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Oxygen release time in leaf discs and thylakoids of peas and Photosystem II membrane fragments of spinach

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The half-time of O_2 release in *Chlorella*, thylakoids and Photosystem II membranes has generally been found to be 0.9 to 3.0 ms, but recently Plijter et al. (Biochim. Biophys. Acta 935 (1988) 299–311) reported it to be between 30 to 130 ms. This work presents a new method, using a Clark electrode, for measuring O_2 release times in leaf discs, thylakoids and Photosystem II membranes. The sample is illuminated with saturating pulses of light of 3 μs to 200 ms duration given at 5 Hz. Presuming the Kok model of four charge transfers per O_2 evolved, the O_2 release time could be calculated from the time needed to observe the evolution of one O_2 less 4-times the turnover time of the charge transfer reaction. Upper bounds for the half times of O_2 release were found to be 6, 11, and 5 ms in thylakoids, leaf discs, and Photosystem II membrane fragments, respectively. In thylakoids, using a bare platinum electrode operated under weak bias conditions, the half-time of O_2 signal rise is 2.7 ms. The rate of rise of this O_2 signal could be slowed by treating the thylakoids with nitrate or formate. The turnover time of the oxygen evolution system, the minimum time between evolution of oxygen molecules, of Photosystem II that is necessary to support light saturated photosynthesis was measured to be 16, 28 and 22 ms in thylakoids, leaf discs and Photosystem II membrane fragments, respectively. The rate limitation for photosynthesis is not the O_2 release time but is the rate of charge transfer through or beyond the plastoquinone pool.

Introduction

The photosynthetic mechanism for splitting water into protons, electrons, and oxygen is driven by the photoreactions that occur at Photosystem II (PS II) in the thylakoid membrane of chloroplasts. This process involves the photooxidation of a special chlorophyll (Chl) molecule, P680, which extracts electrons from water and donates electrons to Photosystem I (PS I) and, ultimately, carbon dioxide (for reviews, see Wydrzynski [1]; Amesz [2]).

The generally accepted kinetic model for oxygen evolution is that of Kok et al. [3], in which the oxygen system undergoes four photoreactions to pass through five different charged states (S_n):

$$S_0 \xrightarrow{h\nu} S_0' \longrightarrow S_1 \xrightarrow{h\nu} S_1' \longrightarrow S_2 \xrightarrow{h\nu} S_2' \longrightarrow S_3 \xrightarrow{h\nu} S_3' \longrightarrow S_4 \longrightarrow S_0 \quad (1)$$

$$2H_2O \quad O_2 + 4H^+$$

where the $S_n \xrightarrow{h\nu} S_n'$ steps represent phototransitions and the $S_n' \rightarrow S_{n+1}$ steps represent the subsequent charge transfer that occurs in the dark and must be completed before the next phototransition is possible. On the donor side of PS II, this charge-transfer reaction is $S_n\ Z^+\ P680 \rightarrow S_{n+1}\ Z\ P680$ where Z is a charge carrier and P680 is the reaction center Chl. On the acceptor side of PS II this is $P680\ Qa^-\ Qb \rightarrow P680\ Qa\ Qb^-$ where Qa and Qb are the primary and secondary quinone acceptors.

Kinetic measurements of the S-state transitions have been made by measuring oxygen evolution with flashes given under different temporal sequences. The $S_0' \rightarrow S_1$ had a half-time of 200 to 400 μs [4], $S_1' \rightarrow S_2$ had a half-time of 200 μs [3], $S_2' \rightarrow S_3$ was sigmoidal, with a

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 2(*N*-morpholino)ethanesulfonic acid; P680, reaction center chlorophyll of Photosystem II; PS II, photosystem II; Qa, primary quinone acceptor of Photosystem II; Qb, secondary quinone acceptor of Photosystem II, Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; Z, charge carrier between P680 and the oxygen-evolving complex.

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100 μs lag [4] and 400 μs [3] and slower [4] components. Based on these transition times, the underlying electron transport reactions that limit the transition can be identified. The $\bar{Q}a\ Qb \rightarrow Qa\ \dot{Q}b$ quinone electron-transfer reaction, which has 200 μs and 2 ms components [5,6], can limit any of these transitions. The $S_0\ Z^+ \rightarrow S_1\ Z$ and $S_1\ Z^+ \rightarrow S_2\ Z$ reactions take place in less than 100 μs [7] and are too rapid to be limiting. The $S_2\ Z^+ \rightarrow S_3\ Z$ has a 400 μs half-time [7] and can possibly limit the $S_2' \rightarrow S_3$ transition.

The kinetics of the $S_3 \rightarrow S_4 \rightarrow S_0$ reaction and the associated release of oxygen have been measured in a number of different ways by various research groups. Using a bare-platinum electrode illuminated with an amplitude-modulated light source, the amplitude and phase shift of oxygen release can be used to determine the kinetics of this reaction. In *Chlorella*, Joliot et al. [8] found the half time of this reaction to be 0.9 ms, while Sinclair and Arnason [9] reported a value of 2 ms. In spinach chloroplasts Arnason and Sinclair [10] observed a half-time of 3 ms. Using a bare-platinum electrode illuminated by a delayed xenon flash method, Bouges-Bocquet [4] found in spinach chloroplasts that this reaction was exponential with a half-time of 1.2 ms. Using an oxygen concentration electrode in a capillary flow apparatus, Etienne [11] found a half-time of 1.5 to 2.2 ms in *Chlorella*, depending on cell diameter. Oxidation of mitochondrial cytochrome *c* indicated an upper bound for the oxygen release half-time of 3 ms in *Chlorella* [12]. In leaf discs, using photobaric methods, flash-induced oxygen release was detected in less than 8 ms [13]. This release time included the diffusion of oxygen out of the leaf so the oxygen release time was less than 8 ms. Taking all of these methods into consideration, the half-time for oxygen release was found to be between 0.9 and 8 ms.

Recently, this kinetic picture has been challenged [14]. Using a bare-gold electrode with −148 mV polarization potential, the kinetics of the $S_3 \rightarrow S_4 \rightarrow S_0$ charge transfer reaction could be differentiated from the kinetics of oxygen release. The authors hypothesized that the following two-step reaction is required for oxygen evolution:

$$\begin{array}{lll} S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0 & \text{half-time 2 to 3 ms} & (2) \\ H_2O \quad\quad H_2O \quad\quad H_2O\ (\text{oxidized}) & & \\ S_n \longrightarrow S_n \longrightarrow S_n & \text{half-time 30 to 130 ms} & \\ H_2O\ (\text{oxidized}) & & \\ 1/2\,O_2 \quad H_2O & n = 0, 1, 2, 3 & \\ +2\,H^+ & & (3) \end{array}$$

where water is oxidized (Eqn. 2) in a reaction that is kinetically distinct from the release of oxygen and binding of water (Eqn. 3). The binding of the water is required before the reaction of Eqn. 2 can occur again. This hypothesis requires that the rate-limitation in photosynthesis be the oxygen-release step with a half-time of 30 to 130 ms.

