Biochimica et Biophysica Acta, 504 (1978) 288—297

BBA 47577

FUNCTIONAL HOMOGENEITY OF *P*-700 IN CYCLIC AND NON-CYCLIC ELECTRON TRANSPORT REACTIONS IN THYLAKOIDS

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(Received January 17th, 1978)
(Revised manuscript received May 31st, 1978)

Summary

The photoinduced turnover of *P*-700 (the reaction center chlorophyll *a* of photosystem I) in higher plant thylakoids was examined at room temperature by observation of the kinetics and amplitude of the transmission signal at 700 nm. The concentration of *P*-700 functional in cyclic and non-cyclic electron transfer reactions was compared. For the cyclic reactions mediated by *N*-methylphenazonium-*p*-methosulfate, 2,3,5,6-tetramethylphenylenediamine, 2,6-dichlorophenolindophenol and *N,N,N',N'*-tetramethylphenylenediamine and non-cyclic reactions utilizing either methylviologen or $NADP^+$ as acceptor, the illuminated steady-state concentration of *P*-700^+ was shown to be similar. The data support the concept of a homogeneous pool of *P*-700 that is capable of interaction in both cyclic and non-cyclic electron transfer reactions and are consistent with previous data obtained in vivo.

The amplitude and kinetics of the *P*-700 signal were found to be very dependent upon the composition of the reaction medium and differences were noted for turnover in the cyclic and non-cyclic reactions. Specifically, at white light saturation, the addition of low concentrations of divalent cations, such as Mg^{2+} or Ca^{2+}, had no effect on the signal amplitude during the cyclic reactions, but, in confirmation of previous work, caused an attenuation of the signal amplitude during non-cyclic flow. At low light intensities, the divalent cations caused a similar reduction in redox level of *P*-700 in the steady-state during non-cyclic flow and also reduced the rate of *P*-700 photooxidation in the cyclic reactions. The concentration of divalent cation that reduced the signal amplitude of *P*-700^+ during non-cyclic flow was compared with that required for the stimulation of the variable component of fluorescence, and it was shown to be similar with half maximal effects at 1 mM Mg^{2+}. The observations confirm that divalent cations control non-cyclic electron transport by an activation of

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1, 1-(dimethylurea); DCIP, 2,6-dichlorophenol indophenol.

Photosystem II in addition to regulating the distribution of excitation energy between the two photosystems.

Introduction

It is now well-established that two photochemical systems drive non-cyclic electron transport in thylakoids in the production of $NADPH_2$ and ATP during photosynthesis [1]. Considerable effort has been directed towards an understanding of the organization of the thylakoid [2], and recently several investigators have reported that a substantial fraction of the long-wavelength Photosystem I is morphologically isolated from Photosystem II [3—6]. Following the work of Jacobi and Lehman [3], Sane et al. [4] characterized various fractions of thylakoids that had been fragmented mechanically. They provided ultrastructural and biochemical data that showed the stroma lamellae and end membranes of the grana stacks comprised exclusively of Photosystem I. The grana membranes were shown to be comprised of both Photosystems, and Photosystem II was claimed to be restricted to such appressed regions of the membranes. These suggestions were supported by Arntzen et al. [5,6] who also showed that the membrane fraction derived from the stroma lamellae was capable of methylphenazonium methosulfate-mediated cyclic photophosphorylation but could not form a measurable trans-membrane ΔH^+. Additional comparative studies of chloroplasts of various developmental stages showed a correlation between reduced proton accumulation and chloroplasts with high concentrations of stroma lamellae [5].

Further support for a functionally isolated Photosystem I has been provided from spectroscopic experiments. In a study of the single turnover flash-induced *P*-700 signal in thylakoids, Haehnel [7] concluded that only 75% of Photosystem I is coupled to Photosystem II in linear transport. Breton reported the existence of two distinct pools of *P*-700 based upon linear dichroism measurements [8] and Gasanov and Govindjee [9] found differences in the low temperature fluorescence spectra and polarization of fluorescence of photosystem I fractions isolated from grana and stroma membranes. More recently Hiyama et al. [10] have reported the existence of two forms of *P*-700 in isolated cyanobacterial membranes following a kinetic analysis of the *P*-700 signal during Photosystem I driven reactions.

