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Voltammetric detection of superoxide production by photosystem II

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Abstract Oxygen radicals play both pathological and physiological roles in biological systems. The detection of such radicals is difficult due to their transient nature and the presence of highly efficient antioxidant mechanisms. In plants the physiological role of oxygen is twofold, oxygen is produced by the oxidation of water and consumed as an electron acceptor. The direct involvement of oxygen in photosynthetic events exposes the photosynthetic apparatus to a high probability of damage by oxygen radicals. We report here a direct, simple and rapid method for the measurement of superoxide in vitro based on voltammetric detection. It has potential applications for other in vitro systems investigating superoxide production. We show that in addition to the well established production of superoxide from photosystem I, under reducing conditions superoxide is also produced by photosystem II, probably from the Q_A site.

Key words: Superoxide; Thylakoid; Photosystem II; Mehler reaction; Quinone

1. Introduction

The generation of oxygen radicals is an intrinsic part of aerobic metabolism. Oxygen radicals play a paradoxical role in biological systems [1]. Such radicals are well known agents of destruction in the diseased state or during stress conditions, yet they also play an important role in coordinating the cellular response to such stresses [2–4]. Superoxide has been implicated in a variety of cellular processes, from aging and cancer to viral pathogenesis and inflammation [4–8]. It has also been implicated in the response of plants to environmental stress [3,9–12]. Detection of superoxide has proved difficult due to its transient nature and the presence of the superoxide scavenging enzyme SOD, as well as low molecular weight antioxidants such as ascorbate, phenols, and nitric oxide. Most detection methods have involved reactions with intermediate compounds that are not always specific for superoxide. ESR spin trapping methods have been used [9] but are not suitable in many systems due to side reactions and instability of the spin trap. Other measurements include the detection of stable intermediates, radical attack on biological molecules and enzyme-linked assays [3,13–17].

The direct involvement of oxygen in photosynthesis exposes the photosynthetic apparatus to a high probability of damage by oxygen radicals. To counter this, photosynthetic oxygenic organisms have well developed mechanisms for dealing with oxidative stress [15,18,19]. The physiological role of oxygen in photosynthesis is twofold. The oxidation of water to molecular oxygen is the source of reducing power for photosynthetic assimilation. In addition, reduction of oxygen can occur in the Mehler reaction, and it has been suggested that this could serve as a mechanism for dissipating excess reducing power in a controlled manner [18,20–23]. Oxygen is able to serve as an electron acceptor from photosystem I [24] and it is well known that superoxide radicals are generated by this reaction [25]. However, the physiological role of the Mehler reaction in vivo remains controversial [18,20–23,26,27].

Due to the pervasiveness of oxygen and its reactive nature, it is inevitable that it is also involved in damaging responses when energy dissipation by the plant is restricted or the system is perturbed. It has been suggested that oxygen radicals are involved in some kinds of photoinhibition [28,29], particularly in the turnover of the D1 protein of PSII [30]. The oxygen evolving complex has been shown to produce H_2O_2 when disrupted by removal of extrinsic proteins or detergent treatment [31,32]. The production of oxygen radicals has also been demonstrated on the acceptor side of photosystem II [33–35] during strong illumination.

In order to clarify the role of superoxide in photosynthesis, here we present a novel method for the detection of this radical and show that it is produced on the acceptor side of photosystem II, probably at the Q_A site.

2. Materials and methods

Peas (*Pisum sativum* cv. 'Greenfeast') were grown in a growth chamber at a constant temperature of 22°C and 700 μmol quanta m^2 s^{-1} for 12 h per day. Thylakoids were isolated [36] from 3 week old leaves. Oxygen consumption was measured polarographically, using 1 mM methyl viologen (MV) as an electron acceptor, in a specially constructed, water jacketed chamber which housed an oxygen electrode (Rank Bros, Bottisham, UK), a glassy carbon, a platinum and an $Ag/AgCl_2$ electrode. Ferricyanide interacted directly with this electrode configuration and therefore was not a suitable electron acceptor

