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The influence of membrane localized protons on energy utilization at the reaction centres of Photosystem II in isolated thylakoids

Alexander M. Ehrenheim, Giorgio Forti and Giovanni Finazzi

Centro di Studio CNR sulla Biologia Cellulare e Molecolare delle Piante. Dipartimento di Biologia, Universitá di Milano, Milano (Italy)

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We have investigated the steady-state photochemical efficiency of PS II under modulated actinic illumination, and the effect of $\Delta\Psi$ and ΔpH on it. Nigericin, valinomycin, nonactin and NH_4Cl were added to suppress $\Delta\Psi$ and/or ΔpH. A correlation between an increase of PS II photochemical efficiency and the suppression of 9-aminoacridine fluorescence quenching, in the presence of the lipophilic ionophores, was found. No effect of NH_4 Cl on PS II activity was observed. The enhancement of PS II photochemical efficiency by the ionophores had its maximum at low light intensity and disappeared when it approached saturation. We conclude that electron transport dependent localization of protons in intramembrane domains lowers the efficiency of light energy utilization at the PS II reaction centre.

Introduction

The primary photochemical events of photosynthesis create a charge separation across the thylakoids membranes, and therefore an electric potential difference across the membrane, negative outside (see review, Ref. 1). Both photochemical reactions contribute equally to the electric field generation [1], which is complete in less than 1 ns [2], while an additional slower field generation is believed to be due to electron transport through the plastoquinone–cytochrome *b*–cytochrome *f* system [3,4]. The oxidation of water and reoxidation of plastoquinone are known to accumulate protons in the thylakoid lumen, so generating a ΔpH across the membrane [1]. However, several authors have reported evidence that the protolytic reactions of electron transport may produce protons localized in specific domains within the membrane, and that their equilibration with the proton pool in the thylakoid lumen is regulated by the ionic and osmotic composition of the medium [5,6].

The role of the electric field and of ΔpH across the membrane (the proton-motive force) as the energy source for ATP synthesis and their effects on electron transport have been extensively studied (see review Ref. 7). However, little is known about the effect of $\Delta\Psi$, ΔpH or both on light harvesting and energy transfer to the reaction centres. Meiburg et al. [8] have shown that an externally applied electric field inhibited the photochemical reduction of Q_a in osmotically swollen chloroplasts ('blebs'), and induced charge recombination between Q_a^- and the positive charge on the inner side of the membrane [8]. More recently, Braun and Malkin [9] reported that uncouplers as well as valinomycin induced imbalance of energy distribution between the two photosystems in favor of PS II [9].

We report here investigations on the role of proton gradients and of $\Delta\Psi$ in regulating the efficiency of energy utilization at the reaction centres of PS II, as measured by electron transport from water to NADP under conditions where PS I activity was saturated with far red light and any kinetic limitation by secondary electron transport was excluded by the low frequency (6 Hz) of the flash excitation [10]. We report that the suppression of $\Delta\Psi$ had no influence on PS-II-limited electron transport, whilst stimulation was observed by the lipophylic ionophore nigericin, which is known to

Correspondence: G. Forti, Centro di Studio CNR sulla Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Universitá di Milano, Via Celoria 26,Milano 20133, Italy.

Abbreviations: PS I, PS II. Photosystems I and II, respectively; DCMU, 3-(3,4 dichlorophenyl)-1,1 dimethylurea; Q_a, the quinone primary acceptor of PS II; 9-aa, 9-aminoacridine; $\Delta\Psi$, electric potential across the thylakoid membrane.

suppress ΔpH through the electroneutral exchange of H^+ against K^+. Valinomycin and nonactin stimulated at rather high concentrations (in the micromolar range), where they affected also ΔpH. The uncoupler NH_2Cl, which stimulated as usually observed electron transport under high-intensity continuous light and abolished ΔpH, had no influence under low-intensity, low-frequency flash illumination.

It appears, therefore, that membrane-localized protons accessible to the lipophylic ionophores but not to NH_3 are important in regulating light energy utilization by PS II.

