FLASH POLAROGRAPHIC DETECTION OF SUPEROXIDE PRODUCTION AS A MEANS OF MONITORING ELECTRON FLOW BETWEEN PHOTOSYSTEMS I AND II

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1. Introduction

In isolated chloroplasts exogenous low potential electron acceptors, such as methyl viologen (MV), have been used to study the chloroplast electron transport involving photosystem I. The reduction of acceptors and their consequent autoxidation [1] can most easily be followed polarographically by the uptake of O_2. The reactions which are believed to occur [2,3] when two electrons are transferred from water to MV are as follows:

$$H_2O + 2MV_{ox} \xrightarrow[h\nu]{\text{chloroplasts}} 1/2\ O_2\uparrow + 2MV_{red} + 2H^+ \quad (1)$$

$$2MV_{red} + 2O_2\downarrow \xrightarrow{\text{spontaneous}} 2MV_{ox} + 2O_2^- \quad (2)$$

$$2O_2^- + 2H^+ \xrightarrow{\text{spontaneous}} H_2O_2 + O_2\uparrow \quad (3)$$

$$H_2O + 1/2\ O_2\downarrow \longrightarrow H_2O_2 \quad (4)$$

where reaction (1) is the oxidation of water and reduction of MV driven by the light reactions of photosystems I and II; reaction (2) is an autoxidation of MV, which produces superoxide ions (O_2^-) [4]; reaction (3) is the spontaneous dismutation of superoxide; and reaction (4) is the summation of these steps, which is equivalent to the Mehler reaction [5].

These reactions have been studied extensively using continuous illumination, and the evolution of O_2 in particular has been studied under flashing light illumination [6]. The reduction of photosystem I acceptors during flash illumination has only been briefly studied [7] using the polarographic technique. This work describes a signal detected from flash-illuminated isolated chloroplasts using a polarographic electrode with the platinum biased positive with respect to the Ag/AgCl junction. Evidence is presented that indicates this signal originates from photosystem I and is due to superoxide ions. MV and ferricyanide both can accept electrons from photosystem I and act as intermediates in the generation of superoxide, but are not themselves directly detected by the electrode. Preliminary results are presented, which show how this technique can be used to study electron flow between photosystems I and II.

2. Materials and methods

In this work, chloroplasts were used that had been isolated from leaves of Alaska peas (*Pisum sativum*, var. Alaska) grown in the laboratory and harvested 10–14 days after germination. The isolation procedure consisted of taking 75 g leaves and homogenizing them for 20 s in a Waring Blendor in 150 ml grinding medium (0.4 M sucrose, 0.1 M N-Tris(hydroxylmethyl)methylglycine (tricine), 5 mM $MgCl_2$, 10 mM NaCl, 20 mM ascorbate and 250 mg bovine serum albumin, at pH 7.8). The homogenate was strained through 8 layers of cheesecloth and centrifuged for 1 min at 1000 × *g*. The supernatant was then centrifuged for 5 min at 5000 × *g* to pellet the chloroplasts, which were resuspended in a 50 mM sodium phosphate

buffer, at pH 7.8, to obtain broken chloroplasts. These chloroplast fragments (thylakoids) were pelleted by a 5000 × *g* 5 min centrifugation and were resuspended in grinding media, without the ascorbate or bovine serum albumin, to a chlorophyll concentration of 1 mg/ml or as otherwise indicated in the results. Chlorophyll concentrations were determined as in [8] using the equations in [9].

The oxygen-exchange rates in chloroplasts under continuous illumination were measured with a Yellow Springs Instrument 5331 Clark electrode, laboratory-built monitoring circuit, and Heath EUW-20A recorder. Saturating continuous illumination was provided by an incandescent lamp through a Corning CS 3-71 glass filter and 4 in. water filter.

