Biochimica et Biophysica Acta, 1057 (1991) 331–336

ADONIS 000527289100106U

BBABIO 43371

Inhibition of electron transfer from A_0 to A_1 in Photosystem I after treatment in darkness at low redox potential

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(Received 4 October 1990)

Key words: P-700; Charge recombination; Flash absorption spectroscopy; Phylloquinone; Potentiometry; EPR

Electron transfer reactions in Photosystem I (PS I) were investigated by flash-absorption spectroscopy under highly reducing conditions in the cyanobacterium *Synechocystis* PCC 6803. Under strong illumination, the inhibition of electron transfer from A_0^- to A_1 has been previously put forward and was attributed to the double reduction of phylloquinone (Sétif, P. and Bottin, H. (1989) Biochemistry 28, 2689–2697). The same inhibition, characterized by the appearance of fast recombination kinetics between P-700$^+$ and A_0^- ($t_{1/2} = 40$ ns) is found, in the present work, to be induced in the dark, when Photosystem I is incubated at a very low redox potential (below −550 mV) in the presence of low-potential redox mediators. The kinetics of appearance of this inhibition process are studied at various pH values and redox potentials, in the presence of different mediators. The inhibition is found to be slowly induced (from minutes to hours) and is slowly reversible at potential values above −350 mV. These results are interpreted by assuming that phylloquinol (the inhibiting species) can be formed in the dark as well as under illumination. They also indicate that in Photosystem I, the redox potential of the semiphylloquinone/phylloquinol couple is higher than that of the phylloquinone/semiphylloquinone couple.

Introduction

The reaction center of Photosystem I (PS I) in oxygen-evolving organisms includes the primary electron donor P-700 and five electron acceptors. The iron-sulfur centers (Fe-S_x, Fe-S_A and Fe-S_B) are thought to reduce soluble ferredoxin which is involved in NADP$^+$ reduction, as well as many in metabolic pathways and probably in cyclic electron flow around PS I. The functional organization of the three iron-sulfur centers is still uncertain. The primary electron acceptor, A_0, has been proposed to be a chlorophyll molecule. A secondary acceptor, A_1, is considered as mediating electron transfer between A_0 and the iron-sulfur centers. It has been tentatively identified with phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone; vitamin K-1) which is present in the PS I reaction center [1] with a stoichiometry of 2 molecules per P-700 [2,3]. The role of the second phylloquinone molecule is still unknown. Some recent experimental evidence supports the identification of A_1 with one molecule of phylloquinone [4]. In these experiments, extraction and reconstitution of phylloquinone show that this molecule is involved in the photoreduction of NADP$^+$ as well as in the oxidation of A_0^- during forward electron transfer. Recently, experiments of extraction of phylloquinone followed by readdition of various quinones gave contradictory results. Iwaki and Itoh [5] show that a large number of quinones (benzo-, naphtho- and anthraquinones) can suppress the recombination reaction between P-700$^+$ and A_0^- in ether-extracted PS I preparations. They concluded that the electron transfer to Fe-S centers was restored, and also that the quinone site shows no strict functional specificity, although the binding is stronger if the phytyl side chain is present. On the other hand, Biggins [6] showed that 1-4-naphthoquinones with a long hydrophobic side-chain (C_{10} or longer) are necessary to restore electron transfer to terminal Fe-S acceptors in phylloquinone depleted PS I preparations, whereas anthraquinones act as exogenous electron acceptors and do not transfer electron to iron-sulfur

Abbreviations: BV, benzyl viologen, 1,1′-dibenzyl,4,4′-bipyridinium dichloride; Fe-S, iron-sulfur centers; MV, methyl viologen; 1,1′-dimethyl,4,4′-bipyridinium dichloride; NHE, normal hydrogen electrode; PP-670, 1-methyl-4(2-pyrimidyl)pyridinium bromide; TQ, Triquat; 1,1′-trimethylene-2,2′-bipyridinium dibromide.

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centers. Confirming the chemical identity of A_1, it has been also suggested that A_1 can be doubly reduced by a strong illumination under highly reducing conditions, and that this reduction is slowly reversible [7]. The present work reports the occurrence of A_1 double reduction in the dark, at low redox potential in the presence of mediators. Strict similarity between the results of the two types of treatment lends further support to the identification of A_1 with phylloquinone, and provides some insight on the electrochemistry of this quinone in situ.

