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THE TURNOVER TIMES OF PHOTOSYNTHESIS AND REDOX PROPERTIES OF THE POOL OF ELECTRON CARRIERS BETWEEN THE PHOTOSYSTEMS

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SUMMARY

The turnover time of photosynthesis was measured by periodic and aperiodic flashes over a wide range of pulse frequencies and background oxygen concentration. The turnover can be described by a sequence of relaxation times and is slowed upon reduction of the pool of electron carrier between the two photosystems.The exclusive reaction of the pool components with non-ionic oxidants supports their identification as plastoquinone. An argument is presented that the first acceptor of the pool is a chromanol.

Several models are discussed that explain the truncation of the oxygen flash yield *versus* flash period in anaerobic repetitive single-flash experiments.

INTRODUCTION

We remarked in the preceding paper[1] that a likely candidate for the electron transport pool, in our kinetic scheme, was plastoquinone. Stiehl and Witt[2] proposed, on the basis of optical absorbance measurements, a pool of 5 plastoquinones in *Chlorella*. Crane *et al.*[3] were able to demonstrate a plastoquinone pool of 5–7 members, in the same organism, which they showed was essential for photosynthetic electron transport. These pools of 10 electrons in the former case and 10–15 in the latter are in fair agreement with our estimate[1] of 20 pool members.

The turnover time of photosynthesis is the time required for the photosynthetic system to recover from photoexcitation. Empirically, it is the length of time required between two successive saturating light flashes for the photosynthetic system to give half-maximal oxygen yield on the second flash. Emerson and Arnold[4], using the repetitive single-flash method, were the first to measure this turnover time. They could only place an upper limit of 40 ms at 25 °C for *Chlorella* because of limitations of their apparatus. They clearly showed that this turnover time was a strongly temperature-dependent process, requiring 0.4 s at 1.1 °C. The maximal oxygen

Abbreviations: PMS, phenazine methosulfate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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yield per flash was shown to be temperature independent over this range. Stiehl and Witt[2] and Witt[5] have made extensive measurements of the kinetics of oxidation and reduction of plastoquinone. Good agreement is obtained between our turnover times for oxygen as measured by repetitive single and double flashing and the kinetics of plastoquinone turnover. Further agreement derives from a similar dependence of turnover rate on the redox state of the pool. We will also offer explanations for the heterogeneity of these turnover times, and for the truncation of the oxygen yield per flash, in anaerobic repetitive single-flash curves, at times longer than can be accounted for by the turnover time.

METHODS

The algae used in these experiments were *Phormidium luridum* and *Chlorella vulgaris*, grown under the conditions described in the preceding paper[1]. The oxygen detection methods used were the oxygen luminometer and the gas flow oxygen polarograph described earlier. The buffers and filters for the lamps were also as described. A Strobrite flash lamp delivering 5-μs flashes and a Stroboslave delivering 4-μs flashes were used in repetitive double-flash experiments. The timing intervals in repetitive single- and double-flash experiments were controlled by Tektronix pulsers (Types 161, 162, and 163). In repetitive single-flash experiments, flashes were given at a fixed time interval until a steady state was reached. In repetive double-flash experiments, a second flash followed the first after a variable delay time. The pair was repeated at a relatively slow rate, ranging from 12 to 1 per s, until a steady state was achieved.

The above oxygen measurements were performed using equipment with a slow time response. Thus the integrated oxygen yields were measured and individual flashes could not be distinguished.

To assure that the increasing yield of the flash pair, in repetitive double-flash experiments, was due solely to the second flash, as the members of the flash pair were separated, we performed the following experiment. *Chlorella* and *Phormidium* were given repetitive double flashes under conditions (similar to Figs 3b and 3c) such that the second flash had attained maximal yield, but where the time between the first and second flashes was still a small fraction (approx. 10%) of the time between the second and the following first flash. Using the oxygen polarograph in the voltage clamp mode with a fast feedback amplifier[1], we measured directly the oxygen yields of the individual members of the flash pair. We found that the oxygen yield of the first flash was constant whether or not it was preceded by the second member of the previous flash pair. Thus we conclude that in the repetitive double-flash experiments, the first flash remains constant and the enhanced yield of the flash pair as the members are separated is due solely to the second flash.