Signals in flash illumination experiments were measured on a Joliot type [10] polarographic electrode using a General Radio 1538-A strobe lamp for excitation and a laboratory-built DC coupled monitoring circuit having a 2 ms rise time. Saturating flashes were given at a rate of 1/s and signals were digitized and stored in a Biomation 805 waveform recorder and then permanently recorded with a Hewlett Packard 7128A recorder. To optimize the signal-to-noise ratio, the electrode was operated in a well-grounded Faraday cage, and the platinum electrode surface was frequently cleaned with concentrated NH_4OH.

In the upper chamber of the Joliot type electrode, a buffer consisting of 100 mM NaCl and 50 mM sodium phosphate, at pH 7.8, was used. For experiments under high O_2 or N_2 tension, this medium was bubbled with the appropriate gas for at least 45 min. Also in these experiments, the O_2 or N_2 was blown over 0.5 ml sample at a chlorophyll concentration of 1 mg/ml in a test tube on ice in the dark for about 15 min.

For experiments with superoxide dismutase (SOD), the enzyme was dissolved immediately before use in 1 mg/ml bovine serum albumin, 0.1 mM ethylenediamine tetraacetic acid, and 50 mM sodium phosphate at pH 7.8 to conc. 20 mg/ml. The SOD was obtained from Sigma.

3. Results

With the platinum biased positive with respect to the Ag/AgCl junction, flash-illuminated chloroplasts gave rise to an amperometric signal corresponding to the platinum electrode accepting electrons from some charge carrier. Figure 1 shows a typical recording of these signals from chloroplasts with $Fe(CN)_6^{3-}$ present as an electron acceptor. The quantity of electrons made available to the platinum, which these signals represent, oscillates with flash number, having a period of two, being maximum on the first flash, and damping by the tenth flash.

To understand the relationship of these signals to electron flow in photosynthesis, it is necessary to identify the source of the signal. With no electron acceptor present, the signals are quite small and do not oscillate, as can be seen in fig.2. With either $Fe(CN)_6^{3-}$ or MV present as an electron acceptor, the signals are greatly enhanced and period-of-two oscillations become apparent (fig.2). Since $Fe(CN)_6^{3-}$ accepts electrons from both photosystem I and II [11] but predominantly photosystem I [12], and MV accepts electrons from photosystem I [13], this signal seems to be originating from photosystem I.

To further test the origin of this signal, chloroplasts were incubated with $HgCl_2$ as in [14], a procedure that inhibits the flow of electrons between the 2 photosystems at plastocyanin [15]. The $HgCl_2$ treatment was shown to be effective by observing electron flow through photosystems I and II under continuous illumination with MV and $Fe(CN)_6^{3-}$ used as acceptors, respectively. As can be seen in table 1 (line 5), electron flow through photosystem I has been com-

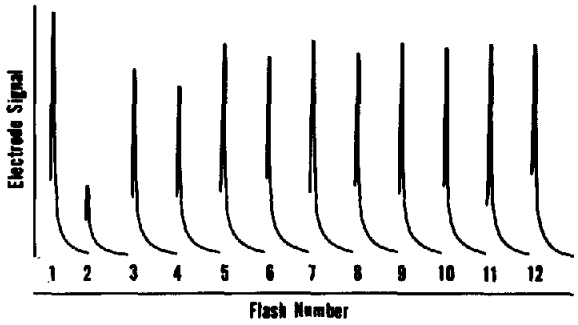

Fig.1. Direct recording of amperometric signals occurring during flash illumination of chloroplasts that were dark adapted for 5 min. $Fe(CN)_6^{3-}$ was present as an acceptor at 0.5 mM, and the platinum electrode was bias 200 mV positive with respect to the Ag/AgCl junction. An upward deflection corresponds to the platinum electrode receiving electrons.