Attempts to monitor superoxide using polarographic techniques have not been successful [37,38]. We have employed a voltammetric technique initially developed to measure the redox state of the ubiquinone pool in mitochondria [39–41] and the plastoquinone pool in thylakoids [42] to measure superoxide. A glassy carbon working electrode was poised at a potential of +360 mV with respect to a $Ag/AgCl_2$ reference electrode and connected to a platinum auxiliary electrode via the reaction medium, this configuration being referred to as the 'Q' electrode [42]. Current flow was measured between the working and auxiliary electrodes and output as a voltage to a recorder. For further details of the system see Cleland [42]. Thylakoids were assayed

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Abbreviations: cyt *bf*, cytochrome *bf* complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DNP-INT, 2-iodo-2′,4′,4′-trinitro-3-methyl-6-isopropyl diphenyl ether; MV, methyl viologen; PQ, plastoquinone 9; PSI-sox, superoxide produced by photosystem I; PSII-sox, superoxide produced by photosystem II; Q_A, primary quinone electron acceptor of PSII; Q_B, secondary quinone electron acceptor of PSII; Q_i, quinone reducing site on cyt *bf* complex; Q_o, quinol oxidizing site on cyt *bf* complex; SOD, superoxide dismutase

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in 50 mM Tricine pH 7.8, 5 mM $MgCl_2$, 5 mM KH_2PO_4, at a Chl concentration of 12 μM. The actinic light intensity was 1000 μmol quanta m^{-2} s^{-1}. A superoxide generating system was used to calibrate the Q signal using 20 μM riboflavin as a photosensitizer and 200 μM TEMED as an electron donor [43,44]. Superoxide dismutase (SOD, EC 1.15.1.1) was obtained from Sigma and used at a concentration of 60 U ml^{-1}. DNP-INT was a kind gift of Prof. A. Trebst.

To monitor electron transport through PSI, the absorbance change at 700 nm was measured during continuous illumination with far red light (702 nm) using repetitive laser flashes at 680 nm. The first-order rate constant for P_{700} rereduction was calculated by taking an exponential fit of the data. Chl concentration was 50 μM. Measurement of cytochrome *b* was carried out as described by Chow and Hope [45].

3. Results

3.1. Detection of superoxide by the Q electrode

The response of the Q electrode to superoxide formation was initially determined using riboflavin/TEMED as a chemical means to generate superoxide [43,44]. Illumination of a solution containing riboflavin as a photosensitizer and TEMED as an electron donor resulted in a rapid rise of the signal to a steady state level (Fig. 1). Concurrently, a small rate of oxygen uptake was observed, indicative of the reduction of oxygen to superoxide. The omission of either riboflavin (Fig. 1) or TEMED (data not shown) from the assay buffer in the electrode chamber, or during illumination, prevented signal formation. The addition of SOD completely suppressed the signal (Fig. 1 and Table 1), but had no effect on the rate of O_2 uptake, confirming that the signal detected by the Q electrode is due to the formation of superoxide. Returning the sample to darkness also resulted in loss of the signal (data not shown). Increasing the irradiance increased the size of the Q signal concomitant with an increase in the rate of oxygen consumption (Fig. 2). There was a linear relationship between these two parameters (Fig. 2, inset). The Q electrode therefore responded in a linear manner to superoxide formation over the concentrations measured, and detected any change in the concentration of superoxide. The xanthine/xanthine oxidase system for generating superoxide could not be used with the Q electrode because the product of the reaction, uric acid, also interacted with the electrode and produced a signal.

The redox potential of O_2/O_2^- couple is −330 mV [46] under standard conditions of 1 atmosphere. NADH, which has a similar redox potential, can also be detected by the Q electrode (J. Wiskich, personal communication). In solution the redox potential of O_2/O_2^- couple is −160 mV [47]. Therefore, it is not surprising that the Q electrode, which is poised at a potential of +360 mV relative to the reference electrode, was able to detect the superoxide radical.