RESULTS

Oxidation of the pool by non-ionic reagents

Repetitive single-flash experiments (Fig. 1) were performed with *Phormidium* coupled to $K_3Fe(CN)_6$ (anionic oxidant) or to $K_3Fe(CN)_6$ *plus* phenazine methosulfate (PMS) (cationic oxidant). We found that there was little difference between

the kinetics of the reductive loss in the presence of these ionic oxidants and that for coupling to CO_2 (Figs 4 and 6 of preceding paper[1]). We conclude that $K_3Fe(CN)_6$ and oxidized PMS are inaccessible to the pool and couple to the photosynthetic electron transport chain on the reducing side of Photosystem I. Because the ionic oxidants do not affect the pool while the non-polar oxidants, benzoquinone (Fig. 1) and oxygen (Fig. 12 of preceding paper[1]) completely eliminate the loss, it seems likely that the pool itself consists of a non-polar oxidant (*e.g.* plastoquinone) in a hydrophobic environment.

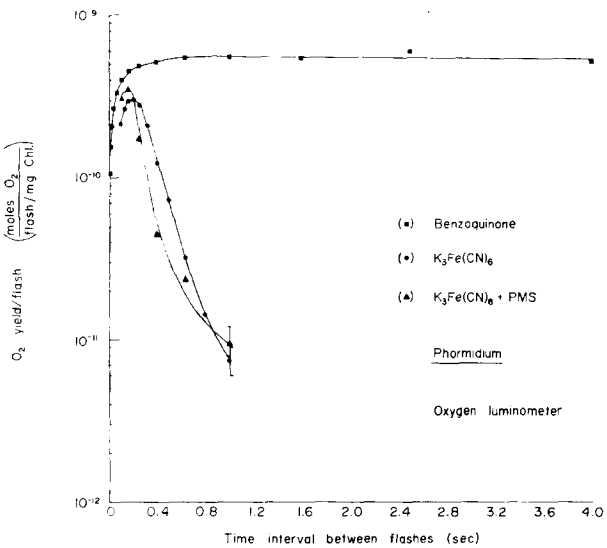

Fig. 1. Oxygen yield on repetitive single flashing of *Phormidium* at 3 ppm O_2 in the presence of 0.25 mM benzoquinone (■), 0.5 mM $K_3Fe(CN)_6$ (●), or 0.25 mM $K_3Fe(CN)_6$ *plus* 25 μM phenazine methosulfate (PMS) (▲). For the $K_3Fe(CN)_6$ run, cells (13.4 μg chlorophyll *a*) were suspended in 6 ml of succinate buffer and given saturating flashes of yellow (>480 nm) light. Cells for the $K_3Fe(CN)_6$+PMS run (5.0 μg chlorophyll *a*) as well as those for the benzoquinone run (4.5 μg chlorophyll *a*) were suspended in 2 ml of the same buffer, and the flashes were >570 m.

Saha *et al.*[6] have recently demonstrated, in spinach chloroplasts, that electrons accepted by polar (Class I) oxidants pass through two phosphorylation sites, while those accepted by non-polar (Class III) oxidants pass through only one. These authors suggest, as we do, that the coupling site for electron transfer is dependent on the polarity of the oxidant.

The variation of the fluorescence yield in spinach chloroplasts with redox potential[7] supports the argument that A and Q are plastoquinone. A potentiometric titration revealed two states of fluorescence quenching, each corresponding to an oxidized species. The characteristic midpoint potentials were −35 mV and −270 mV at pH 7.0 and corresponded to one electron transitions. In addition, the pH dependence of the midpoint potentials corresponds to −60mV per pH unit. This pH dependence is characteristic of the quinone–hydroquinone system[8]. The possibility that Q is a chromanol will be considered in the discussion.

Turnover time and the redox state of the pool. Conditions that oxidize or reduce

the pool (pulse rate, O_2 background) also effect the turnover time of photosynthesis. The dependence of the turnover kinetics on the pool oxidation state is in agreement with correlations that Witt[5] has made between the oxidation and reduction rates of plastoquinone and the oxidation state of the plastoquinone pool. We will turn now to a discussion of these measurements.

