Biochimica et Biophysica Acta, 546 (1979) 175—182

BBA Report

BBA 41314

ANOMALOUS OXYGEN UPTAKE FROM ISOLATED CHLOROPLASTS INHIBITED IN PHOTOSYSTEM II AND WITHOUT EXTERNAL ELECTRON DONORS

AVNER MUALLEM and SHMUEL MALKIN

Biochemistry Department, The Weizmann Institute of Science, Rehovot (Israel)

(Received September 5th, 1978)

Key words: Oxygen uptake; Oxygen electrode; Superoxide ion-radical; Photosystem II; Electron donor; (Chloroplast)

Summary

Light induced modulated signal of oxygen uptake by isolated chloroplasts in the presence of methyl viologen, when Photosystem II activity was inhibited and in the absence of any electron donors, was detected by a modulated oxygen Pt electrode, polarized negatively. Evidence is brought to show that an electrochemical process which takes place on the surface of the negatively polarized Pt-cathode produces an intermediate which serves as an electron donor to Photosystem I. Attempts to identify this intermediate show that it may be very probably the superoxide radical generated by the electrochemical reduction of oxygen which continuously diffuses from the external circulating medium to the electrode.

The modulated oxygen electrode [1—3] was used by us to measure oxygen uptake signals from isolated chloroplasts in the presence of methyl viologen as mediator [4]. In these measurements the oxygen evolution signal is largely nullified by proper phase adjustment of the reference signal to the lock-in amplifier prior to the addition of the methyl viologen; such adjustment causes the oxygen evolution signal to be orthogonal to the reference. The addition of methyl viologen to the chloroplasts, under such conditions, produces a signal which has the characteristics of pure oxygen uptake.

While working on the general properties of such a system we noticed a puzzling effect that oxygen uptake could persist for quite a long time even in the presence of DCMU, which cuts the supply of electrons from Photosys-

Abbreviations: PS I and PS II, Photosystem I and Photosystem II, respectively; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

tem II. We then noticed that similar reports exists in the literature: Fork already noted a similar effect with a d.c. (unmodulated) polarographic system using DCMU-inhibited Swiss-chard chloroplasts [5] or chloroplasts from red algae from which phycobilin pigments had been removed [6], but he attributed this effect to oxygen uptake prompted by an endogenous electron donor. Recently Kleinen Hammans et al. [7] using a modulated polarographic system, measured oxygen-uptake induced by illuminated whole cells of *Anacystis nidulans* (a blue-green alga) which were inhibited by DCMU. They also related this reaction to the existence of native reductants.

Using the modulated polarographic method we show that the Pt cathode, when polarized in the adequate potential for polarographic measurements of oxygen, can act as an electron donor for photoreduction of oxygen to chloroplasts in which Photosystem II was inhibited.

The modulated electrode was similar as described in [2]. Lettuce chloroplasts were prepared as in [8] and were stored usually at liquid-air temperature [9]. Fresh chloroplasts behaved very similarly. A drop of chloroplasts (~ 4 mg/ml chlorophyll) was placed on the Pt electrode forming a thin layer (~ 0.1 mm) then covered by a dialysis membrane. The other side of the membrane was in contact with a circulating buffer medium, containing usually 30 mM tricine (pH 7.3) and 100 mM KCl at a flow rate of a few ml/min. Concentrated solutions of methyl viologen, DCMU, or H_2O_2 were added to the circulating medium as the experiment required.

Two light sources were used: modulated light (a.l.), and constant direct light (d.l.) which was used as a background light of high intensity. Cut-off filters (> 450—500 nm) were used for spectral definition. A lock-in amplifier (PAR-128A) was used to measure and record (time resolution ~ 0.3 s) the amplitude and phase of the polarographic output signals. A reference signal was provided by diverting a small part of the modulated light to a photoelectric transducer.

Under conditions when the oxygen evolution was inhibited it was possible to determine the amplitude and phase of the uptake signal: in the steady-state, by maximizing the signal through the phase-shifter on the lock-in amplifier; in the transient by recording the in-phase and quadrature components (relative to the steady-state), separately.