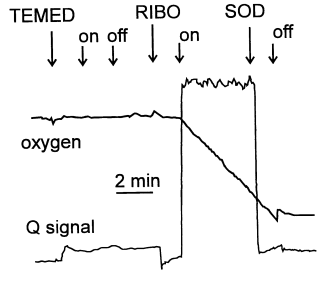

Fig. 1. Simultaneous measurement of oxygen uptake and the signal from the Q electrode, arising from superoxide production induced by light in the presence of riboflavin as the photosensitizer and TEMED as the electron donor, and the effect of SOD on the signal.

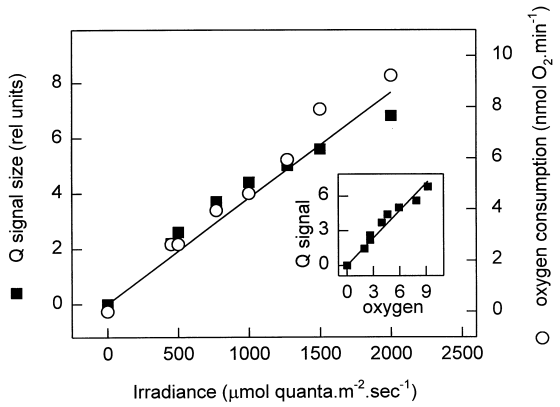

Fig. 2. The response of oxygen uptake (○) and the Q signal (■) to increasing irradiance; $r^2 = 0.98$. Inset: the relationship between the Q signal and oxygen uptake; $r^2 = 0.96$.

3.2. Superoxide formation by thylakoids

Illumination of isolated thylakoids in the presence of MV as an electron acceptor resulted in a rapid increase in the signal detected by the Q electrode (AFig. 3). This was paralleled by a constant rate of oxygen uptake. The signal declined slightly during continuous illumination, possibly due to the presence of residual antioxidants in the thylakoids. The addition of SOD completely suppressed the Q signal, although the rate of oxygen uptake was unaffected. The sensitivity of the Q signal to SOD suggests that the signal arises from the formation of superoxide in the thylakoids. The reduction of MV is known to generate superoxide radicals at PSI [25,48], and this was detected by the Q electrode. Addition of an uncoupler caused an increase in the rate of oxygen uptake and an increase in the Q signal (data not shown).

3.3. Superoxide formation from sites upstream of PSI

The Q signal and oxygen evolution were both abolished by the photosystem II inhibitor DCMU (Table 1), indicating that the signal arose as a result of photosynthetic electron transport. DNP-INT is a non-redox active inhibitor of the cytochrome *bf* complex that inhibits turnover of the complex by binding to the quinol reducing site [49–51]. The Q signal was not completely abolished by DNP-INT, although oxygen uptake was completely inhibited and, in its place, oxygen evolution was observed (Fig. 3B). The latter was attributed to residual catalase present in the thylakoid preparations since it was inhibited by azide (data not shown). Evidence that O_2

Table 1
The size of the Q signal before and after various additions (2.5 μM DNP-INT, 5 μM DCMU)

	Light	Dark	+SOD	+DCMU	+DNP-INT
Thylakoids	7.7	0	0	0	1.15
Ribo/TEMED	3.0	0	0	–	–

The Q signal is expressed in relative units. The rate of oxygen uptake was 275 μmol O_2 consumed mg chl^{-1} h^{-1} for thylakoids and 11.182 μmol O_2 consumed min^{-1} for the riboflavin/TEMED system.

