FLASH-INDUCED Mn^{2+} OXIDATION OBSERVED BY ESR SPECTROMETRY IN LETTUCE CHLOROPLASTS

Yona SIDERER and Shmuel MALKIN
Biochemistry Department, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

The requirement of manganese for the process of photosynthetic oxygen evolution has been recognized for many years [1]. It has been suggested that manganese acts as an electron carrier between water and photosystem II [2]. Moreover it has been proposed that Mn is part of the positive charge accumulator [3] which is involved in the formation of the S-states postulated by the model for oxygen evolution [4].

In [5] we used electron spin resonance (ESR) spectroscopy to study manganese, in lettuce chloroplasts. We showed an ESR signal, originating from bound manganese, which decreased in amplitude upon continuous illumination of the chloroplasts. The on–off kinetics of the signal of control chloroplasts was compared to that of chloroplasts incubated with Tris, treated with heat, or to which specific inhibitors (DCMU, FCCP) or artifical electron donors (NH_2OH, phenylene diamine) were added. We have concluded from these studies that the decrease in the amplitude of the Mn^{2+} ESR signal reflects oxidation of Mn^{2+} by photosystem II, and the dark restoration is rereduction to Mn^{2+}.

Comparing our results with those of others showed some differences: the manganese ESR signal from active spinach chloroplasts was too weak [6] to be able to work on, but still it was distinguishable (cf. fig.1 in [6]). The signal increased tremendously upon Tris treatment [6,7] as well as upon washing with chaotropic agents [7]. In this case a light-sensitive decrease of the Mn ESR signal was demonstrated although oxygen evolution was inhibited [7]. On the other hand, a respectable Mn ESR signal was obtained [8] in intact Chlorella cells, which was light sensitive. We repeated our measurements with positive results on *Tetragonia expansa* and also on lettuce in San Diego, CA. (This experiment was done in G. Feher's laboratory, UCSD). Our previous results were therefore not specific to our lettuce or instrument.

It is not easy to answer the question whether the manganese that we observe is not exogenous to the O_2 evolving system, even in its bound form. Indeed it was shown that, in lettuce chloroplasts, added external manganese acts as an electron donor in photosystem II (e.g., when NADP is used as an electron acceptor) and its photooxidation was verified [9]. This result has been confirmed by ESR spectroscopy [10]. However there were significant differences in the behaviour of endogenous and added manganese.

A possible approach is to check the behaviour of the Mn^{2+} ESR signal toward periodic flashes. Measurements of proton NMR relaxation time (T_2) of water performed with chloroplast suspensions after exposure to a series of light flashes showed an oscillatory pattern of $1/T_2$ as a function of the flash number [11]. The rationale of this experiment was based on the fact that $1/T_2$ is very sensitive to the concentration of the paramagnetic Mn^{2+} and therefore it was suggested that the change in $1/T_2$ of the protons as a function of flash number indirectly measures Mn^{2+} concentration after each flash [12].

2. Materials and methods

Broken lettuce chloroplasts were prepared by the procedure in [12]. The chlorophyll concentration for

Address correspondence and reprint requests to: S. Malkin

each experiment was ~1 mg/ml. ESR measurements were carried out on a Varian F-12 spectrometer operating at 9.3 GHz (x band) with an E_{102} cavity equipped with a slit in its front to allow light to reach the chloroplasts in the cavity. The optical path of this cuvette was 0.02 cm. Continuous illumination was obtained using a slide projector (Brown). For the flashes a Rhodamine 6G dye laser giving a wavelength range between 570–618 nm was used (Phase R 1200). The light was dispersed with a lens so that a nearly homogenous beam was directed to cover the front face of the ESR cavity. The laser firing was timed at will with a home-made timer which triggered both the laser and a signal averager (CAT ≡ computer of average transients, Hewlett-Packard 5480 A) on which the ESR signals were progressively accumulated. A compromise between the need to obtain good signal/noise ratio and relatively short time of experiment (total time of one experiment could take several hours with alteration of samples in between) forced us to choose a resolution time of ~1 s.

