PROTON RELEASE INTO THE INTERNAL PHASE OF THYLAKOIDS DUE TO PHOTOSYNTHETIC WATER OXIDATION

On the periodicity under flashing light

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Received 2 May 1977

1. Introduction

Photosynthetic electron transport in green plants causes the inwardly directed transport of protons across the thylakoid membrane. The release of protons inside is well documented [1–6]. Amongst the various indirect techniques, for measuring pH changes in the submicroscopic internal phase, the one with the permeating pH, indicating dye neutral red, is distinguished by sensitivity and time resolution for the small pH changes induced under flash excitation of chloroplasts [5]. Based on spectrophotometric studies with this dye, we reported previously that proton release inside under flashing light at low frequency (less than 0.2 Hz) is kinetically biphasic, with the rapid phase (half-rise time about 300 μs) attributable to the oxidation of water by photosystem II and the slower one (about 20 ms) due to the oxidation of plastohydroquinone by photosystem I [5]. It was furthermore shown, that this rapid phase can be isolated if the electron transport between the two photosystems is interrupted by the plastoquinone antagonist DBMIB [7]. Proton release due to water oxidation under these conditions is the subject of this communication.

The oxidation of water to produce oxygen follows a characteristic kinetic pattern. When dark-adapted chloroplasts or algae are excited with a series of short flashes the oxygen yield per flash reveals damped oscillation with a period of four [8,9]. Kok et al. [9] interpreted this as follows: the water oxidizing enzyme system cycles stepwise through five oxidation states ($S_0, \ldots, S_4$) with S_1 most stable in the dark-adapted system. Oxygen is produced during the transition $S_4 \rightarrow S_0$, only. The chemistry of the water oxidizing enzyme system is largely unknown. There is evidence that manganese is involved [10] and that it may change its spin state during transitions between the above S-states [11].

Two types of hypothetical models for the role of manganese may be distinguished:

(i) Under successive flashes an aggregate involving manganese goes stepwise through the oxidation states. Only after having accumulated four oxidizing equivalents, it interacts with two molecules of water to produce O_2.

(ii) Each of the successive oxidation states of the enzyme system involves water and its oxidation products, respectively. The role of manganese may be to trap these oxidation products as ligands, as proposed by Renger [12].

These two types of mechanisms would differ in the pattern of proton release but not in the pattern of oxygen release under successive flashes. A mechanism of type (i) would imply that proton release stricly follows oxygen evolution, while mechanism (ii) implies deviations of the latter from the former.

Fowler and Kok [13] studied proton release by the water oxidizing enzyme system by means of a rapid glass electrode. Protons released inside were detected in the external phase after passage across the thylakoid membrane (which was accelerated by an uncoupler). With ferricyanide as electron acceptor between the two photosystems, proton production seemed to follow a damped oscillation quite similar to the one of oxygen evolution (see fig.5 in [13]). In Fowler's recent paper [14] the characteristic features of this pattern, namely almost no proton release in flashes 1 and 2, were not reproduced (see figs 4b and 4b′ in [14]).

In this communication we report on oxygen yield and proton release under excitation of chloroplasts with repetitive flash groups. Polarographic experiments on the oxygen yield and spectrophotometric experiments on proton release inside were carried out in parallel. To reinstall the dark equilibrium between the *S*-states during the repetition time of the flash groups (5 s) the relaxation between the *S*-states was accellerated by addition of an Adry agent (see [15]).

Under conditions were the oxygen yield follows the known periodical pattern, with high yield in the third flash and very little yield in the first two flashes, we observe proton release in all flashes. This is discussed in the light of the above models.

2. Experimental

Spinach grown in a phytotron was kindly supplied by Dr Kötter (Schering AG). Chloroplasts were prepared according to Siggel et al. [16]. They were used freshly. Unless otherwise indicated, chloroplasts were suspended in the following standard reaction medium: KCl 20 mM, $MgCl_2$ 1.3 mM, ferricyanide 260 μM; neutral red 13 μM; bovine serum albumin (BSA) 1.3 mg/ml, dibromothymoquinone (DBMIB), 3 μM, the Adry agent 2(3,4,5-trichloro)-anilino-3,5-dinitrothiophene (ANT2s) 0.6 μM, imidazole, if added was present at 5 mM. The average chlorophyll concentration was 50 μM in the oxygen yield experiments and 10 μM in the experiments on internal proton release. The pH was titrated to 7.2 and was stable within 0.1 units for 10 min.

DBMIB was kindly supplied by Dr Draber (Bayerwerk Wuppertal-Elberfeld), and ANT2s was a gift from Prof. Dr Bücher (Bayerwerk Leverkusen).