Several workers have attempted to localize the various Photosystems on the thylakoids by the light-dependent deposition of electron-dense material followed by electron microscopy. Hall et al. [11] showed the deposition of copper ferrocyanide on the stroma lamellae as well as on the grana stacks and questioned the proposal of Sane et al. [4]. Nir and Pease [12] performed similar experiments and concluded that their data were ambiguous but, in a more recent investigation, Kirchanski [13] showed the deposition of material in the stroma areas to be non-specific for Photosystem II and supports the proposal advanced by Sane et al. [4].

Armond and Arntzen [14] have now demonstrated the existence of Photosystem II in the stroma lamellae using labelling techniques. They also showed that the Photosystem II activity in the stroma lamellae is 20—25% that of the

grana, requires higher light intensities for saturation and is of a smaller photosynthetic unit size.

Work previously reported from this laboratory on the participation of cytochrome *f* [15] and *P*-700 [16] in the in vivo cyclic and non-cyclic electron transfer reactions in algae supported a homogeneous array of Photosystem I units capable of interaction in both electron transfer reactions in contrast to the proposal of Sane et al. [4]. The present studies extend this work to isolated thylakoids, and the participation of *P*-700 in the electron transfer reactions was investigated by spectrophotometry. It will be shown that the determination of the functional pool of *P*-700 is complicated by the effect of divalent cations on the photoreactions. The data presented are consistent with the concept of a functionally homogeneous pool of *P*-700 in support of the previous results obtained in vivo [15,16]. The data also confirm the dual role of divalent cations in the regulation of the photoreactions of thylakoids by controlling the distribution of excitation energy between the two Photosystems and by activation of Photosystem II.

Materials and Methods

Thylakoids were isolated from peas by the procedure described by Gross [17] and were shown by electron microscopy to be morphologically similar [18]. The preparations comprised mainly of envelope-free chloroplasts showing well-defined grana and stroma lamellae and were stored in 100 mM sucrose, 1 mM Tris-HCl, pH 8. The reaction mixtures contained 100 mM sucrose, 5 mM Tris-HCl (pH 8.0), 6 mM KCl and, where indicated, 100 μM methylviologen, 10 μM methylphenazonium methosulfate, 10 μM DCIP and 5 μM DCMU. For the reactions using 10 mM $NADP^+$ as acceptor saturating amounts of spinach ferredoxin (Sigma Chemical Company) were included.

The *P*-700^+ signal was measured spectrophotometrically using a single beam instrument as described previously [16] and the fluorescence of variable yield was measured using the same instrument as follows. The sample was excited by a weak 430 nm measuring beam modulated at 1 kHz. The modulated fluorescence signal at 680 nm isolated by a Baird Atomic Interference filter was detected by a 2.54 cm diameter photodiode (United Detector Technology) placed at right angles to the excitation beam in the sample compartment. After current to voltage conversion the modulated signal was recovered using a Princeton Applied Research Model 120 Lock-in amplifier. A broad band direct current actinic beam of wavelength range 420—600 nm was presented from beneath the sample and controlled by means of a manual shutter. The overall instrument response time was 1 s and was limited by the strip chart recorder.

Thylakoids were used at a concentration equivalent to 10 μg chlorophyll/ml for the *P*-700^+ determinations and 1 μg chlorophyll/ml for the fluorescence measurements. Optical path lengths of 1 cm were used for both the 700 nm transmission measuring beam and the 430 nm fluorescence excitation.

Results

Functional homogeneity of P-700 in cyclic and non-cyclic transport

The participation of *P*-700 in cyclic and non-cyclic electron transport reac-

tions was determined spectrophotometrically by observing the photoinduced signal at 700 nm in thylakoids undergoing electron transfer reactions mediated by a variety of cofactors and electron acceptors. The experimental protocol permitted steady-state determinations of the concentration of $P\text{-}700^+$ and induction effects were eliminated. It became immediately apparent that the composition of the medium markedly modified the signal obtained in each class of reaction with respect to amplitude and kinetics. Extremely slow turnover of *P*-700 was observed in all reactions in solute concentrations of less than 5 mM, but, in agreement with Shavit and Avron [19], high rates of electron transport were established at concentrations greater than 10 mM KCl, NaCl or sucrose.