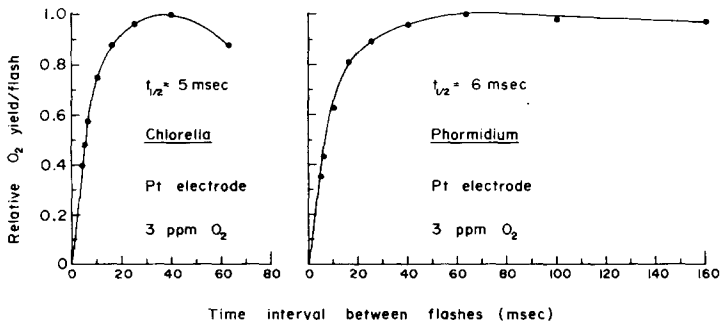

Fig. 2. Oxygen yield on repetitive single flashing of *Chlorella* and *Phormidium*, coupled to CO_2, at 3 ppm O_2 and standard conditions for the oxygen polarograph[1].

Anaerobic repetitive single-flash experiments for *Chlorella* coupled to CO_2 give a half-turnover time of 5 ms at 24 °C (Fig. 2) and 25 ms at 5.4 °C (Fig. 5 of preceding paper[1]), thus showing a $Q_{10}=2.2$. *Phormidium* assayed under the same conditions shows a similar 6-ms half-turnover time at 24 °C (Fig. 2).

Using the repetitive double-flash method, turnover times were measured for *Chlorella* and *Phormidium*. Under nearly aerobic conditions (2% O_2 in argon) the half-turnover time is the same, 0.6 ms, at both 1 flash pair/s (Fig. 3a) and flash pairs/s (not shown). Under anaerobic conditions, however, we find that the turnover time is dependent on how rapidly the flash pairs are given. For *Chlorella* (Fig. 3b) 1 pair of flashes/s results in a turnover of 4.2 ms, while that at 1 pair/80 ms is 0.65 ms. Similar results are observed for *Phormidium* (Fig. 3c), 2.4 ms at 1 pair/s and 0.54 ms at 1 pair/0.2 s.

Thus we find that, under anaerobic conditions, very rapid and very slow flashing give approximately the same turnover time, whereas moderate flash rates give 7–8-fold more rapid turnover. These results, however, are readily understandable if the turnover time is determined by the redox state of the pool, which in turn, establishes the rate-determining step. We have shown (preceding paper[1]) that infrequent flashes, given under anaerobic conditions, permit the reduction of the pool of electron carrier, between the photosystems. A moderate rate of flashing results in oxidation of the pool, and very rapid flashing again reduces the pool. Possible explanations for the latter are (a) that the rate limiting step between the two photoreactions is the oxidation of the reduced pool or (b) an increase in the feedback to the pool of the reduced product of photoreaction P–X, as discussed in the preceding paper[1]. Thus, the above results strongly suggest that the turnover time is dependent on the redox state of the pool.

We pointed out above that there was much evidence for identifying the kinetic pool with the plastoquinone pool of photosynthesis. In aerobic repetitive