In this work we describe a new method, using an oxygen concentration electrode, for measuring oxygen release times in leaf discs, thylakoid membranes, and PS II membrane fragments. Use of the concentration electrode avoids the low redox potential, H_2O_2 and OH^- production, and undefined diffusion times that take place at the cathode of a bare-platinum electrode, all of which may distort the kinetics of oxygen production. Maximum values for half-times of oxygen release are 11, 6 and 5 ms in leaf discs, thylakoids, and PS II membrane fragments, respectively. Also, in thylakoids, using a bare-platinum electrode, the oxygen signal half-time of rise is 2.7 ms. Clearly, these oxygen release times are not rate-limiting for photosynthesis.

Materials and Methods

Spinach (*Spinacia oleracea* L.) was purchased from the local market. Dwarf pea seedlings (*Pisum sativum* L. var. Wando) were grown in vermiculite-filled trays in a growth chamber (16 h day; 25/20 °C; 70 W/m^2 irradiance from a combination of cool-white fluorescent and incandescent lamps). The peas were harvested 18 to 21 days after germination. Thylakoids (broken chloroplasts) were isolated from leaves as described previously [15]. PS II membrane fragments were prepared by Triton X-100 washing of spinach thylakoids as previously described [16] for BBY-M particles. The reaction medium for thylakoids was 400 mM sucrose, 50 mM Tes, 10 mM NaCl, and 5 mM $MgCl_2$ at pH 7.5 and for PS II membrane fragments was 400 mM sucrose, 50 mM Mes, 10 mM NaCl, and 5 mM $MgCl_2$ at pH 6.5.

Oxygen evolution in thylakoids and PS II membrane fragments was measured with a concentration electrode (Clark electrode) in a 2 ml chamber surrounded by a water-jacket maintained thermostatically at 20 °C. Oxygen measurements of leaf discs were made with a Hansatech model LD2 leaf chamber and Clark electrode as previously described [17]. Pulsed-light excitation was provided by a 150 W tungsten halogen lamp, focused by a dichroic reflector and lens, and whose light path was interrupted by an electronic shutter (Uniblitz model 214L0A0W5HB) with a 0.8 ms opening time. Saturating pulses were given at 5 Hz. The shutter was driven by a laboratory-built pulse generator with variable pulse length. Flash excitation was provided by an EG&G model FX-200, xenon strobe lamp operated with 4.5 J (1500 V with 4 μF capacitance) input energy. The flashes had a duration of 5 μs at half intensity [18], were saturating, and given at 5 Hz.

Measurements of the kinetics of oxygen evolution by thylakoids following excitation by a xenon flash were

made with a bare-platinum electrode [19] of design similar to that of Joliot and Joliot [20]. The sample is applied directly on the platinum surface in a channel 1.5 × 15 mm and 0.18 mm deep. This channel is covered by a piece of dialysis membrane, which keeps the sample in the channel. Above the dialysis membrane is a 3 ml chamber filled with the following medium: 50 mM sodium phosphate and 100 mM NaCl at pH 7.5, unless noted otherwise. A ring of silver, the Ag|AgCl electrode, is located in the upper chamber in a position shielded from the excitation light. Flash excitation was provided by a General Radio Stroboslave 1539-A xenon flash. Signals were detected by a laboratory-built, AC-coupled transimpedence amplifier that had a 0.5 ms risetime. Analog signals were digitized with a Biomation 2805 waveform recorder and plotted for signal analysis as described in Appendix 2. The sample was incubated in the dark for 5 min on the electrode surface with the bias off. Bias was applied 10 s prior to the measurement. The AC-coupling eliminated signal drift that occurs during the first few minutes after application of the electrode bias. The platinum surface was scrubbed with a paste of $CaCO_3$ between each application of thylakoids to the electrode. This was necessary to maintain maximum signal size and most rapid signal kinetics. Bias voltages are given relative to the standard hydrogen electrode. The platinum was biased at −415 mV unless noted otherwise.

Chl *a* fluorescence was measured by the two-flash method [21]. Excitation light was provided by a General Radio Stroboslave 1539-A or the shuttered tungsten-halogen lamp described above passed through Corning CS 4-96 and CS 3-71 glass filters. The analytic flash was provided by a General Radio Stroboslave xenon flash through a Corning CS 4-96 glass filter and a 0.5% transmittant neutral density filter. Chl *a* fluorescence was detected by a Hamamatsu R928 photomultiplier that was shielded with a Corning CS 2-64 glass filter. The optical axis of the actinic and analytic flashes was at 90° to that of the photomultiplier. The analog output of the photomultiplier was digitized by a Biomation 2805 waveform recorder. Digitized data were transferred to a Hewlett Packard HP87 minicomputer, which had been programmed to determine peak heights.

Results

The present models for oxygen evolution require that PS II reaction centers undergo four charge separations and then molecular oxygen is released with a half-time of 2 to 3 ms, according to the model of Kok et al. [3], and 30 to 130 ms half-time, according to the model of Plijter et al. [14]. These very different kinetics were tested here by giving pulses of actinic light that were of saturating intensity and of sufficient duration to allow the PS II reaction center to undergo four or more charge separations.

For an actinic pulse of short duration compared to the recovery time of the reaction center after charge separation, each pulse will generate one charge separation and 0.25 oxygen molecules per PS II reaction center since four charge separations are needed for every oxygen evolved [3]. This is accomplished with a xenon flash and is indicated in Figs. 1–3 as a pulse of zero duration. As the pulse duration is increased and becomes comparable or greater than the PS II reaction center recovery time after charge separation, multiple charge separations and consequent S-state advances will occur. One oxygen molecule will be evolved per PS II reaction center per pulse when the pulse duration is equal to $t_{01} + t_{12} + t_{23} + t_{34}$ where t_{01}, t_{12}, t_{23} and t_{34} are the times to pass through the various S-states transitions ($S_0 \rightarrow S_1$, etc.). See Appendix 1 for a derivation of this equation and a description of this cyclic reaction and its individual steps. Diffusion time of oxygen across the thylakoid membrane or out of the leaf is not a problem with this method, since the diffusion time will be shorter than the 200 ms between illumination pulses. The oxygen release step will occur in the dark interval between pulses.

As the pulse duration of the actinic light is lengthened, more oxygen will be evolved per pulse. Two oxygen molecules will be evolved when the pulse duration is equal to $\Delta T(2) = 2(t_{01} + t_{12} + t_{23} + t_{34}) + t_{rel}$ where t_{rel} is the time for the release of molecular oxygen (see Appendix 1). The difference in the pulse duration times, $\Delta T(2) - \Delta T(1)$, for the release of two versus one oxygen molecule will be the following:

$$\Delta T(2) - \Delta T(1) = t_{01} + t_{12} + t_{23} + t_{34} + t_{rel} \quad (4)$$

or

$$t_{rel} = \Delta T(2) - \Delta T(1) - t_{01} - t_{12} - t_{23} - t_{34}$$

A difference in the t_{rel} time of 2.9 to 4.3 ms (2 to 3 ms half-time) in the model of Kok et al. [3] compared to 43 to 188 ms (30 to 130 ms half-time) in the model of Plijter et al. [14] will be easily observed.

