutralred were recorded at 552 or, as earlier, at 524 nm, does not make too much difference, due to the broad absorption band of the dye.)

RESULTS

The effect of preillumination with OPDM on the proton conductivity of the membrane

The effect of preillumination in the presence of OPDM on the decay of the electrochromic absorption changes at 524 nm is documented in Fig. 2. The chloroplast suspension was excited with a short flash of light at zero time. While the decay of the absorption changes after the flash was slow in control chloroplasts (preilluminated in the absence of OPDM), about half of the extent of decay was accelerated if chloroplasts were preilluminated in the presence of OPDM. If chloroplasts were incubated with OPDM in the dark, the influence on the decay was negligible. In several experiments like those in Fig. 2 the acceleration was restricted to between 30 and 50 % of the total extent of the flash-induced absorption change at 524 nm. This can be interpreted in two ways: either OPDM affected only 50 % of the thylakoids, or it induced some

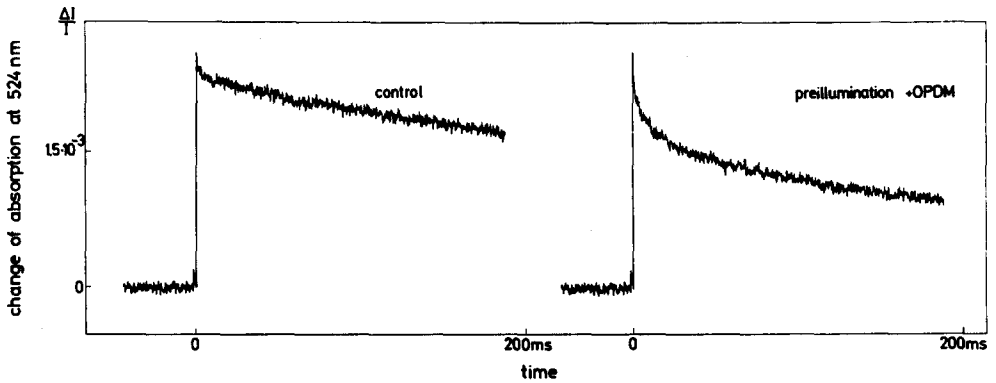

Fig. 2. Time course of the electrochromic absorption changes at 524 nm in the control (left) and in chloroplasts preilluminated in the presence of OPDM (right). Chloroplasts were excited with a short flash of light at $t = 0$.

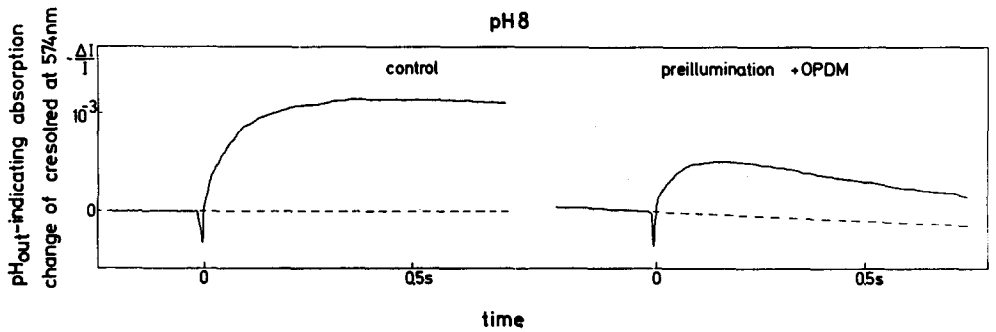

Fig. 3. Time course of the pH_{out}-indicating absorption changes of cresolred at 574 nm after excitation of chloroplasts with a short flash of light at $t = 0$. Left: control; right: chloroplasts preilluminated with OPDM.

