Studies on the proton release pattern of the donor side of system II

Correlation between oxidation and deprotonization of donor D_1 in Tris-washed inside-out thylakoids

G. Renger and M. Voelker

Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Straße des 17. Juni 135, 1000 Berlin 12, Germany

Received 30 September 1982

The protonization pattern of the endogenous donor component D_1 which feeds electrons directly into chl-a_{II}^{+} has been analyzed in Tris-washed inside-out thylakoids with the aid of appropriate pH-indicators. It was found that under repetitive flash excitation the amount of protons released is proportional to the extent of D_1-oxidation, depending on the time between the flashes. The kinetics of D_1-oxidation (being practically the same as in normal Tris-washed chloroplasts) are faster than the proton release by two orders of magnitude. The results lead to the conclusion that D_1 is protonized in the reduced state with $pK(D_1^{red}) < 5$ and becomes deprotonized in the oxidized state with $pK(D_1^{red}) \gtrsim 8$. The proton release is kinetically limited by a transport barrier. Implications on the interpretation of the proton release pattern in preparation with intact water oxidation are discussed.

Photosynthesis *System II* *Endogenous donor, to chlorophyll-a_{II}* *Proton release* *Wateroxidation* *Inside-out thylakoids*

1. INTRODUCTION

Photosynthetic water oxidation to molecular oxygen occurs via a 4-step univalent reaction sequence which gives rise to a characteristic oscillation pattern of oxygen evolved in dark-adapted chloroplasts after excitation with a flash train [1,2]. Redox equivalents of sufficient oxidizing power are produced by electron ejection from the excited singlet state of a special chlorophyll-*a* complex, referred to as chl-a_{II} [3]. The cooperation of 4 oxidizing redox equivalents takes place within the water splitting enzyme system Y. Water oxidation to molecular oxygen is necessarily coupled with proton release. As the patterns of oxygen evolution and proton release significantly differ, redox transitions in system Y preceding O_2 formation are inferred to be coupled with deprotonization [4–6]. Kinetic EPR measurements indicate that a redox carrier, symbolized by D_1, provides the functional connection between chl-a_{II} and system Y [7]. Indirect evidence led to the conclusion that in normal chloroplasts the reduction kinetics of chl-a_{II}^{+} is regulated by the pH of the inner thylakoid space [8]. Likewise, in Tris-washed chloroplasts, completely deprived of their oxygen evolving capacity, the rate of chl-a_{II}^{+} reduction was found to depend on the pH [9]. These results favor the idea that either D_1 itself or a protein responsible for the functional connection between D_1 and chl-a_{II} is protonizable. Recently, a deprotonization was

Abbreviations: BCP, bromocresol purple; chl-a_{II}, chlorophyll-a_{II}; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; D_1, endogenous donor to chl-a_{II}; ISO thylakoids, inside-out thylakoids; MES, morpholinoethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

reported to be coupled with the transitory oxidation of D_1 in normal chloroplasts in the presence of DCMU [10]. However, some ambiguity remains in the interpretation of the data, because there has been neglected the existence of a cyclic reaction path around system II taking place in DCMU-treated chloroplasts as detected by measurements of the reoxidation kinetics of the primary plastoquinone acceptor via fluorescence [11] or absorption changes [12]. In order to clarify the protonization properties of D_1, experiments were performed with Tris-washed class II chloroplasts and Tris-washed inside-out (ISO) thylakoids. These data indicate that D_1 becomes deprotonized in its oxidized state, D_1^{ox}, in Tris-washed thylakoids. The pK-value of the reduced state, D_1^{red}, is estimated to be ~8. Furthermore, D_1 is inferred to be separated from the aqueous phase by a transport barrier. This causes a retardation of proton release so that no information could be obtained about a deprotonization of D_1 during its fast transient oxidation in normal chloroplasts with intact system Y.

2. MATERIALS AND METHODS

Class II chloroplasts and inside-out thylakoids have been prepared as in [13,14]. Tris-washing of normal class II chloroplasts and inside-out thylakoids was performed as outlined in [15], trypsinization as in [16]. The standard reaction mixture contained chloroplasts (10 μM chlorophyll), 10 mM KCl, 2 mM $MgCl_2$ and 100 μM phenyl-*p*-benzoquinone as electron acceptor. Other additions as indicated in the figure legends.

