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Mechanism of action of anions on the electron transport chain in thylakoid membranes of higher plants

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Abstract With an aim to improve our understanding of the mechanisms behind specific anion effects in biological membranes, we have studied the effects of sodium salts of anions of varying valency in thylakoid membranes. Rates of electron transport of PS II and PS I, 77K fluorescence emission and excitation spectra, cyclic electron flow around PS I and circular dichroism (CD) spectra were measured in thylakoid membranes in order to elucidate a general mechanism of action of inorganic anions on photosynthetic electron transport chain. Re-distribution of absorbed excitation energy has been observed as a signature effect of inorganic anions. In the presence of anions, such as nitrite, sulphate and phosphate, distribution of absorbed excitation energy was found to be more in favor of Photosystem I (PS I). The amount of energy distributed towards PS I depended on the valency of the anion. In this paper, we propose for the first time that energy re-distribution and its valence dependence may not be the effect of anions *per se.* The entry of negative charge (anion) is accompanied by influx of positive charge (protons) to maintain a balance of charge across the thylakoid membranes. As reflected by the CD spectra, the observed energy re-distribution could be a result of structural rearrangements of the protein complexes of PS II caused by changes in the ionic environment of the thylakoid lumen.

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Abbreviations

Chl *a*	chlorophyll *a*
CD	circular dichroism
DCMU	3-(3, 4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenol indophenol
FITC	fluorescein isothiocyanate
F_M	maximum transient fluorescence of chlorophyll *a* for dark adapted samples
F_{MAX}	maximum fluorescence of chlorophyll *a* for dark adapted samples in the presence of DCMU
F_O	minimum fluorescence of chlorophyll *a* for dark adapted samples
F_V	variable fluorescence of chlorophyll *a*
Hepes	N-2-hydroxyethyl-piperazine-N, 2-ethanesulphonic acid
LHCII	light harvesting complex of photosystem II
MV	methyl viologen
OEC	oxygen evolving complex
PPBQ	phenyl-p-benzoquinone
PQ	plastoquinone
PS I	photosystem I
PS II	photosystem II
RC	reaction center
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine

Introduction

The energy conversion and storage capabilities of chloroplast thylakoid membranes may be traced directly to the ability of chlorophyll, ligated to thylakoid protein compo-

nents of the two photosystems to trap radiant energy and to convert it into chemical energy. The light energy absorbed by the light harvesting complexes, LHC II and LHC I, associated with the two photosystems, PS II and PS I, respectively is transferred, in the form of excitation energy, to the 'core' of the reaction center supercomplexes and ultimately to the photochemical reaction centers, where the primary charge separations take place. The photosynthetic pigment protein complexes embedded in the thylakoid membrane are structurally complex, highly organized and dynamically regulated structures (Tikkanen et al. 2008).

Photosynthesis depends on a set of complex protein molecules that are located in and around a highly organized membrane system. The ionic environment of the thylakoid membrane influences and regulates many functions, such as the electron transport, energy transfer, photophosphorylation, photosynthetic carbon reduction etc. According to the Hofmeister effect, a series of salts have consistent effects on the solubility and stability of proteins; anions are known to have a larger effect than cations and the effects are proposed to be related to the interaction of proteins interactions with interfacial solute and water molecules (Dér 2008). Thylakoid stacking and unstacking depends on ionic conditions of suspension (Izawa and Good 1966; Murakami and Packer 1971; Gross and Prasher 1974; Chow et al. 2005). The correlation between the thylakoid stacking and cation induced decrease in photosystem I (PS I) electron transport has been suggested by Barber and Chow (1979). The effects of ions on the electron transport chain and the transfer of the absorbed excitation energy between the two interacting photosystems has been of keen interest (Jajoo et al. 2001). Most of the studies of ions on these processes were directed on evaluating the effects of specific cations because of their ionic charge (mono, di, or polyvalent nature) and size. Mono and divalent cations affect several primary processes in thylakoids: initial energy distribution, or 'spillover' from PS II to PS I and rate constant of thermal dissipative transitions (Wong and Govindjee 1979). Some effects may be co-current, with the largest effect being on the excitation energy redistribution process. Since most of these experiments were performed in thylakoids suspended in low salt medium, the role of anions went unrecognized for quite some time. Anions have a profound effect on both the structures and function of PS II. Anions can remove proteins (e.g., under chloride depletion) (Kuwabara and Murata 1982), influence surface charges (Richter and Homann 1984) and have the protective role during heat stress as in case of chloride (Tiwari et al. 2007). Apart from these, there exist specific anion binding sites in PS II. Anions like chloride and formate, have been known to affect both the acceptor and donor sides of PS II (Jajoo et al. 2005). The surface charge forces redistribution of ions in the liquid phase in contact with the membranes (Jajoo et al. 1994).