Fig. 1 shows photoinduced transients obtained at white light saturation indicating the turnover of *P*-700 during non-cyclic transport (upper trace) and two cyclic reactions (lower two traces). The rate constants for the recovery of $P\text{-}700^+$ during the cyclic reactions were limited by the reaction conditions. In anaerobic conditions of the presence of ascorbate, the reduction of $P\text{-}700^+$ in the methylphenazonium methosulfate-mediated reaction was about 10-fold faster than shown. The same was found for the DCIP reaction and much faster recoveries were observed when the reactions were conducted with the DCIP in the reduced form obtained either by reduction with Pt/H_2 or photoinduced in situ by the thylakoids prior to the addition of DCMU. In both instances, however, the signal amplitudes obtained in the Photosystem I cyclic reactions were similar to those found in the methylviologen-catalyzed non-cyclic reaction. Es-

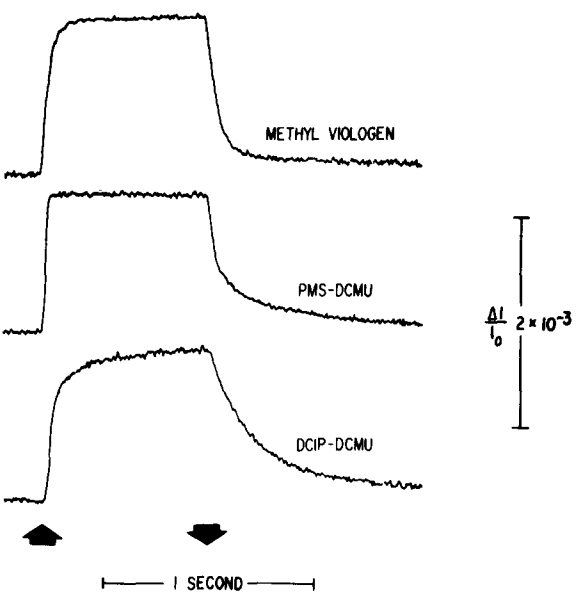

Fig. 1. Increase in transmission due to *P*-700 in non-cyclic ($H_2O \rightarrow MV$) and cyclic (with methylphenazonium methosulfate or DCIP with DCMU) electron transfer reactions in isolated thylakoids. The white activation light was 800 ms in duration and of an incident intensity of 10^5 ergs · cm^{-2} · s^{-1}. The light was provided by a tungsten halide source filtered through heat glass. The time between light flashes was 2 s. The transient as presented represent the average of 64 repetitions and an increase in transmission at 700 nm represents an increase in concentration of $P\text{-}700^+$.

sentially similar results were obtained (not shown) for the cyclic electron transfer reactions mediated by 2,3,5,6-tetramethyl-*p*-phenylenediamine and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and non-cyclic electron transport using $NADP^+$ as acceptor. It is concluded, therefore, that the functional pool of *P*-700 in the thylakoids is homogeneous and is capable of participation in both electron transfer pathways in vitro.

As mentioned above, the transmission signal due to $P\text{-}700^+$ was very dependent upon the ionic composition of the reaction medium and particularly to low concentrations of divalent cations. Accordingly, a detailed study of the effects of the cations on the $P\text{-}700^+$ signal was considered essential to establish the validity of the conclusion drawn from the data presented in Fig. 1.

Effect of divalent cations on the redox state of P-700

Fig. 2 shows the effect of Mg^{2+} on the signal obtained at light saturation. The signal amplitude was reduced 28% and the recovery time was accelerated by the addition of Mg^{2+} during non-cyclic flow (upper two traces) but the absorption transient obtained during the cyclic reaction (lower two traces) was

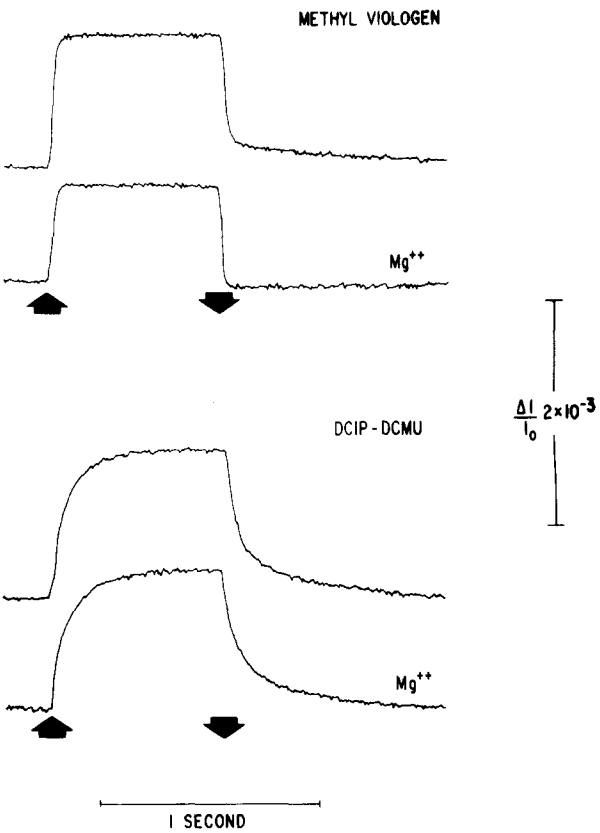

Fig. 2. Effect of 3 mM $MgCl_2$ on the photoinduced increase in transmission of *P*-700 in non-cyclic electron transfer using methylviologen as acceptor (upper two traces) and cyclic electron transfer mediated by DCIP (lower two traces). The activation flash protocol was as described in Fig. 1.