Materials and Methods

Biological samples

The cyanobacterium *Synechocystis* PCC 6803 was grown photoautotrophically on BG-11 medium [8] in 16 litre batch culture. Cells were collected at late logarithmic phase. Pellets were washed once in distilled water and frozen. PS I was prepared essentially as described by Biggins and Mathis [4]. Cells were thawed and resuspended in 0.4 M sucrose, 10 mM KCl, 50 mM Tricine-NaOH (pH 7.6), 10 mM EDTA and 1 mM of the proteinase inhibitors: phenylmethylsulfonyl fluoride, ϵ-amino-*n*-caproic acid and benzamidine. The suspension was passed three times through a French pressure cell at 20000 lb/in^2. The homogenate was centrifuged 10 min at $10000 \times g$ and the resulting supernatant 1 h at $200000 \times g$ to sediment the thylakoid membranes. The last centrifugation step was repeated once after resuspension of the pellet. The thylakoid pellet was resuspended in the buffer and solubilized for 45 min in the presence of 30 mM *n*-octyl β-D-glucopyranoside and 10 mM sodium cholate at a chlorophyll concentration of 1.5 mg/ml. The suspension was centrifuged at $10000 \times g$ for 10 min, and the supernatant at $200000 \times g$ for 1 h. The PS I pellet was resuspended in a small volume of buffer, in which EDTA and proteinase inhibitors were omitted, and frozen.

Potentiometric measurements

Potentiometric measurements were done directly in the optical cuvette, using platinum plate and saturated calomel electrodes. The suspension medium was thoroughly de-aerated and the cuvette was continuously stirred and flushed with pure argon gas. Redox potential was adjusted and kept constant by addition of small volumes of buffered 1 M sodium dithionite or diluted solution of potassium ferricyanide. Redox mediators were methyl viologen (MV; E_0', $= -0.44$ V), benzyl viologen (BV; $E_{m7} = -0.311$ V), triquat (TQ; $E_0' = -0.55$ V) and PP-670 ($E_0' = -0.67$ V, $E_0 = -0.72$ V at pH 7.8) [9,10].

Absorption kinetic measurements

Nanosecond absorption changes at 820 nm were measured as described by Van Best and Mathis [11] as modified by Brettel and Sétif [12]. Excitation laser pulses at 532 nm were of 30 ps duration, and the measuring light provided by a laser diode (820 nm) was detected by a rapid-response silicon photodiode (Lasermetrics 3117). After amplification, the signal was recorded by an IN 7000 transient digitizer equipped with a 1 GHz amplifier (Intertechnique). This digitizer was preferred to the system previous used [7,12], since it was much more efficient in signal averaging. However, its use was found to present some drawbacks due to distortion of the signal. Distortion is apparent in the slow rise time for about 25% of the signal and in a small lengthening of the fast decay ($t_{1/2} = 40$ ns). In a few experiments performed with the older system, it was checked that this slow rise was absent whereas the fast decay exhibited a halftime of about 30–35 ns, as previously reported.

EPR measurements

EPR spectroscopy was carried out either at 4 K or at 10 K with an X-band Bruker ER200D spectrometer equipped with an Oxford ESR 900 helium cryostat. A standard cavity (TE 102 mode) was used. Continuous illumination was provided by an 800 W tungsten-iodine lamp whose beam was filtered to remove infrared light (water cuvette + Calflex filters) and concentrated onto the cavity window by means of a plexiglass light pipe.

Results

In PS I reaction centers incubated in darkness in the presence of an excess dithionite, the absorbance increase measured at 820 nm following a laser flash is due to P-700$^+$ formation and decays slowly in the microsecond to millisecond time range (in the absence of any rapid electron donor to P-700). Depending upon the pH value (between 8 and 11), this treatment results either in the reduction of Fe-S_A and Fe-S_B (pH > 10) or leaves these iron-sulfur acceptors partially in the oxidized state (pH < 10). For pH values higher than 10, the charge separation state which is formed after a flash excitation decays with a $t_{1/2} = 320$ μs in *Synechocystis*. Until recently, it has been accepted that this phase ($t_{1/2} = 250$ μs in spinach) originated from the back reaction between P-700$^+$ and Fe-S_x^- [13]. However, this attribution has been recently questioned by Brettel [14], who proposed that the 250 μs recombination phase observed in *Synechococcus* sp. PS I preparations is due to the back-reaction between P-700$^+$ and A_1^-. At lower pH values, the decay is due mostly to the back reaction between P-700$^+$ and the iron-sulfur centers Fe-S_A^- and Fe-S_B^-. Its half-time is of the order of 100 ms in *Synechocystis* PS I. Similar treatments were performed in total dark-