It has been reported that structural perturbation of the thylakoid membrane by the anions may be a physical cause for the observed inhibition of the photo induced electron transport (Papageorgiou 1989). Addition of anions may restore balance of positive and negative charges resulting in an increased rate of electron transport through PS I (Jajoo and Bharti 1995). Since many anions stimulate PS I mediated electron transport, the stimulation may be explained by the increased negative surface charge potential. Barber and Chow (1979) proposed that electrostatic forces can control the integrity of chloroplast membrane structure. Anions, in theory, might alter electrostatic forces between the membrane components by acting as counter ions regions of the chloroplast membrane bearing a fixed net positive charge. Anions may also increase the density of negative charges causing a force of repulsion acting between Chl-protein complexes and causing them to redistribute within the plane of the chloroplast membrane which may divert more energy in favor of PS I (Sinclair 1987).

Our recent work (Singh-Rawal et al. 2010) has demonstrated the role of luminal protons in regulating the migration of LHC II between the two photosystems. Earlier, anions were also shown to regulate distribution of absorbed excitation energy in a valency dependent manner (Jajoo et al. 1998), although the mode of action was not reported. This evoked our interest to investigate the mechanism of action of anions in relation to protons in the thylakoid membranes. Our results suggest that energy re-distribution observed with inorganic anions is probably the effect of proton influx to balance the negative charge of the anion uptake. Since biological membranes, including thylakoid membranes, are constituted of a number of proteins, anions may show Hofmeister effects in their action here too.

Materials and methods

Deveined market spinach (*Spinacea oleracea* L.) was used for the isolation of thylakoid membranes using the method of Kuwabara and Murata (1982). These were stored in 30% glycerol in liquid nitrogen until further use. The chlorophyll concentration was estimated by the method of Porra et al. (1989). The stored thylakoid membranes were thawed on ice and washed with shock medium to remove glycerol and to give an osmotic shock. These membranes were incubated for 3 min in the dark and centrifuged at 8200 × g for 15 min. The resultant pellet was resuspended in suspending medium. The constituents of the suspending medium were 0.33 M sucrose, 50 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$ and 1 mM NaCl. The shock medium contained 50 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$ and 1 mM NaCl. Anion treatment was given by addition of the respective anion to the suspension medium and incubating it in dark for 30 min.

Measurement of PS II electron transfer rates

PS II activity was measured in terms of μmoles of oxygen evolved $mgChl^{-1}$ h^{-1} using a Clark type electrode (Oxygraph, Hansatech. King's Lynn, UK). The reaction mixture (1 ml) contained 0.1 mM PPBQ, 0.1 mM potassium ferricyanide, 0.33 M sucrose, 50 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$ and 1 mM NaCl. A Thylakoid suspension equivalent to 20 μgChl/ml was used. Photosynthetically active light of intensity 150 Wm^{-2} (~950 μmol photons m^{-2} s^{-1}) was used.

Measurements of PS I electron transport rates

PS I activity was measured in terms of μmoles of oxygen consumed $mgChl^{-1}$ h^{-1} with the $TMPD_{(red)}$→MV reaction. These measurements were carried out with a Clark type electrode (YSI 5300 model with 5357 probe) with a constant temperature bath (25±1) °C. The reaction mixture contained 100 mM sucrose, 20 mM HEPES-NaOH (pH 7.5), 3 mM ascorbate, 0.1 mM $TMPD_{(red)}$, 5 μM DCMU, 5 mM sodium azide, 0.1 mM methyl viologen, and the thylakoid suspension equivalent to 20 μgChl/ml was constantly stirred using a magnetic stirrer. The light intensity used was 150 Wm^{-2} (~950 μmol photons m^{-2} s^{-1}).