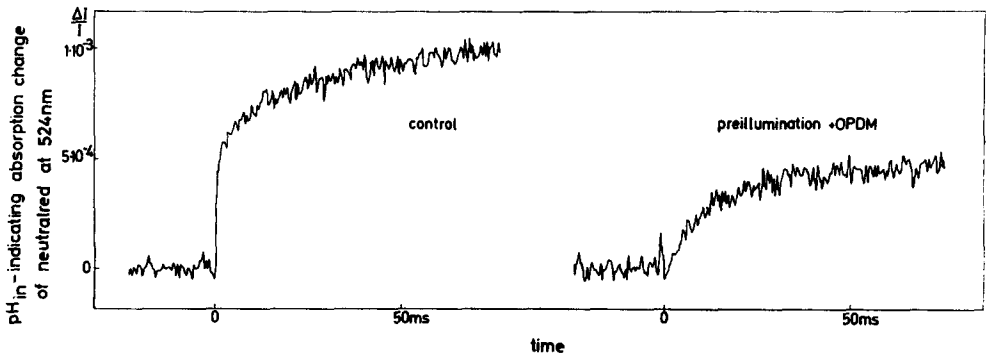

Fig. 4. Time course of the pH_{in}-indicating absorption changes of neutralred at 524 nm after excitation of chloroplasts with a short flash of light at $t = 0$. Left: control; right: chloroplasts preilluminated with OPDM.

conducting pathway which is switched off after passage of one half of the charges or at half of the voltage (but see below).

The effect of OPDM on the flash induced pH changes in the external phase is illustrated in Fig. 3. The rise of absorption in control chloroplasts with benzylviologen as terminal electron acceptor corresponds to the uptake of two protons per electron from the outer phase as shown previously [22, 23]. Due to the low intrinsic proton permeability of the thylakoid membrane in the absence of ADP the flash-induced alkalinization does not relax significantly during the measuring interval. As is obvious from the right-hand trace in Fig. 3, preillumination of chloroplasts in the presence of OPDM abolishes about 50 % of the proton uptake from the external phase. This could reflect the deactivation of one of the two photosystems. That this is not so is, however, evident from Fig. 2. The extent of the flash-induced electric potential difference (with approximately equal contributions from both photosystems according to ref. 22) remains unchanged. In addition we found that neither the extent of the absorption changes at 700 nm (indicative of Photosystem I activity) nor the oxygen evolution under continous light (ferricyanide as acceptor, uncoupled conditions, indicative of Photosystem II) were affected. We have to conclude that OPDM treatment deactivates one of the sites of proton uptake from the external phase without deactivating the underlying electron transport.

Proton uptake from the outer phase was previously associated with the reducing sites of both photosystems [22–24]. To find out whether the deactivation of one of the two sites for proton uptake is specific for either photosystem, we repeated the experiment shown in Fig. 3, but with ferricyanide (300 μM) instead of benzylviologen as terminal electron acceptor. In contrast to benzylviologen, ferricyanide does not bind one proton per electron on reduction by Photosystem I. Under these conditions (not shown) we observed half of the proton uptake for control chloroplasts and virtually no proton uptake for chloroplasts preilluminated in the presence of OPDM. This can be interpreted in two ways: either pretreatment with OPDM abolishes proton uptake at Photosystem II, or it abolishes the uptake totalling one proton per electron.

The effect of OPDM on the flash-induced pH changes in the internal phase is illustrated in Fig. 4. Chloroplasts were excited with a short flash of light at time zero.

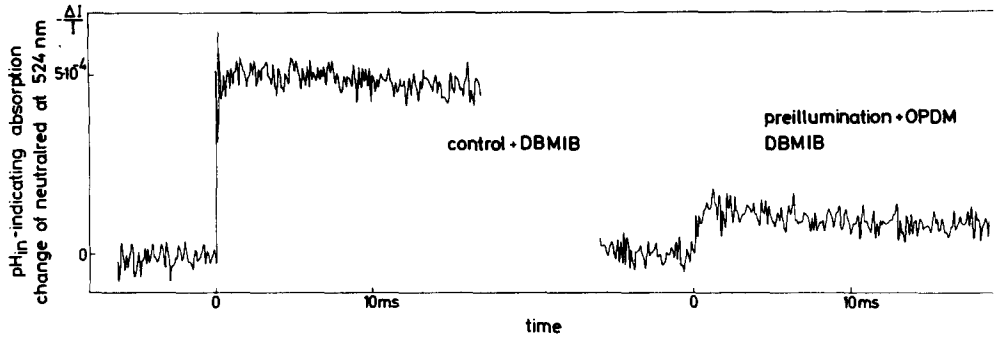

Fig. 5. Time course of the pH_{in}-indicating absorption changes of neutralred at 524 nm after excitation of chloroplasts with a short flash of light at $t = 0$. Photosystem II is the only active one in proton transport [21] due to the presence of DBMIB, which blocks electron transport between plastoquinone and Photosystem I. Left: control; right: chloroplasts preilluminated in the presence of OPDM.