ness at various pH values (pH 8, 9, 10 or 11), in the presence of sodium dithionite and of different low potential electron mediators. During the course of incubation, samples were periodically submitted to absorption kinetic measurements. At the beginning of the incubation, each sample showed a slow decay phase which cannot be analysed on this time scale (Fig. 1a). This slow reduction of P-700$^+$ can be ascribed to the electron donation by reduced mediators, which, under the present conditions, is faster than the recombination reactions between P-700$^+$ and the secondary electron acceptors. During the course of incubation, the kinetics of decay of P-700$^+$ were largely modified. Fig. 1b shows the appearance of a fast decay of $t_{1/2} = 40$ ns together with the decrease of the amplitude of the slow component. It also shows that the total amplitude of the signal is increased. Such a decay has already been observed in *Synechocystis* PS I reaction centers under strong illumination and highly reducing conditions, but in the absence of electron mediators [7]. It was interpreted as originating from the recombination reaction between P-700$^+$ and A_0^- due to the inhibition of the electron transfer between A_0^- and A_1. It has also been described in PS I reaction centers in which secondary electron acceptors are lacking or inactivated [4,12,15,16]. The increase in initial amplitude can be attributed to the positive absorbance contribution of the reduced chlorophyll A_0^- at 820 nm. This charge recombination reaction produces some triplet state of the primary donor P-700, which relaxes with a $t_{1/2} = 4.5$ μs [7]. The signal measured at the beginning of incubation can be totally recovered after overnight dialysis of the incubated sample against fresh, non-deaerated buffer.

Fig. 2 represents the amplitude of the 40 ns decay component as a function of the incubation time in darkness for three pH values in the presence of MV,

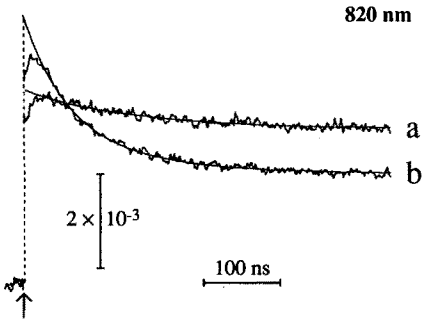

Fig. 1. Kinetics of absorbance changes measured at 820 nm and induced by a saturating laser flash (λ = 532 nm; duration 30 ps; frequency 1 Hz). *Synechocystis* PS I preparation (100 μg Chl/ml) is suspended in 0.2 M glycine/NaOH (pH 10), 10 mM KCl, 0.3% (w/v) sodium deoxycholate, BV, MV, TQ and PP-670 40 μM each and 10 mM sodium dithionite. Kinetics were measured after different times of dark incubation: (a) 40 s and (b) 34 min after addition of dithionite, respectively. The sample is contained in a closed square cuvette of 1 cm optical path. Each trace is the average of 50 experiments.

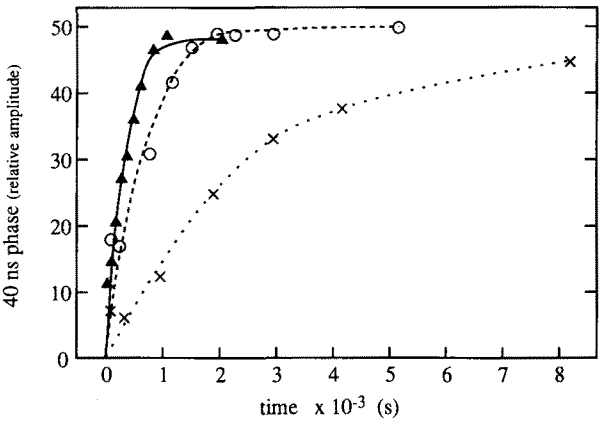

Fig. 2. Amplitude of the 40 ns decay phase as a function of the incubation time in darkness measured at 820 nm. *Synechocystis* PS I preparation (100 μg of chl/ml) is suspended in 0.2 M glycine/NaOH in the presence of 10 mM KCl, 0.3% (w/v) sodium deoxicholate, BV, MV, TQ and PP-670 40 μM each and sodium dithionite 10 mM. ×, pH 9; ○, pH 10; ▲, pH 11. The sample is contained in a closed square cuvette of 1 cm optical path. Other conditions as in Fig. 1.