Measurement of chlorophyll *a* fluorescence

Chl *a* fluorescence induction kinetics was measured at 25°C using a Plant Efficiency Analyzer (PEA), Hansatech, King's Lynn, Norfolk, England. Excitation light of peak wavelength at 650 nm from an array of three light-emitting diodes was focused on the surface of the leaf disc to provide a homogeneous illumination light spot of about 4 mm in diameter. Light intensity used was 3,000 μmol photons m^{-2} s^{-1} to generate maximal fluorescence intensity (Fm) for all the treatments. The fast fluorescence transients were recorded and digitized on line with 12-bit resolution from 10 ms to 1s, with a time resolution of 10 ms for the first 200 data points. The fluorescence signal at 40 μs the fourth digitized data point was considered as a reliable value for the initial fluorescence intensity. The fluorescence signal is received by the sensor head during recording and is digitized in the control unit using a fast digital converter.

The low temperature (77K) Chl *a* fluorescence emission and excitation spectra of thylakoid membranes were measured in a Jasco (FP-6300 model, Tokyo, Japan) spectrofluorometer equipped with a liquid nitrogen dewar with continuous flow of nitrogen gas at room temperature from beneath, to avoid ice crystal formation from the water vapor. Sample was put in a quartz capillary which was placed in the dewar. The reaction mixture used was 0.33 M sucrose, 50 mM HEPES–NaOH (pH 7.5), 1 mM NaCl, 1 mM $MgCl_2$, thylakoid suspension 10 μgChl/ml and 30% glycerol. The excitation wavelength was 435 nm with a 2.5 nm slit width of the excitation emission monochromator. Figure 1 and 2 were normalized to area.

Fluorescence excitation spectra at 77K were monitored at 735 nm whilst scanning the excitation light from 635 nm to 715 nm to estimate the changes in the absorption cross section of PS I. The reaction mixture and thylakoid suspension were the same as for the emission spectra. Wavelength calibration of the equipment was done using FITC (Fluorescein isothiocyanate, Isomer I, Sigma Chemicals) and wavelengths of the spectra have been corrected.

Measurement of dark re-reduction of $P700^+$

The redox state of P700 was monitored by measuring the absorbance changes at 820 nm using PAM-101 fluorimeter (Walz-Germany). The samples were illuminated for 30 s by far red light in order to oxidize P700. Re-reduction of $P700^+$ in the dark was then recorded.

Measurement of circular dichroism (CD)

CD spectra were recorded at room temperature on a Jasco J-815 spectropolarimeter. Thylakoid suspension equivalent to 20 μgChl/ml was used. The spectra were measured at room temperature between 400 and 750 nm with an optical pathlength of 1 cm and a bandwidth of 2 nm.

Table 1 Changes in PS II electron transport rates (H_2O→DCPIP reaction), PS I mediated electron transport rate as monitored by the oxygen consumption measurements ($DCPIPH_2$→MV) in saturating (150 Wm^{-2}) light for control, nitrite (20 mM), sulfate (10 mM), phosphate (6.66 mM). The data show averages of 5 values±S.D Normalized values are given in parentheses

Parameter	Control	Nitrite (20 mM)	Sulphate (10 mM)	Phosphate (6.6 mM)
PS II rates	105±2 (100)	70±3 (68)	54±2 (52)	43±2 (41)
PS I rates	124±5 (100)	156±9 (125)	213±4 (171)	272±5 (219)
F_V/F_M	0.53±0.01 (100)	0.40±0.01 (76)	0.37±0.01 (70)	0.31±0.03 (59)
Fv/Fo	1.13±0.02 (100)	0.88±0.03 (78)	0.60±0.04 (53)	0.45±0.01 (40)

