Cation Effects on System II Reactions in Thylakoids: Measurements on Oxygen Evolution, the Electrochromic Change at 515 Nanometers, the Primary Acceptor and the Primary Donor

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Divalent cations are known to decrease the "spillover" of excitation energy from photosystem II (PS II) to PS I. At low light intensities, this does lead to an increased quantum yield of photoreactions in PS II. Contrary to some suggestions that PS II is activated at high light intensities, or in strong flashes of light by the addition of Mg^{2+}, we suggest here that Mg^{2+} ions may not activate reaction center II, P 680. After brief light flashes, we do not find any significant enhancement in (a) O_2 yield/flash, (b) proton yield/flash, (c) the amplitude of charge separation in PS II, as monitored by 515 nm absorbance change, and (d) the amplitude of the reduced primary acceptor, as monitored by 320 nm absorbance change. Data on absorbance change at 820 nm suggest that the absence of Mg^{2+} does not lead to inactivation of electron donation to $P\,680^+$.

Mono- and divalent cations regulate excitation energy distribution between the two photosystems (PS) of photosynthesis (see reviews by Barber [1], Williams [2], and Wong [3]). Since this regulation may be of significance to the optimization of *in vivo* photosynthesis at low light intensities, it deserves further study. In thylakoids, the higher activity of PS II in the presence of low (3 – 10 mM) concentration of divalent cations is suggested to be due to a reduced energy transfer from PS II to I (see Murata [4]), and to a better coupling of antenna complex II to its reaction center complex (see Arntzen and Ditto [5]; Wong and Govindjee [6]). Some authors have suggested that divalent cations may also increase system II activity by activating reaction center II (Rurainski and Hoch [7], and Bose and Arntzen [8]). In this paper, we suggest that divalent cations may not activate reaction center II based on the following observations. Addition of divalent cations to thylakoids (a) leaves almost unchanged the oxygen or proton yield per flash, with ferricyanide as the electron acceptor; (b) does not change the extent of charge separation in PS II, as monitored by an electrochromic absorbance increase at 515 nm in the presence of ferricyanide; and (c) does not affect the formation of the plastoquinone anion radical (labelled as A_1^- or A_2^-, where A_1 is the primary electron acceptor of PS II), as measured by an absorbance increase at 320 nm. Finally, measurements of an absorbance increase at 820 nm are consistent with our suggestion that the absence of a divalent cation does not inactivate electron donation to $P\,680^+$.

Methods

Thylakoids were prepared from the leaves of 8 – 10 day old pea plants as described either by Gross [9] or by Bose and Arntzen [8]. In the first method, referred to as the sucrose-washing method, the grinding medium contained 350 mM sucrose and 50 mM Tris-HCl at pH 7.5, the washing medium was 100 mM unbuffered sucrose and the resuspension medium was 100 mM sucrose and 0.4 mM Tris-HCl at pH 7.8. Thylakoids were washed twice or thrice in this method. Three samples were used in the experiments: cation-depleted (sucrose-washed), plus 5 or 10 mM NaCl (referred to as the Na^+ sample), and plus 5 or 10 mM NaCl plus 5 or 10 mM $MgCl_2$ (referred to as the Mg^{2+} sample). In the second method, to be referred as the sorbitol method, the grinding medium contained 0.4 M sorbitol and 0.1 M tricine, pH 7.5, the washing medium simply contained 10 mM NaCl, and the suspension medium had 0.4 M sorbitol, 10 mM NaCl and 10 mM tricine, pH 7.5. In this method, only one wash was given. The two sam-

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ples used were: thylakoids without $MgCl_2$ (referred to as the minus Mg^{2+} sample) and with 5 or 10 mM $MgCl_2$ added (referred to as the plus Mg^{2+} sample).

The oxidation of the reaction center chlorophyll a was measured as an absorbance increase at 820 nm, as described by Van Best and Mathis [10]. The actinic flash was from a ruby laser (wavelength, 694 nm; saturating intensity; duration of flash, 8 ns). For measurement, a fresh dark-adapted sample was used. The measuring light was 820 nm (half-bandwidth, 3 nm). The time constant of the instrument was approximately 1 µs. The temperature of the sample was 21 °C, and the Chl concentration was 20 µg/ml of suspension. Four or 8 mM ferricyanide was used to block absorbance changes due to the reaction center Chl a of PS I, P 700, when specified.