BV, TQ and PP-670. For the three pH values, the same maximal amplitude of the 40 ns decay phase was reached at the end of incubation. It must be noted that the four electron mediators used here were tested separately and are not equivalently efficient in promoting the appearance of the 40 ns decay phase (MV > TQ > BV > PP-670) (not shown) and that their efficiency is not related to their midpoint potentials (BV > MV > TQ > PP-670). Therefore, efficiency seems to be governed, besides redox potential, by other parameters which could be the hydrophilicity of the mediators, their chemical stability at high pH values or their accessibility to the phylloquinone site. Incubation in the dark in the presence of dithionite and MV was also performed at pH 8 (not shown) and resulted in a very slow appearance of the 40 ns phase ($t_{1/2}$ of appearance > 10 h), whereas this phenomenon could not be detected at pH 7. However, if samples (excess dithionite ± mediators, 8 < pH < 11) are submitted to a strong illumination during the course of the dark incubation, the amplitude of the 40 ns phase is rapidly increased to its maximum value. Similar experiments of dark incubation were done in the presence of MV alone at three different pH values. Half-times of appearance of the 40 ns phase under different conditions, together with the measured redox potentials which are reached under such conditions are summarized in Table I. Fig. 3 shows the results of incubation experiments performed in the presence of various concentrations of MV alone at pH 10. These experiments show that a MV concentration as low as 1.6 μM (P-700 being 0.6 μM) is still rapidly inducing the appearance of the 40 ns decay, and that the rate is maximum at MV 40 μM. This indicates that the inhibition of the electron transfer from A_0^- to A_1 can be easily induced, even with a MV/P-700 stoichio-

TABLE I

Half-time of appearance of the 40 ns decay as a function of pH and low-potential mediators

Same conditions as in Fig. 2. The concentration of each mediator was 40 μM.

pH	MV alone	MV, BV, TQ and PP-670	
	$t_{1/2}$ (min)	$t_{1/2}$ (min)	E_m (NHE) (mV)
9	> 60	35	−575
10	5–6	12	−635
11	4	6	−655

metric ratio as low as 3. The absence of actinic effect due to the excitation laser flash has been tested in repeating the previous experiments but in doing flash spectroscopic measurements at only a few incubation times. Results were completely identical to those presented above, i.e., the half-time of appearance of the fast decay phase is not influenced by the number of flashes applied to the sample.

In order to discriminate between direct pH effects on the appearance of the 40 ns decay phase and pH effects on the redox poising due to dithionite in excess, incubation experiments were performed at pH 11 and redox potential was progressively lowered. Fig. 4 shows the time-course of a dark incubation at pH 11 (MV + TQ) as done before. In the present case, dithionite was not added in excess, but the E_m was decreased step by step, by successive additions of concentrated dithionite. It indicates that the half-time of appearance of the fast kinetic phase does not depend directly upon the pH value, but upon the E_m of the suspension medium. At $E_m = -455$ mV, the 40 ns phase is almost totally absent. If the E_m is lowered to −555 mV, the fast phase

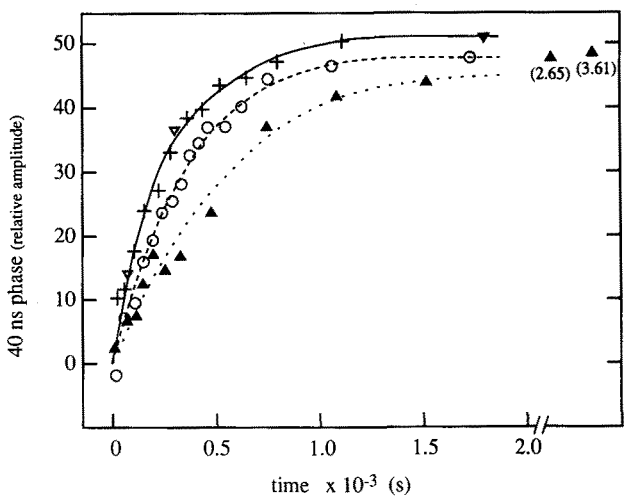

Fig. 3. Time-course of the appearance of the 40 ns decay phase measured at 820 nm in *Synechocystis* PS I preparation. Same conditions as Fig. 2 except buffer is glycine/NaOH 0.2 M (pH 10) and MV is the only mediator. MV concentrations are: ▲, 1.7 μM; ○, 8.3 μM; ▽, 42 μM; +, 100 μM.