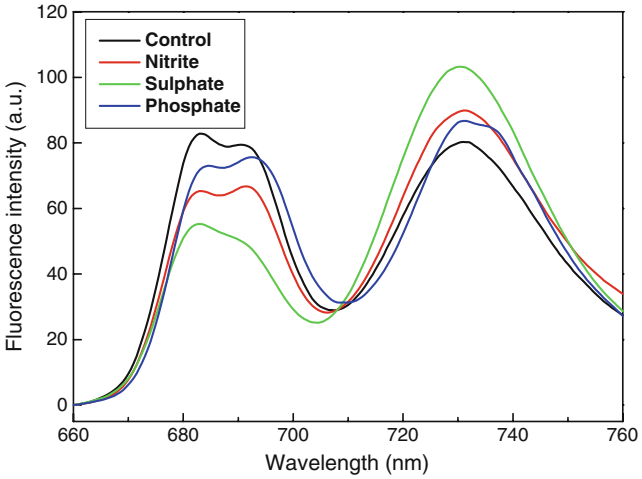

Fig 1 Chl *a* fluorescence emission spectra (measured at 77K) of thylakoid membranes treated with nitrite (monovalent), sulfate (divalent) and phosphate (trivalent). Emission spectra were recorded at excitation wavelength of 435 nm and were normalized at 705 nm

Results and discussion

To study the mechanism of anions, some inorganic anions like nitrite (mono-valent), sulphate (di-valent) and phosphate (tri-valent) were selected. To curb the effects of sodium (Na^+) ion, equimolar Na^+ concentration was chosen. Hence, 20 mM NO_2^-, 10 mM SO_4^{2-} and 6.66 mM PO_4^{-3} were chosen for further study.

Effect of anions on PSII and PS I electron transport rates

The rates of electron transfer through PS II ($H_2O \rightarrow$DCPIP reaction) showed 32%, 48%, 59% decrease with 20 mM NO_2^-, 10 mM SO_4^{2-} and 6.66 mM PO_4^{3-} respectively. There was a simultaneous increase in the rates of PS I catalyzed electron transfer (Table 1), where the anions showed an increase of 25%, 71% and 119% in PS I rates with 20 mM NO_2^-, 10 mM SO_4^{2-} and 6.66 mM PO_4^{3-} respectively. The results are in agreement with earlier reports (Jajoo et al. 1998). These results indicated a valency dependent pattern in the action of anions. The fluorescence induction kinetics at room temperature showed an increase in F_O and decrease in F_M. The resultant F_V/F_M ratio indicated a 24%, 30% and 41% decrease in NO_2^-, SO_4^{2-} and PO_4^{3-} respectively (Table 1). There was a decrease in F_{MAX} in the anion treated samples in the presence of DCMU also (data not shown). This implied a decrease in photochemistry of PS II in the presence of anions. Anions have been reported to cause redistribution of energy towards PS I at the expense of PS II, as indicated by a increase in F_O and decrease in F_M (absence of DCMU) and F_{MAX} (presence of DCMU). The decrease in F_V/F_O ratio also supported this contention.

Fluorescence emission and excitation spectra at 77K

The role of anions in causing energy re-distribution has been confirmed by studying Chl *a* fluorescence emission and excitation spectra at 77K. Three distinct peaks are observed in control thylakoids: A peak at 685 nm has been attributed to Chl *a* in CP43 (PS II), a peak at 695 nm is related to Chl *a* in CP47 (PS II) and peak at 735 nm is attributed to PS I. A change in the F735/F685 ratio is regarded as a sign of energy distribution between PS II and PS I, as F735 originates from PS I while F685 from PS II (Govindjee 1995). As evident from Fig. 1, there is a decrease in F685 and a concomitant increase in F735 as the valency of the anion is increased. The change in the ratio F735/F685 with different anions is shown in Table 2. To clarify whether it is a specific anion effect or the effect due to the negative charge carried by the anion in general, fluorescence emission at 77K spectra were measured for phosphate anion in its three different valency states (Fig. 2). The ratio F735/F685 changes as the valency of phosphate changes, being highest in trivalent phosphate. Thus, we could see that the anion-induced effects depended on the valency of the anion and not on any specific anion *per se*.

These results clearly indicate that anions caused an increase in the rates of PS I in a valency dependent manner. Whether they perform this function by increasing the antenna size of PS I or by increasing the rates of cyclic electron flow around PS I, needs to be investigated. Antenna size of PS I may be estimated by measuring the absorption cross section area of PS I (by fluorescence excitation spectroscopy) or by measuring the rate of $P700^+$ reduction in the presence of DCMU and MV.