Fig. 1 shows data for this type of experiment in pea thylakoids, using two different electron acceptor systems. The additional pulse duration required for 2 versus 1 oxygen evolved per PS II reaction center per pulse is 11 ms for ferricyanide and DCBQ and 22 ms for ferricyanide alone. Fig. 2 shows measurements on PS II membrane fragments and Fig. 3 for leaf discs from pea plants. The additional pulse duration for 2 vs. 1 oxygen per PS II reaction center per pulse in these cases is 12 and 18 ms, respectively. In Figs. 1 to 3, the slope decreases at pulse duration greater than 10 ms. This is

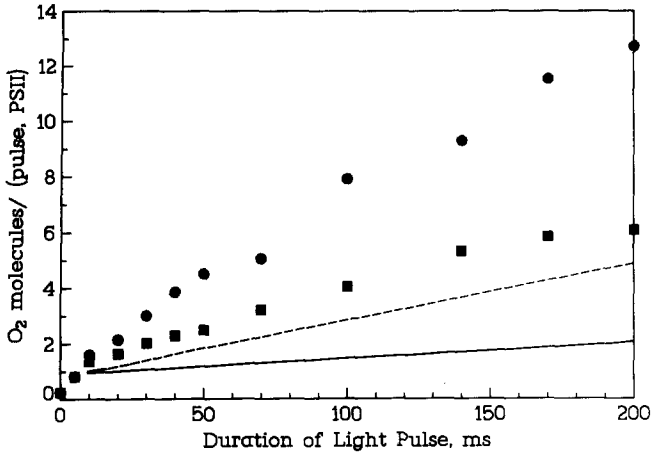

Fig. 1. Oxygen evolved per illumination pulse versus the duration of the pulse. Pea thylakoids at a concentration of 25 μg Chl/ml were measured with the electron acceptor system of 1 mM ferricyanide, 0.5 μM gramicidin, and 100 μM DCBQ (●) or no DCBQ (■). Pulses are given at 5 Hz, so a pulse duration of 200 ms is continuous illumination. The ordinate value of 0.25 is equivalent to 0.25 O_2/(460 Chl·pulse). This value was obtained by illuminating the sample with saturating xenon flashes [38] and is indicated as 0 ms duration on this figure. Theoretical curves are shown for a limitation in PS II reactions by an oxygen-release step of 43 ms (- - - - -) and 188 ms (————); see Appendix 1 for the method of calculation.

because of limited electron flow rate on the acceptor side of PS II.

For the pulse duration experiment to be valid, the oxygen system and the PS II reaction center must turnover as many times as possible during the light pulse without limitation by quantum absorption. In other words, the individual light pulses must be saturating. This was shown to be the case by doing light intensity curves for all the different duration light pulses.

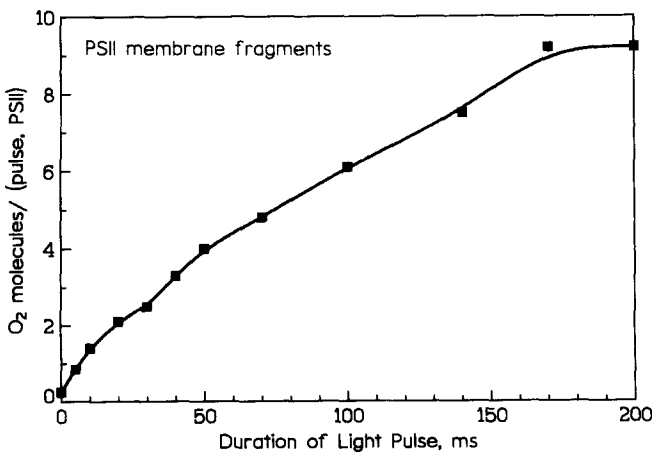

Fig. 2. Oxygen evolved per illumination pulse versus the duration of the pulse. PS II membrane fragments at a concentration of 25 μg Chl/ml were measured with the electron acceptor system of 1 mM ferricyanide, 0.5 μM gramicidin and 100 μM DCBQ. The ordinate value of 0.25 is equivalent to 0.25 O_2/(383 Chl·pulse). Other details are as in the legend of Fig. 1.

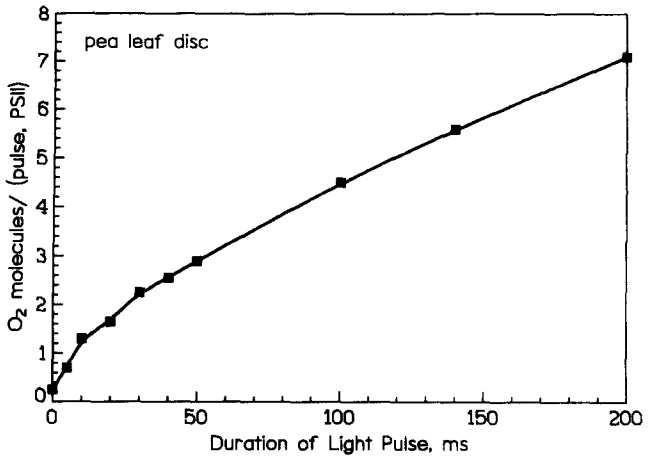

Fig. 3. Oxygen evolved per illumination pulse versus the duration of the pulse. Measurements were made on a leaf disc of 2.5 cm diameter cut from a pea leaf. The Chl concentration in the leaf disc was 450 mg/m^2. The ordinate value of 0.25 is equivalent to 0.25 O_2/(440 Chl·pulse). Other details are as in the legend of Fig. 1.

Fig. 4 shows this type of light intensity curve for a light pulse of 10 ms duration. The curve saturates at about 50% full pulse intensity, giving 1.2 oxygen molecules per reaction center. This saturation behavior indicates that light intensity is not limiting.

The rate of charge flow on the reducing side of PS II can limit photochemistry by the following reaction: P680 Qa^- Qb → P680 Qa Qb^- where the P680 Qa^- Qb form of the reaction center is unable to undergo additional charge separations. The Qa^- Qb → Qa Qb^- electron transport reaction can be monitored by the decay of Chl *a* fluorescence after a flash [5,22,23]. Decay data are shown for thylakoids in Fig. 5. This

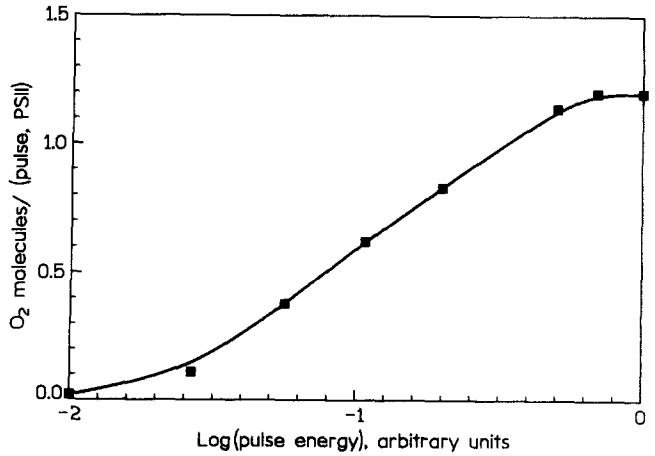

Fig. 4. Oxygen evolved per illumination pulse versus the intensity of the pulse. Measurements were made in pea thylakoids. Illumination pulses were 10 ms in duration, were given at 5 Hz, and were varied in intensity with neutral-density filters. The ordinate value of 0.25 is equivalent to 0.25 O_2/(460 Chl·pulse). Other details are as in the legend of Fig. 1.

