Biochimica et Biophysica Acta 848 (1986) 274–278
Elsevier

BBA 41928

The slow rate of proton consumption at the reducing side of Photosystem I is limited by the rate of redox reactions of extrinsic electron acceptors, but not by a diffusion barrier for protons *

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(Received July 30th, 1985)

Key words: Photosystem I; Proton consumption; Superoxide dismutase; Ferredoxin; NADP$^+$

Absorption changes of cresol red at 575 nm which indicate proton consumption in the external bulk phase were measured under repetitive flash excitation of pea thylakoids. In the presence of methyl viologen as terminal electron acceptor, proton consumption due to the activity of both photosystems was slow with a half-rise time of 130 ms. If either superoxide dismutase was added or the natural acceptor system was complemented by addition of ferredoxin and NADP$^+$, the rise of the alkalization was accelerated and two phases became distinguishable: a slow phase which was due to Photosystem II activity ($t_{1/2} \approx 100$ ms) and a fast phase which was due to Photosystem I activity. In the presence of the artificial electron acceptor methyl viologen, the rise of the fast phase was accelerated in proportion to the amount of added superoxide dismutase to a limit of $t_{1/2} = 10$ ms. From these results we concluded that the slow rate of proton consumption at the reducing side of Photosystem I was limited by the nature of the supplied acceptor system, but not predominantly by a diffusion barrier for protons.

Introduction

During photosynthesis Photosystem II and Photosystem I cooperate in series to transfer electrons from H_2O to NADP$^+$ (for a recent review, see Ref. 1). In vivo this process is coupled with proton consumption from the stroma by two reactions: (1) reduction of plastoquinone at the reducing side of Photosystem II; and (2) reduction of NADP$^+$ at the reducing side of Photosystem I. After isolation of ruptured chloroplasts the stromal components of photosynthesis are lost, in particular, NADP$^+$, the terminal electron acceptor and ferredoxin, intermediate electron carrier [2]. Historically, electron transport in isolated thylakoids was not observed, unless Hill introducted artificial electron acceptors [3].

Bipyridylium herbicides (i.e., methyl viologen) are commonly used as terminal acceptors. After their reduction by Photosystem I, they react with oxygen to produce the superoxide anion [4]:

$$\text{methyl viologen}^{\cdot} + O_2 \rightarrow \text{methyl viologen} + O_2^{\cdot -}$$

The superoxide radical is a natural side product in photosynthetic processes [5–7]. Its dismutation is catalysed by superoxide dismutases, which are common to aerobic cells [8]. During this process

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* A short report on this subject was presented by us (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 261–264, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands.

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylene-diamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NADP$^+$, oxidized form of nicotinamide adenine dinucleotide phosphate.

one proton per electron is consumed (Ref. 9):

$$2\,O_2^{-} + 2\,H^{+} \rightarrow H_2O_2 + O_2$$

In green tissues most of the superoxide dismutase activity is located in the stroma of chloroplasts. Only a minor part is membrane bound and is still present in isolated, ruptured chloroplasts [10,11].

In the presence of viologen acceptors, flash-induced pH changes in the suspending medium of thylakoids are observed which corresponded to the disappearance of 2 H^{+}/e^{-}: one proton taken up at the reducing side of Photosystem II and one proton consumed at the reducing side of Photosystem I (reviewed in Ref. 12). Surprisingly, the rise of the transient alkalization, although induced by the two different protolytic reactions, is seemingly monoexponential with a rather long half-rise time of 60 ms [13] or even longer when monitored spectrophotometrically via pH-indicating dyes. In comparison with electron transport (rate-limiting step $t_{1/2} = 10$ ms) both proteolytic reactions are retarded (for a review, see Ref. 12). From these results a diffusion barrier for proton uptake at the reducing sides of both photosystems was proposed [13].

We investigated whether the rate of proton binding at the reducing side of Photosystem I was retarded by a diffusion barrier for protons or whether it was dependent on the nature of terminal acceptor system.

Materials and Methods

Pea seedlings (*Pisum sativum*) were grown under artificial illumination (Osram 65W/77R Flura) in a cycle of 16 h light and 8 h dark.