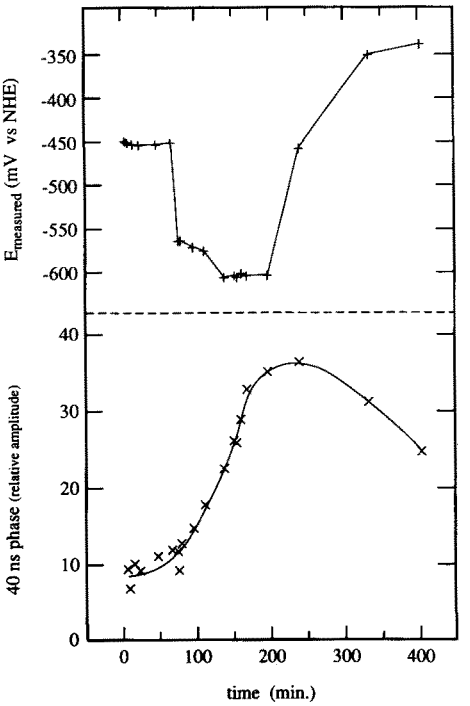

Fig. 4. Amplitude of the 40 ns phase (bottom) and measured redox potential (top) as a function of time and of redox titrant additions. *Synechocystis* PS I preparation (100 μg of chl/ml) is suspended in 0.2 M glycine/NaOH (pH = 11), 10 mM KCl, 0.3% (w/v) sodium deoxycholate, 100 μM MV and TQ each; sodium dithionite was 200 μM at the beginning of the dark incubation and then the redox potential was adjusted by small additions of concentrated dithionite. Potentiometric measurements were done directly in the optical cuvette (optical path: 1 cm) using platinum plate and saturated calomel electrodes. The cuvette was continuously stirred and kept under a small pressure of pure argon gas.

appears to increase, but fairly slowly. It develops rapidly and completely when the potential of the medium reaches the value of −605 mV. If the potential is then increased to −350 mV, a decrease of the amplitude of the 40 ns phase initiates but is slow ($t_{1/2}$ = 200 to 250 min). The same result was observed after an increase of the potential to −80 mV. These results clearly show that the redox potential of the medium must be lower than −500 mV to induce the inhibition of electron tranfer between A_0^- and A_1.

The results also demonstrate that the reversibility of the phenomenon can be observed without extensive dialysis of the sample, but the reoxidation reaction is a slow process. Overnight dialysis against fresh buffer of a sample incubated in the dark, in which the 40 ns phase has totally developed, results in the total restoration of the initial slow signal (similar to Fig. 1a). Such a dialysed sample can be submitted again to a dark incubation in the presence of dithionite and low-potential mediators, and shows again electron transfer inhibition between A_0^- and A_1 as indicated by the appearance of the 40 ns decay phase. The electron transfer restoration observed here is similar to that of samples sub-

mitted to strong illumination in the presence of dithionite and then dialysed [7]. Both treatments induce the same inhibition which can be reversed by the removal of reductant. The reversibility of the electron transfer inhibition has been tested in some other ways. The inhibition was induced by a strong illumination at pH 8 in the presence of dithionite in excess. The decay kinetics of P-700$^+$ were then measured and show a $t_{1/2} = 40$ ns phase of maximum amplitude. The sample was then kept in the dark, and kinetics were periodically measured. No disappearance of the 40 ns phase was observed, even after 25 h. Addition of MV either following or during the strong illumination did not change the result. Thus the potential imposed by dithionite in excess at pH 8 ($E_m = -500$ mV), although not low enough to impede the electron transfer after A_0, is likely too reducing to allow the restoration of the electron transfer from A_0^- to A_1.