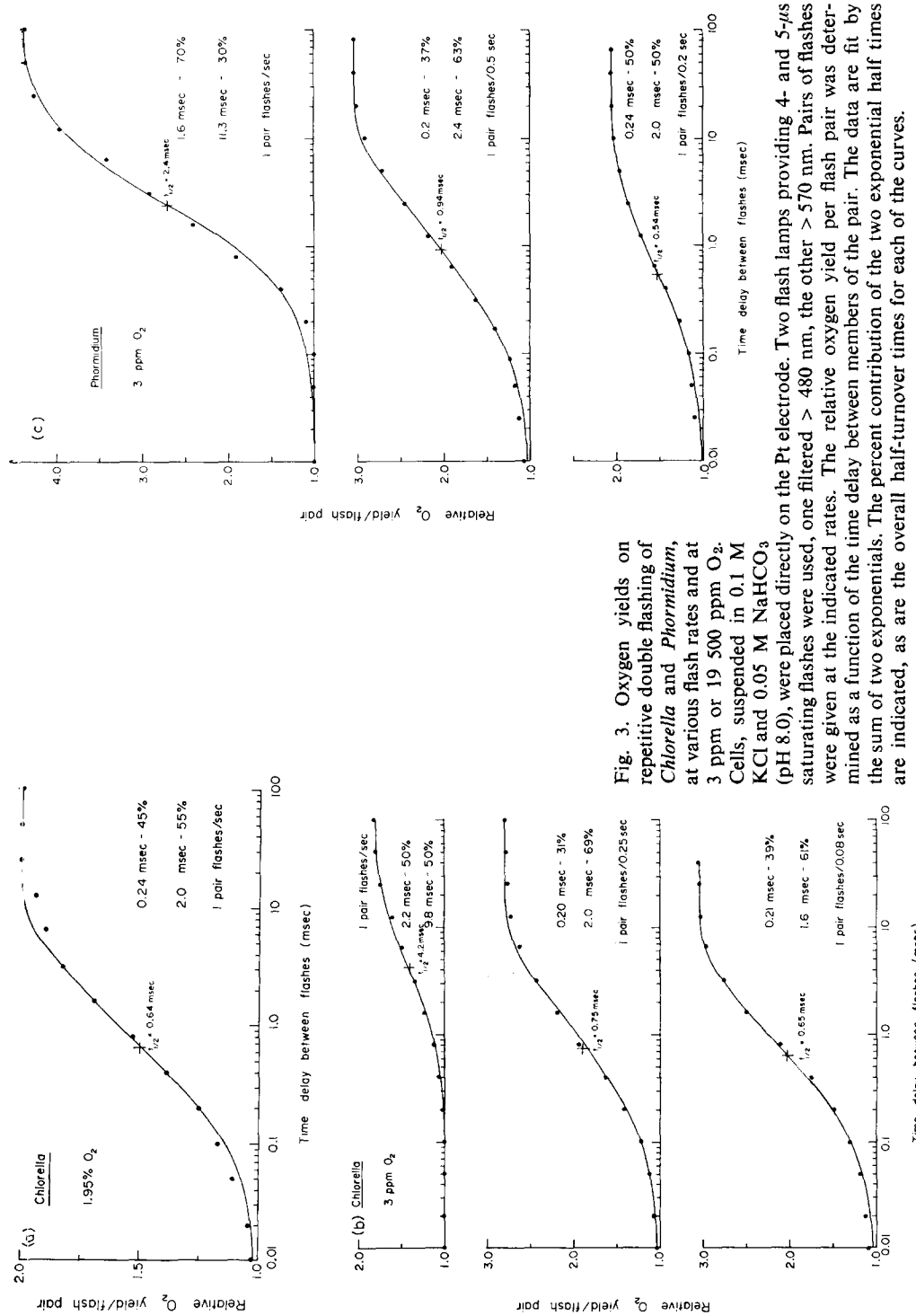

Fig. 3. Oxygen yields on repetitive double flashing of *Chlorella* and *Phormidium*, at various flash rates and at 3 ppm or 19 500 ppm O_2. Cells, suspended in 0.1 M KCl and 0.05 M $NaHCO_3$ (pH 8.0), were placed directly on the Pt electrode. Two flash lamps providing 4- and 5-μs saturating flashes were used, one filtered > 480 nm, the other > 570 nm. Pairs of flashes were given at the indicated rates. The relative oxygen yield per flash pair was determined as a function of the time delay between members of the pair. The data are fit by the sum of two exponentials. The percent contribution of the two exponential half times are indicated, as are the overall half-turnover times for each of the curves.

single long-flash experiments in spinach chloroplasts, Witt[5] estimated that the time for the reduction of plastoquinone, following a light flash, was about 1 ms if the pool is oxidized. By repetitive double-flash experiments, using benzylviologen and $K_3Fe(CN)_6$ as electron acceptors, Vater *et al.*[9] showed that this time corresponded to the half-turnover time for oxygen production (approx. 0.6 ms). These are the same times we observe under aerobic or rapid flash conditions. Witt[5] has estimated that when the pool is fully reduced by saturating light under aerobic conditions, oxidation of a pool member takes 4.5 ms. This result is in approximate agreement with our 4.2- and 5-ms turnover time for oxygen production in *Chlorella* measured under anaerobic conditions by repetitive double and single flashes, respectively. The similarity of our kinetic results with those of Stiehl and Witt[2] and Witt[5] for plastoquinone, confirms our belief that pool A and plastoquinone are one and the same.