not modified. Other divalent cations such as Ca^{2+} and the aliphatic amine, spermidine (not shown) had similar effects on the signal during non-cyclic flow. These results are in agreement with those of Rurainski et al. [20] and Henkin [21] who also reported that Mg^{2+} reduces the steady state signal amplitude due to *P*-700$^+$ in non-cyclic electron transport.

Several mechanisms could account for the observed attenuation of the P-700$^+$ signal in non-cyclic transport but the fact that the addition of Mg^{2+} results in an acceleration of the overall reaction [20—26] must be accommodated. As suggested by Henkin [21] the most plausible mechanism is to postulate that Mg^{2+} increases the activity of Photosystem II relative to Photosystem I so that the redox state of *P*-700 shifts toward a more reduced level. This most likely occurs by activation of Photosystem II rather than by inactivation of Photosystem I because the overall reaction velocity increased and Mg^{2+} does not appear to affect the Photosystem I cyclic reaction (Fig. 2, lower traces). Thus, the increased rate of linear transport must be due to relief of inhibition at the level of photosystem II or the intersystem chain. Wydrzynski et al. [27] and more recently Bose and Arntzen [28] and Wong et al. [29] have reported that Mg^{2+} causes an activation of Photosystem II.

Fig. 3 shows data obtained by activating the thylakoids with low intensities of 640 nm monochromatic light. The lower two traces show that the addition of Ca^{2+} significantly decreased the rate of photooxidation of *P*-700 in the cyclic reaction. This implies that the Photosystem I photochemistry was inhibited by addition of the divalent cation and is consistent with numerous

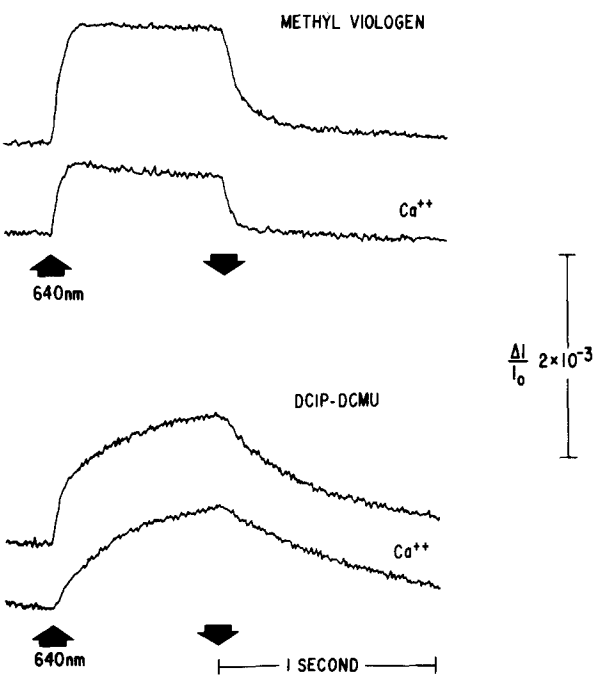

Fig. 3. Effect of 3 mM $CaCl_2$ on the photoinduced increase in transmission of *P*-700 in non-cyclic and cyclic electron transfer reactions driven by weak 640 nm monochromatic light. Conditions as in Fig. 1 except that the light intensity was $5 \cdot 10^3$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}.

reports that divalent cations decrease the quantum efficiency of Photosystem I reactions and increase the efficiency of Photosystem II reactions [21,22,24, 30—32]. The upper two traces show that the addition of Ca^{2+} in the non-cyclic reaction resulted in a 50% decrease in concentration of *P*-700$^+$ in the steady state. This effect was much greater than that observed at high intensities of white light (Fig. 2, upper traces) and may be explained by the combination of the decrease in photochemical activity of Photosystem I (as in Fig. 3, lower traces) and the activation of Photosystem II (Fig. 2, upper traces). These data confirm that the effect of the divalent cations is 2-fold: one being the extensively documented effect on the distribution of excitation between the two Photosystems [21,30,32—38] and an additional effect at the level of electron transport that appears to control the flux of reductant generated by Photosystem II [27—29].