Absorption measurements were performed with a repetitive flash spectrophotometer [17]. Flash-induced oxygen evolution was detected with a Clark-type electrode as in [18]. For the chl-a_I measurements at 703 nm (measuring light intensity 25 $\mu W.cm^{-2}$) additional far-red background illumination ($I = 450\,\mu W.cm^{-2}$) was applied (see [19]). To prevent a significant disturbance of the D_1^{ox}-reduction in Tris-washed chloroplasts, the measuring light beam at 570 nm was pulsed as in [20] (light pulse 30 ms, $I = 50\,\mu W.cm^{-2}$, exciting flash was fired 10 ms after switching on the measuring light pulse). At 834 nm a continuous measuring light beam (1 $mW.cm^{-2}$) can be used, because of the very low absorption in this spectral region and the insufficient energy of the light quanta for chl-a_{II} photooxidation.

3. RESULTS

Absorption changes at 834 nm and 570 nm measured in Tris-washed class II chloroplasts and inside-out (ISO) thylakoids are depicted in fig. 1. The time between flashes was long enough (10 s) to allow D_1 reduction in the dark, and the total number of quanta absorbed due to the measuring light beam was sufficiently small to assure that <5% of the centers are excited before the flash. The fast relaxation kinetics ($t_{1/2} = 5-10\,\mu s$) of 834 nm absorption changes reflecting chl-a_{II}^{+} reduction by D_1 [9] are practically identical in both preparations. These results show that the functional connection between D_1 and chl-a_{II} remains invariant to inverted membrane polarity.

A completely different behavior is expected for the pH-detecting absorption changes of bromocresol purple (BCP) at 570 nm because of reverted membrane anisotropy. This was found to be the case. In suspension of Tris-washed class II chloroplasts a rather small positive absorption change with slow rise kinetics is observed, which reflects a proton uptake from the outer aqueous phase, probably coupled with reduction to the quinol redox state of the secondary plastoquinone B [21]. As the proton transport from the outer side is limited by a transport barrier in normal chloroplasts [22], only part of the total signal can be resolved within 10 ms. In contrast, in Tris-washed ISO thylakoids a pronounced negative 570 nm absorption change is induced with a half-risetime of 1 ms, indicating a proton release into the outer aqueous phase. A calibration shows that the signal amplitude corresponds to ~0.85 protons/600 chl. The failure to obtain a 1:1 stoichiometry with the concentration of the system reaction centers (~1:600 chl) is very likely due to some contamination of ISO thylakoids with right-side-out (RSO) particles. Regardless of these stoichiometric subtleties, the data of fig. 1 reveal a flash-induced proton release of almost 1:1 stoichiometry in Tris-washed ISO thylakoids. As the average oxygen yield/flash in Tris-washed ISO thylakoids was <5% of that in normal ISO thylakoids, the proton release in fig. 1 cannot be due to water oxidation. To prove that this proton

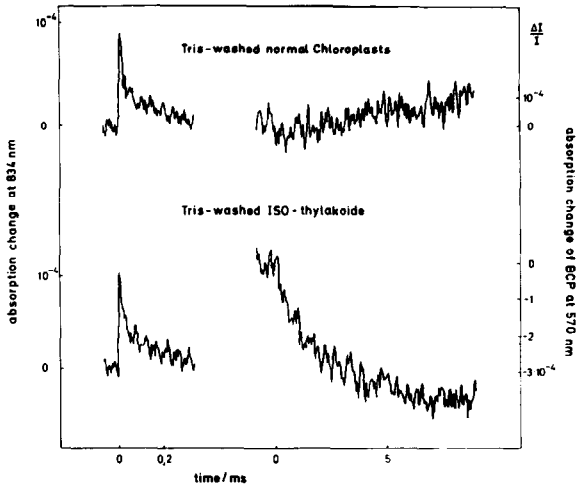

Fig. 1. Absorption changes at 834 nm (left) and 570 nm (right) as a function of time in Tris-washed class II chloroplasts (top) and inside-out thylakoids (bottom). $K_3[Fe(CN)_6]$ (0.5 mM) and 20 mM MES/NaOH (pH = 6.5) were added for measurements of 834 nm absorption changes. The absorption changes at 570 nm were not affected by 0.5 mM $K_3[Fe(CN)_6]$, the pH was adjusted to 6.5 by injection of small amounts of HCl or NaOH, respectively. Repetitive excitation: 0.1 Hz. Other additions and experimental conditions as in section 2.