Table 2 Measurements of the ratio F735/F685 in the thylakoid membranes treated with various anions in the presence and absence of 5 mM NH_4Cl (uncoupler). In the third column is shown the rate of re-reduction of $P700^+$ in anion treated thylakoid membranes in the presence of DCMU and MV

Treatment	F735/F685 ratio (no uncoupler)	F735/F685 ratio (with 5 mM NH_4Cl)	Rate of re-reduction of $P700^+$ (in sec)
Control	1.03±0.05	0.93±0.03	23.73±0.30
20 mM nitrite	1.15±0.02	1.01±0.02	21.03±0.50
10 mM sulphate	1.29±0.04	1.13±0.03	18.78±0.41
6.66 mM phosphate	1.59±0.07	1.31±0.05	16.04±0.30

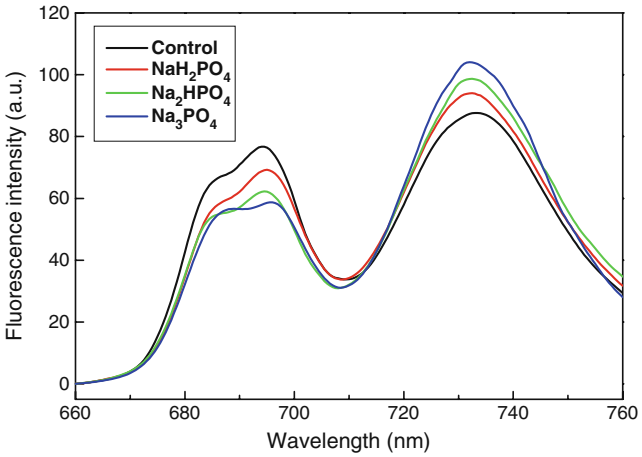

Fig. 2 Chl *a* fluorescence emission spectra (measured at 77K) of thylakoid membranes treated with phosphate in different valence forms. Emission spectra were recorded at excitation wavelength of 435 nm and were normalized at 705 nm

Fluorescence excitation spectra (Fig. 3) were monitored and an increase in the absorption cross section area of PS I in the presence of various anions was measured. An increase in excitation spectra of PS I, a consistent increase in shoulder at 650 nm (attributed to Chl *b*) and 680 nm (attributed to LHC II) showed that anions caused energy re-distribution (Canaani et al. 1984). The fluorescence excitation spectra at 77K, normalized at 705 nm, showed an increase around 683–685 nm and 654–655 nm region indicating clear rise in the association of LHC II with PS I in the anion treated samples. The extent of increase in the absorption cross section of PS I was again found to be dependent on the valency of the anion, being least in nitrite treated membranes and highest in phosphate treated membranes.

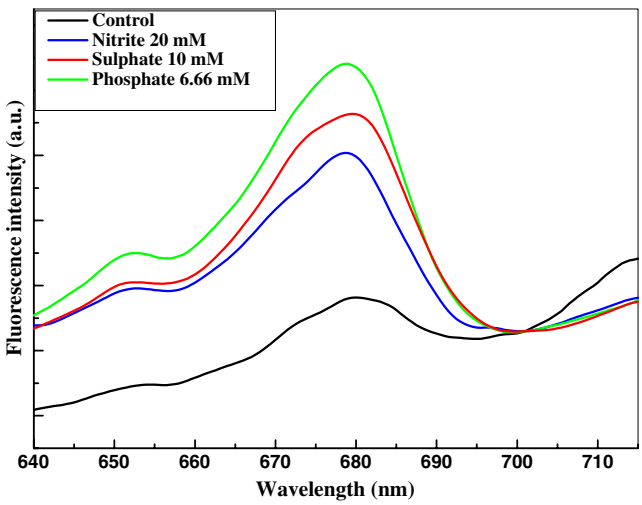

Fig. 3 Low temperature (77K) F735 fluorescence excitation spectra of thylakoid membranes treated with different anions. Spectra were normalized at 705 nm

This was further confirmed by measuring flash-induced absorbance changes of P700 with different anions and its re-reduction after the light is turned off (Table 2). The rate of reduction was found to be fastest in the presence of phosphate treated samples. The half life of the $P700^+$ signal was found to be 21.03±0.09 s for nitrite, 18.78±0.07 s for sulphate and 16.04±0.03 s in case of phosphate. This suggests a relative faster rate of supply of electrons to $P700^+$ (leading to its faster decay) in phosphate-treated samples as compared to sulphate and nitrite treated samples. Since in these experiments, flow of electron from PS II was inhibited by using DCMU, the supply of more electrons to PS I is possible only if its antenna size increases, leading to trapping of more energy. These results confirm that antenna size of PS I has increased in the presence of anions in a valency dependent manner.