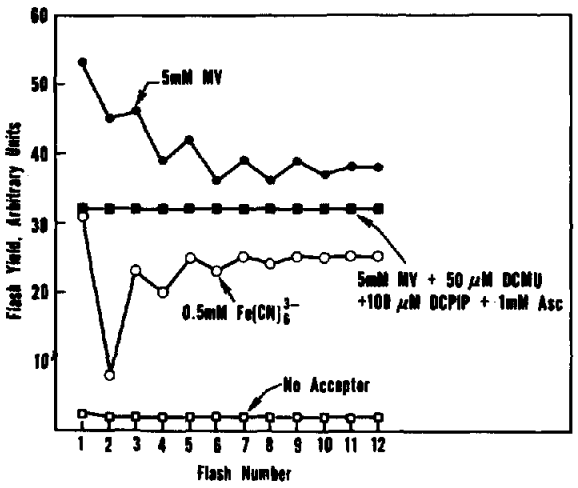

Fig.2. A plot of the maximum yield per flash versus flash number from chloroplasts dark adapted for 5 min on an electrode with the platinum bias 200 mV positive with respect to the Ag/AgCl junction. Yields are plotted under the following sample conditions: (□–□–□) no additions; (○–○–○) 0.5 mM $Fe(CN)_6^{3-}$; (●–●–●) 5 mM MV; (■–■–■) 5 mM MV + 50 mM DCMU + 100 μM DCPIP + 1 mM ascorbate.

pletely eliminated, while photosystem II is still functioning; this was also demonstrated by normal flash-illuminated O_2 evolution (data not shown). The low rate of O_2 production seen in $HgCl_2$-treated chloroplasts (table 1, line 4) confirms the belief that $Fe(CN)_6^{3-}$ accepts elctrons inefficiently from photosystem II. The $HgCl_2$ treatment of chloroplasts completely eliminates the positive bias electrode signals, again implying that they originate from photosystem I. Addition of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) to chloroplasts to stop electron flow eliminates the signal entirely; however, addition of 2,6-dichlorophenolindophenol (DCPIP) plus ascorbate to restore photosystem I electron flow [16] also restores the signals (fig.2). Note that while the signals are restored, their oscillatory behavior is not.

If the signal is due to electrons from photosystem I and acceptors are needed for a large signal, then MV_{red} and $Fe(CN)_6^{4-}$ might be giving up their electrons directly to the platinum. Another possibility is that O_2^- is generated and is the origin of the signal. If the latter is correct, the signal will be sensitive to oxygen tension since O_2^- generation is dependent upon oxygen (see reaction (2) in section 1). Figures 3 and 4 show that, indeed, as the oxygen tension drops so does the positive bias signal.

SOD catalyses the removal of O_2^- (reaction (3) in section 1) and should inhibit the positive bias signal if it originates from O_2^-. The activity of the SOD when added to these chloroplasts was demonstrated by the procedure in [17]. Table 1 (lines 1, 2 and 3) show, as expected for active SOD, that the trebling of oxygen consumption when ascorbate is added to chloroplasts with MV as an acceptor is

Table 1

Line	Conditions	O_2 exchange rate (μmol O_2/mg Chl-h)
	Control chloroplasts	
1	5 mM MV + 10 mM MA + 10 mM NaN_3	−170
2	5 mM MV + 10 mM MA + 10 mM NaN_3 + 10 mM Na ascorbate	−416
3	5 mM MV + 10 mM MA + 10 mM NaN_3 + 10 mM Na ascorbate + 1 mg SOD	−185
	$HgCl_2$ treated chloroplasts	
4	5 mM $Fe(CN)_6^{3-}$	+18
5	5 mM MV + 10 mM MA + 10 mM NaN_3	0

Oxygen exchange rates measured with a Clark electrode under continuous illumination as in section 2. Isolated chloroplasts at a chlorophyll concentration of 50 μg/ml were used with additions as listed: Methyl viologen (MV), methyl amine (MA) and superoxide dismutase (SOD)