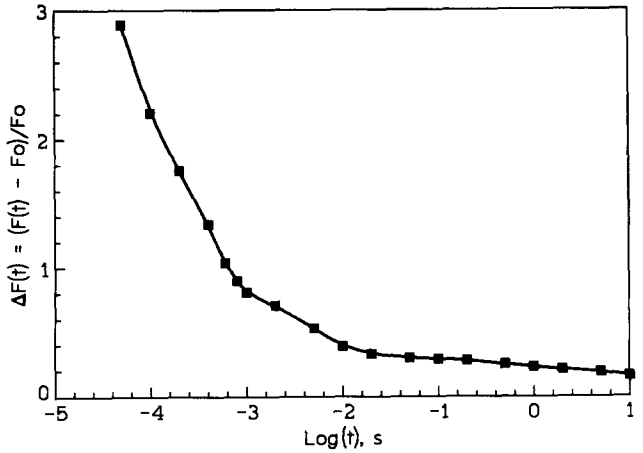

Fig. 5. Variable Chl *a* fluorescence, $\Delta F(t) = (F(t) - F_0)/F_0$, as a function of time after an excitation flash. Xenon excitation flashes were given at 5 Hz and the fluorescence, $F(t)$, was measured at various times, t, after the final flash in a series of 40 flashes. F_0 is the fluorescence from a sample that was dark-adapted for 10 min or longer and that had received no excitation flashes. The fluorescence decay is shown for pea thylakoids at 10 μg Chl/ml with 400 μM ferricyanide and 0.5 μM gramicidin present.

decay is multiphasic and has been analyzed as a sum of four exponential components:

$$\Delta F(t)/\Delta F(\max) = \sum_{i=1}^{4} C_i \exp(-k_i t)$$

where $\Delta F(t)$ is the variable fluorescence at time t after a flash, $\Delta F(\max)$ is the variable fluorescence extrapolated to time zero, C_i is the coefficient of the ith component, and k_i is the rate constant of the ith component. The maximum rate, $k(\max)$, of $Qa^- Qb \rightarrow Qa Qb^-$ can be estimated as the value of $d(\Delta F(t)/\Delta F(\max))/dt$ at $t = 0$, which is the following:

$$k(\max) = -\sum_{i=1}^{4} C_i k_i.$$

The minimum lifetime for this reaction is the inverse of the maximum rate, $t(\min) = 1/k(\max)$. These values are also shown in Table I. This minimum lifetime, $t(\min)$, value represents the highest rates at which $Qa^- Qb \rightarrow Qa Qb^-$ charge transfer can occur after repetitive flashes. We assume that $t(\min)$ is a reasonable estimate for the lowest value for t_{01}, t_{12}, t_{23} or t_{34}.

By applying Eqn 4, one can estimate a maximum value for the release of oxygen: $t_{rel} = \Delta T(2) - \Delta T(1) - 4 \times t(\min)$. Values are shown in Table I for the various samples measured and range from 8 ms (5 ms half-time) in PS II membrane fragments to 21 ms (15 ms half-time) in pea thylakoids with ferricyanide as an electron acceptor. In all of our samples the half-times of oxygen release are 15 ms or less, which is significantly different than the 30 to 130 ms value reported by Plijter et al. [14].

A very simple and compelling result is shown in Fig. 1. With saturating continuous light, 13 oxygen molecules are evolved in 200 ms, which is equivalent to 1 oxygen molecule per 15 ms. This clearly rules out a limitation in the 30 to 150 ms range for an oxygen release reaction.

If oxygen release, Eqn. 3, were rate-limiting, then a slow rise in oxygen per pulse per PS II versus pulse duration would occur that would be independent of the electron acceptor used. In Fig. 1, theoretical curves are shown for rate-limiting oxygen release times of 43 ms (- - - - -) and 188 ms (———), see Appendix 1 for

TABLE I

Values for chlorophyll a fluorescence decay and oxygen evolution parameters for various samples

Experimental details and methods of analysis are given in the text, the legend of Fig. 5, and Appendix 1. The error range is the S.D. for seven repetitions of the measurement.

Sample	$k(\max)$ (s^{-1})	$t(\min) = 1/k(\max)$ (μs)	$\Delta T(2) - \Delta T(1)$ (ms)	t_{rel} (ms)
Pea thylakoids (0.5 μM gramicidin, 400 μM ferricyanide)	2720 ± 300	367 ± 40	22 ± 3	21 ± 3
Pea thylakoids (0.5 μM gramicidin, 400 μM ferricyanide, 10 μM DCBQ)	1730 ± 240	597 ± 81	11 ± 2	9 ± 2
PS II membrane fragments (0.5 μM gramicidin, 400 μM ferricyanide 10 μM DCBQ)	936 ± 179	1070 ± 210	12 ± 2	8 ± 2
Pea leaf discs (5% CO_2)	2020 ± 290	495 ± 70	18 ± 3	16 ± 3

the method of calculation. The data in Figs. 1 to 3 do not support a limitation in PS II reactions by an oxygen release time of more than 48 ms. These data rise more rapidly with pulse duration than expected if oxygen release was limiting. Also, the oxygen release per pulse (Fig. 1) is dependent upon the electron acceptor used, which indicates a limitation on the acceptor side of PS II, not a limitation by oxygen release. For steady-state excitation, DCBQ supports higher rates of oxygen evolution than ferricyanide. This is because ferricyanide is predominantly a Photosystem I acceptor and oxygen evolution is limited by the electron transport rate through the plastoquinone pool. DCBQ accepts electrons from Qa and Qb prior to the plastoquinone pool. Therefore, DCBQ circumvents the plastoquinone pool rate limitation and supports higher rates of electron flow and oxygen evolution.

As the pulse duration of the excitation light is made longer than 7 to 10 ms the oxygen evolved per reaction center per pulse exceeds unity in Figs. 1 to 3. The maximum value of oxygen per reaction center per pulse occurs with a pulse duration of 200 ms, which is continuous light for pulses given at 5 Hz. This represents the rate-limiting step for photosynthesis. Values for this rate limitation are calculated and shown for our samples in Table II. The 7 ms rate-limitation for PS II turnover in leaf discs of peas agrees well with 8 ms found for leaves of rapeseed at 25 °C [17] and 10 ms for *Chlorella* at 14 °C [24].