2.1. *Photometric detection of pH transients inside the thylakoids*

The chloroplast suspension in an optical absorption cell (2 cm) was mounted in a flash spectrophotometer (for instrumentation, see [17,18]. The sample was excited periodically (results invariant for repetition times between 5 s and 10 s) with groups of short flashes (half-width 15 μs) spaced 10 ms apart. The energy of each flash (0.5 mJ $\cdot$ cm^{-2} above 600 nm) was about saturating photosystem II. Changes of absorption were monitored at a wavelength of 552 nm (and 524 nm, respectively). To avoid excitation of the sample by the measuring light during the relatively long repetition periods, the measuring light was passed through a photoshutter which was triggered open 40 ms before the first flash and closed 160 ms thereafter. The total energy of the measuring light, to which the sample was exposed during one cycle, was then 20 μJ (intensity X opening time = 100 μJ $\cdot$ cm^{-2} X 200 ms). At 552 nm this was negligible in comparison with the energy of a single flash above 600 nm.

The response of neutral red to pH transients in the internal phase was extracted from the observed absorption changes at 552 nm as previously [5]: absorption changes were recorded twice, first from a suspension without and then from a suspension with the permeating buffer, imidazole. In both cases the external phase was strongly buffered by BSA. As previously demonstrated, the subtraction of the latter signal from the former produces the 'pH_{in}-indicating absorption changes of neutral red' while eliminating response of the dye to events other than internal pH changes (plus underlying absorption changes of intrinsic chloroplasts pigments). If the pH changes inside are small, as under flashing light at low repetition rate, the response of neutral red is pseudo-linear [5,20]. However, problems arise if, as under continuous illumination, neutral red redistributes and accumulates in the internal phase under the influence of a larger pH difference across the thylakoid membrane [19]. It has to be pointed out that complications arising under these conditions are not relevant under flashing light at low repetition rate, as the inwardly directed diffusion of the dye under the influence of a small pH difference (in the order of 0.1 unit) is small as compared to the amount of neutral red accumulated inside already in the dark (see [19,20]).

2.2. *Polarographic detection of oxygen production*

Oxygen production under flashing light was detected by a polarographic method as previously [21]. A chloroplast suspension was excited with repetitive flash groups (repetition period 5 s). The distance between successive flashes in each group was 10 ms. The oxygen evolution caused by the *n*th flash was evaluated by subtracting the oxygen yield by a group of *n*-1 flashes from the one by a group of *n* flashes (see [22]). It is noteworthy that the chlorophyll concentrations were different in the experiments on oxygen yield and in those on proton release. This, however, does not explain the difference in the respective yields (see fig.3). Our experiments strongly rely on the activity of the Adry agent ANT2s. The higher chlorophyll concentration in the oxygen yield experiments is rather unfavourable for its activity. The observation of a pronounced oscillation with period of four under these conditions implies the existence of such an oscillation under the conditions of the pH experiment at lower chlorophyll concentration as well.

The concentration of ANT2s required to accellerate the relaxation of the *S*-states was higher than in similar experiments in the absence of BSA. This may have been caused by adsorption of ANT2s to BSA. The periodical pattern of the oxygen yield, however, proves that a sufficient amount of the Adry agent acted on the chloroplasts.

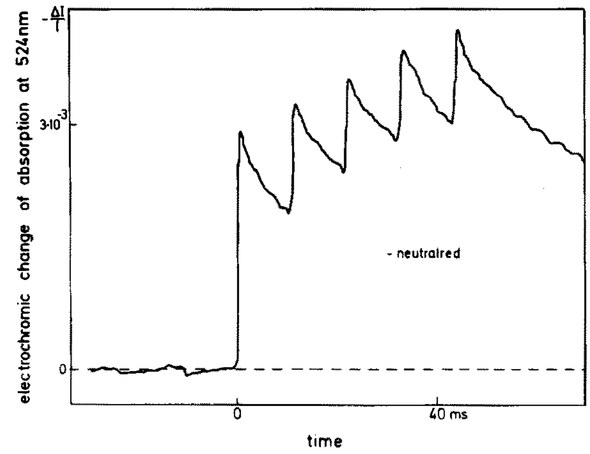

Fig.1. Time course of the electrochromic change of absorption at 524 nm. Chloroplasts were excited with a group of five short flashes. Standard reaction medium except for the absence of neutral red.

3. Results and discussion

Figure 1 shows the time course of the electrochronic absorption changes at 524 nm (no neutral red present) under excitation of chloroplasts with periodical groups of short flashes (five flashes/group). The extent of these absorption changes lineary indicates the electric potential across the thylakoid membrane (for reviews, see [22,23]). Both photosystems contribute about equally to the total extent if excited by a short flash [24]. The pattern shown in fig.1 was obtained in the presence of DBMIB which interupts the electron transfer between the two photosystems at the level of plastoquinone [7]. Based on our previous work the pattern in fig.1 can be explained as follows: As photosystem I is re-reduced in the dark-time between flash groups (5 s) by an as yet unidentified endogeneous donor, the first flash activates both photosystems. As this re-reduction is slow (half-rise time about 1 s [25]) photosystem I is not regenerated between the flashes of one group. Consequently flash numbers 2–5 activate photosystem II, only. The relative extent of the flash-induced rise of absorption in successive flashes is not quite 100 : 50 : 50 : 50 : 50 as expected but 100 : 46 : 40 : 39 : 39 (see fig.1). This deviation is not too relevant in the context of the effects discussed in this communication (possible reasons are the about 13% higher abundance of Photosystem I [26], partial saturation etc.). Photosystem II activity varies no more than between 100% and 80% during flashes 1–5. As previously shown [5,25] the reduction of photosystem I by the unknown endogenous donor is not coupled to proton release inside. Hence proton release inside should be entirely due to water oxidation under these conditions.