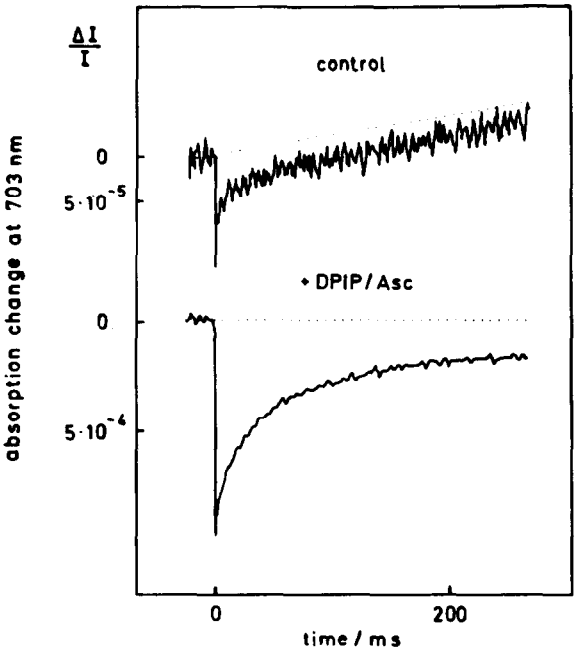

Fig. 2. Absorption changes at 703 nm as a function of time in Tris-washed inside-out thylakoids in the absence and presence of 15 μM DCIP and 0.5 mM ascorbate. The chloroplast suspension contained: 5 μM chl, 50 μM phenyl-*p*-benzoquinone and 20 mM MES/NaOH (pH 6.5). Other additions and experimental conditions as in section 2.

ejection is coupled with oxidation of the donor D_1, it remains to be shown that:

(i) Under the experimental conditions of fig. 1, only system II is functionally active in the Tris-washed ISO thylakoids;

(ii) The amplitudes of the 570 nm absorption change correlate with the extent of flash-induced D_1 oxidation.

The former problem has been solved by measurements of absorption changes at 703 nm. The data depicted in fig. 2 reveal, that the extent of the 703 nm absorption changes is $< 10\%$ in the absence compared to that observed in the presence of DCIP/ascorbate. Accordingly, system I reactions cannot be responsible for the proton release detected in Tris-washed ISO thylakoids (see fig. 1). The second question can be answered by comparing (under repetitive flash excitation) the extent of D_1 oxidation and proton release as a function of the time t_d between the flashes. The amplitude of the 5–10 μs component of the 834 nm absorption change, referred to as $\Delta A^{5\,\mu s}_{834}(t_d)$, detects the extent of D_1 oxidation by chl-a_{II}^{+} [9], which depends on the slow recovery of D_1 during the dark time, t_d, between the flashes. Fig. 3 shows that the normalized amplitudes of D_1 oxidation expressed as $\Delta A^{5\,\mu s}_{834}(t_d)/\Delta A^{5\,\mu s}_{834}(t_d = 10\,s)$ and of proton release described by $\Delta A_{570}(t_d)/\Delta A_{570}(t_d = 10\,s)$ nearly coincide in their dependencies on the t_d between the flashes. The result confirms that D_1 oxidation in Tris-washed ISO thylakoids is coupled with proton ejection into the outer aqueous phase. A completely different pattern is expected to arise in ISO thylakoids with intact water-splitting enzyme system Y. In this case D_1^{ox} becomes reduced in the 0.1–1 ms range [7], and the overall reaction of system II is limited by the electron transfer to the exogenous acceptor (phenyl-*p*-benzoquinone) which occurs in the ms range. Accordingly, the extent of H^+ release should be independent of the t_d down to ~100 ms (see fig. 3, ---). A striking phenomenon of the data in fig. 1 is the significant divergence in the kinetics of flash-induced D_1 ox-