The reduction of the primary electron acceptor A_1 was measured as an absorbance increase (ΔA) at 320 nm, as described by Mathis and Haveman [11]. The actinic flash was from a xenon lamp (wavelength of excitation, 390 – 700 nm; saturating intensity; duration, 3 µs). For each measurement, a fresh dark-adapted sample was used; measurements were made with two successive flashes separated by 70 ms. The measuring light was 320 nm (half-bandwidth, 10 nm; at the intensity used, DCMU* had no influence on ΔA at 320 nm at the first flash). The time constant of the instrument was 100 µs. Samples containing 20 µg/ml chlorophyll were maintained at 9 °C.

Changes in absorbance at 515 nm, reflecting charge separation in the thylakoid membrane, were measured with a single beam spectrophotometer linked to a transient recorder (Data Lab DL 905) and a minicomputer as described by Saphon and Crofts [12]. The actinic flash was from a xenon lamp (wavelength > 630 nm; saturating intensity, as checked by the use of neutral density filters; duration, 18 µs). To ensure a fresh aliquot for every measurement, a stop-flow system was used. The dark time between the flashes was 16 seconds. The signal-to-noise ratio was improved by averaging 8 traces. The measuring beam was at 515 nm (half-bandwith, 1 nm; intensity, 10 µW/cm²). The time constant of the instrument was 10 µs.

Oxygen evolution was measured, using repetitive (> 200) flashes from a xenon (Stroboslave, Radiotechnic) lamp, by a Clark electrode. These flashes (white light; saturating intensity; duration, 5 µs) were separated by 200 to 300 ms. The temperature of the sample was 20 °C, and the Chl concentration was 10 µg/ml. The suspension contained 1 mM ferricyanide and 1 µM gramicidin D in addition to the sorbitol-tricine buffer described above at pH 7.5; experiments were also made at pH 6.5 and 7.0. The size of the photosynthetic unit was calculated as originally described by Emerson and Arnold [13]. The proton yield/flash was also measured with repetitive light flashes (same conditions as above) as the acidification produced (4 H^+ are produced/O_2evolved), using a pH meter (Radiometer 22) equipped with a combination electrode. In these measurements, the medium was 40 mM sorbitol, 0.5 mM ferricyanide, 0.1 mM tricine (pH, 6.5), 10 mM NaCl and 1 µM gramicidin D. The proton concentration was determined by comparison with known aliquots of HCl.

Results and Discussion

Oxygen and proton yield. Independent of the presence of 5 or 10 mM $MgCl_2$, we found, in all the ten experiments performed (pH, 7.5), the maximum O_2 yield per flash to be same within the experimental errors and variations between samples: 1 O_2 molecule per 2,300 ± 200 chlorophyll molecules present. In order words, there are 575 ± 50 total chlorophyll molecules per 1 active reaction center II plus 1 active reaction center I molecule, with or without 5 or 10 mM $MgCl_2$. The O_2 yields in steady state saturating light, as measured with a Clark electrode, were also unaffected by the addition of 5 or 10 mM $MgCl_2$ to the Na^+ samples **. The above results were obtained with both sucrose and sorbitol-containing suspensions. Independent and unpublished experiments of Rita Khanna are consistent with all of the above observations on the Mg^{2+} effects on O_2 yield per flash, and on the steady state rates of O_2 evolution, measured in the pH range of 6.5 to 7.5. The proton yield per flash, in five experiments, at pH 6.5, was 1 H^+ per 500 ± 70 chlorophyll molecules in the minus Mg^{2+} sample, and it remained unchanged upon the

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*DCMU, 3-(3,4-Dichlorophenyl)1,1 dimethylurea.