Relation between the redox level of P-700 and the magnitude of the fluorescence of variable yield

In view of the low concentration of divalent cation required to modify the *P*-700$^+$ signal as described above and the implication that the cations affect Photosystem II, it was thought necessary to directly compare the *P*-700 response with a Photosystem II parameter. Fig. 4 shows the experimental protocol employed for acquisition of the variable component of fluorescence (F_v) that is widely accepted as originating from Photosystem II [33] and increases upon addition of Mg^{2+}. Fig. 5 illustrates the dependence of the *P*-700$^+$ and F_v signals on concentration of Mg^{2+}. The same preparation of thylakoids was used for the samples in both measurements. The data indicate that

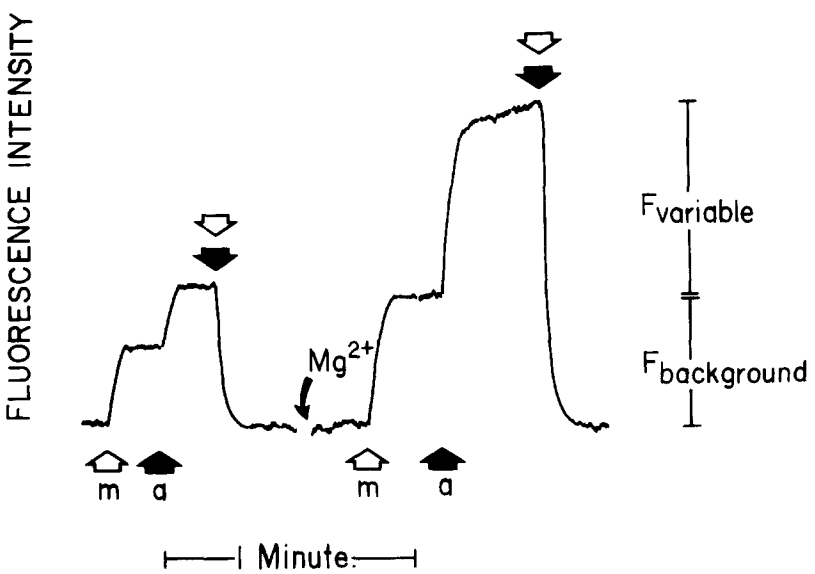

Fig. 4. Effect of Mg^{2+} on the modulated fluorescence signal in thylakoids. The modulated measuring beam (m) of 430 nm and activation beam (a) were turned on and off as indicated. $MgCl_2$ was added to give a final concentration of 3 mM. The fluorescence of variable yield (F_v) was taken as the amplitude of the modulated signal that occurred in response to the activation beam (a).

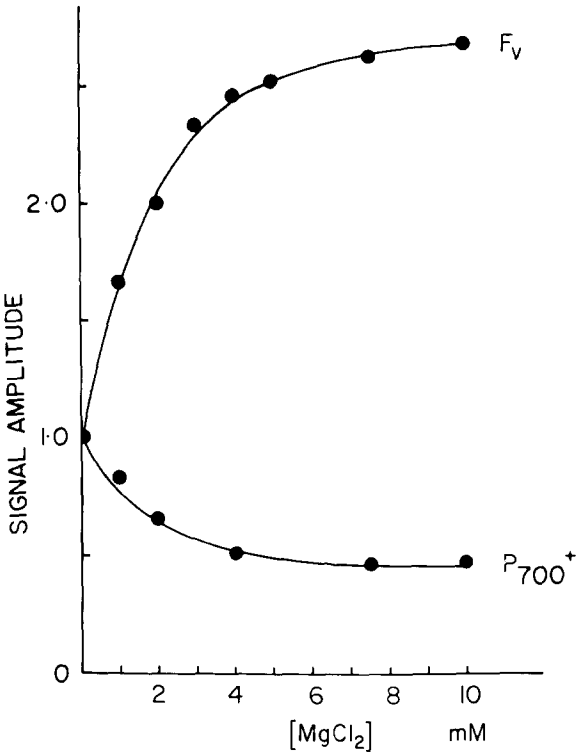

Fig. 5. Dependence of the steady state amplitude of the $P\text{-}700^+$ signal and fluorescence of variable yield (F_v) on concentration of $MgCl_2$. Reaction conditions as in Fig. 3 for the methylviologen supported non-cyclic electron transfer reactions were used for the $P\text{-}700^+$ determination and as in Fig. 4 for the determination of F_v. The control amplitudes of the two signals (minus $MgCl_2$) were adjusted to unity for comparison of the two activities.