Materials and Methods

Stroma-free thylakoids were isolated as previously described [11] from spinach leaves grown in a growth chamber. The leaves were kept 2 h in darkness at room temperature, to allow dephosphorylation of LHC-II by the endogenous phosphatase [12]. The medium for extraction and storage of the thylakoids always contained $MgCl_2$ 5 mM and NaCl 10 mM in order to preserve membrane stacking and the grana structure. The reaction medium consisted of 0.1 M sucrose, 30 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 10 mM NaCl, 10 mM NaF and 20 mM KCl. NADP was 0.5 mM and ferredoxin 3 μM, unless otherwise stated. NADP reduction was measured as previously described [10] at room temperature (22-23 °C). Actinic light was produced by a xenon flashlamp (EG and G) providing single turnover flashes (peak at 3 μs, more than 90% of the energy delivered in 12 μs, see Ref. 10), and the frequency was as indicated. Not less than 500 flashes were fired, so that we measured steady-state electron transport. The flashes were filtered through a heat filter and attenuated by neutral filters. A 722 nm continuous beam (10 nm half-bandwidth) was provided from the opposite side of the cuvette. The experiments were terminated within 3 h after the extraction of thylakoids. Chlorophyll was estimated according to Arnon [13]. $\Delta\Psi$ was measured as the absorbance increase at 518 nm, due to actinic illumination [1]. The actinic illumination was performed under the same conditions described above for NADP reduction (722 nm continuous beam and red light flash illumination at 6 Hz). The signal was measured with a Sigma ZWS spectrophotometer and acquired by a transient recorder software at the rate of 1 kHz. 300 acquisitions were averaged and stored in a personal computer. Acquisition began after 100 single turnover flashes which established the steady state. The instrument limitation of 1 kHz did not allow the kinetic analysis of the fast component of the signal [4], but was adequate for our purposes.

The electron-transport-dependent quenching of 9-aminoacridine fluorescence was measured as an indication of ΔpH [14], through the reliability of this method for a quantitative estimation of ΔpH has been challenged [15]. The fluorescence of 9-aa has been excited through a broad filter transmitting from 320 to 395 nm, and measured at 460–480 nm.

Results

The influence of valinomycin (at the rather high concentration of 1.8 μM) on the reduction of NADP with water as the electron donor was measured at pH 8 under low-intensity white light (410 to 700 nm) modulated at 6 flashes/s, supplemented with a continuous beam at 722 nm of intensity high enough to saturate PS I activity [10]. Under these conditions, light capture by PS II was strictly limiting electron transport [10], and the addition of valinomycin stimulated about 22% electron transport, whilst the addition of ethanol (the solvent for valinomycin) had no effect (fig. 1). The effect of valinomycin as a function of the intensity of modulated (6 flashes/s) light is shown in Fig. 2. Stimulation was observed to increase when the energy flux increased from 1 to about 5 W m^{-2}, then decreased progressively at higher light intensities, to disappear completely under saturating light. A similar effect was observed upon addition of nonactin, an ionophore endowed with the same K^+ specificity as valinomycin (Table I). Nigericin was active at much lower concentrations, whilst the uncoupler NH_4Cl had no effect (Table I). This was observed in a large number of experiments, using thylakoid preparations from many spinach cultures over a period of 1 year (see Table I). The stimulation observed ranged from 20% to 40%.

The stimulation of PS-II-limited NADP reduction as a function of the concentration of the ionophores was

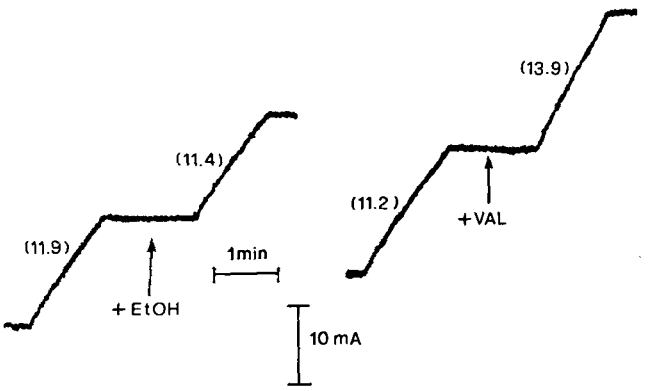

Fig. 1. Effect of valinomycin on NADP photoreduction under modulated actinic illumination. Conditions: see Materials and Methods. The intensity of modulated (6 Hz) light of 410 to 700 nm was 1.14 W m^{-2}. The background light (722 nm) was 17.5 W m^{-2}. Chl concentration was 15.5 μg/ml. Valinomycin was added at the final concentration of 1.8 μM, dissolved in 1 μl ethanol. The same volume of ethanol was added in the control. The figures on the graph represent absorbance increase, in mA/min.