Fig. 1 illustrates a typical experiment: Isolated chloroplasts with oxygen as the sole acceptor [5] give rise simultaneously to light-induced oxygen evolution from Photosystem II coupled with oxygen uptake from Photosystem I. In constant (d.c.) light the uptake ($O_2 \rightarrow H_2O_2$) rate usually exceeds the evolution ($H_2O \rightarrow O_2$) rate by a factor of 2 yielding a net O_2 exchange in the direction of uptake. The modulated signal, however, does not have any obligatory relations between evolution and uptake (since its average in time is always zero) and as the frequency increases the uptake signal is much more damped compared to the evolution. At high frequency (> 5 c.p.s.) the net a.c. signal is largely due to O_2 evolution (Fig. 1 left hand side; light on). This signal decayed ultimately in about 15—20 min (Fig. 1. dashed curve), due to the loss of PS II activity (cf. later). Such decay was also observed when methyl viologen was present initially and its photoreduction was directly monitored as a function of time with a positively polarized [2] Pt electrode.

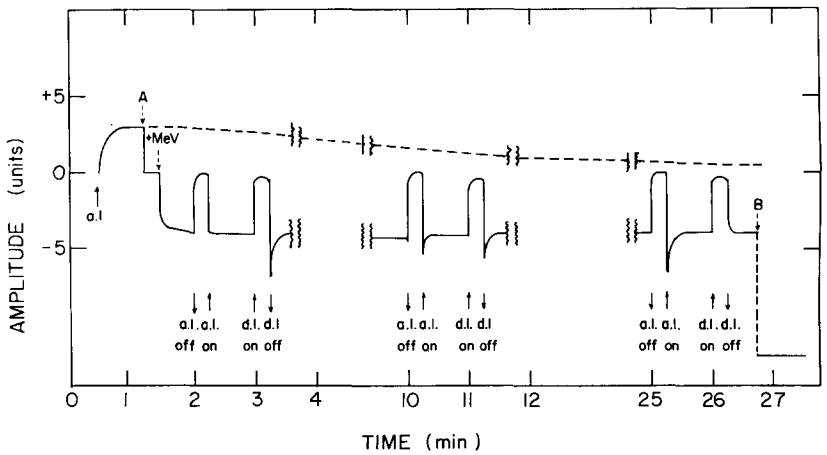

Fig.1. Signals from a typical experiment. Chloroplasts with no added electron donors or acceptors show mainly O_2 evolution signals upon a.l. excitation (left hand side). This signal decays gradually with time (dashed curve). At point A the O_2 evolution signal is nullified by proper phase-shift of the reference. Upon addition of methyl viologen (MeV) O_2 uptake signals are obtained. At the right hand-side, where the O_2 evolution signal has died off, a phase adjustment (at point B) shows the maximal amplitude of the uptake signal. The transients upon switching on and off the a.l. and d.l. lights are explained in the text. The concentration of methyl viologen was 10^{-4}M; The modulation frequency was 12 Hz and the Pt electrode potential was set to −0.65 vs. saturated calomel electrode.

The experiment was now repeated with fresh chloroplasts; the reference adjusted by phase rotation to nullify the output (point A in Fig.2) by bringing the signal into quadrature position.

Addition of methyl viologen, which greatly enhances oxygen uptake, caused the appearance of a negative signal, representing mainly oxygen uptake. The