Based on the oxygen evolution per pulse data presented here, there is no evidence for an oxygen release limitation of photosynthesis. Chl *a* fluorescence is another method that can be used to check for an oxygen release limitation. If PS II charge separation is driven by a series of saturating light pulses and oxygen release, Eqn. 3, is rate-limiting, then charge separation will cease during the excitation pulse because electron donors, newly bound water molecules, will not be available. This lack of electrons during the pulse will stop the reduction of Qa to Qa^- and Chl *a* fluorescence will not be driven to the maximum level of fluorescence, which corresponds to 100% Qa^-. Instead, Chl *a* fluorescence will remain at a constant level or will decrease with increased pulse duration until the pulse length exceeds the time of oxygen release. This type of measurement is shown in Fig. 6. Clearly, by a pulse duration of 10 ms Qa is largely reduced and by 30 ms it is fully reduced, the Chl *a* fluorescence is at the maximum level. These data are inconsistent with the hypothesis that oxygen release limits photosynthesis by restricting electron availability at PS II. There is no indication of a plateau in the rise of Chl *a* fluorescence in the 30 to 130 ms range as expected based on the work of Plijter et al. [14]. Instead, Chl *a* fluorescence rises to maximum level with a 30 ms pulse length, which indicates an electron flow limitation on the reducing side of PS II.

Another method for determining the kinetics of oxygen release is to measure the oxygen signal from a bare-platinum electrode that is illuminated with a series of saturating flashes [25,26,14]. A typical signal is shown in Fig. 7 and we believe it is generated by the following process. In our electrode, the flash excitation is saturating and as such causes all of the thylakoid reaction centers in the sample channel to react simultaneously. The rise in the signal is the rate at which the concentration of oxygen near the platinum surface, increases after

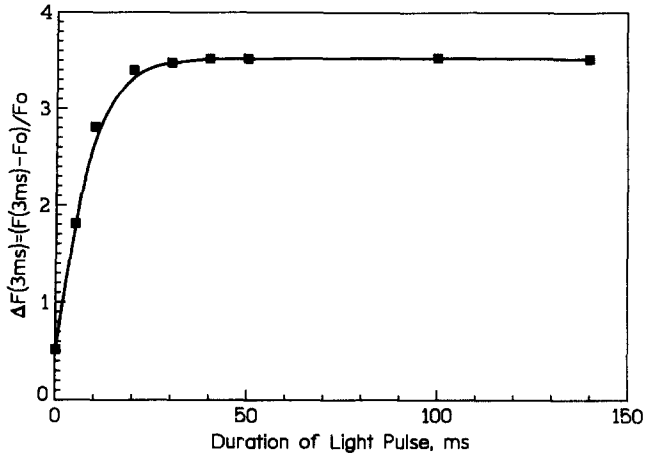

Fig. 6. The Chl *a* variable fluorescence, $\Delta F(3\text{ ms}) = (F(3\text{ ms}) - F_0)/F_0$, as a function of duration of the excitation pulse. The Chl *a* fluorescence is measured 3 ms, $F(3\text{ ms})$, after the end of a saturating light pulse and F_0 is the fluorescence level in a sample that has been dark-adapted for 10 min. Excitation light pulses are given at 5 Hz with durations between 5 and 140 ms. For the 0 ms duration point, saturating xenon flashes were used for excitation. The fluorescence is measured when steady state is established, which is after 25 or more pulses have been given. This measurement was made on pea thylakoids at 10 μg Chl/ml with 400 μM ferricyanide and 0.5 μM gramicidin present. In the presence of 10 μM DCMU, the maximum value for $\Delta F(3\text{ ms})$ is found to be 3.55.

TABLE II

Rate limitation for photosynthesis under saturating continuous light

The turnover time per oxygen evolving center is calculated by presuming four electrons or charge separations are required for every oxygen evolved [4]. $V(\text{con})$ is the oxygen per pulse per oxygen evolving center at a pulse duration of 200 ms, which at 5 Hz is continuous light, obtained from Figs. 1–3. T is the turnover time, the minimum time between charge separations, of PS II that is necessary to support $V(\text{con})$: $T = 200\text{ ms}/(4 \times V(\text{con}))$. The error range is the S.D. for five repetitions of the measurement.

Sample	$V(\text{con})$	T (ms)
Leaf discs, 5% CO_2	7.2±1.1	6.9±1.2
Thylakoids, 0.5 μM gramicidin, 1 mM ferricyanide	6.0	8.3
Thylakoids, 0.5 μM gramicidin, 1 mM ferricyanide, 100 μM DCBQ	12.7	3.9
PS II membrane fragments, 0.5 μM gramicidin, 1 mM ferricyanide, 100 μM DCBQ	9.2	5.4

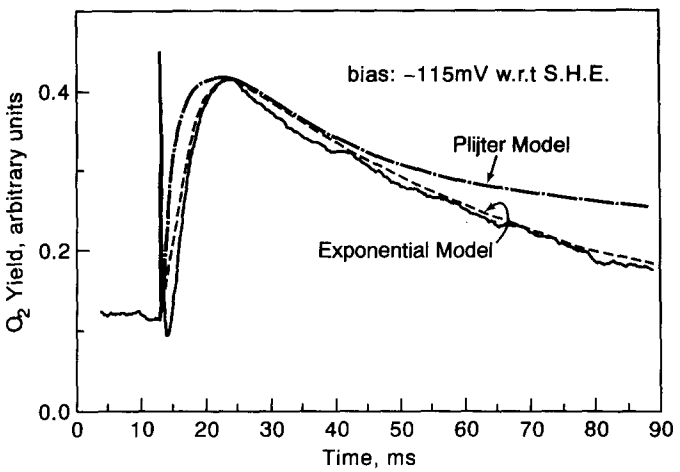

Fig. 7. Oxygen signal (solid line) from a bare-platinum electrode illuminated with saturating xenon flashes. The signal is shown for control thylakoids after 25 or more flashes have been given. The trace is with the platinum biased at −115 mV. Kinetic analysis of the data is given in Table III, according to the exponential method of Appendix 2. Theoretical curves as shown for the exponential analysis (- - - - - -) and one-dimensional diffusion analysis of Plijter et al. [14] (— · —) described in Appendix 2. The sharp spikes at the beginning of the oxygen signal are electronic artefacts from the discharge of the flash lamp.

an excitation flash. This is the reaction of Eqn. 1 or the

$$S_3 \xrightarrow{h\nu} S_3' \longrightarrow S_4 \longrightarrow S_0$$
$$2\,H_2O \quad O_2 + 4\,H^+$$

combined reactions of Eqns 2 and 3. Also, the rise in the signal includes the diffusion time of oxygen across the thylakoid membrane and any buffer-filled distance to the platinum surface. Since these diffusion times are not known, the rise time of the oxygen signal is at best an overestimate of the time for oxygen release. Simultaneously, the oxygen is being depleted. The oxygen concentration is dropping due to the oxygen consuming reaction at the platinum as well as the diffusion of oxygen out of the channel across the dialysis membrane into the upper chamber. Mathematical analysis of these signals is described in Appendix 2.