Broken chloroplasts were prepared from 14–16 days old plants as described by Förster et al. [14]. Chloroplasts were frozen in liquid nitrogen in the presence of 30% ethylene glycol [15] and stored for up to two months before use. They gave similar results as freshly prepared chloroplasts.

Thylakoids equivalent to 10 μM chlorophyll were added to an assay medium containing 25 mM KCl, 3 mM $MgCl_2$ and 15 μM Cresol red, pH 7.9. Further additions are given in legends of the figures.

Absorption changes were measured spectrophotometrically in the same set up as described by Förster et al. [14]. The pH_{out}-indicating absorption changes of cresol red at 575 nm were obtained by subtraction of signals recorded in the absence of the dye from those recorded in the presence of the dye.

Electrochromic absorption changes, which indicated the activity of Photosystem I and II were measured at 522 nm [16,17].

Absorption changes which indicated Photosystem I activity were recorded in the near infrared at 819 nm [18]. Absorption changes due to Photosystem II activity did not contribute to the latter signal, because the reduction of P-680^{+} is too fast to be resolved at the shortest dwell time (50 μs), which we used [19,20].

Samples were excited with saturating light flashes (xenon flash lamp, wavelength longer than 610 nm; FWHM, 15 μs; 1 mJ/cm^2). The measuring beam was gated open only during the actual sampling interval. Its energy per repetition was kept below 10 μJ/cm^2. Typically, 20–40 samples were averaged. The detector was a photomultiplier EMI 9558 with six dynodes. The flash repetition rate was 0.1 Hz.

NADP^{+}, ferredoxin and superoxide dismutase were purchased from Sigma. The activity of superoxide dismutase from bovine blood was 2800 units/mg protein. Ferredoxin from spinach contained 0.15 M Trizma buffer (pH 7.5). The buffer was removed by elution of ferredoxin with 5 mM $MgCl_2$/10 mM NaCl (pH 7.8) over a PD-10 column from Pharmacia. This was necessary to diminish quenching of the pH transients in the assay medium.

Results

Fig. 1 shows the pH_{out}-indicating absorption changes of cresol red at 575 nm under repetitive flash excitation of pea thylakoids. If methyl viologen was used as terminal electron acceptor (Fig. 1A), proton consumption occurred by two different reactions: (1) during the reduction of plastoquinone at the reducing side of photosystem II, and (2) during the dismutation of superoxide which was formed by Photosystem I via methyl viologen. The half-rise time of the pH signal was 130 ms. Kinetic analysis by exponentials revealed that the rise of the absorption change was not uniphasic. This was more expressed, if freshly prepared thylakoids were used for the experiment and less pronounced, if freezed/thawed thylakoids were used (as in Fig. 1A). Fig. 1B shows the pH_{out}-indicating absorption changes of cresol red when only Photosystem I was active. Photosystem II was inhibited by DCMU and Photosystem I activity was restored by addition of the donor DAD. Then the half-rise time of the signal was much longer ($t_{1/2}$ = 390 ms) than that of the control (Fig. 1A).

We investigated whether the rate of proton consumption might be limited by the dismutative oxidation of superoxide. For this purpose we added superoxide dismutase to the assay medium (Fig. 1C and D). Two clearly separated phases became immediately apparent (Fig. 1C). 50% of the signal rose slowly ($t_{1/2}$ = 100 ms) and 50% was 10-times faster ($t_{1/2}$ = 10 ms). When Photosystem II was inhibited and Photosystem I was reactivated, only the fast phase reappeared (Fig. 1D). Obviously, addition of superoxide dismutase accelerated proton consumption which occurred at the reducing side of Photosystem I.