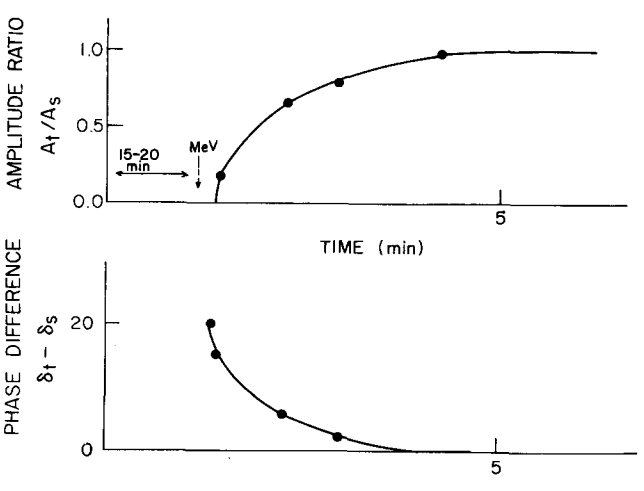

Fig.2. The development of the a.c. uptake vector (amplitude and phase) after the addition of MeV (10^{-4}M) to the circulating medium. DCMU ($2 \cdot 10^{-5}$M) inhibited chloroplasts. A_t, δ_t: A_s, δ_s amplitude and phase as a function of time before steady level is reached and at the steady state, respectively. Other conditions as in Fig.1.

signal indeed showed characteristic behavior expected from PS I, when PS II serves as an electron donor. Thus, when strong background d.l. light was applied the signal was inhibited (presumably due to the complete closure of PS I reaction centers by *P*-700 oxidation). When the d.l. light was stopped there was first an overshoot to a larger signal, decaying ultimately back to the steady-state. This is consistent with the transitory full reduction of *P*-700 (full opening of the reaction centers), which occurred immediately after the strong light had been switched off. The reducing power originated in this case from the pool of electron carriers located between the two photosystems, which had been fully photoreduced by PS II in the strong light. The a.l. light eventually slowly oxidized part of the *P*-700, until a steady-state was reached. This effect of overshoot decayed, signaling a gradual inhibition of PS II (Fig.1, middle and right). In parallel, another effect was developed: When the a.l. light was first turned off and then resumed again, an overshoot to large signal occurs (Fig.1, middle). This indicates that for such "aged" chloroplasts reaction centers can be now restored by a dark reaction but not by a light reaction (i.e. after d.l.), thus allowing a transitory large signal before a steady-state is achieved.

An oxygen uptake signal, completely similar to the one observed for "aged" chloroplasts was observed also from chloroplasts which were inhibited either by DCMU or mild heating. This signal occurred when methyl viologen was added to the medium and with no addition of any apparent exogenous donor. Fig. 2 shows the development of such an "anomalous" O_2 uptake signal upon addition of methyl viologen. The slow rise of the amplitude and the correlated decrease of the phase delay reflect the diffusion from the circulating medium. Control experiments with denatured chloroplasts (by acidification or strong heating) showed no output signal, which proved that the uptake signal must be attributed to the activity of the chloroplasts. Comparison of a rough action spectrum of this uptake signal with the corresponding absorption spectrum (Fig.3) indicates that Photosystem I pigments alone are involved.

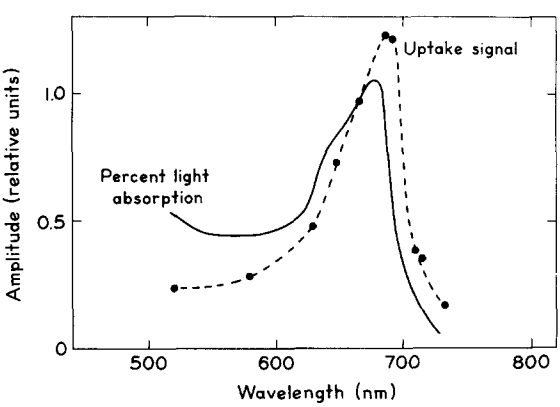

Fig.3. Action spectrum of O_2 uptake (signal/light intensity) in comparison with the absorption spectrum. ——, percent absorption; •- - -•, action spectrum. The action-spectrum points were obtained by the use of interference filters. The light intensity was measured by a calibrated silicon cell and the effect of intensity was checked to be in the linear range. Other conditions as in Fig.1.

TABLE I

COMPARISON OF MEASUREMENTS OF O_2 EXCHANGE AT NEGATIVE POLARIZATION POTENTIAL AND METHYL VIOLOGEN (MeV) REDUCTION AT POSITIVE POLARIZATION POTENTIAL

	O_2 uptake*	MeV reduction*
Active chloroplasts (PS II and PS I)	100	45
"Aged" chloroplasts (only PS I)	120	0

*Arbitrary units; the same sensitivities for both measurements.