A control experiment was made by EPR spectroscopy. *Synechocystis* PS I preparations (600 μg of chl/ml) were suspended in 0.2 M glycine/NaOH (pH 11), 0.3% sodium deoxycholate, 300 μM of each low potential mediator (MV, BV, TQ and PP-670) and 10 mM sodium dithionite. Samples were frozen either immediately or after a period of dark incubation at room temperature, by rapid immersion in a solid CO_2/ethanol bath and then transferred into liquid nitrogen. Characterization at 10 K of samples frozen immediately or incubated for 2 h show that in both conditions iron-sulfur centers Fe-S_A and Fe-S_B were almost totally in the reduced state (at least 90%), whereas iron-sulfur center Fe-S_x was almost totally in the oxidized form. EPR spectra recorded at 4 K under illumination show the presence of a characteristic P-700 triplet spectrum [17,18] in the sample submitted to a long dark incubation, whereas this feature is totally absent in the sample immediately frozen. The amplitude of the ^{3}P-700 low-field z peak is about 82% of the maximum amplitude, which can be observed in a sample frozen under illumination in the presence of dithionite in excess at pH 11 but in the absence of any redox mediators. It is known that, at low temperature, the recombination reaction between P-700$^+$ and A_0^- induces the formation of the P-700 triplet state. Therefore, the EPR results are consistent with the room-temperature absorption experiments and can be explained as well by the inhibition of electron transfer from A_0^- to A_1, tentatively attributed to the formation of A_1^{2-}.

Discussion

Identical kinetic behavior of P-700$^+$ re-reduction in PS I reaction centers can be observed either after a strong illumination in the presence of dithionite, or following a long dark incubation in the presence of dithionite and low potential mediators. The effect of strong illumination under reducing conditions has already been tentatively attributed to the formation of A_1^{2-}, which inhibits electron transfer further than A_0^-. More surprising is the fact that MV, at a potential lower than -550 mV, can promote in the dark an electron transfer inhibition between A_0 and A_1. Kinetics of P-700$^+$ back-reaction after either a strong illumination of the sample or its incubation in the dark with reduced mediators are identical. This analogy between the results of the two treatments in terms of electron transfer favors the proposal of the formation of the phylloquinol in the dark. In purple bacteria as well as in PS II reaction center, the disappearance of the $Q_A^-Fe^{2+}$ EPR signal under highly reducing conditions was interpreted by the double reduction of the quinone Q_A [19,20]. The latter authors proposed that the double reduction of Q_A in PS II can occur in the dark at pH 7, in the presence of dithionite, and is accelerated by the addition of benzyl viologen. The second reduction step (quinol formation) happened to be very slow and no true redox equilibrium could be observed. However, in PS II the semiquinone species (Q_A/Q_A^-; $E_m = -100$ mV) is stable and is transformed into quinol at a lower potential (< -350 mV). The reduction of phylloquinone in PS I is shown to be due to the mediators (MV alone or MV + BV + TQ + PP-670), since dithionite alone does not induce the appearance of the fast P-700$^+$ decay phase unless a strong illumination is provided to the sample. In PS I, the formation of the inhibiting species (ascribed to quinol formation) occurs at a very low potential ($\simeq -600$ mV), whereas it disappears for potential values as high as -350 mV. As the redox potential of A_1/A_1^- is thought to lie around or below that of Fe-S_x/Fe-S^-_x ($E_m = -705$ mV) [21], our results indicate that the quinone/semiquinone transition occurs at a potential much lower than the formation of the quinol, therefore indicating that the semiquinone is unstable. Possibly due to severe reaction rate limitations, no titration corresponding to reversible reactions was possible. Furthermore, in our interpretation, reoxidation of the quinol takes place at a high potential (about -350 mV) as compared to its formation from the quinone. This reoxidation reaction appears to be also a very slow process, impeding any titration to be done. However, one can safely propose that the value of the potential of the two electrons transition (phylloquinone/phylloquinol) takes place between -550 mV and -350 mV.

Our results provide further explanation (i.e., double reduction of A_1) for the work of Inoue et al. [22] in which they describe a similar electron tranfer inhibition in isolated spinach chloroplasts, named 'PS I photoinhibition'. They show that a strong illumination of PS I at pH 8 or pH 10, under anaerobic conditions and in the presence of dithionite results in a large inhibition of MV or NADP$^+$ reduction by PS I. However, since PS I

photoinhibition is observed under drastic experimental conditions, it is difficult to imagine that this phenomenon is physiologically relevant.

The electrochemical behavior of phylloquinone in PS I reaction centers appears to be similar to the general redox behavior of quinones in protic solvents and in cytochrome b_6/f and b/c_1 complexes, where the quinol species is likely less reducing than the semiquinone species (Ref. 23 and references therein).

The present results also show that great care must be taken when performing redox titrations of functional properties in PS I at very low potential, since the use of viologens appears to block the electron transfer in this reaction center.

Acknowledgements

We would like to thank Dr. Elstner for his kind gift of PP-670.

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