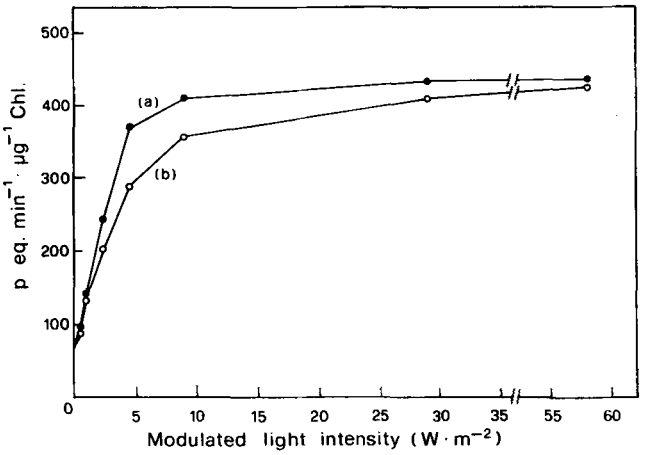

Fig. 2. Effect of the intensity of modulated light on the stimulation of NADP reduction by valinomycin. Conditions: see Materials and Methods. Chlorophyll concentration was 40 μg/ml. Actinic illumination was modulated at 6 flashes/s, filtered through a heat filter and a 630 nm long-pass filter. The continuous beam of 722 nm was of 17.5 W m^{-2}. This intensity was high enough to saturate PS I activity (see Ref. 10).

correlated with their inhibition of 9-aminoacridine fluorescence quenching (Table II, A). When light was modulated at 6 flashes/s the uncouplers had no effect at saturating intensity, proving that any kinetic limitation by the inter-system electron transport was abolished at this frequency of excitation of the primary photochemical reactions (Table II, B).

However, at high intensity of continuous light both uncouplers nigericin and NH_4Cl produced the usually observed large stimulation of electron transport (Table III), which is known to be regulated under these conditions by the rate-limiting reoxidation of PQH_2. It can be seen (Table II) that valinomycin did inhibit the 9-aa fluorescence quenching at concentrations above 200 nM, where it stimulated electron transport. The same correlation was found between electron transport stimulation and decrease of 9-aa fluorescence quenching in the case of nonactin (Table II), an ionophore of similar K^+ specificity. The stimulation by nigericin was observed at lower concentrations (in the nanomolar range), and was similarly correlated with the suppression of the 9-aa fluorescence quenching. NH_4Cl had no effect on PS-II-limited electron transport, although it suppressed 9-aa fluorescence quenching (Table II).

As reported in the case of valinomycin (see Fig. 2), the stimulation of PS-II-dependent electron transport was observed only at low intensity flash illumination and disappeared at high intensity in the case of the other lipophylic ionophores as well (not shown).

In order to investigate the role of $\Delta\Psi$ in the observed stimulation of NADP reduction by the lipophilic ionophores, we have measured their effect on the $\Delta\Psi$ – indicating 518 nm absorbance change, under the same conditions of actinic illumination. We have observed that valinomycin and nonactin completely inhibited $\Delta\Psi$ at the concentration of 100 nM (not shown), where no effect of these ionophores was found on NADP reduction (see Table II). NH_4Cl had no influence at any concentration, whilst nigericin enhanced

TABLE I

Effect of valinomycin, nigericin, nonactin and NH_4Cl on NADP photoreduction

Conditions: as in Fig. 2. Modulated light intensity was 4.54 W m^{-2}. Standard error is indicated.

	Ionophore or uncoupler added			
	valinomycin	nigericin	nonactin	NH_4Cl
Reaction rate ratio	(1.8 μM)	(100 nM)	(2 μM)	(3 mM)
Ionophore/control	1.24 ± 0.075	1.22 ± 0.08	1.21 ± 0.05	1.00 ± 0.03
Number of experiments	27	10	4	5

TABLE II

Concentration dependence of the ionophore effect on NADP photoreduction and on 9-aa fluorescence quenching

Conditions: as in Fig. 2. Modulated light intensity was 4.54 W m^{-2} for part A of the table. In part B, modulated light intensity was saturating (58 W m^{-2}).