Do chloroplasts contain unknown endogenous electron donors? Measuring methyl viologen reduction in positive potential [2] gave negative results. We were able, however, to monitor the reduction of methyl viologen in positive potential when Photosystem II was still active, or when artificial electron donors to System I were added to chloroplasts inhibited in Photosystem II (Table I). Also the steady level of the uptake signal could last for several hours, indicating an unreasonably large pool of electron donors. It was unavoidable to assume that the Pt cathode itself, when polarized properly (only in negative potential, Table I) serves as an intermediate in supplying electrons to PS I. One could think of the electrode as an "external electron donor", due to the electrochemical process which takes place on its surface. Fig.4 demonstrates, in a simplified way, the loop of electron transfer made by the chloroplasts, oxygen and products of oxygen cathodic reduction, one of which (E^-) serves as the active donor to the chloroplasts.

The following experiments show that the "anomalous" uptake signal responds to changes in the electrochemical or the surfacial conditions of the cathode. Fig.5 shows the difference between the polarogram of modulated "normal" signal of oxygen evolution from illuminated fresh chloroplasts, and the polarogram of the "anomalous" oxygen uptake produced by the same sample. The outstanding "abnormality" of the later appears in a region where the normal polarogram gives a plateau. There is a marked decrease of amplitude and a parallel increase in the phase-delay as the polarization potential is made more negative than about —0.525 V. Fig.6 shows how the signal

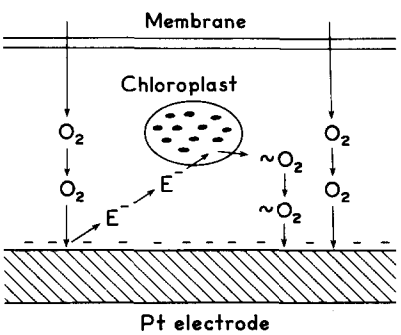

Fig.4. Schematic representation of electron flow involving chlororoplasts and the negative Pt electrode. The sign ~ indicates the modulated flow.

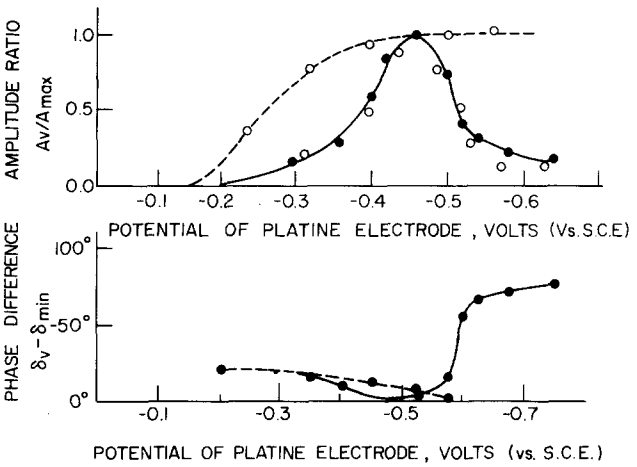

Fig.5. Amplitude and phase-lag (absolute value) of the a.c. O_2 uptake or evolution as a function of cathodic polarization voltage vs. saturated calomel electrode (S.C.E.). o- - -o, O_2 evolution in the absence of electron acceptors. •——•, O_2 uptake after inhibition by added DCMU and addition of methyl viologen. — o —, The same sample but with addition of H_2O_2 (10^{-4} M). A_v, δ_v, amplitude and phase. A_{max}, the maximum value of the amplitude. δ_{min}, the minimum phase lag obtained: For O_2 production $180° < \delta_{min} < 270°$; For O_2 uptake $0° < \delta_{min} < 90°$.