both signals respond similarly to the addition of Mg^{2+} and the effect was half maximal at 1.2 mM. Saturation occurred at 5 mM Mg^{2+} and this value is in excellent agreement with those reported previously for the increase in F_v [21, 30,32—38] and modification of electron transport reactions [21,22,24,30—32]. The data strongly suggest that the observed response of *P*-700 to Mg^{2+} is very closely correlated with events at the level of Photosystem II.

Discussion

The data presented show that divalent cations greatly modify the $P\text{-}700^+$ signal in thylakoids with respect to the steady-state amplitude and the kinetics of photooxidation and reduction. The effects of cations on the signal observed during cyclic and linear electron transport were different and depend upon intensity. It is clear that cognizance of such effects is required in a determination of the concentration of functional *P*-700 in the electron transport reactions. The true concentration of functional *P*-700 may only be accurately determined from such $P\text{-}700^+$ amplitude measurements if in the illuminated steady-state the rate of *P*-700 photooxidation far exceeds its rate of reduction

[39]. This criterion has been met in the data shown in Fig. 1 in the cyclic reactions, where limitations on $P\text{-}700^+$ reduction have been imposed by employing oxidizing conditions, and in the non-cyclic reaction by the exclusion of divalent cations from the reaction medium. Under such conditions, it is apparent that the concentration of *P*-700 that participates in cyclic and linear electron transfer is the same. It is therefore proposed that all of the *P*-700 is accommodated in the linear electron transfer chain (Z-scheme) and may undergo Photosystem I cyclic flow under appropriate conditions. These conclusions are consistent with those arrived at previously following similar studies in vivo [15,16].

The conclusions are at variance with those of Haehnel [7] who reported that 25% of Photosystem I in thylakoids is functionally isolated. However, Haehnel used single turnover flash experiments for the determination of functional *P*-700 in the two pathways rather than the steady-state technique exploited here and the data are not directly comparable. It should be noted, however, that 1 mM Mg^{2+} was included in the reaction mixture utilized by Haehnel and the effect of this on the *P*-700 response following brief flashes is not known.

Hiyama et al. [10] reported that two kinetically distinguishable forms of *P*-700 occur in isolated membranes from *Nostoc muscorum* as measured during the Photosystem I dependent reduction of $NADP^+$ by various electron donors in the presence of ascorbate. The possibility of multiple sites of electron donation to $P\text{-}700^+$ and simultaneous cyclic electron flow could account for the complex kinetic response but, otherwise, the discrepancy with the results reported here and our previous work in vivo that included cyanobacteria [16] is not readily apparent.

The data confirm that divalent cations exert two separate effects in the regulation of the thylakoid reactions. In the experiments conducted at high light intensities (Fig. 2) the effect of Mg^{2+} is best interpreted as relieving a limitation in rate at the level of Photosystem II. This supports the work of Wydrzynski et al. [27], Bose and Arntzen [28] and Wong et al. [29] who showed the reversible activation of Photosystem II by Mg^{2+}. At lower intensities (Fig. 3) the effects of divalent cations on energy distribution became apparent as indicated by the reduction in rate of *P*-700 oxidation in the cyclic reaction and the much greater attenuation of the steady-state amplitude of $P\text{-}700^+$ in the non-cyclic reaction. These observations are consistent with the reports that the addition of divalent cations decreases the quantum efficiency of reactions driven by Photosystem I and increases those driven by Photosystem II [20,24, 30—32]. These observations, together with those on the stimulation of fluorescence by divalent cations, have been variously interpreted in support of the spillover of excitation from Photosystem II to Photosystem I as proposed by Murata [32,34], transfer of excitation from Photosystem I to Photosystem II [25], an increase in Photosystem II fluorescing units [36] or an increase in absorption cross section of the Photosystem II antenna [30]. It is not possible to choose between these postulates on the basis of the experiments reported here. However, the effect of Mg^{2+} on the redox level of *P*-700 is directly related to the events sustained in Photosystem II as demonstrated by the dependence of the two signals on the concentration of added divalent cation (Figure 5).

Acknowledgements

This work was supported by the National Science Foundation (BMS-74-19700). Susan R. Quinones and Jan Svejkovský are thanked for their assistance with some of the experiments.

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