Addition		Reaction rate (pequiv. min^{-1} μg Chl^{-1})	Ionophore/control ratio–ΔFl	9-aa fluorescence quenching, $\Delta Fl/Fl$
Part A				
Control		269	–	0.27
Valinomycin	0.1 μM	268	1	0.27
	0.2 μM	298	1.11	0.23
	0.4 μM	322	1.20	0.14
	1.0 μM	315	1.17	0.15
	1.8 μM	335	1.25	0.14
Nonactin	0.1 μM	270	1.00	0.27
	0.5 μM	278	1.03	0.19
	1.0 μM	293	1.09	0.15
	2.0 μM	307	1.14	0.10
	5.0 μM	336	1.25	0.06
	10.0 μM	336	1.25	0.05
Nigericin	2 nM	282	1.05	0.23
	20 nM	308	1.15	0.00
	50 nM	340	1.26	0.00
	100 nM	336	1.25	0.00
	1000 nM	328	1.22	0.00
NH_4Cl	1 mM	269	1.0	0.00
	3 mM	268	1.0	0.00
	10 mM	237	0.88	0.00
Part B				
Control		431	–	0.40
Nigericin	1.0 μM	432	1	0.00
NH_4Cl	3.0 mM	430	1	0.00

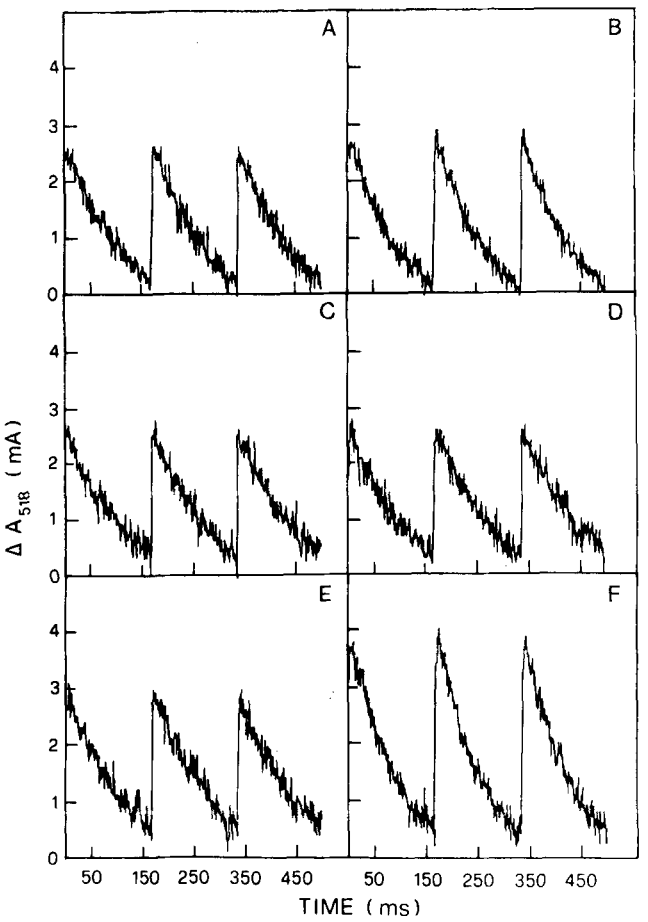

Fig. 3. Effect of nigericin and NH_4Cl on the 518 nm absorption change. Conditions: as in Fig. 2. The intensity of modulated red light was 8.9 W m^{-2}. (A) Control; (B) 3 mM NH_4Cl; (C) control; (D) 2 nM nigericin; (E) control; (F) 100 nM nigericin.

$\Delta\Psi$ in the concentration range from 50 to 1000 nM (Fig. 3), the same concentration range over which it stimulated PS-II-limited electron transport (Table II).

The possibility was investigated that the observed stimulation of PS II by lipophylic ionophores might be attributed to the suppression by them of the 'high-energy fluorescence quenching [16], a process which dissipates excitation energy in the antenna of PS II. We have therefore measured the effect of nigericin on room temperature fluorescence, under the same illumination regime as that adopted to measure NADP reduction, 9-aa fluorescence quenching and $\Delta\Psi$. No effect of nigericin on F_0 was observed under our conditions of low-intensity modulated actinic illumination, whilst a small decrease of steady-state fluorescence (F_{ss}) was observed (Table IV). F_m was measured upon illumination with a high intensity white light flash of 5 s duration: under these conditions, nigericin depressed F_m (Table IV), an expected consequence of the stimulation by the uncoupler of the interchain electron transport. The same effects of nigericin reported above were observed when methylviologen was used instead of NADP as the electron acceptor (not shown).