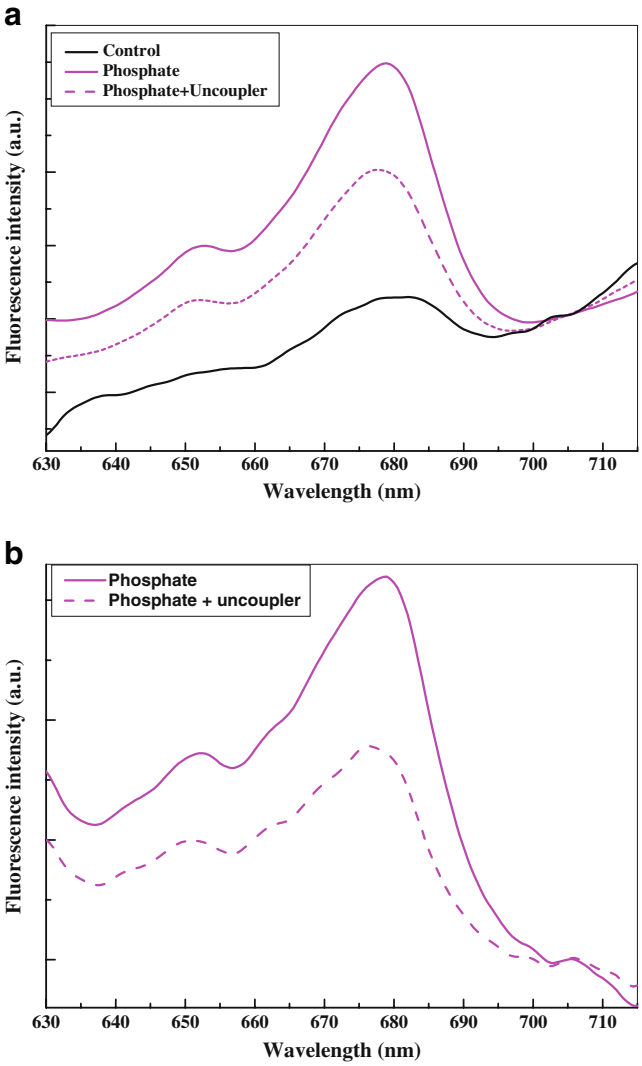

Fig. 4 Chlorophyll *a* spectra (at 77K) of samples treated with inorganic phosphate in the presence of uncoupler 5 mM NH4Cl. **a** Excitation spectra **b** Absorption cross section area

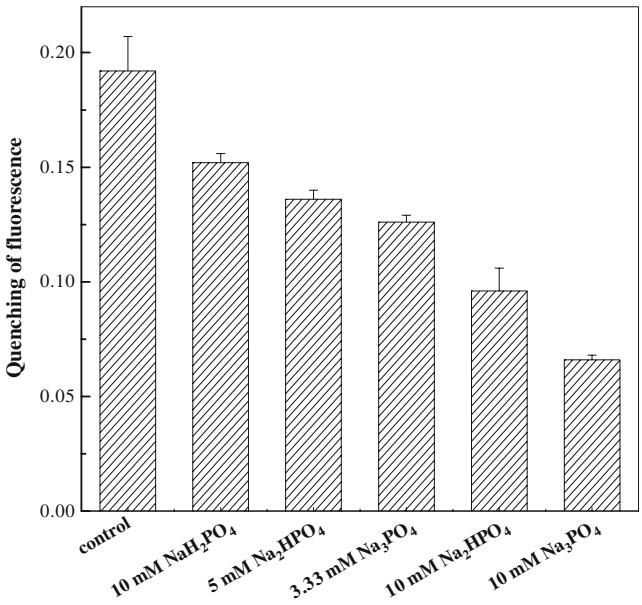

Fig. 5 Changes in quenching of fluorescence 9-aminoacridine in the presence of phosphate at different valency states

So far it is demonstrated that anions are causing a change in energy redistribution between the two photosystems. The extent of energy redistribution was more in favour of PS I and depended on the charge carried by the anion. The ability to cause energy redistribution was in the order PO_4>SO_4>NO_2. However, how change in the charge carried by an anion can regulate energy redistribution is still obscure. Our work on the effects of pH on thylakoid membrane organization (Singh-Rawal et al. 2010) led us to assume that influx of anions in the thylakoid lumen is accompanied by efflux of protons (H^+) in order to counter balance the charge on the membrane. If anions action is related to proton influx, then uncouplers, such as NH_4Cl, should influence the action of anions.