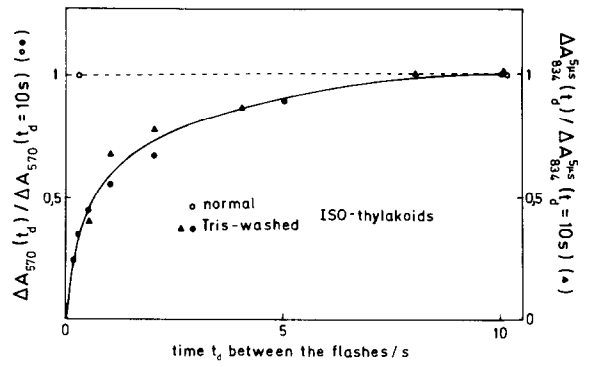

Fig. 3. Relative extent of proton release $\Delta A_{570}(t_d)/\Delta A_{570}$ (t_d = 10 s) (○, ●) and relative amplitude of the 5–10 μs relaxation kinetics, $\Delta A^{5\mu s}_{834}(t_d)/\Delta A^{5\mu s}_{834}(t_d = 10\,s)$ as a function of time t_d between the flashes in normal (○) inside-out and Tris-washed (●, ▲) inside-out thylakoids. Experimental conditions as described in fig. 1, except of variable repetition frequency.

idation (5–10 μs) and H^+ release (~1 ms). A free diffusional proton transport to the indicator molecules is expected to be much faster than 1 ms. Accordingly, it is reasonable to assume that the functional group of D_1 is covered up by surroundings which act as a transport barrier to protons.

The results of fig. 1–3 support the idea that, at neutral pH, D_1 is protonized in the reduced state and becomes deprotonated in the oxidized state. Preliminary data (not shown) further indicate that the extent of proton release declines at higher pH. On the basis of these data a rough estimation can be given of the pK-values of D_1^{red} and D_1^{ox}:

$$pK(D_1^{red}) \sim 8;\ pK(D_1^{ox}) < 5$$

4. DISCUSSION

This study leads to the conclusion that in Tris-washed ISO thylakoids the donor D_1 contains a functional group carrying a protonizable site with a pK-value which depends on the redox state. Furthermore, this group is embedded in a matrix which functions as a transport barrier to protons. If one takes into account the estimate of $pK(D_1^{red}) \sim 8$, the pH dependency of the reduction kinetics of chl-a_{II}^+ in normal [8] as well as in Tris-washed chloroplasts [9] regulated by protonizable groups with pK 5–6 is probably caused by another effect (e.g., allosteric effect), not related to possible differences in the electron-transfer kinetics of the functional group of D_1 in the protonized and deprotonized state, respectively.

Interestingly enough, the electron-transfer kinetics from D_1 to chl-a_{II}^+ is not markedly affected by mild trypsinization, neither in Tris-washed, class II chloroplasts [23] nor in Tris-washed ISO thylakoids (unpublished). Therefore, further experiments are required in order to characterize the D_1 matrix.

In normal ISO thylakoids a lag phase has been observed for proton release [24]. However, in Tris-washed ISO thylakoids hardly any lag is detected (in preparation). The absence of a lag in Tris-washed ISO thylakoids can be easily understood, because of the fast formation (5–10 μs) and the long lifetime of the oxidized state of D_1, which becomes deprotonized. Likewise, the results with normal ISO thylakoids can also be explained by a barrier for H^+-transport from D_1^{ox} which prohibits proton release into the outer aqueous phase during the lifetime of D_1^{ox}, so that only the S_i-state transition in system Y (see [1,2]), characterized by a lag of a few 100 μs, gives rise to formation of species stable enough to act as source for H^+-ejection. Another explanation could be offered by the assumption of markedly different protonization properties of D_1 in normal compared to Tris-washed ISO thylakoids. For the time being we cannot decide whether the protonization properties of D_1 are modified by Tris-washing. However, regardless of these details, the possibility of protonization/deprotonization reactions coupled with the transient oxidation of D_1 implies a serious consequence for the interpretation of proton release kinetics of the system II donor side. If D_1 oxidation in normal chloroplasts is also coupled with deprotonization, then the detected rise kinetics of proton release would reflect either the transport barrier to the outer aqueous phase or the deprotonization of oxidized D_1, depending on the site of the proton sensor. Under these circumstances, a correlation with the S_i state-transition in the water-splitting enzyme system Y can be achieved only by use of an indicator dye which selectively binds to Y but does not monitor the transient proton release from D_1. Other theoretical problems arising with the proton release pattern of system Y have been discussed [25].