Heterogeneity of turnover times

Figs 3a, 3b and 3c show the data on which the repetitive double-flash turnover times are based. It is apparent that not only do the anaerobic curves shift to shorter time as the pair repetition rate increases, but that the shapes of curves change as well. In fact, none of the curves shown for *Chlorella* and *Phormidium* represent a pure exponential turnover. Each of these curves is heterogeneous and may be represented by a mixture of two out of three possible exponential rates ($t_{\frac{1}{2}}$ approx. 0.2 ms, 2 ms, and approx. 10 ms).

Probably what is most striking about these curves is the 0.2-ms component of the turnover at high pulse rate or high background oxygen. The maximum contribution of this component in any of the curves is 50%, the other 50% is contributed by a component of 2 ms. As the pulse rate is decreased, under 3 ppm O_2 background, the percent contribution of the fast component decreases until at low pulse rate (1 pair/s) it is absent altogether. A new component of 10 ms appears at these low pulse rates which is probably contributing appreciably to the 5–6-ms half-turnover time observed under repetitive single-flash conditions.

Relative O_2 yield of second flash

We note that the gain factor in these turnover curves, *i.e.* the ratio of the yield of O_2 per flash pair at long pair intervals to that when the pair members are superimposed, can be quite large, *e.g.* >4 in Fig. 3c. This is predicted by our model of the preceding paper. The factor can be read from the "loss" curves (Figs 4 and 12 of preceding paper[1]) as the ratio of the yield at half the time interval to that at the full time interval between the flash pairs. Only when the yield is constant with period, as under nearly aerobic conditions, or with *Chlorella* at long times under anaerobic conditions, does one get a simple factor of 2 (Figs 3a, 3b and 3c).

Turnover measurements when coupled to Hill oxidants

Turning to results given in Table I, it is apparent that the turnover, as measured by repetitive single-flash experiments for *Phormidium* cells coupled to $K_3Fe(CN)_6$ (30–36 ms, Table I) is 5–6 times longer than that for the same cells coupled to CO_2, 6 ms (Fig. 2). This difference in turnover time is sufficient to account for half of the 10-fold difference in the light-saturated oxygen rates observed under

TABLE I

TURNOVER TIMES OF *PHORMIDIUM* BY REPETITIVE SINGLE FLASH COUPLED TO HILL OXIDANTS AT 3 ppm O_2 IN OXYGEN LUMINOMETER

Phormidium	$t_{1/2}$ (*ms*)	*Oxidant*
Cells	29	0.25 mM benzoquinone
	36	5.0 mM $K_3Fe(CN)_6$
	30	5.0 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$
Cell-free preparation	35	0.67 mM $K_3Fe(CN)_6$

these same conditions[10]. The remaining factor of 2 derives from the 2-fold greater flash yield for oxygen production in CO_2-coupled cells over those coupled to $K_3Fe(CN)_6$ (compare Fig. 1, this paper and Fig. 6, preceding paper[1]).

Repetitive double-flash experiments with *Phormidium* cells, coupled to $K_3Fe(CN)_6$ (Table II), show a dependence of turnover on the rate at which pairs of flashes are given. These results support our earlier contention (Fig. 1) that even in the presence of $K_3Fe(CN)_6$ the redox state of the pool is dependent on the flash rate and is rather insensitive to the presence of this oxidant. Furthermore, the turnover time in *Phormidium* cells is independent of the $K_3Fe(CN)_6$–$K_4Fe(CN)_6$ redox potential. We stress that it is unique feature of *Phormidium* that intact cells are able to evolve oxygen with $K_3Fe(CN)_6$ as the ultimate electron acceptor. Apparently, the plasma membranes are more permeable to $K_3Fe(CN)_6$ than those for eukaryotic algae.

The turnover times for repetitive single and double flashes for the *Phormidium* cell-free preparation[10] coupled to $K_3Fe(CN)_6$ are 35 and 32 ms, respectively (Tables I and II). The similarity of these half-turnover times are understandable in light of the linear light intensity dependence and lack of reductive loss in this preparation and its insensitivity to (3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[10], *i.e.* $K_3Fe(CN)_6$ couples directly to photoreaction Z–Q, bypassing the pool. Thus, the turnover time is determined only by the rate of electron transfer to $K_3Fe(CN)_6$.