TABLE III

Stimulation of electron transport by uncouplers under continuous illumination

Conditions: as in Fig. 2. Continuous light intensity was saturating (200 W m^{-2}). The concentration of ferredoxin was 9 μM.

Addition		Reaction rate (μequiv. min^{-1} μg Chl^{-1})	Ionophore/control-ΔFl	9-aa fluorescence quenching, ΔFl/Fl
Control		0.812	-	0.31
Nigericin	100 nM	3.888	4.46	0.00
	1000 nM	4.222	5.2	0.00
NH_4Cl	1 mM	3.803	4.68	0.00
	3 mM	4.176	5.14	0.00

TABLE IV

Lack of effect of nigericin on fluorescence upon low-frequency actinic illumination

Actinic illumination and other conditions as in Fig. 2. Electron transport (water to NADP) was measured as O_2 evolution in a Clark electrode (Hansatech); fluorescence was measured simultaneously using a PAM fluorimeter (Walz, Effeltrich, F.R.G.) applied to the O_2 electrode cell. The low intensity excitation with 1 μs LED flashes (at 650 nm) was modulated at 1.6 kHz; the modulated emission was measured at $\lambda > 700$ nm. F_0 minimal fluorescence; F_{ss}: steady-state fluorescence; F_m: maximal fluorescence, attained with a saturating white light 5 s flash.

Addition	Electron transport (nmol O_2/min)	Fluorescence (arbitrary units)		
		F_0	F_{ss}	F_m
None	2.04	19	26	76
Nigericin	2.80 (+37%)	19	23.8	66

Discussion

Our observations demonstrated that the addition of the lipophylic ionophores nigericin, valinomycin and nonactin led to enhanced utilization of excitation energy at the reaction centres of PS II, under actinic illumination of low frequency (6 Hz). This effect was correlated with the ability of the ionophores to inhibit the 9-aminoacridine fluorescence quenching (Table II). No correlation was observed with the inhibition of the electric field across the membrane, monitored by the 518 nm absorption change. Rather, the 518 nm change was increased in the presence of nigericin, indicating an enhanced charge separation, which we interpret as an additional evidence for the stimulation of photochemical activity of PS II (Fig. 3). This is in agreement with the observation by Meiburg et al., who have shown that an externally applied electric field inhibited the photochemical reduction of Q_a and increased charge recombination in swollen chloroplast [8]. The

dependence of the effects of the ionophores on light intensity (see Fig. 2) indicates that their action enhanced energy utilization at the PS II reaction centres: indeed, the effect disappeared at high intensity of modulated light, when PS I was saturated with a 722 nm continuous beam. The correlation between the ionophore-dependent electron transport stimulation and the inhibition of the 9-aminoacridine fluorescence quenching indicated that the proton gradient is likely to be involved in this effect, and it was remarkable that NH_4Cl had no effect at any concentration (though its usual large stimulation under continuous high intensity light was observed). This suggests that the lipophilic character of the ionophore as well as the localization of protons in domains within the membrane were required to observe PS II activity enhancement.

Our results differ from those reported by Braun and Malkin [9], who found that uncouplers enhanced the imbalance of energy distribution in favour of PS II at low as well as high light intensity. In their experiments, gramicidin was the most effective uncoupler, but NH_4Cl was also active (at the concentration of 10 mM) as well as other uncouplers and valinomicyn 2 μM. However, under their conditions, pH 7.3 and high frequency (100 Hz) of actinic illumination, the two mechanisms of the uncoupler action, namely the effect on interchain electron transport and that on energy utilization at the reaction centres which we report here, could not be resolved. In our experiments the low-frequency actinic light condition was such that any kinetic limitation by secondary electron transport had been abolished as demonstrated by the observation that neither nigericin nor NH_4Cl stimulated electron transport at saturating energy flux (see Table II).

We can therefore conclude that, due to localized proton domains which are generated during electron transport, the efficiency of energy utilization at the PS II reaction centre is lowered. The elimination of those localized protons by the lipophilic ionophores restored more efficient energy utilization at the PS II reaction centres. Work is in progress to investigate whether this effect is related mainly to the photochemical primary process or to the energy transfer efficiency from the antenna to the reaction centre.

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