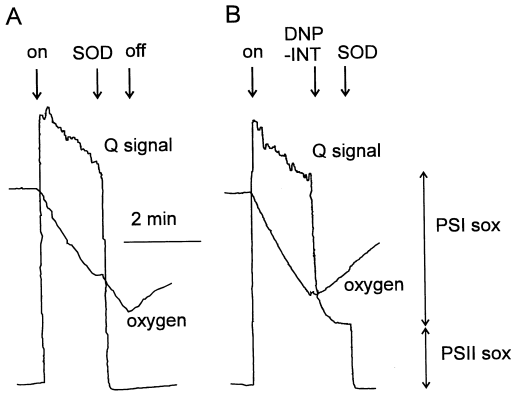

Fig. 3. A:) The response of isolated thylakoids to actinic light and the effect of SOD, measured as oxygen consumption and the signal from the Q electrode. The rate of O_2 consumption using 1 mM MV as an electron acceptor was 273 μmol O_2 mg chl^{-1} h^{-1}, the light intensity was 1000 μmol quanta m^2 s^{-1}. B: The response of isolated thylakoids to actinic light and the effect of 2.5 μM DNP-INT, measured as oxygen consumption and the signal from the Q electrode. PSI-sox refers to superoxide generated by PSI; PSII-sox refers to DNP-INT insensitive superoxide production which is primarily PSII generated (see text).

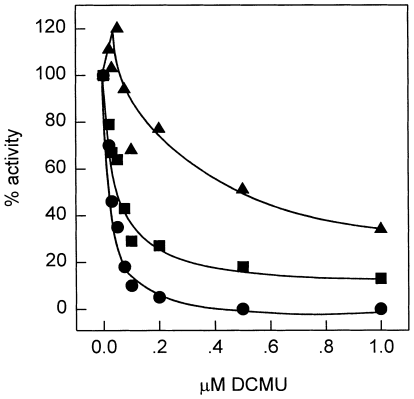

Fig. 5. The effect of increasing concentrations of DCMU on oxygen uptake (●), superoxide production attributed to PSI (PSI-sox ■) and DNP-INT insensitive superoxide production attributed to PSII (PSII-sox ▲). Activity is expressed as a percentage of the control measured in the absence of any inhibitors. 100% oxygen uptake = 245 μmol O_2 mg chl^{-1} h^{-1}; 100% PSI-sox = 6.2 rel units; 100% PSII-sox = 1.2 rel units.

photoreduction persists in the presence of DNP-INT was also obtained in studies of the superoxide-dependent oxidation of epinephrine [17] and light-driven formation of the ascorbate radical in isolated thylakoids [52]. That DNP-INT did not completely abolish the Q signal suggests that there may be a site of superoxide production prior to the binding site of this inhibitor, i.e. at or before the Q_o site on the cyt *bf* complex. The superoxide signal produced by thylakoids can therefore be divided into two components. The DNP-INT sensitive component, designated PSI-sox, arises from PSI, while the DNP-INT insensitive component, designated PSII-sox, may arise, at least partially, from PSII.

To determine if the superoxide produced in the presence of DNP-INT was due to leakage of electrons to P_{700} through the DNP-INT block, we measured the rereduction kinetics of P_{700} (Fig. 4). Titration of the rate constant for P_{700} rereduction with DNP-INT showed that at a concentration of 1.33 μM only 6% of PSI activity remained. In contrast, the Q signal remaining in the presence of DNP-INT was 31% of the original signal size (Fig. 3). Therefore it is unlikely that superoxide produced in the presence of DNP-INT arises from PSI. Measurement of cytochrome *b* turnover by the absorbance change at 563 nm showed incomplete oxidation and total inhibition of rereduction in the presence of DNP-INT, indicating the absence of Q cycle operation (data not shown).

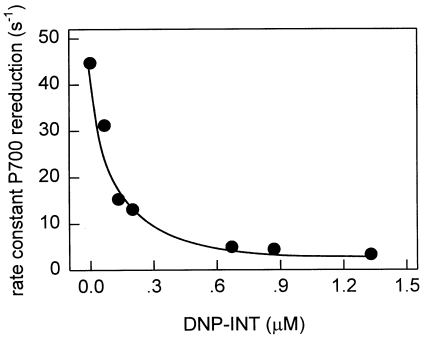

Fig. 4. The effect of increasing concentrations of DNP-INT on the rate constant for P_{700} rereduction.