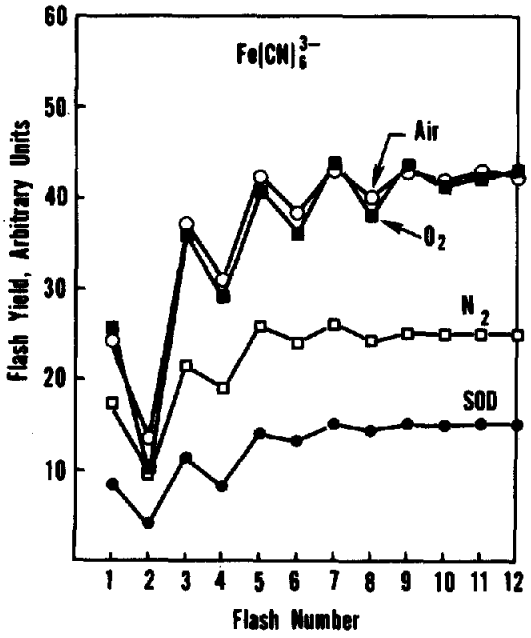

Fig.3. A plot of the maximum yield per flash versus flash number from chloroplasts dark adapted for 5 min on an electrode with the platinum bias 250 mV positive with respect to the Ag/AgCl junction. For all cases, 0.5 mM $Fe(CN)_6^{3-}$ was present with the buffers equilibrated with (o–o–o) air, (■–■–■) O_2, (□–□–□) N_2 or (●–●–●) air and SOD added.

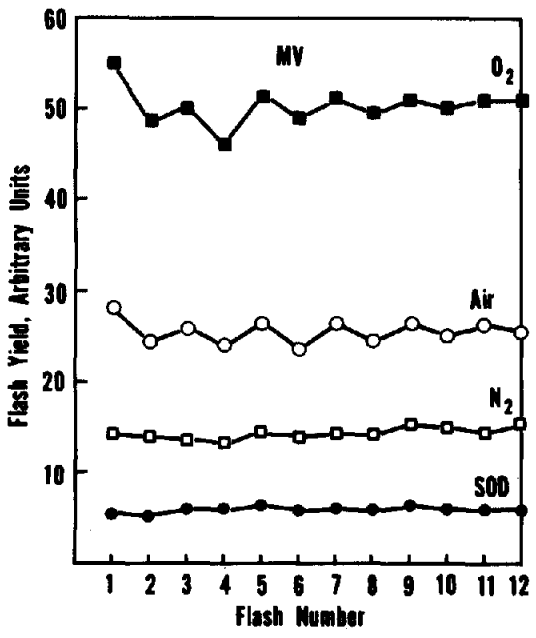

Fig.4. Same conditions as in fig.3 except 5 mM MV was present in all samples instead of 0.5 mM $Fe(CN)_6^{3-}$.

eliminated when SOD is present. The presence of SOD decreases the positive bias signal size as can be seen in fig.3, 4.

4. Discussion

The signals described here correspond to electrons transferred after flash illumination to a platinum electrode biased positive by a few hundred mV with respect to an Ag/AgCl junction. The observation of these positive bias signals is not unique and has been reported [7], but only with MV as an acceptor. It was also suggested [7] that MV interacted directly with the platinum electrode. Data presented here demonstrate that $Fe(CN)_6^{3-}$ can also be used as an acceptor when observing these signals and that these signals are dependent upon photosystem I activity. More specifically, it is demonstrated that these signals originate from O_2^-, not from the reduced acceptor directly, based on interpretations of experiments at high and low oxygen tension and with SOD present.

The results with air, O_2 and N_2 equilibrated buffers need further comment. The residual positive bias signal seen in fig.3, 4 under N_2 conditions is believed due to small amounts of oxygen, which unavoidably reached the sample when it was transferred and loaded onto the electrode in air. Also, it appears that the O_2 content of air is sufficient to saturate the O_2 generating reaction with $Fe(CN)_6^{3-}$ as an acceptor (fig.3) but not with MV (fig.4).