The two methods of analyzing the oxygen signals of Fig. 7, which are given in Appendix 2, give different results. The one-dimensional diffusion model does not give as good a fit to the data as does the exponential model. Based on this goodness of fit, the exponential model is used in our analysis.

The sharp spike at the beginning of the oxygen signal shown in Fig. 7 is an electronic artefact from the discharge of the flash lamp. This distorts the rising edge of the oxygen signal and is easily seen as a deviation from the theoretical curve, shown as a dashed line. The theoretical curve is based on average data shown in Table III for −115 mV bias of the electrode and the exponential model of Appendix 2.

TABLE III

Kinetic parameters for oxygen signals from a bare-platinum electrode as shown in Fig. 7

t_p is the time after the flash at which the peak in the oxygen signal occurs. τ_d is decay time of the oxygen signal following the peak. τ_r is the rise time of the oxygen signal, which is calculated by the exponential method in Appendix 2. The error range is the standard deviation for five repetitions of the measurement.

Line	Measurement conditions	t_p (ms)	τ_r (ms)	τ_d (ms)
1	bias = −415 mV	7.9±0.5	3.9±0.3	26±5
2	bias = −115 mV	9.6	4.0	48
3	bias = −365 mV, 200 mM NaCl	7.8	3.4	30
4	bias = −365 mV, 100 mM NaCl	7.6	3.3	30
5	bias = −365 mV, 50 mM NaCl	7.9	3.2	35
6	bias = −365 mV, 20 mM NaCl	7.9	3.5	30
7	treated with 50 mM NaCl	7.3	3.3	27
8	treated with 50 mM $NaNO_3$	9.0	5.0	25
9	treated with 20 mM $NaHCO_2$	12.2	7.0	33
10	treated with 20 mM $NaHCO_2$ and 10 mM $NaHCO_3$ added	9.2	4.8	28

This proposed operation of the electrode is confirmed by the measurements in Figs. 7–9 and the data in Table III. Fig. 8 shows how the amplitude of the oxygen signal changes with bias voltage. From −115 to −365 mV the amplitude increases, since with the more negative potential the probability of oxygen reduction at the platinum increases. At potentials more negative than −365 mV the oxygen signal is unchanged, which means the reduction of oxygen molecules at the platinum has reached a maximum value. This polarogram is virtually identical to that of Bader et al. [27] but quite different from that of Plijter et al. [14]. The inhibition of the signal amplitude at potentials below −480 mV [14] was not observed with our electrode.

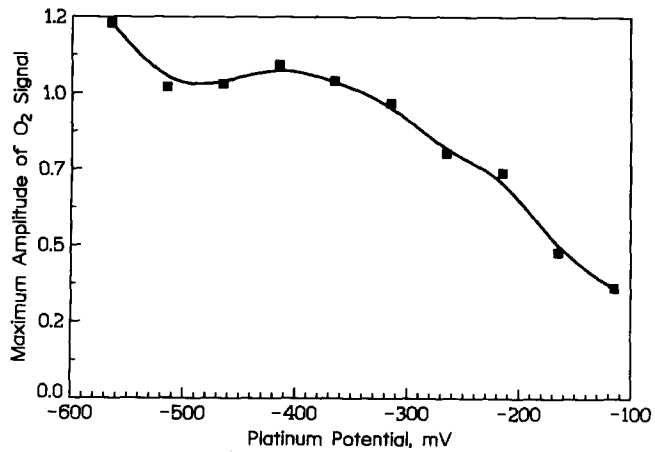

Fig. 8. Maximum amplitude of the oxygen signal from a bare-platinum electrode versus bias potential. Relative amplitudes are plotted and have been normalized to the maximum amplitude at −365 mV. The amplitudes are shown for control thylakoids after 25 or more flashes have been given. The sample is allowed to settle on the electrode for 5 min before the measurement is made. The bias is applied 10 s prior to the beginning of flash illumination. Analysis of the oxygen signals is made with the exponential model described in Appendix 2.

The dependence of the rise time, τ_r, and decay time, τ_d, on electrode bias is shown in Fig. 9. τ_r is about 3.9 ms and within experimental error is independent of bias voltage in the −115 to −585 mV range. Between −115 to −285 mV τ_d is about 46 ms and decreases to 11 ms at a bias of −585 mV. In the −115 to −285 mV range, the signal decay is mostly due to diffusion of oxygen out of the channel into the upper chamber. At potentials more negative than −285 mV, the consumption of oxygen at the platinum surface becomes large enough to be significant compared to diffusion and τ_d becomes smaller. It is important to note that at weak bias potentials (−115 to −285 mV), where τ_d is maximal and invariant, τ_r remains approx. 3.9 ms and does not become 10-fold or more greater as predicted from the work of Plijter et al. [14].

Work by Meunier and Popovic [26] indicated that the conductivity of the electrolyte could alter the response time of the bare-platinum electrode. To test for this possibility the upper chamber of the electrode was filled with solutions of various conductivities: 50 mM phosphate (pH 7.5) and NaCl at 200, 100, 50 and 20 mM. The results are shown in Table III, lines 3 to 6. We found no significant change in signal kinetics with these different conductivities, which indicates our electrode had no kinetic limitations due to solution conductivity.

Treatment of thylakoids with the anion NO_3^- was shown by Sinclair [28], using the phase shift method, to slow the oxygen evolution reaction, Eqn. 1. Here thylakoids at 200 μg Chl/ml were incubated for 1 h in the dark at 20 °C in the following medium: 400 mM sucrose, 50 mM Tricine, 1 mM $MgCl_2$ and 50 mM NaCl or 50 mM $NaNO_3$ at pH 7.9. The oxygen signal kinetics were measured and are shown in lines 7 and 8 of Table III. The $NaNO_3$ treatment increased the τ_r from 3.3 ms to 5 ms, which is in good agreement with the results of the phase shift method used by Sinclair [28]. This supports our analysis and interpretation of these flash induced signals that are detected by a bare-platinum electrode.

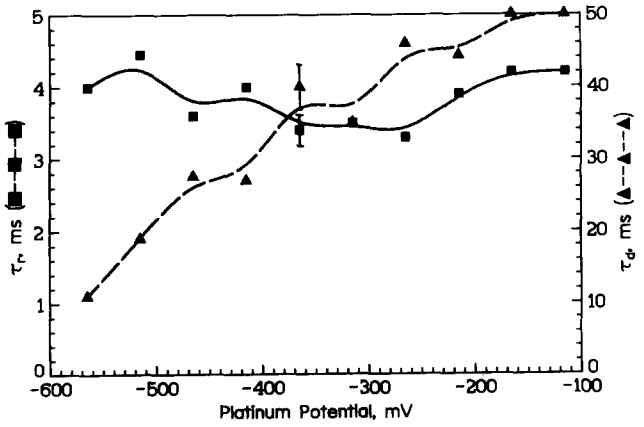

Fig. 9. The rise time, τ_r, and decay time, τ_d, of the oxygen signal from a bare-platinum electrode versus bias potential. These rise and decay time are shown for control thylakoids after 25 or more flashes have been given. The sample is allowed to settle on the electrode for 5 min before the measurement is made. The bias is applied 10 s prior to the beginning of flash illumination. Analysis of the oxygen signals is made with the exponential model described in Appendix 2. Error bars shown at −365 mV are for five repetitions of the measurement.