The rise of absorption indicates acidification of the internal phase [21]. In control chloroplasts the rise is biphasic, as reported previously. Preillumination in the presence of OPDM virtually eliminates the rapid phase. The rapid phase was previously attributed to proton release in consequence of water oxidation by Photosystem II [21]. It seems as if OPDM abolishes the release of protons by the water oxidizing enzyme system without deactivating its ability to produce oxygen. Another experiment where proton release into the internal phase was entirely due to Photosystem II (caused by the presence of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, DBMIB, at 3 μM) seemed to support the possibility that the effect of preillumination in the presence of OPDM is specific for protons resulting from the water oxidizing enzyme system. This experiment is documented in Fig. 5. As in previous work [21] only the rapid phase of proton release is observable in the presence of DBMIB. It is greatly reduced after preillumination of chloroplasts with OPDM.

Are the observed effects related to the interaction of OPDM with CF1?

According to Weiss & McCarty (private communication) preillumination of chloroplasts in the presence of OPDM causes the binding of this reagent to CF1. This affects the activity of photophosphorylation. Under our preillumination conditions the rate of the linear phosphorylation decreased by about 50 % (not shown). The experiments above described seem to suggest a specific effect of OPDM on protons resulting from Photosystem II activity. It has to be questioned whether this is caused by the above cited action of OPDM on CF1 or on an additional direct effect on Photosystem II.

Fig. 6 again shows the effect of preillumination in the presence of OPDM on the proton release into the internal phase. The upper two traces correspond to those shown in Fig. 4. The lower two traces were obtained in the presence of ATP and of GTP, respectively, during the preillumination period. It is obvious that the presence of ATP largely prevented the effect of OPDM on the rapid phase of proton release while GTP did not. If ATP was present in the measuring interval only, but not during the preillumination period, no such protection was observed. In the light of the well known specificity of CF1 for ATP as compared to GTP in binding experiments

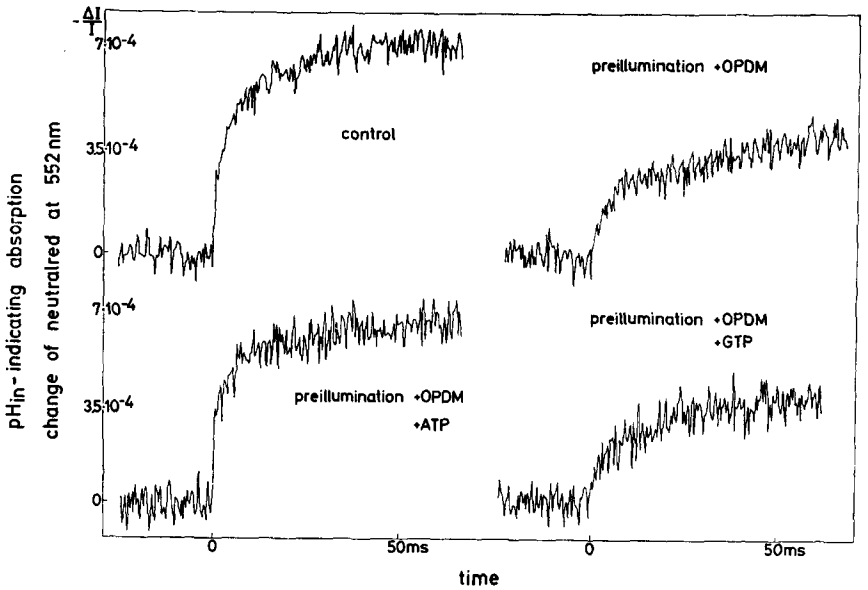

Fig. 6. The influence of the presence of nucleotides (ATP, GTP) during preillumination of chloroplasts with OPDM on the pH_{in}-indicating absorption changes of neutralred at 524 nm. The upper two traces are analogous to those in Fig. 4. The lower two traces show that ATP does and GTP does not prevent the abolition of the rapid phase of proton release into the internal phase, respectively.

[27–29] this suggests that the observed effects of preillumination in the presence of OPDM are due to its specific interaction with CF1.

The observed effects were prevented also in the presence of uncoupling agents (e.g. NH_4Cl, FCCP) during the preillumination period (not shown). This strongly suggests that they are attributable to a modification of CF1, which is brought forth by an energy linked conformational change of the enzyme.