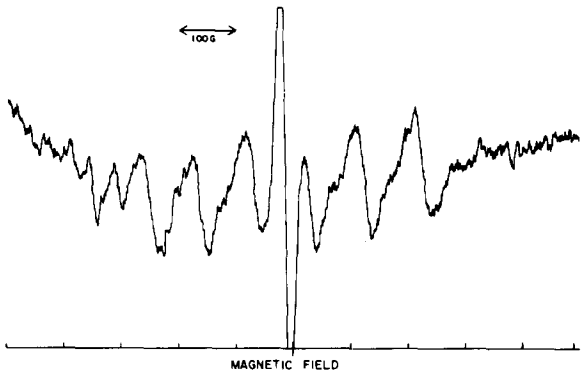

Fig.1. ESR spectrum of lettuce chloroplasts, showing typical sextuplet Mn signal and the typical large central signal. The magnetic field center is at 3440 G; modulation amplitude 12.5 G; microwave power 200 mW; response time 1 s.

3. Results and discussion

The Mn^{2+} ESR signal of lettuce chloroplasts is shown in fig.1. To see its response to light, the manganese ESR signal was monitored by fixing the magnetic field on the maximum of one of its hyperfine bands. The change in the signal amplitude with time after turning the light on and off or after a flash were recorded on the CAT. The response to continuous light is shown in fig.2A. The fraction of decrease in the light was different in different samples, and ranged between 0.2–0.8 of the signal amplitude. Figure 2B shows the change in the manganese signal amplitude after each flash, in a series of relatively closely spaced flashes. In this case a steady state in the S-states was probably achieved and was the same for each flash. Although noise interferes, the decrease in the Mn^{2+} signal amplitude can be clearly distinguished. It occurs exactly at the moment when the flash was fired. In order to verify that the decrease in the signal amplitude was caused by the light and was not an electronic artefact two control experiments were carried out:

(1) Monitoring the ESR signal (signal I) located in the center of the manganese sextuplet. Upon continuous illumination this signal which indicates free radical(s) and not Mn (very probably *P*-700), grows in the light. In the flashing experiments this signal indeed increased in opposite manner to the Mn^{2+} signal (fig.2C).

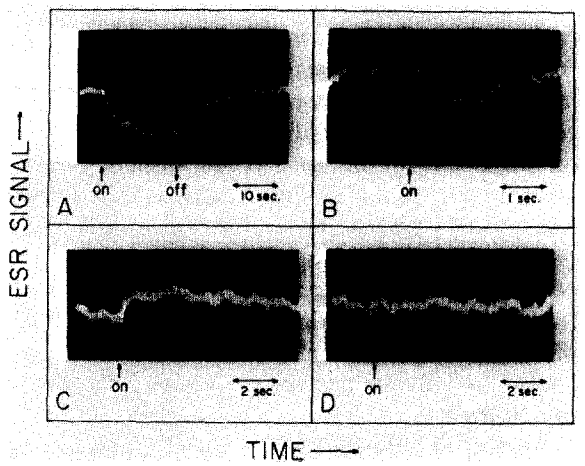

Fig.2. (A) Kinetic behaviour of the Mn signal (the 5th peak from left in fig.1) towards continuous strong light. Magnification on the CAT is × 8. The experiment was repeated every 15 s. Number of repetitions 10. (B) Kinetic behaviour of the Mn signal towards single flashes. Magnification on the CAT is × 64. The experiment was repeated every 7 s. Number of repetitions 180. (C) The same as B except that the magnetic field was fixed at the position of the central signal. 90 repetitions every 20 s. (D) The same as B except that the magnetic field was fixed outside the range of ESR signals (3850 G). 90 repetitions every 24 s.

(2) Measuring the response to a flash when the magnetic field was fixed in higher field than that of the manganese resonance. Here the optical and electronic properties of the system were the same and there was no change in the amplitude upon flashing (fig.2D).