**Wong [3] has, however, observed in sucrose-washed thylakoids a small (upto a maximum of ~ 25%) Mg^{2+}-induced enhancement of steady state O_2 evolution (at pH 7.0) under saturating light conditions, with ferricyanide, but *not* with dichlorophenol indophenol as electron acceptor. Explanation of these differences requires further experimentation.

addition of 5 or 10 mM $MgCl_2$ in each experiment examined. The large (~ 2 fold) enhancement of O_2 yield/flash upon Mg^{2+} addition, obtained with a rate electrode by Bose and Arntzen [8], could not be supported here by measurements made with a concentration electrode. We note that these differences are, perhaps, not due to differences in the pH of the samples used; Bose *et al.* [14] had used a pH of 6.8 ± 0.1 (see their Table IV).

The 515 nm absorbance change. The flash-induced absorption change at 515 nm is used as an indicator of the primary charge separation in the reaction center II complex when the P 700 is kept oxidized by 5 mM ferricyanide (see Witt [15]). Experiments were made on sucrose-washed, Na^+, and Mg^{2+} samples. Fig. 1 shows the cumulation of 8 traces each for the Na^+ and Mg^{2+} samples (equivalent to the minus and plus Mg^{2+} samples, respectively). These results were confirmed in a separate set of experiments. Since the samples contained 5 mM ferricyanide, most of the observed changes are due to charge separation in system II. Fig. 1 (a, b) shows that the initial amplitude of the 515 nm change, after single flashes, is the same for the Na^+ and the Mg^{2+} samples. This similarity in the amplitude implies that cations do not activate the charge separation in PS II. Fig. 1 (c, d) shows that Mg^{2+} did not activate charge separation in system II even in the presence of DCMU. However, an approximately 30% decrease in the amplitude of the 515 nm change caused by the presence of DCMU remains unexplained. Perhaps, a DCMU-induced shift in equilibrium from A_2^-, present in the dark, toward A_1^- (see Ref. [16]) could lead to some A_1^- being present before the flashes under our experimental conditions, thereby reducing the charge separating capacity (also see ref. [17]). An analysis of the decay of the 515 nm absorbance change by the method of Provencher [18] gives two components for the Na^+-(60%, 1.1 ms; 40%, 6.4 ms) and the Mg^{2+}-(73%, 0.4 ms; 27%, 3.5 ms) samples. The 515 nm decay kinetics is accelerated by the addition of Mg^{2+}. Such an acceleration as well as the absence of change in the amplitude of the 515 nm absorbance change were independently observed by Vredenberg and Schapendonk [19] and by the present authors [*cf.* 20].

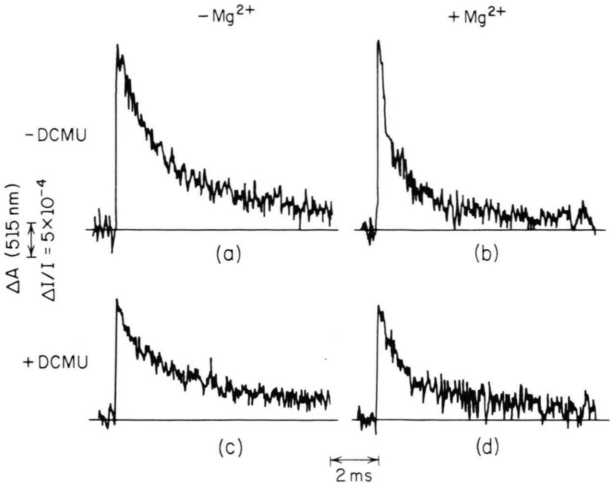

Fig. 1. Flash induced absorbance change at 515 nm. Cumulation of 8 decay traces. Pea thylakoids suspended in 100 mM sucrose + 1 mM tris-HCl; pH, 7.2; [Chl] = 30 μg/ml; temperature = 23 °C; measuring-beam bandpass = 1 nm. Top traces (a, b) were in the absence of DCMU; lower traces (c, d) were in the presence of 4 μM DCMU; left column without and right column with 9.7 mM Mg^{2+}. All samples contained 9.7 mM NaCl and 5 mM ferricyanide. (Similar results were obtained with sorbitol-containing thylakoids; pH, 7.5.)