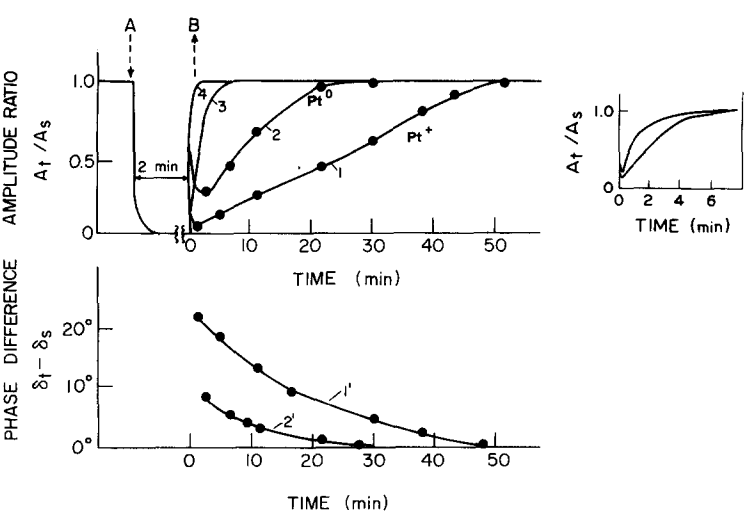

Fig.6. The time course of the uptake signal (measured at a polarization of —0.65 V after preanodization of the Pt electrode). 1,1', after 2 min anodization at +0.6 V; 2, 2', after 2 min anodization at 0.0 V; 3, after 2 min anodization at +0.6 V, but in the presence of H_2O_2 (10^{-4} M); 4, after 2 min anodization of PV=0.0 V, in the presence of H_2O_2. At point A the negative polarization potential was reversed to the proper anodic potential. At point B the negative polarization was resumed. Insert: curves 3 and 4 resolved in shorter time-scale.

depends on the previous polarization of Pt electrode prior to measurements. The experiment was started after achievement of steady-state uptake signal. The polarization of the electrode was changed to zero or positive voltage for 2 min and was resumed to the previous negative value (—0.65 V). Nearly the same previous steady-state level was again reached, even when this experiment was repeated several times. However, the time required to build up the steady-state level differed according to the value of the previous polarization potential and its duration. Curves 1 (1′) and 2 (2′) show that the build-up period after such anodization (+0.6 V) is quite long and significantly longer ($t_{1/2}$ about 25 min) than after polarization of the Pt in zero volts for the same period ($t_{1/2}$ about 7.5 min). A consideration of all possible reactions shows that this build-up time cannot be due to the accumulation of E^- per se and that it probably arises from changes in the electrode surface properties caused by changes in the polarization, which are such that the production of E^- is inhibited after anodization. (Examples of such surface changes are well known [10].)

A candidate for the role of E^- could be H_2O_2 (formed by electrochemical reduction of oxygen [10]. This possibility was eliminated: adding H_2O_2 externally through the circulating medium, we expected that a change of polarization in the manner of Fig.6 should result in an immediate response upon the return of the normal potential, which is not the case. The build-up time of the signal was considerably and dramatically shortened (Fig. 6, curves 3,4), but it was not as short as the response time of the instrument. Another evidence is based on the fact that no reduction of methyl viologen was detected in positive potential when H_2O_2 was added. Also, since the form of the polarogram in Fig. 5 is probably due to limitation in E^- (i.e. the decay at the high negative potential indicates slower E^- formation or faster E^- destruction by electrode processes), the fact that H_2O_2 addition does not change the shape of the polarogram (Fig.5) indicates that it cannot be identified as E^-.

Considering electrochemical processes which are known to occur one of them being the cathodic reduction of oxygen, it is almost unavoidable to consider the pair O_2^- or $HO_2\cdot$ for the role of E^-. $HO_2\cdot$ is relatively an unstable species but O_2^- is much more stable, having an apparent life time of many seconds [11]. The superoxide anion is formed electrolytically in detectable levels [12]. The assumption that O_2^- may interact with PS I can find support by the evidence [13, 14] that cytochrome *c* can be univalently reduced by this radical.