TABLE II

TURNOVER TIMES OF *PHORMIDIUM* BY REPETITIVE DOUBLE FLASH COUPLED TO HILL OXIDANTS AT 3 ppm O_2 IN OXYGEN LUMINOMETER

	$t_{1/2}$ (*ms*)	*Oxidant*
Cells		
1 pair flashes/200 ms	1	5.0 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$
1 pair flashes/s	4	0.5 mM $K_3Fe(CN)_6$, 0.17 mM $K_4Fe(CN)_6$
Cell-free preparation		
1 pair flashes/500 ms	32	0.2 mM $K_3Fe(CN)_6$
1 pair flashes/500 ms	32	0.2 mM $K_3Fe(CN)_6$, 8.0 mM $K_4Fe(CN)_6$

DISCUSSION

Although the kinetic model derived in the preceding paper qualitatively explains the turnover time data, two quantitative points require elaboration. The first is the truncation (plateau) of the oxygen yield per flash in anaerobic repetitive single-flash experiments (Fig. 2 and Figs 6, 12 and 13 preceding paper[1]) at short time intervals between flashes. As flashes are given rapidly and then at progressively lower frequencies, one would expect that once the oxygen yield per flash were no longer limited by the turnover time, at 10–15 ms between flashes, that these curves would immediately show a sharp transition to the 160 ms ($t_{\frac{1}{2}}$) decreasing flash yield kinetics. Instead (Fig. 2) the flash yields remain constant out to 50 ms between flashes for *Chlorella* and 175 ms between flashes for *Phormidium*. Only at these time points does the rapid fall begin. Thus, some factor other than the turnover time is responsible for this truncation. This phenomenon will be discussed in terms of two models: one where the pool need only be half oxidized to obtain maximum flash yield, and another where the pool must be fully oxidized to obtain maximum yield.

The second quantitive point requiring elaboration of the simple model is the heterogeneity of the turnover times and their dependence on the redox state of the pool.

Truncation

The first model is a simple chemical interpretation of the model (Fig. 4) presented in the previous paper[1] and is based on the assumption that Q and A are both plastoquinone. We postulate that Q can accept no more than 1 equivalent per excitation. In that case, the maximum yield per flash would be attained when Q is either in the semiquinone or quinone form. The former case would correspond to half oxidation of the pool, since the pool members can conceivably exist in three oxidation states: quinone, semiquinone and hydroquinone and are in equilibrium with Q. Thus more than half-oxidation of the pool would not affect the yield of

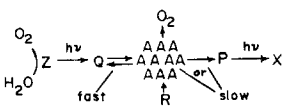

Fig. 4. Model 1.

oxygen which would be truncated at one-half the maximum level predicted by our simple theory. Moreover, this limiting yield would occur whenever the pool is half oxidized, whether determined by the flash frequency or by the relative rates of electron input (R) and output (O_2). In fact the best fit between our kinetic theory and the experimental flash yields (Fig. 12 of ref. 1) occurs for a maximum yield at half oxidation of the pool. Michaelis *et al.*[11] showed that alkaline conditions were necessary to stabilize the semiquinone anion which would otherwise disproportionate to the quinone and hydroquinone. One might argue that the inside of a membrane provides a proton-free environment which should retain the semiquinone. However, its small dielectric is unlikely to support any high concentration of semiquinone

anion or hydroquinone dianion in the pool. These anions would be protonated to remove the charge. It is possible though that a single quinone, such as Q, could be stabilized as a semiquinone at a sufficiently polar specialized site.

An intriguing way to lock Q into a one electron reaction is to have it in the form of a plastochromanol, which can be reversibly oxidized to plastochromanoxyl. Further oxidation, however, would require C–O bond cleavage. Kohl *et al.*[12] have obtained evidence for a chromanoxyl in the broad slow ESR signal found in green plants. In addition, Dunphy *et al.*[13] found a ratio of about 1 plastochromanoxyl to 15 plastoquinones in chloroplasts. Cramer and Butler[7] observed considerable hysteresis in their redox titration of fluorescence yield changes. If the species they titrated were plastochromanol, oxidation would first reversibly yield plastochromanoxyl. Further oxidation would produce the open ring plastoquinone. If the ring closure were slow, then the reductive titration would proceed through the semiquinone and hydroquinone. The starting and final redox species would differ and thus account for the difference in redox potentials they obtained on oxidative and reductive titrations.