Another treatment that has been reported to slow the oxygen-release reaction is that of the anion, formate [29]. This slowing by formate binding could be reversed by addition of bicarbonate. In this action, formate behaves as an anion [30,31] just as NO_3^- and disrupts oxygen evolution reactions. There has also been a report in the literature [32] that showed no effect of formate on the rate of oxygen evolution. Here, thylakoids at 200 μg Chl/mg were incubated in the dark at 20 °C for 15 min in 50 mM phosphate, 180 mM NaCl, and 20 mM formate at pH 6.4, which is a known procedure [33] for binding formate. Addition of 10 mM bicarbonate was used for a control sample. The oxygen signal kinetics were measured and are shown in lines 9 and 10 of Table III. Formate treatment increased the τ_r to 7 ms and 10 mM bicarbonate partially reversed this giving a τ_r of 4.8 ms.

Many measurements of control samples gave an oxygen signal τ_r of 3.3 ± 0.3 ms. We believe this rise in the oxygen signal corresponds to the $S_3 \rightarrow S_3' \rightarrow S_4 \rightarrow S_0$ oxygen evolving reaction of Eqn. 1. Based on this concept $t_{rel} = \tau_r - t_{34}$ or $t_{rel} = 3.3$ ms − 0.37 ms = 2.9 ms where the value of t_{34} was estimated by t_{min} from Table I for pea thylakoids with gramicidin and ferricyanide present. This value for t_{rel} from bare-platinum electrode data is high, since the time for oxygen diffusion has not been subtracted. Both the oxygen pulse duration method and the flash oxygen signal method indicate an oxygen release time in pea thylakoids, t_{rel}, of significantly less than 43 to 188 ms.

Discussion

In this work, a new method is described that uses an oxygen-concentration electrode for measuring oxygen release times in leaf discs, thylakoids, and PS II membrane fragments. Maximum (upper bound) values for the half-times of oxygen release are found to be between 5 and 11 ms for these various samples. Table IV is a comparison of values for the release time reported in the literature. All reports, except Plijter et al. [14], find the oxygen release time to be a few milliseconds.

Our concentration-electrode data are different than the gush of oxygen observed upon saturating continuous illumination by Joliot [34]. We give a series of saturating pulses of excitation light and thus avoid the lag in oxygen evolution from S-state activation [3]. Also, we find in Figs. 1–3 more than 1 oxygen molecule released per PS II center per pulse. By 130 ms, the

TABLE IV

Summary of material, methods and measured half-times for the oxygen release reaction of photosynthesis as reported by various research groups

Reference	Method	Material	Half-time (ms)
8	bare-platinum electrode, modulated light	*Chlorella*	0.9
11	concentration electrode, capillary flow	*Chlorella*	1.5 to 2.2
4	bare-platinum electrode, delayed xenon flashes	thylakoids	1.2
9	bare-platinum electrode, modulated light	*Chlorella*	2.0
10	bare-platinum electrode, modulated light	thylakoids	3.0
13	photobaric detection	leaf discs	< 8.0
14	gold-screen electrode, oxygen signal kinetics	thylakoids, PS II membrane fragments	30 to 130
12	oxidation of mitochondrial cytochrome *c*	*Chlorella*	< 3.0
This work	concentration electrode, variable duration light pulses	leaf discs, thylakoids, PS II membrane fragments	5.1 to 11.0
This work	bare-platinum electrode, oxygen signal kinetics	thylakoids	2.9

longest half-time of oxygen release of Plijter et al. [14], we find 9 oxygen molecules released per PS II per pulse.

One of the consequences of an oxygen-release step of 30 to 130 ms would be that this step would be rate-limiting for photosynthesis. This is slower than the previously accepted rate limitation, the 10 ms half-time for electron turnover or 40 ms for oxygen evolution at 15°C measured by Emerson and Arnold [24]. This temperature-dependent 10 ms rate limitation of photosynthesis has been hypothesized to be located between the two photosystems, since under very strong irradiation Qa is driven to its reduced state and P700 to its oxidized state [35].

In Figs. 1–3, rate limitation occurs when the slope decreases at a pulse duration of 10 ms or longer. The turnover times required to support these maximum rates of photosynthesis are calculated in Table II and are in good agreement with the 10 ms value found by Emerson and Arnold [24] but are much shorter than expected if an oxygen release step of 130 ms was rate-limiting. Also, the rise in Chl *a* fluorescence to a maximum value with a pulse duration of 20 ms (Fig. 6) is consistent with a rate limitation in charge transfer in the plastoquinone pool and inconsistent with restricted electron availability due to slow oxygen release.

Our measurements with a bare-platinum electrode are in agreement with the concentration electrode results, compare t_{rel} of Table I with τ_r of Table III. Plijter et al. [14] criticized the use of bare-platinum electrodes when high, negative-potential bias was used and oxygen consumption at the platinum surface was at such a high rate that the signal was severely truncated and distorted. Here, we measured the rise-time of oxygen signals with various bias potentials and found no significant variations. Our lowest bias potential was −115 mV, which was sufficiently low for oxygen consumption at the platinum surface to be small compared to diffusion of oxygen out of the sample channel, see Fig. 9. This low potential along with our exponential method of data analysis, Appendix 2, we believe allowed us to avoid the problems raised by Plijter et al. [14] and to determine the rise-time of the oxygen signal with reasonable accuracy. This was confirmed by our rise-times being sensitive to sample treatment with $NaNO_3$ and $NaHCO_2$, Table III. Earlier work by Miyao et al. [36], using a bare-platinum electrode similar to that used in this work, showed that removal of the 33 kDa extrinsic protein slowed the release of oxygen. Applying our exponential analysis to the data of Miyao et al. [36] gave a control with $\tau_r = 3.5$ ms and a 33 kDa-depleted sample with a $\tau_r = 12$ ms. The control sample is in good agreement with the data presented here in Table III.

We feel the gold-screen electrode as used by Plijter et al. [14] is distorting the oxygen-rise signal by an unidentified problem of electrode chemistry. Gold electrodes in particular are subject to electrochemical 'poisoning' by protein absorption [37], which may significantly slow the electrode response. We have found that for maximum signal size and rapid signal kinetics our platinum surface had to be scrubbed with a paste of $CaCO_3$ before every sample application. Thorough scrubbing of a gold screen would be virtually impossible, which may lead to protein absorption and surface contamination.