Fig. 7 shows the effect of preillumination with OPDM on the proton release

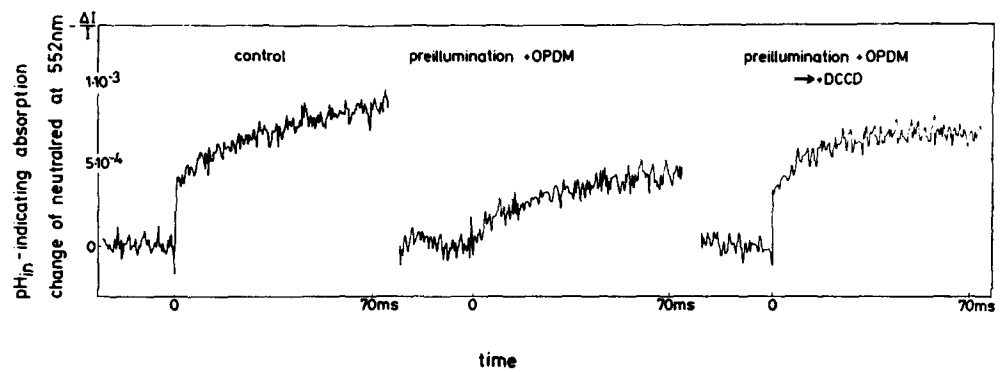

Fig. 7. The effect of DCCD added after the preillumination period on the pH_{in}-indicating absorption changes of neutralred at 524 nm (below) and on the electrochromic absorption changes at 524 nm (above). Left: control; middle: chloroplasts preilluminated with OPDM; right: chloroplasts preilluminated with OPDM and DCCD added (final concentration 1 μM) after the end of preillumination.

into the internal phase if dicyclohexylcarbodiimide (DCCD) is added (left trace) after the preillumination period. It is obvious that DCCD reverses the effect of OPDM. It has been shown previously that DCCD closes the proton channel opened up in the thylakoid membrane by extraction of the coupling factor [30–32].

The experiments so far seem to suggest that preillumination of chloroplasts in the presence of OPDM modifies CF1 in a way to shortcircuit protons resulting from Photosystem II across the thylakoid membrane.

Is there any "site-specificity" of the proton channel through modified CF1?

The subsequent experiments question into the apparent specificity of the proton channel through modified CF1 for protons from Photosystem II. Fig. 8 shows proton release into the internal phase due only to Photosystem I. Pyocyanine (3 μM) was used as electron donor for Photosystem I with Photosystem II blocked by the addition of 3,(3,4-dichlorophenyl)1,1-dimethylurea (DCMU) after the preillumination period. It is noteworthy that proton release inside due to the oxidation of pyocyanine by Photosystem I is by orders of magnitude more rapid than by the oxidation of plastohydroquinone in the intact system (in freshly prepared chloroplasts). Comparison of the two traces in Fig. 8 shows that preillumination in the presence of OPDM abolishes the rapid proton release into the internal phase caused by the oxidation of pyocyanine by Photosystem I. The activity of Photosystem I under these conditions was confirmed by measuring the absorption changes at 700 nm (not shown). This demonstrates that the abolition of proton release is not restricted to protons attributable to Photosystem II activity.

There was a faint possibility that the effect shown in Fig. 8 was specific for the electron donor pyocyanine. However, if this were so one should expect, that preillumination in the presence of OPDM would abolish the release of two protons per electron, if the Photosystem II proton was released by the water oxidizing system and the Photosystem I proton during oxidation of pyocyanine. Fig. 9 shows the effect of preillumination with OPDM on the proton release under these conditions. In contrast to the situation underlying Fig. 8, Photosystem II was not inhibited by DCMU. It is evident that the modified CF1 shortcircuits only one proton, as previously. This excludes any