The site of superoxide production prior to PSI was explored by titrating the Q signal and the rate of oxygen uptake with DCMU. PSI-dependent superoxide production and oxygen uptake showed a similar inhibition pattern with increasing concentrations of DCMU (Fig. 5). However, PSII-dependent superoxide production showed quite a different response. Low concentrations of DCMU (less than 0.1 μM) caused a slight increase in PSII-sox whereas higher concentrations had an inhibitory effect. However, the PSII superoxide production was inhibited to a lesser extent than PSI superoxide production or oxygen evolution. This suggests that the site of PSII superoxide production may be Q_A^- (see Section 4). However, we cannot rule out that DNP-INT itself induces the formation of superoxide by causing over-reduction of the PQ pool. DNP-INT has been shown to cause reduction of the PQ pool [42].

4. Discussion

We have shown that the Q electrode can be used to directly measure superoxide production in isolated thylakoids. This method has significant advantages over more commonly used spectrophotometric or ESR detection methods since it is simple, rapid and involves no intermediate compounds. It has potential applications for other in vitro systems investigating superoxide production. However, some caution must be exercised since a number of biological compounds with reducing properties (e.g. ascorbate, NAD(P)H, ferredoxin) can react directly with Q electrode.

In thylakoids the predominant site of superoxide production is PSI. This site is operative in vivo when other electron acceptors such as CO_2 are limited. Here we show that there is a second site for superoxide production outside PSI. The most likely candidate for this site of superoxide production is plastosemiquinone. Semiquinones, particularly those derived from

compounds with the hydroquinone structure, react rapidly with molecular oxygen, with second order rate constants above 10^7 M^{-1} s^{-1} [53]. In mitochondria ubisemiquinone has been shown to be a source of electrons for superoxide formation [54].

There are four sites of plastoquinol/plastosemiquinone formation that are possible sites for superoxide production. These are Q_A^-, Q_B^-, the PQH_2 pool and plastosemiquinone formed during the Q cycle. We favor Q_A^- as the site of superoxide formation. The apparent increase in PSII superoxide production associated with low concentrations of DCMU (Fig. 5) suggests that the plastosemiquinone generating superoxide may be Q_A^-. This can be explained in the following manner: the presence of low concentrations of DCMU restricts electron flow out of Q_A, causing further reduction, over and above the restriction already imposed by DNP-INT. This causes an increase in superoxide formation to dissipate the accumulating excitation energy. The inhibition of PSII-sox production at higher concentrations of DCMU may be caused by damage within PSII induced by the superoxide itself.

It is unlikely that Q_B^- is the site of superoxide production because DCMU binds to that site, effectively displacing the quinone. The presence of low concentrations of DCMU therefore would not be expected to cause an increase in superoxide production if the radical were generated at this site. Plastoquinol is only slowly oxidized by oxygen in the dark [42,55–57] with a $t_{1/2}$ of over 1 min. In contrast, superoxide production in the thylakoid system measured with the Q electrode was instantaneous with the onset of illumination. Plastoquinol is therefore unlikely to be the site of superoxide production observed here. The other possible site of superoxide generation is the quinol oxidizing site, Q_o, on the cyt *bf* complex. Cytochrome *b* has been suggested to be the site of H_2O_2 production in mitochondria [58]. DNP-INT binds to this region of the cyt *bf* complex. Our measurements showed an inhibition of cyt *b* rereduction in the presence of DNP-INT, therefore it is unlikely that any plastosemiquinone is formed either at the Q_o or Q_i sites. It is also difficult to explain how low concentrations of DCMU would induce an increase in superoxide production from this site, given that any such effect must be transmitted through the PQ pool.

In summary, we have demonstrated the formation of superoxide in isolated thylakoids by a novel voltammetric method. The results presented here suggest that when the photosynthetic electron transport chain becomes over-reduced, superoxide production may be generated at the Q_A site on PSII. The implications for PSII-driven formation of reactive oxygen species in vivo, and its possible role in photoinhibition, remain to be explored.

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