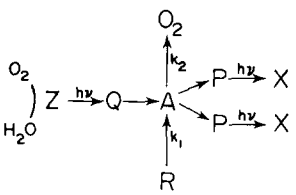

Fig. 5. Model 2.

A second model for the truncation of the oxygen yield is to have two P–X photoreactions (Fig. 5) coupled to each pool. As long as the pool is partially reduced, excitation results in the removal of two equivalents from A. When the pool is fully oxidized, each P can accept an equivalent, one of which would be contributed by Q and the other by R. The pool is retained in an oxidized state for a longer time than predicted by the simpler model. Thus the oxygen yield is constant from time $1/k_1 R$ until the turnover time becomes limiting. Under conditions of fully oxidized pool, the reduction of P could occur in two steps: a fast one with the (photo) equivalent from Q and a slow one with an equivalent from R.

Two additional models were considered, one in which Q and A were related by a redox equilibrium constant, K, greater than one, *i.e.* the redox potential of A oxidizing with respect to Q, and another in which several Q served as acceptors to Photosystem II. Theoretical curves were calculated for these models using Eqn 5, Fig. 10 of the preceding paper[1] and were found to be considerably more shallow than the experimental curves (Fig. 12 of ref. 1), and are thus unacceptable.

A test of our models would be to compare the redox state of the pool, by optical means, to the oxygen flash yield. Also desirable would be a quantitative *in vivo* study by ESR of the semiquinones in Q and A.

Truncation would be observed if there were a 2-fold turnover of Photosystem I in a single flash. The most rapid component reported for the rereduction of P700 following flash excitation is about 15 μs[14,15]. This component accounts for only 30% of the total recovery and occurs in a time range appreciably longer than the

5-μs flashes used here. The fact that repetitive single-flash curves for *Chlorella* and *Phormidium* using 10-ns N_2 laser flashes[16] were identical to those using 5-μs xenon strobe flashes, assures that there is little, if any, 2-fold turnover of either photosystem.

Heterogeneity and shift of turnover times

We noted in Figs 3a, 3b and 3c, the increase in the turnover time upon anaerobic reduction of the pool. These curves were fit with a sequence of exponential times, the respective contributions of which are indicated. We have not yet carried out a detailed analysis of the relaxation kinetics of our model. We thus do not give a quantitative interpretation to the heterogeneous times of the turnover curves. Nonetheless, we point out that this dependence of the turnover time on pool redox state is consistent with our model (Fig. 4) if the rate of electron transfer into the pool is 0.2–2 ms, while the output of the pool is about 10 ms. When the pool is oxidized (high O_2, rapid flash rate) the turnover time (*i.e.* the reoxidation of Q) is determined by the rate of electron transfer from Q to A. When the pool is reduced (low flash rate under anaerobic conditions), the Q to A transfer is slowed by the low probability of finding oxidized A in the pool. The relaxation time for Q is then determined by the reoxidation time of A which, from our kinetics, would be about 10 ms. Whether the rate-limiting step for reoxidation of A is within the pool or between the pool and P cannot be decided from our studies.

Other evidence for 0.2 -and 2-ms contributions to the turnover times comes from transient measurements. A 0.2-ms turnover time is seen in measurements of the oxygen yield from individual flashes in a series[17] as a function of the delay between the first and second flashes. Similarly, the decay of the increased quantum yield of fluorescence, following a saturating flash[18] shows about 3/4 of the 0.2-ms component and 1/4 of the 2-ms component. Because of the similar kinetic data[2] for plastoquinone we have tended to ascribe our rapid turnover under oxidized conditions to the reoxidation of Q. However, we cannot rule out the possibility that the oxidizing side is responsible for the rapid and heterogeneous turnover times.

Döring *et al.*[19] have observed a 0.2-ms relaxation time following the bleaching of chlorophyll a_{II} in a short flash. However, the exact relationship between this absorbance change and the oxidation of water remains unclear.

If the oxidizing side were indeed responsible for the heterogeneous turnover times, our results would require that biphasic kinetics be observed for the reduction of reaction center chlorophyll in Photosystem II following a light flash. Inability to observe such kinetics would support the reducing side model by default.

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