Absorbance changes at 320 nm. Fig. 2 shows the absorbance changes at 320 nm in thylakoids with and without Mg^{2+}. The amplitude of this absorbance

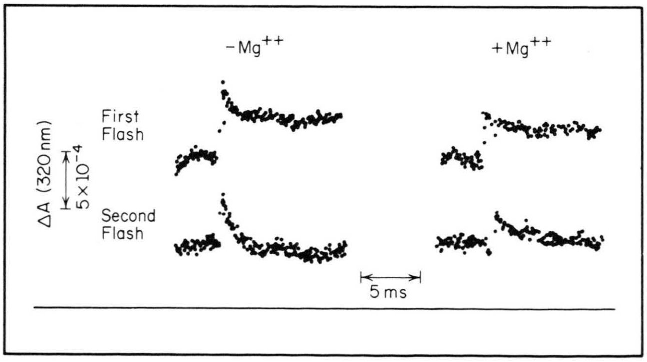

Fig. 2. Absorption changes at 320 nm induced by first or second flash in a group of xenon flashes (Δt between flashes = 70 ms) given to dark-adapted thylakoids from peas (temperature: 9 °C). Average of 60 measurements. Chlorophyll concentration: 20 μg/ml; sucrose-washed thylakoids + 5 mM Na^+ or 5 mM Na^+ + 5 mM Mg^{2+}; pH 7.5. (Similar results were obtained with spinach thylakoids and with 10 mM cations, and also with sorbitol-containing thylakoids at pH, 7.5.)

increase was not enhanced by the addition of Mg^{2+} (60 measurements in two series of experiments). In fact, the amplitude of the absorbance change ($\Delta I/I \sim 5 \times 10^{-4}$) was decreased by about 20% in the presence of 10 mM $MgCl_2$. This decrease in amplitude cannot be due to the flash being non-saturating since Mg^{2+} favors the excitation of system II [4, 5]. However, this decrease can be easily explained by changes in the "sieve effect" and scattering by the sample when $MgCl_2$ is added. Addition of $MgCl_2$ indeed increases the scattering of 320 nm light by a factor of ~ 1.4. If a correction is applied for the "sieve" effect, as proposed by Pulles *et al.* [21], then the small difference in the amplitude of absorbance change at 320 nm between plus and minus Mg^{2+} samples disappears. However, a small difference in the decay kinetics of the 320 nm change remains: The half-time of the decay in two series of experiments, 60 measurements each, was 690 μs for the minus Mg^{2+} sample and 1,000 μs in the plus Mg^{2+} sample. This decay is thus slowed down slightly (about 30%) by Mg^{2+} addition. On a slow (500 ms) time scale, the absorbance change at 320 nm observed in the minus Mg^{2+} sample was normal, as it showed the usual behaviour in a sequence of 4 flashes as published by Mathis and Haveman [11] for cation-containing chloroplasts. Wong *et al.* [22] have observed a Mg^{2+}-induced slight acceleration of the decay of the chlorophyll a fluorescence yield after a flash. In apparent contrast to this result and that on the 515 nm absorption change, where Mg^{2+} also accelerates its decay, the decay of the absorption change at 320 nm was slowed down by Mg^{2+} addition. Besides reflecting the decay of A_1^- (Q^-), these three measurements may also reflect different additional reactions. (a) The decay of the 515 nm absorbance change is not necessarily linked only to the electron transport phenomena; it is also affected by the movement of ions across the thylakoid membrane as well as by other changes in the intrinsic forward and backward reactions in the photosystem. (b) The 320 nm change may be affected by electron flow from A_1^- to A_2 (B or R; [16, 23]) but more likely by the conversion of two semiquinones ($A_1^- + A_2^-$) into a hydroquinone ($A_1 + A_2^{2-}$) [24] or a back-reaction ($A_1^- + P\,680^+ \rightarrow A_1 \cdot P\,680$) – the electron flow from A_1^- to A_2 is not expected to cause any absorbance changes as both are semiquinones; in addition, in the present study, a fast component (100 – 200 μs) of A_1^- decay may have been missed. (c) Fluorescence decay may reflect mainly the transfer of electrons from A_1^- (or Q^-) to A_2 or to $P\,680^+$. Further research is necessary for the complete understanding of these effects. What is clear, however, is that the amplitudes of ΔA-320 (reflecting A_1^- and A_2^-) and 515 nm (reflecting the charge separation) remain unaffected by cations.