In order to test this hypothesis, experiments were done with anions in the presence of ammonium chloride, which led to a decline in the F735/F685 ratio (Table 2). This result indicated that the action of anions was not independent but related to the H^+ concentration in the lumen. The excitation spectra of PS I (Fig. 4a) and measurement of its absorption cross section (Fig. 4b) at 77K showed a decrease in phosphate treated thylakoid membranes treated with uncoupler. It was intriguing to note that this decrease was also valency dependent. Nitrite and sulphate showed similar effects but to a lesser extent (data not shown). The dependence of rate constant for proton uptake on ionic strength was explained by suggesting that the rate limiting step is the reaction of a proton with a univalent anion in an aqueous medium. The following mechanism was proposed by Hopes and Mathew (1983):

$$PQ_B{}^{2-} + 2HA \rightarrow PQ_BH_2 + 2A^-$$

$$2A^- + 2H^+(aq) \rightarrow 2HA(aq)$$

where $Q_B{}^{2-}$: proton acceptor, A: anion

We propose that energy re-distribution observed with anions may not be the effect of anions *per se* but the influx of protons to counteract the anion influx into the thylakoid lumen. An indication of the change of the luminal pH can be obtained through the measurement of the quenching in the fluorescence of 9-aminoacridine. At the steady state, the transmembrane concentration gradient of amine cation will be equivalent to the protons. Thus, in principle, ΔpH can

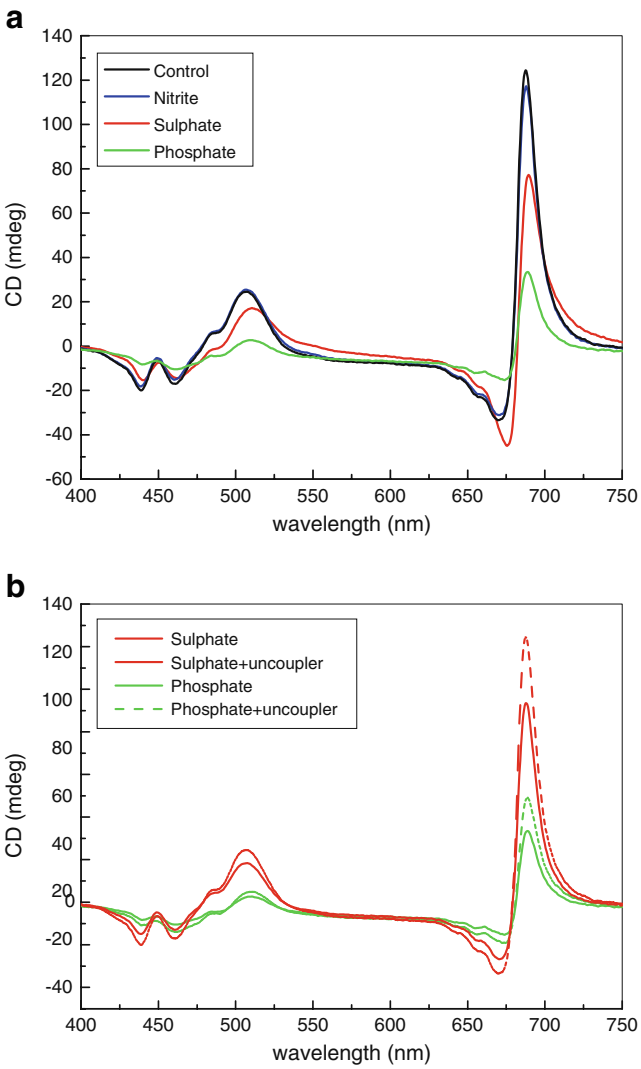

Fig. 6 **a** CD spectra in thylakoid membranes treated with equimolar concentrations of nitrite, sulphate and phosphate. **b** CD spectra of phosphate and sulphate treated thylakoid membranes in the presence of 5 mM NH_4Cl