These data show that the donor component D_1 contains a protonizable site close to its functionally active redox group, and therefore the question arises as to the chemical nature of this group. Experiments are in progress to solve this problem.

ACKNOWLEDGEMENTS

The authors would like to thank Dipl. Phys. W. Weiss for invaluable contributions to this work, Dr P. Gräber and Dipl. Phys. G. Dohnt for critically reading the manuscript and C. Behm for technical assistance. The financial support by Deutsche Forschungsgemeinschaft is gratefully acknowledged.

REFERENCES

[1] Joliot, P., Joliot, A., Bouges, B. and Barbieri, G. (1971) Photochem. Photobiol. 11, 287–305.
[2] Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 10, 457–475.
[3] Döring, G., Renger, G., Vater, J. and Witt, H.T. (1969) Z. Naturforsch. 24b, 1139–1143.
[4] Junge, W., Renger, G. and Ausländer, W. (1977) FEBS Lett. 79, 155–159.
[5] Saphon, S. and Crofts, A.R. (1977) Z. Naturforsch. 32c, 810–816.
[6] Velthuys, B.R., Crowther, D. and Hind, G. (1979) Biochim. Biophys. Acta 547, 138–148.
[7] Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289.
[8] Renger, G., Gläser, M. and Buchwald, H.E. (1977) Biochim. Biophys. Acta 461, 392–402.
[9] Conjeaud, H. and Mathis, P. (1980) Biochim. Biophys. Acta 590, 353–359.
[10] Hong, Y.Q., Förster, V. and Junge, W. (1981) FEBS Lett. 132, 247–251.
[11] Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363.
[12] Renger, G. and Weiss, W. (1982) FEBS Lett. 137, 223–228.
[13] Akerlund, H.E., Anderson, B. and Albertson, P.A. (1976) Biochim. Biophys. Acta 449, 525–535.
[14] Anderson, B., Akerlund, H.E. and Albertson, P.A. (1977) FEBS Lett. 77, 141–145.
[15] Yamashita, T. and Butler, W.L. (1968) Plant Physiol. 43, 1978–1988.
[16] Renger, G. (1977) in: Membrane Bioenergetics (Packer, L. et al. eds) pp. 339–350, Elsevier Biomedical, Amsterdam, New York.
[17] Rüppel, H. and Witt, H.T. (1969) Methods Enzymol. 316–380.
[18] Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439.
[19] Haehnel, W. (1976) Biochim. Biophys. Acta 423, 499–509.
[20] Renger, G. and Wolf, Ch. (1975) Z. Naturforsch. 30c, 161–171.
[21] Fowler, C.F. (1977) Biochim. Biophys. Acta 459, 351–363.
[22] Ausländer, W. and Junge, W. (1974) Biochim. Biophys. Acta 357, 285–298.
[23] Renger, G. and Eckert, H.J. (1981) Biochim. Biophys. Acta 638, 161–171.
[24] Tiemann, R., Renger, G. and Gräber, P. (1981) Proc. 5th Int. Congr. Photosynthesis (Akoyunoglou, G., ed.) Vol. 3, pp. 85–95, Balaban Int. Sci. Service, Philadelphia PA.
[25] Renger, G. (1978) in: Photosynthetic Oxygen Evolution (Metzner, H. ed.) pp. 229–248, Academic Press, London, New York.