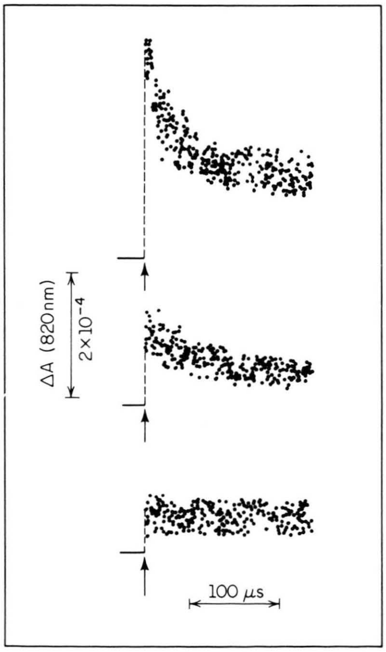

Fig. 3. Absorption changes at 820 nm induced by a saturating ruby laser flash given to dark-adapted thylakoids from peas (temperature: 20 °C). Average of 4 measurements. Chlorophyll concentration: 20 μg/ml; pH, 7.5; sucrose washed thylakoids + 5 mM Na^+. Top trace: + 1 mM sodium ascorbate + 10 μM DCPIP, no Mg^{2+}; middle trace: + 5 mM potassium ferricyanide, no Mg^{2+}; and bottom trace: + 5 mM potassium ferricyanide, + 5 mM Mg^{2+}. (Similar results were obtained with spinach thylakoids and with 10 mM cations, and also with sorbitol-containing thylakoids at pH 7.5.)

Absorbance changes at 820 nm. In the presence of reduced 2,6-dichlorophenolindophenol (10 μM DCPIP and 1 mM sodium ascorbate), the 820 nm absorbance increase decays biphasically after a flash, the major component decaying with a $t_{1/2}$ of ~ 20 μs (Fig. 3, top trace). There was no measurable effect of divalent cations on the amplitude and the decay kinetics of this absorbance change (data not shown). That is, sucrose-washed, Na^+ and Mg^{2+} samples, and plus and minus Mg^{2+} sorbitol samples gave identical results. Upon addition of 4 mM ferricyanide, the signal was much smaller: down to about 30% (minus Mg^{2+}) or 15 – 20% (plus Mg^{2+}). Without Mg^{2+}, half of the signal decays with $t_{1/2} \cong 40$ µs and the remaining signal decays very slowly (Fig. 3, middle). With Mg^{2+}, only a slow phase is present (Fig. 3, bottom). Upon further addition of 4 mM ferricyanide the signal without Mg^{2+} becomes indistinguishable from that with Mg^{2+}. Since ferricyanide largely oxidizes P 700 chemically and is supposed to have little effect on PS II, our data suggest that most of ΔA at 820 nm is due to P 700, there is no significant Mg^{2+} effect on it, and that P 680^+ is largely re-reduced within the time resolution of our measurements, in agreement with previous observations [10]. Thus, the difference noted between the minus Mg^{2+} (Fig. 3, middle) and the plus Mg^{2+} (Fig. 3, bottom) is, perhaps, due to a slightly less efficient chemical oxidation of P 700 by ferricyanide in the absence of Mg^{2+} (see Itoh [25]). If a significant part of electron donation to P 680^+ were inactivated by the absence of Mg^{2+}, then we would have observed a major slow phase as observed earlier by other inactivation procedures ([10]; Conjeaud *et al.*, [26]). It seems that the absence of Mg^{2+} does not cause a poor connection between P 680 and the oxygen evolving system and, hence, sample must have a fast ($\ll 1$ µs) reduction of P 680^+, found under physiological conditions, instead of the slow (> 1 µs) reduction found under other conditions [10, 26]. We, therefore, suggest that in the absence of Mg^{2+}, a significant fraction of electron donation to P 680^+ is not inactivated.

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