From these controls we concluded that the response to the flash was a real one resulting from the oxidation of Mn^{2+} similar to that in continuous illumination. There was a significant difference however: A comparison of the amplitudes shows that in a single saturating flash only ~1/8th of the manganese which reacted in continuous light was oxidized. Estimation of the manganese concentration in photosystem II gives numbers in the vicinity of 8 atoms/reaction center [13]. This fits also with our estimates from the quantitation of the Mn^{2+} ESR signal after acidification [5]. Thus there is a single Mn^{2+} oxidation corresponding to each turnover of the reaction center, and there is a pool of ~8 Mn^{2+} which are oxidized only by the continuous light.

Figure 3 shows the result of an experiment in which 4 flashes were given 2.5 s apart with 52 s dark periods before the repetition of the experiment. In this series the maximal decrease in the amplitude of the Mn signal was obviously after the third flash.

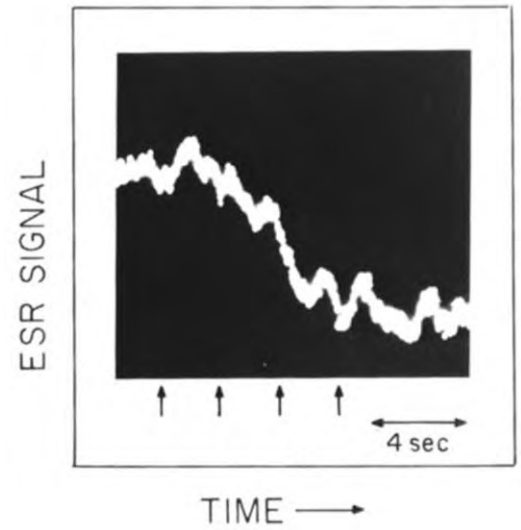

Fig.3. Kinetic behaviour of the Mn signal (the 5th peak) towards a group of 4 flashes given 2.5 s apart. 100 repetitions every 60 s, with replacement of sample every 30 min. Magnification on the CAT × 32.

There was only a small decrease after the second flash and the change after the fourth flash is within the noise level so that it is difficult to distinguish any changes there. This experiment attempted to correlate the changes in the Mn with the changes in the S-states showing that there may be a correlation of the transition $S_3 \rightarrow S_4$ with Mn^{2+} oxidation. If the ESR signal is a reflection of the internal Mn of photosystem II then in the first 4 flashes we accumulated Mn in higher oxidation states and the next flash, after the first 4, should relax the Mn back to Mn^{2+} by its photoact. Unfortunately this experiment is yet to be done.

Another possibility is that the ESR signal is due to exogenous manganese which is oxidized by photosystem II. Our experiment then shows that such manganese is oxidized by S_4. In any case these results apparently are not in agreement with the proton $1/T_2$ relaxation measurements [11]. In particular after 3 flashes it seems that we have a lower concentration of Mn^{2+} whereas the proton relaxation measurements suggest a maximum in Mn^{2+} concentration after 3 flashes. This discrepancy should be cleared in future work.

Perhaps the difference in the dark restoration kinetics of Mn has some relevance. After continous light the kinetics is clearly biphasic with approximately equal amplitudes and life-times of ~1 s and 5 s, respectively. After a single flash there is a single decay of ~1–2 s life-time. After 4 flashes the decay becomes much slower. However we feel that this aspect is too preliminary to comment on intelligently.

So far we have not succeeded in obtaining a complete and clear picture of the response of the manganese signal to various numbers of flashes. The main difficulty comes from the smallness of the response which is buried (in a single experiment) under noise. This called for many repetitions and therefore for a compromise in the dark interval given for complete relaxation (only a minute instead of the optimal several minutes) and the time resolution of our apparatus. We have calculated that to obtain a satisfactory pattern the time resolution should increase to $\geqslant 0.3$ s (in order to obtain a better separation of the response of each flash). Combining all this with a signal/noise ratio better than that achieved in the present report means that the number of repetitions should increase by two orders of magnitude, which

means many days-long experiments with frequent replacement of samples. This goal calls for a different planning of the present set-up.

In conclusion, more work is needed and a better signal/noise ratio in the manganese ESR signal in order to be able to verify the conjecture of the involvement of the manganese in forming the S-states This report serves to demonstrate the feasibility to observe Mn oxidation by flashes and to see differences in flash pattern.

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