A rough estimation was made for the maximum range of concentration under our experimental conditions that the superoxide anion can reach in the chloroplasts chamber, by considering the rate of diffusion from the outer circulating medium of O_2 to the electrode, the bimolecular decay rate of O_2^- of $3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ [15] in the pH range of 7.3, the diffusion out to the circulating medium, assuming for simplicity that the efficiency of converting O_2 to O_2^- is 1 and that the electrochemical reduction rate of O_2 was limited by the diffusion of O_2. The range of superoxide anion concentration thus obtained was about $10^{-7}-10^{-5}$ M (depending on what one assumes about the diffusion rate within the enclosing dialysis membrane) which is quite a plausible value for the interaction with the chloroplasts.

We can speculate on the specific role of H_2O_2 in reducing the build-up time after anodization (Fig.6). Immediately after resumption of the negative polarization, O_2 reduction does not yield O_2^- in any significant quantity until the proper electrode surface is built. The presence of H_2O_2 furnishes a possible alternative way to form O_2^- starting with its electrochemical reduction, as in the following example [16, 17]:

$$H_2O_2 \xrightarrow{e^-} H_2O_2^- \longrightarrow \text{"}\cdot OH\text{"} + OH^- \quad (1)$$

$$\text{"}\cdot OH\text{"} + \text{"}H_2O_2\text{"} \longrightarrow H_2O + \text{"}HO_2\cdot\text{"} \quad (2)$$

(The quotation notation isused to indicate a possible participation of equivalent species, i.e. O^-, HO_2^- and O_2^-.)*

It appears, in conclusion, that in measurement with the oxygen electrode one should be aware of the possibility that electrochemical reactions on the electrode surface produce materials that interact with chloroplasts, an effect especially important in the rate-electrode, with its large surface/volume ratio.

Kinetically, the system is probably very complicated as one should bear in mind the possibility of oxygen "evolution" from the superoxide ion radical, when it donates its electrons, not to mention the usual complexities concerning the electron transfer in the chloroplast.

References

1 Joliot, P., Hoffnung, M. and Chabaud, R. (1966) J. Chim. Phys. 63, 1423—1441
2 Joliot, P. and Joliot, A. (1968) Biochim. Biophys. Acta 153, 625—634
3 Joliot, P., Joliot, A. and Kok, B. (1968) Biochim. Biophys. Acta 153, 635—652
4 Mehler, A.A. (1951) Arch. Biochem. Biophys. 33, 55—77
5 Fork, D.C. (1963) Plant Physiol. 38, 323—332
6 Fork, D.C. (1961) Carnegie Inst. Wash. Yearb. 60, 369—370
7 Kleinen Hammans, J.W., Hendriks, G.M. and Teerlink, R. (1977) Biochem. Biophys. Res. Commun. 74, 1560—1565
8 Avron, M. (1960) Biochim. Biophys. Acta 40, 257—272
9 Bekina, R.M. and Krashovsky, A.A. (1968) Biokhimiya, 178—179
10 Hoare, J.P. (1968) The Electrochemistry of Oxygen, Interscience, New York
11 Behar, D., Czapski, G., Rabani, J., Dorfman, L.M. and Schwartz, H.A. (1970) J. Phys. Chem. 74, 3209—3213
12 Forman, H.J. and Fridovich, I. (1972) Science 175, 339
13 McCord, J.M. and Fridovich, I. (1968) J. Biol. Chem. 243, 5753—5760
14 McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049—6055
15 Bielski, B.H.J. and Saito, E. (1971) J. Phys. Chem. 75, 2263—2266
16 Haber, F. and Weiss, J. (1934) Proc. R. Soc. A, 147, 332—351
17 Rabani, J. and Nielsen, S.O. (1969) J. Phys. Chem. 73, 3736—3744

*While these reactions occur at the surface of the electrode, there is in addition [16] a chain propagation mechanism, which involves the radicals in the solution: $\text{"}HO_2\text{"} + H_2O_2 \rightarrow O_2 + H_2O$, with the continuous regeneration of the $HO_2\cdot$ radical occurring by Reaction 2.