In previous work it was observed that each photosystem contributed approx. 50% to the magnitude of the pH_{out}-indicating absorption changes of cresol red at 575 nm when both photosystems worked in series [21]. Here, however, when only Photosystem I activity was restored by addition of DAD about 70% (Fig. 1B and D) of the magnitude of control signals (Fig. 1A and C) reappeared. We examined whether the surplus was due to Photosystem I activity. We measured the activity of both photosystems via the electrochromic absorption change at 522 nm and of only Photosystem I via the absorption change in the near infrared at 819 nm. Table I summarizes the results. When Photosystem II was inhibited by DCMU and the electron flow through Photosystem I was reactivated by the donor DAD, the extent of the electrochromic absorption change showed 80% of the control (instead of 50%). The initial magnitude of the absorption change at 819 nm was correspondingly enhanced by 25–30%. This result indicated that the surplus was due to Photosystem I centers, which were activated via the artificial donor DAD, but were not involved in linear electron flow. This was in agreement with data of Melis and Brown [22] who showed that the ratio of Photosystem II/Photosystem I was 0.77 in mature pea chloroplasts. It is apparent from Table I that addition of superoxide dismutase had no significant effect on the activities of Photosystem I and II.

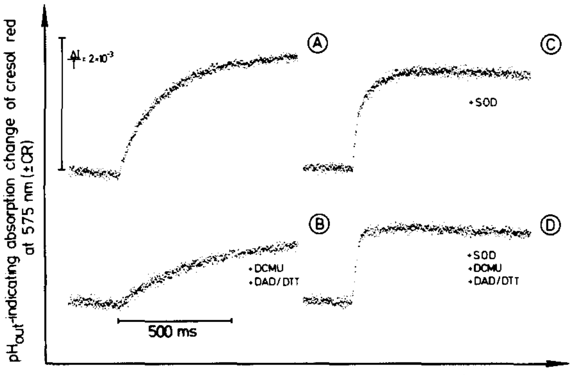

Fig. 1. pH_{out}-indicating absorption changes of cresol red in the suspending medium of thylakoids. Thylakoids suspended in the standard assay medium with methyl viologen (10 μM) as electron acceptor, further additions: (A) none; (B) DCMU (5 μM), DAD (10 μM) and dithiothreitol (DTT) (40 μM); (C) 2800 units of superoxide dimutase/mg chlorophyll; (D) as in (B) plus 2800 units of superoxide dimutase/mg chlorophyll. 40 sweeps were averaged; dwell time, 1 ms; electrical bandwidth, 3 kHz.

Fig. 2 shows the dependence of the half-rise times of proton consumption at the reducing side of Photosystem I on the amount of added superoxide dismutase. The half-rise times were mea-

TABLE I

RELATIVE ACTIVITIES OF PHOTOSYSTEM I AND OF BOTH PHOTOSYSTEMS, MEASURED VIA THE EXTENT OF THE ABSORPTION CHANGES AT 819 nm AND 522 nm, RESPECTIVELY

Assay media as in Fig. 1. 20 signals were averaged, dwell time, 50 μs/point; electrical bandwidth, 300 kHz. DTT, dithiothreitol; SOD, superoxide dismutase.

Additions to the assay medium	Relative extent at 522 nm	Percentage at 819 nm
None	100	100
SOD	98	105
DCMU, DAD, DTT	81	129
DCMU, DAD, DTT, SOD	83	125

sured under conditions when only Photosystem I was active (as in Fig. 1B and 1D). After the addition of 280 units of superoxide dismutase/mg chlorophyll the rate of proton consumption reached its saturation. The half-rise time was 10 ms then.

We questioned whether fast proton binding at the reducing side of Photosystem I was a special feature of the artificial acceptor system or whether it also occurred in the presence of the natural acceptor. We measured flash-induced proton uptake from the external bulk phase of freshly prepared thylakoids after the addition of the terminal acceptor NADP$^+$ and the intermediate electron carrier ferredoxin. Fig. 3 shows the result. Addition of ferredoxin and NADP$^+$ accelerated proton consumption. Two main phases were distinguishable: a slow phase with a half-rise time of 100 ms and a faster with a half-rise time of 20 ms. However, the magnitude of the pH signal was reduced by 50% ($\Delta I/I_0 = 1 \cdot 10^{-3}$ instead of $2 \cdot 10^{-3}$ in the control). We found that the pH changes in the external phase were diminished by increased buffering capacity which was introduced by ferredoxin.