Figure 2 shows the pH_{in}-indicating absorption changes of neutral red at 552 nm under the same conditions as in fig.1. Each flash produces some protons in the internal phase. The extent of proton release inside/flash (together with the oxygen yield/flash) is given in fig.3 as function of the flash number. The relative yields are normalized to the respective yield of the third flash, which is arbitrarily taken as 100%. The oxygen yield (full circles) reveals the characteristic pattern with a very low yield in flash numbers 1 and 2. In contrast to this flash numbers 1 and 2 release more than 70% of the protons which are released by

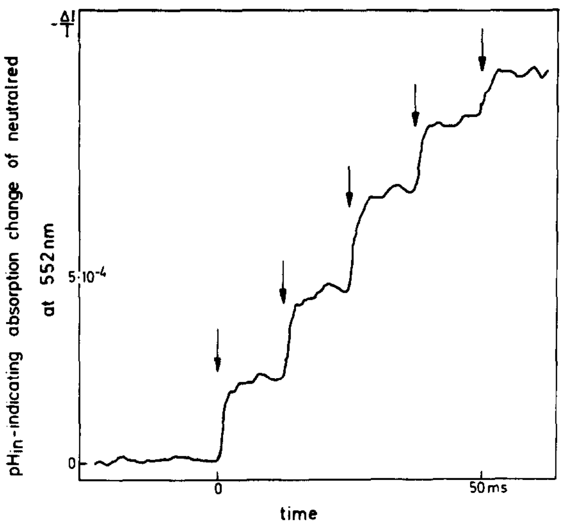

Fig.2. Time course of the pH_{in}-indicating absorption changes of neutral red at 552 nm. Chloroplasts were excited with a group of five short flashes. Standard reaction medium. Average over 32 signals obtained in the absence of the permeating buffer imidazole minus the average over 32 signals obtained in the presence of imidazole.

flash number 3 (open squares). If the oxygen pattern is interpreted to reveal that O_2 is produced only during the dark-transition between states S_4 and S_0 (with S_1 most stable in the dark), then the pattern of the proton release inside indicates proton production also during the transition from S_1 to S_2 and from S_2 to S_3. This favours the possibility that the S-states involve water and its oxidation products, which are 'shielded' by ligation to manganese.

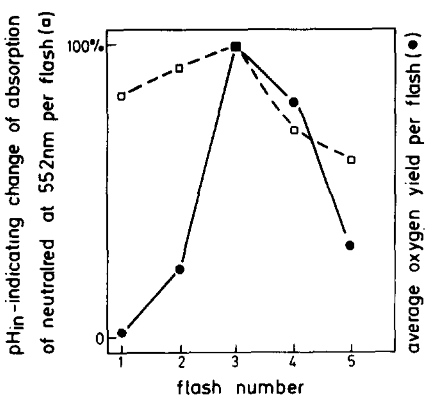

Fig.3. Oxygen yield/flash (circles) and proton release inside/ flash (squares) as function of the flash number. Both patterns were obtained in the presence of an Adry agent (ANT2s) to accellerate the relaxation between the S-states of the water oxidizing enzyme system.

A hypothetical model for this was proposed by Renger [12]. In this model the transition $S_0 \xrightarrow{h\nu} S_1$ represents the univalent oxidation of an unidentified component M, a process not accompanied by proton release. The transitions $S_1 \xrightarrow{h\nu} S_2$ and $S_2 \xrightarrow{h\nu} S_3$ are visualized as the formation of a 'cryptohydroxyl'-radical and of 'cryptoperoxide', respectively, from water. Each of these steps would be accompanied by the release of one proton/electron, provided that the pK values of these species did not deviate too much from the ones of their counterparts in aqueous solution. The last oxidation step, equivalent to the transition $S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0$ in the terminology of Kok et al. [9], would then be:

$$\text{crypto-}H_2O_2 + h\nu + M^+ \longrightarrow O_2 + 2H^+ + M.$$

This step would be the only one producing oxygen (and two protons as well).

A quantitative correlation of the proton yield/flash with this model will be presented elsewhere.

Acknowledgements

The authors are very grateful to Dörte DiFiore and to Ilse Columbus for technical assistance. One of us (GR) acknowledges financial support from the ERP (Sondervermögen), another one (WJ) acknowledges financial support from the EC(Solar Energy Program) and from the Deutsche Forschungsgemeinschaft.

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