be estimated if the extent of amine uptake and the thylakoid internal volume is known. The uptake of 9-aminoacridine into thylakoids is associated with the loss of fluorescence of amine taken up. A calibration of 9-aminoacridine at different pH was prepared and it was observed that the higher the fluorescence, the lesser the amine (here, 9-aminoacridine) uptake. This implies that higher fluorescence reflects higher pH. Furthermore, this fluorescence quenching was studied in the presence of the same anion in three different valency states. It was observed that when 10 mM mono, di and trivalent phosphate were used, fluorescence quenching was highest in trivalent phosphate (Fig. 5). But at the same time, when equimolar anionic (here phosphate) concentration was used the quenching did not show significant change. More quenching of fluorescence represents lower pH of the lumen. In other words, in trivalent anion, more proton influx took place in the lumen making it more acidic and hence more quenching of the fluorescent probe was observed. This led to the conclusion that apart from its functional requirement, state1- state2 transitions observed with anions and its valence dependence may not be the effect of anion *per se*. Thus, when the anions permeate the membrane, the influx of protons to balance the resultant charge causes the observed increase in PS I activity or in other words, state transitions.

These reorganizations were further confirmed by CD measurements. CD spectroscopy is a sensitive and non-destructive technique to study reorganizations of the complexes and the conformation of different pigment-protein complexes (Garab 1996; Garab and van Amerongen 2009). CD signals originate from different levels of structural complexity. CD provides information about the molecular architecture of the pigment system of the antenna and the reaction center complexes, as well as their macro-assemblies. The CD spectra in isolated thylakoid membranes is determined by 3 factors: i) intrinsic CD signal of photosynthetic pigment molecules, which are very weak ii) short range excitonic coupling between the chromophores, showing bands e.g. around (−)650 and (+)450 nm, iii) chirally organized systems, such as *psi* (polymer and salt induced) type signal, which are generally very strong and show peaks at (+)690 nm, (−)675 nm and (+)510 nm (Garab 1996). The magnitude of the psi type CD signal is controlled by the volume (size), chromophore density, and pitch of the helically organized macrodomains (Kim et al. 1986). The band at (−)675 nm is associated with stacking of the thylakoid membranes (Garab et al. 1991; Holm et al. 2005).

The results (Fig. 6a) showed that intensity of the psi-type (ψ) CD, which depends on the size and the long-range order of the components in the aggregates, gradually decreased with different anions suggesting that there is a distinct anion induced change in the macro-organization of thylakoid membranes. A decrease in the amplitude of peaks at (+)690 nm, (−)675 nm and (+)510 nm indicated that anions caused a reorganization of the complexes in the membranes. This strongly indicated that in the presence of anions, aggregation of PS II particles is inhibited and more unstacking of the thylakoid membranes is taking place. In the presence of uncoupler, there was further change in the effects caused by anions (Fig. 6b). The effects induced by negative charges are diminished in the presence of an uncoupler bringing the membranes back to a normal state to some extent. These data suggest that anions action on membrane reorganization is somehow regulated by the proton concentration in the lumen.

Thus, the anions seem to cause changes in the membrane properties by disturbing the charge distribution and initiating counter-ion transportation (most probably H^+). It has also been shown that the phase properties, via influencing the structure of bulk water and lipid/water interface, exhibited a graded response of anions of the Hofmeister series (Sanderson et al. 1991). An association of anions with the bilayer-lipid interfaces occurs which largely followed the Hofmeister series (Aroti et al. 2007).

It is well established that the organization of the thylakoid membrane is integrally associated with the regulation of energy flow in the reaction centers (Neilson and Durnford 2010). We conclude that apart from its functional requirement, energy redistribution observed with anions and its valence dependence may not be the effect of anion *per se*. Thus, when the anions permeate the membrane, the influx of protons to balance the resultant charge causes structural reorganization in thylakoid membranes resulting in energy re-distribution. The action of anions is most probably regulated by their position in the Hofmeister series. Protons in the thylakoid lumen regulate the migration of antenna, and hence the energy distribution, between the two photosystems where a low luminal pH favors antenna migration from PS II to PS I by causing protonation and subsequent detachment and migration of LHC II (Singh-Rawal et al. 2010).

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