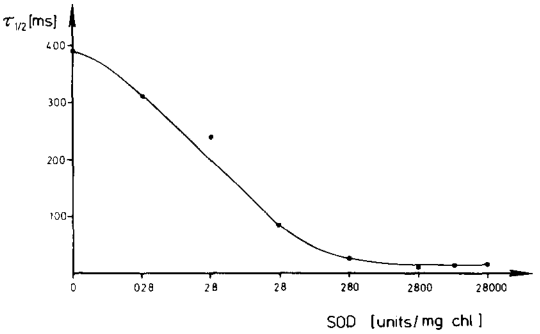

Fig. 2. Half-rise time, $\tau_{1/2}$, of proton uptake at the reducing side of Photosystem I as function of the concentration of added superoxide dismutase (SOD). The half-rise times for proton consumption were obtained from pH$_{out}$-indicating absorption changes of cresol red at 575 nm, which were measured as in Fig. 1D.

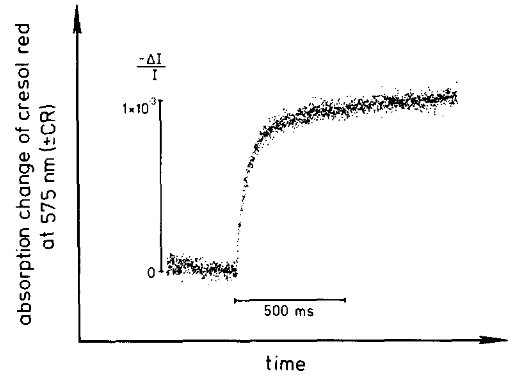

Fig. 3. pH$_{out}$-indicating absorption changes of cresol red at 575 nm in the suspending medium of thylakoids in presence of ferredoxin (40 μg/ml) and NADP$^+$ (200 μM). Standard assay medium, 20 sweeps were averaged; dwell time, 1 ms; electrical bandwidth, 3 kHz.

Discussion

We investigated the rate of proton uptake from the suspending medium of thylakoids under repetitive flash excitation as a function of the terminal electron acceptor. When methyl viologen is reduced, it transfers rapidly electrons to dioxygen to yield O_2^{-} [23]. The pK of O_2^{-} is 4.8 [24]. Although our experiments were performed at pH 7.9, we observed slow proton consumption ($t_{1/2}$ = 390 ms) (Fig. 1B) at the reducing side of Photosystem I, where the oxygen radicals were produced. We attributed this to low catalytic activity of residual superoxide dismutase or of traces of cations as, e.g., Cu^{2+}, Mn^{2+} [25] in the suspending medium.

When superoxide dismutase was added to the assay medium, proton consumption was accelerated in dependence on the enzyme concentration (Fig. 2). The maximal acceleration ($t_{1/2}$ = 10 ms) was observed, when the concentration of superoxide dismutase was in the same range as in the chloroplast (250–500 units/mg chlorophyll) [10,26]. Then the rate of proton consumption at the reducing side of Photosystem I might have been limited by preceeding redox reactions.

We showed that accelerated proton consumption was not restricted to the acceptor system methyl viologen/superoxide dismutase. It occurred also, if NADP$^+$ and ferredoxin were supplemented (Fig. 3). According to Forti et al. [27] a concentration of 10 μM ferredoxin/mg chlorophyll was saturating electron-transfer rates. The present experiments were performed under suboptimal conditions (approx. 4 μM of ferredoxin/mg chlorophyll), because the high buffering capacity of ferredoxin quenched the pH signal drastically. Therefore it was debatable whether the fast phase of proton consumption showed maximal acceleration already at half-rise time of 20 ms.

When both photosystems were active, we observed a slow phase ($t_{1/2} = 100$ ms) of protons uptake which was not variable in dependence on the terminal acceptor. This was attributable to proton uptake at the reducing side of Photosystem II. The mechanism which delayed proton uptake at the reducing side of Photosystem II is subject of other communications [28,29].

The present data show that the rate of proton consumption at the reducing side of Photosystem I was variable with the applied acceptor system. Since the shortest half-rise time was 10 ms, we concluded that slow proton consumption at the reducing side of Photosystem I was not mainly limited by a diffusion barrier for protons.

Acknowledgements

We thank H. Lill and Dr. U. Kunze for computer software and H. Kenneweg for drawings and photographs. Financial aid has been provided by the Deutsche Forschungsgemeinschaft (Ju97/11-7 and Sonderforschungsbereich 171, Projekt A2).

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