In summary we conclude that the upper bound for the oxygen release time is 8 ms, oxygen release is not rate limiting for photosynthesis, and photosynthesis is limited by electron flow through the plastoquinone pool and beyond.

Acknowledgements

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Appendix 1

A method is desired for calculating the average time for evolving various numbers of oxygen molecules. The equations are dependent on the kinetic model assumed for oxygen evolution.

The Kok model

If one assumes the model of Kok et al. [3], see Eqn. 1., then all the steps, including the evolution of molecular oxygen, must be completed sequentially.

For one oxygen molecule to be evolved per reaction center per pulse, the following reactions must take place during the excitation pulse:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4.$$

This requires a pulse duration of $\Delta T(1) = t_{01} + t_{12} + t_{23} + t_{34}$ with the one oxygen molecule being released during the dark interval between pulses,

$$S_4 \rightarrow S_0$$
$$2H_2O \quad O_2 + 4H^+$$

in time t_{rel}.

For two oxygen molecules to be evolved per reaction center per pulse, the following reactions must take place during the excitation pulse:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \longrightarrow$$
$$S_4 \rightarrow S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4.$$
$$2H_2O \quad O_2 + 4H^+$$

This requires a pulse duration of $\Delta T(2) = 2\ (t_{01} + t_{12} + t_{23} + t_{34}) + t_{rel}$ with the second oxygen molecule being evolved during the dark interval between pulses.

The recursion relationship can be written for n molecules of oxygen evolved per reaction center per flash: $\Delta T(n) = n(t_{01} + t_{12} + t_{23} + t_{34}) + (n-1)\ t_{rel}$.

If there are no other rate limitations, then the number of oxygen molecules evolved per reaction center per pulse of duration ΔT will be:

$$n = \frac{\Delta T + \tau_{rel}}{\tau_{01} + t_{12} + t_{23} + t_{34} + t_{rel}}$$

The Plijter model

If one assumes the model of Plijter et al. [14], see Eqns. 2 and 3, then all the reaction steps except

$$S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0$$

can occur twice before oxygen is evolved.

For one oxygen molecule to be evolved per reaction center per pulse, the following reactions must take place during the excitation pulse:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4$$
$$H_2O \qquad H_2O \qquad H_2O \qquad H_2O \qquad H_2O$$

This requires a pulse duration of $\Delta T(1) = t_{01} + t_{12} + t_{23} + t_{34}$ with the one oxygen molecule being released during the dark interval between pulses,

$$S_4 \rightarrow S_0 \qquad \rightarrow S_0$$
$$H_2O \quad H_2O(ox) \qquad H_2O$$
$$1/2\ O_2$$
$$+2\ H^+$$

in time t_{rel}.

For two oxygen molecules to be evolved per reaction center per pulse, the following reactions must take place during the excitation pulse:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu}$$
$$H_2O \qquad H_2O \qquad H_2O$$
$$S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3$$
$$H_2O \quad H_2O \quad H_2O(ox) \quad H_2O(ox) \quad H_2O(ox) \quad H_2O(ox)$$
$$\downarrow t_{rel} \quad \downarrow t_{rel} \quad \downarrow t_{rel} \quad \downarrow t_{rel}$$
$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3$$
$$H_2O \qquad H_2O \qquad H_2O \qquad H_2O$$
$$\xrightarrow{h\nu}$$

Where t_{rel} is the time for release of oxygen and rebinding of water. This requires a pulse duration of $\Delta T(2) = 2\ (t_{01} + t_{12} + t_{23} + t_{34}) + t_{rel}$ with the second oxygen molecule being evolved during the dark interval between pulses. In the scheme shown above, S-state transitions and oxygen release can occur simultaneously.

The recursion relationship can be written for n molecules of oxygen evolved per reaction center per flash: $\Delta T(n) = n\ (t_{01} + t_{12} + t_{23} + t_{34}) + (n-1)t_{rel}$.

If there are no other rate limitations, then the number of oxygen molecules evolved per reaction center per pulse of duration ΔT will be:

$$n = \frac{\Delta T + \tau_{rel}}{\tau_{01} + t_{12} + t_{23} + t_{34} + t_{rel}}$$

Appendix 2

Exponential analysis

Typical oxygen signals from the bare platinum electrode are shown in Fig. 7. It is found empirically that

the rise and decay of the signals are exponentials. The rise function is $1-\exp(-k_r t)$ where k_r is the rate constant. The decay function is $\exp(-k_d t)$ where k_d is the rate constant. The dependence of signal on time will be $S(t) = S_{max} \exp(-k_d t)\ (1-\exp(-k_r t))$ where S_{max} is the maximum amplitude of the signal. The peak of the signal will occur when $dS(t)/dt = 0$ or

$$-k_d \exp(-k_d t_p) + (k_d + k_r) \exp(-(k_d + k_r) t_p) = 0$$

$$-k_d + (k_d + k_r) \exp(-k_r t_p) = 0$$

$$(k_r/k_d + 1) = \exp(k_r t_p) \quad (5)$$

where t_p is the time at which the peak in the signal occurs.

Analysis of oxygen signals such as in Fig. 7 are carried out as follows: t_p is determined, k_d is determined from a semilogarithmic plot of the signal decay tail, and k_r is determined by iteration of Eqn. 5. The rise time, τ_r, of the oxygen signal is equal to $1/k_r$ and the decay time, τ_d, is $1/k_d$. The maximum amplitude of the signal is calculated as:

$$S_{max} = S(t_p)/(1-\exp(-k_r t_p) \exp(-k_d t_p)).$$

One-dimension diffusion analysis

Plijter et al. [14] described the operation of the bare-platinum electrode with differential equations for one-dimensional diffusion. For a sample in a layer of finite thickness on a strongly polarized electrode, the electrode response function is:

$$I_{strong}(t) = \exp(-t/\tau) \quad (6)$$

where τ is the time constant for oxygen release.

For a sample in a layer of finite thickness on a weakly polarized electrode, the electrode response is:

$$I_{weak}(t) = \exp(-t/\tau) \int_0^{(t/\tau)^{1/2}} \exp(y^2)\, dy \quad (7)$$

for small t and $(1/t)^{1/2}$ for large t. Eqn. 7 is equal to the infinite series:

$$I_{weak}(t) = (t/\tau)^{1/2} - \frac{2(t/\tau)^{3/2}}{3} + \frac{4(t/\tau)^{5/2}}{15} + \ldots + \frac{(-1)^{n-1}(2)^{2n-1}(t/\tau)^{n-1/2}(n!)}{(2n)!}$$

$n = 1$ to ∞

Our strongest polarization occurred at -585 mV, which gave signals with a decay time of approx. 10 ms, see Fig. 9. So, τ of Eqn. 6 was estimated as 10 ms. The weak polarization condition occurs at potentials more positive than -165 mV, when τ_d is invariant with bias potential, see Fig. 9. The transition between short and long times occurs at $t = 2\tau$, since at times longer than this, the infinite series becomes extremely sensitive to the truncation of terms.

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