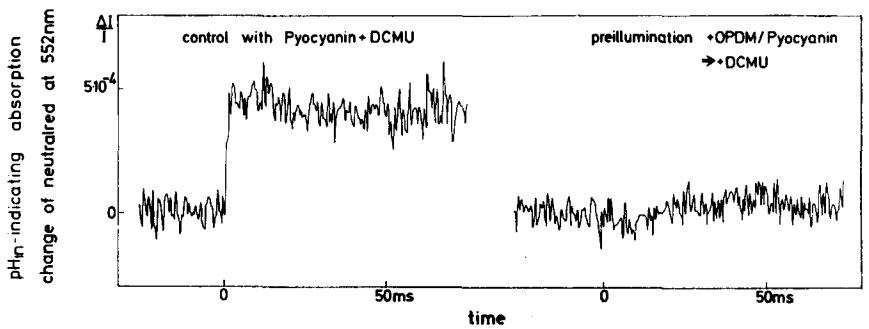

Fig. 8. Time course of the pH_{in}-indicating absorption changes of neutralred at 552 nm with pyocyanine (3 μM) as cofactor for cyclic electron transport around Photosystem I, Photosystem II is blocked by DCMU (0.2 μM). Left: control; right: chloroplasts preilluminated with OPDM (DCMU added after the preillumination period).

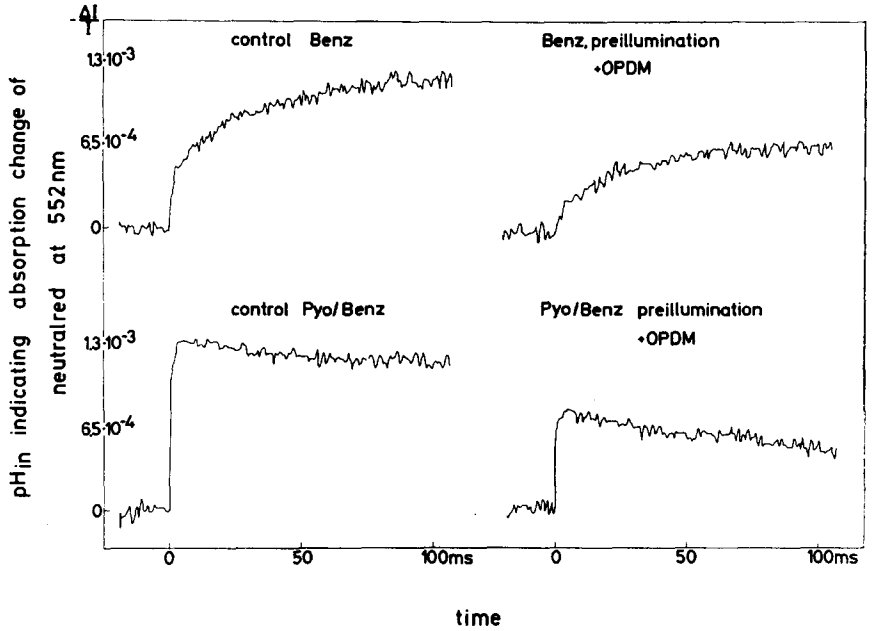

Fig. 9. Time course of the pH_{in}-indicating absorption changes of neutralred at 552 nm with pyocyanine (3 μM) as cofactor for cyclic electron transport around Photosystem I. Photosystem II is also active in contrast to the situation shown in Fig. 8. Left: control; right: chloroplasts preilluminated with OPDM.

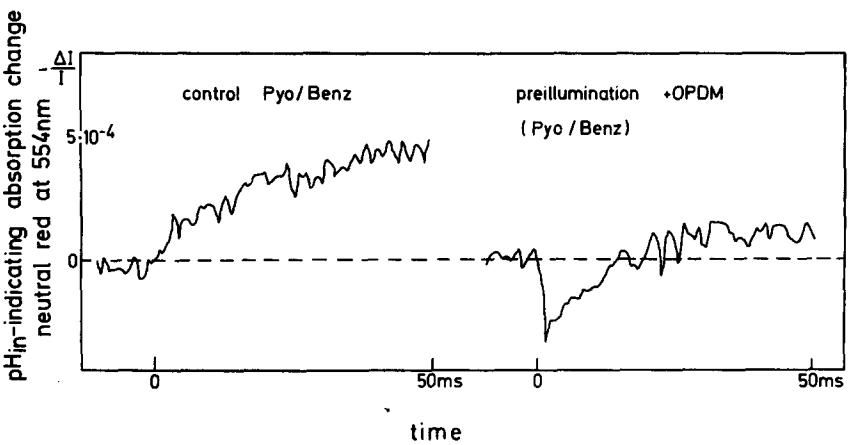

Fig. 10. Time course of the pH_{in}-indicating absorption changes of neutralred at 552 nm with pyocyanine (3 μM) as cofactor for cyclic electron transport around Photosystem I. Photosystem II is blocked by addition of DCMU (0.2 μM) after the preillumination period. In contrast to the situation in Fig. 8 stock chloroplasts instead of freshly prepared ones were used. Left: control; right: chloroplasts preilluminated with OPDM.

special role of pyocyanine which might have overcome an eventual site specificity of modified CF1.