$Fe(CN)_6^{3-}$ acting as an intermediate in O_2^- production and giving rise to the positive bias signal at approx. one-half the amplitude of that produced by MV (fig.2) is somewhat surprising, since $Fe(CN)_6^{4-}$ is less readily autoxidized than MV_{red} [1]. There is evidence that in chloroplast [18] ferrocyanide is oxidized to some extent, presumably by oxygen. Nevertheless, in these experiments, oxygen is more efficiently reduced to O_2^- by $Fe(CN)_6^{4-}$ than directly by the photosystem I primary acceptor, X^-. Presumably, the small signal observed when no acceptors are

present (fig.2) is due to the direct interaction of O_2 with X^-, which is known to be quite slow [5].

One of the most intriguing aspects of the positive bias signal is its oscillatory behavior. It is believed that this signal monitors the period-of-two gating of electron flow between photosystems I and II by an electron carrier 'B' [19,20] after Q, the primary acceptor of photosystem II. The amplitude of the positive bias signal reflects the concentration of X^- generated during the flash, which is dependent on the amount of reduced *P*-700 that exists prior to the flash and consequently the number of electrons that move between photosystems II and I. The oscillations are more pronounced with $Fe(CN)_6^{3-}$ as an acceptor (fig.2) since, with its 420 mV reduction potential, it oxidizes the intersystem pool in the dark. The large amplitude signal resulting from the first flash (fig.1) means that $Fe(CN)_6^{3-}$ does not oxidize *P*-700 to any great extent under these conditions. With MV as the acceptor, small amplitude oscillations are still observed and are due to partial oxidation of the intersystem carrier pool in the dark by oxygen [21]. This would also explain the loss of oscillatory behavior under high N_2 tension with MV as an acceptor (fig.4), but not when $Fe(CN)_6^{3-}$ was an acceptor. The suggestion that oscillations of these signals are due to B is also supported by the lack of oscillations when DCPIP and ascorbate are used to donate electrons to photosystem I (fig.2) without going through B. SOD also appeared to eliminate the oscillations when MV was the acceptor (fig.4) but not when $Fe(CN)_6^{3-}$ was the acceptor (fig.3). It is believed that when MV is an acceptor and SOD is present the oscillations are not resolvable due to the small signal size.

Another difference between the positive bias signal when MV or $Fe(CN)_6^{3-}$ is the acceptor is that the MV signals tend to decrease while the $Fe(CN)_6^{3-}$ signals increase (fig.2). The increase of the $Fe(CN)_6^{3-}$ signal can be explained by the intersystem pool being largely oxidized in the dark by $Fe(CN)_6^{3-}$, and the intersystem becoming progressively reduced with flash number. This occurs due to the variation in numbers of photosystem I and II reaction centers that turn over on any flash. For flashes 2, 4 and 6, more photosystem II reaction centers than photosystem I reaction centers turn over, thus causing the intersystem pool to become reduced. With MV as the acceptor, it appears that more photosystem I reaction centers turn over than photosystem II reaction centers. Perhaps, with a reduced intersystem pool prior to illumination (the condition with MV as an acceptor), the photosystem II activity is reduced compared to photosystem I and what is being seen here is a demonstration of the spillover mechanism proposed in [22]. Work is now being carried out to further test this hypothesis.

A variety of methods have now been used to detect the cycle-of-two gating of electrons between the 2 photosystems including: polarographic techniques [19], chlorophyll *a* fluorescence [20], and absorption spectroscopy at 700 nm [23] and 320 nm [24]. The polarographic method reported here is different from that in [19], since the signals are detected after individual excitation flashes, while electron flow between photosystems I and II was measured [19] by illuminating with modulated far red light after different numbers of preillumination flashes. Further study of the phenomena reported here is now underway, and this technique is being used to understand the control of electron flow between the 2 photosystems.

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