We have to conclude that modified CF1 shortcircuits one proton per electron no matter whether the selected proton be released by Photosystem I or II. If this is correct, modified CF1 should rapidly conduct outwards even the one proton which is already present in the internal phase (buffered, of course) provided that proton release following excitation with a flash of light can largely be retarded. This is realized in Fig. 10. The chemical conditions are as in Fig. 8 (pyocyanine and DCMU). However, stock chloroplasts were used (stored under liquid N_2 for about one year) instead of freshly prepared ones. For a reason which will be the subject of another communication, the oxidation of pyocyanine and the concomitant internal proton release is slow in this system. Due to the presence of DCMU the slow proton release totalled only one proton per electron. Comparison of the right-hand trace, which was obtained after preillumination in the presence of OPDM, shows along with the left-hand one that modified CF1 rapidly eliminates one proton from the internal phase before any proton is released by consequence of the electron transport.

DISCUSSION

The above experiments have shown that preillumination of chloroplasts in the presence of OPDM affects the proton conductivity of the thylakoid membrane. The effect of OPDM was nullified by the presence of ATP but not of GTP during the preillumination period. This suggests that OPDM acts on the coupling factor CF1. Our indirect evidence for a specific action on CF1 is greatly strengthened by Weiss & McCarty's (private communication) chemical proof for the binding of OPDM to CF1 after energization of the thylakoid membrane.

After CF1 reacts with OPDM during the preillumination period a special proton channel is introduced into the membrane, which, after flash excitation of chloroplasts, rapidly binds one proton per electron while ignoring any further proton. This became evident from the observation, by means of the indicator dye neutralred, of the flash induced pH changes in the internal phase. As is evident from the time course of the electrochromic absorption changes and from the pH changes in the external phase, the one proton per electron bound by modified CF1 inside is translocated outwards across the thylakoid membrane.

It is important to note that the apparent specificity of modified CF1 for protons resulting from Photosystem II activity, which could be suggested from information based on Figs. 2–5, can be rejected on the basis of Figs. 8–10. The proton channel through modified CF1, although selective for only one proton per electron, is not otherwise specific (for references on the still controversial "site specificity" of photophosphorylation, see ref. 33).

The opening of the proton channel (through modified CF1) or its proton conductance are counteracted by the carbodiimide DCCD, an agent which closes the proton channel through the counterpart of CF1 in the membrane (after extraction of CF1) [30–32]. It is impossible to correlate the above proton-conducting properties of modified CF1 with any of the intrinsic activities of this enzyme so far described. Unfortunately this is common to almost all experiments with labels for conformational changes of CF1. Even energy-linked nucleotide binding studies so far have appeared

to hit at binding sites remote from those processing ADP during the synthesis of ATP [34]. This may justify further investigation of the modified CF1, although we must be aware that the observed behaviour may be totally irrelevant to photophosphorylation.

The observed behaviour of modified CF1 poses two interesting questions. 1. What activates modified CF1 to rapidly bind protons from inside and to translocate them outwardly across the thylakoid membrane? 2. What closes the proton channel through modified CF1 after passage of only one proton per electron?

Three possible activators of the proton channel through modified CF1 are: (a) some step of the electron transport, (b) the light-induced electric potential difference; and (c) the protons released inside. The fact that modified CF1 can take up one proton per electron before any proton be released inside (see Fig. 10) allows only the first two possibilities. The fact that the rapid proton release attributed to water oxidation (average half-rise time 300 μs [21]) is virtually eliminated by modified CF1 reveals that the time required to switch the proton channel open must be less than 300 μs. Experiments investigating further the opening of the channel are in progress.

The most striking feature of the proton channel through modified CF1 is perhaps that it is virtually open for one, and only one, proton per electron transport chain while being closed for any further proton. From the experimental results in this paper the possibility that the channel is closed if the voltage drops below a certain level can be excluded. It is evident from Fig. 2 that the channel closes at half the extent of the voltage which is induced by stimulation of both photosystems. This, according to ref. 22, is just the voltage induced by stimulation of one photosystem only. As shown in Fig. 8, however, the proton channel is open if only one of the two photosystems is activated.

One can only speculate about other mechanisms for closing the proton channel. One possibility will be followed up by further experiments. It is conceivable that the modified coupling factor once opened up for protons closes after the passage of one proton per activated CF1. A certain recovery time may prevent reactivation whilst the necessary voltage is still there (about say 50 ms after the first activation). This possibility gains some support from the available data on the stoichiometries of protons released per chlorophyll and CF1 per chlorophyll, respectively. Although the precision of the CF1/chlorophyll stoichiometry is still low, a value centered around 1 : 800 is probable. This, however, implies a stoichiometry of two protons released inside per CF1 (both photosystems active). Let us briefly review the data on stoichiometries. From the literature we calculated figures for the stoichiometry of CF1 over chlorophyll ranging from 1 : 200 [35] over 1 : 850 [36] to 1 : 1200 [37]. We would tend to credit the biochemical assays [38, 39, 36] more than the electron microscopic attempts [35, 37, 40], as the latter are loaded with the additional problem that CF1 is non-homogeneously distributed between stacked and unstacked lamellar regions (see refs. 35 and 37). Lien and Racker [38], by extracting chloroplasts in the presence of EDTA, obtained a yield of CF1 corresponding to about 1CF1 per 1300 chlorophylls. However, according to Nelson [39] this treatment removes only between 30 and 70 % of the total amount. If we correct the stoichiometry for this we obtain a figure ranging between 1 : 400 and 1 : 900 CF1 : chlorophyll. Strotmann et al. [36] used another extraction procedure which probably removes 50 % of CF1 from the membrane. This was backed by Miller and Staehelin [37], who adopted this extraction procedure for their electron microscopy work. Strotmann et al. [36] determined a stoichiometry of between 1 :

830 and 1 : 890 CF1 : chlorophyll for spinach chloroplasts. A recent titration of the membrane-bound CF1 in lettuce chloroplasts by a specific inhibitor (binding stoichiometry to the isolated CF1 is 1 : 1) by Steele et al. [43] led to a stoichiometry of one CF1 per 500 chlorophylls.

We determined previously the stoichiometry of protons released per electron transferred (single turnover of both photosystems under excitation by short flash) as $2H^+/e^-$ [22–24]. This figure was recently challenged by Fowler and Kok [41] who reported a stoichiometry of $4H^+/e^-$ under flashing light, without, however, any direct assay for the number of electrons transferred. However, when repeating our previous experiments now using three chemically different pH indicating dyes spanning the pH range from 6 to 8, and using two independent methods for assaying the number of electrons, we found no deviation from a stoichiometry of $2H^+/e^-$ under the following conditions: measuring light intensity below 30 $\mu W/cm^2$, period of flash light 10 s, benzyl viologen as terminal electron acceptor [42]. The stoichiometry of protons released inside per chlorophylls was then $2H^+$ per 770 chlorophylls on average.

This leads us to seriously consider the possibility that the observed selectivity of modified CF1 for one proton per electron might reflect the stoichiometry of about 1 CF1 per 2 protons released inside. If this were so, the observed behaviour may be visualized as follows. After energization of the membrane modified CF1 partly detaches from its counterpart, thus opening a proton channel, which is closed again after the passage of one proton under the driving force of the electric field.

NOTE ADDED IN PROOF (Received August 22nd, 1977)

Under their preillumination conditions (90 s, saturating light) Weiss and McCarty [44] observed that crosslinking of OPDM within the γ-subunit of CF1 occurred in only 10–16% of the total CF1 population. If this were the same under preillumination with flashing light, it is difficult to explain why only one proton per CF1 is shortcircuited across the thylakoid membrane, unless each crosslinked CF1 translocated several protons before self-closing again. This certainly deserves further investigation.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Dr. McCarty for suggesting OPDM, for providing a first sample of this agent together with his unpublished data and finally for many helpful comments. Thanks are due to Ilse Columbus for technical assistance and to Allison McGeer for critical comments on the manuscript. Winfried Ausländer's advice during the experiments was very much appreciated. This work was financially supported by the Deutsche Forschungsgemeinschaft.

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