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REDOX TITRATION OF ELECTRON ACCEPTOR Q AND THE PLASTOQUINONE POOL IN PHOTOSYSTEM II

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Summary

The primary photochemical quencher Q and the secondary electron acceptor pool in Photosystem II have been titrated. We used particles of *Scenedesmus* mutant No. 8 that lack System I and allowed the system to equilibrate with external redox mediators in darkness prior to measurement of the fluorescence rise curve.

The titration of Q, as indicated by the dark level of F_i, occurs in two discrete steps. The high-potential component (Q_h) has a midpoint potential of +68 mV (pH 7.2) and accounts for ~67% of Q. The pH sensitivity of the midpoint potential is —60 mV, indicating the involvement of 1 H^+/e. The low-potential component (Q_l) accounts for the remaining 33% of Q and shows a midpoint potential near—300 mV (pH 7.2).

The plastoquinone pool, assayed as the half-time of the fluorescence rise curve, titrates as a single component with a midpoint potential 30—40 mV more oxidizing than that of Q_h, i.e., at 106 mV (pH 7.2). The E_m shows a pH sensitivity of —60 mV/pH unit, indicating the involvement of 1 H^+/e. The observation that all 12—14 electron equivalents in the pool titrate as a single component indicates that the heterogeneity otherwise observed in the secondary acceptor system is a kinetic rather than a thermodynamic property.

Illumination causes peculiar, and as yet unclarified, changes of both Q and the secondary pool under anaerobic conditions that are reversed by oxygen.

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Abbreviations: PS I, Photosystem I; PS II, Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol; E_m, midpoint potential (at specified pH); Mops, 3-(N-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; chl, chlorophyll.

Introduction

Variations in the fluorescence emission of isolated chloroplasts are best explained by assuming that the primary electron acceptor of PS II (Q) functions as a fluorescence quencher in its oxidized but not in its reduced state [1]. Q^-, formed by light, is oxidized by a large pool of secondary oxidant (A) consisting of plastoquinone. If isolated chloroplasts are illuminated with relatively weak light in the absence of an exogenous electron acceptor (each Q reduced 1—10 times per s), the fluorescence yield rises from a minimum value (F_0, a residual emission that occurs despite the oxidation of Q) to a maximum value (F_{max}, where Q, and therefore also A, has undergone complete photoreduction).

During the slow light-driven rise of the fluorescence from F_0 to F_{max}, the various intermediates between Q and *P*-700 maintain chemical equilibrium so that the shape of the rise curve is a function of the number of intermediates and their equilibration [2]. After a long (10-min) darktime, the rise curve is complex, apparently reflecting the reduction of two pools (A_2, A_1) with different equilibrium constants, each containing 5—8 equivalents [2,3].

The inhibitor DCMU isolates Q from the A pool. In its presence, the rise from F_0 to F_{max} is greatly accelerated and reflects exclusively the photoreduction of the fluorescence quencher, Q.

Recently another component, R [4] or B [5], has been shown to exist between Q and A, functioning as a charge accumulator by accepting electrons singly from Q and transferring them in a pair to the plastoquinone pool. According to this evidence, in aerobic darkness, the PS II acceptor system does not return to the fully oxidized state; instead, one-third of the centers assume the state QR^-A. The equilibrium constant for electron transfer between Q and R has been estimated to be 15—20 [6].

Several attempts have been made to utilize the fluorescence yield and the rise curve to determine the midpoint potentials of the electron acceptors on the reducing side of PS II. The first one was made in this laboratory [7] and employed particles of *Scenedesmus* mutant No. 8 that lacked Photosystem I [8]. The initial fluorescence yield (F_i), observed at a given potential after a long period of dark equilibration, was used as a measure of the redox state of Q, and the area encompassed by the rise curve served to measure the redox state of the secondary pool.

In other studies concerning the effect of redox potential upon the fluorescence yield [9,10], the technical approach was different; spinach chloroplasts (containing both photosystems) were used, and the fluorescence was monitored continuously with a weak exciting beam. As well be discussed in more detail below, this type of measurement is liable to interference by (a) the strong reducing power generated by Photosystem I, and (b) the photochemical action of the exciting beam.

In this study, we reexamined the relationship between the quantitative aspects of the fluorescence rise curve (F_i, F_{max}, area) and redox potential in a system where Q and the secondary acceptor pool were dark-equilibrated with external redox mediators. The initial level of fluorescence (F_i) upon illumination of a dark-adapted sample served to indicate the level of reduction of Q,

and the half-time of the rise curve ($t_{1/2}$) was used to calculate the degree of reduction of the A pool. The data permitted the determination of the oxidation-reduction midpoint potential of Q and the secondary electron pool.

Materials and Methods

Chloroplast fragments were prepared from *Scenedesmus* mutant No. 8 according to the procedure of Kok and Datko [11]. Following the last spin, the pellet was resuspended to a chlorophyll concentration of 4 mg/ml in 50 mM Tris buffer (pH 7.5) containing 0.4 M sucrose and 10 mM NaCl (STN buffer) and used either directly or stored frozen in liquid nitrogen until use. One freeze-thaw cycle did not cause any alteration of the fluorescence rise curve. Mutant No. 8, which lacks the entire Photosystem I apparatus [8,12], was used because a functional Photosystem I would withdraw electrons from the pool during illumination, upsetting the equilibrium with the redox mediators. In spinach chloroplasts, in the presence of the chosen redox mediators, the fluorescence barely rises beyond F_0 during illumination.

The experiments were performed in a specially-constructed plexiglass cell (diameter 18 mm, 2 mm thick) containing a small measuring electrode and a large working platinum electrode for use in electrochemical titrations. The cell was stirred internally with a Teflon-covered magnet which was coupled to a rotating magnet located just beyond the rear face of the cell. Two channels (diameter 2 mm, 20 mm long) serves as agar bridges; one was connected to a 0.1 M $Fe(CN)_6^{-3}/Fe(CN)_6^{-4}$ half cell, the other to a saturated calomel electrode. A small, resealable opening located on the top of the cell was used for the addition of reagents. The front face of the cell was transparent for unimpeded passage of the blue actinic illumination and the resulting fluorescence.

To measure fluorescence, the sample was illuminated with blue light isolated from a mercury lamp (H4AB) with a Corning 4-96 filter. The resulting fluorescence, after passage through a RG-8 Schott filter, was detected by a PIN diode, and after suitable amplification, recorded on a Brush Model 250 recorder. During each illumination, the stirring was interrupted; the magnet always assumed the same rest position. Spurious light accounted for ~4% of the total fluorescence signal. The potential of the cell was measured with a platinum electrode and a standard calomel electrode. The system was calibrated with quinhydrone at pH 6.0. All potentials are reported relative to that of the normal hydrogen electrode.

Electrochemical reductions were performed according to the following protocol: 10 μl of chloroplast fragments (4 mg/ml) were diluted to 2 ml in 50 mM buffer (Mops, pH 7.2; Tricine, pH 8.4; or Mes, pH 6.2) containing 0.4 M sucrose, 1 mM NaCl, 5 mM Mg^{2+}, and 1% glucose; the 0.5-ml fluorescence cell was completely filled with the mixture. The chloroplasts were stirred aerobically in the dark for 5 min (to ensure that the A pool was >90% oxidized, see Fig. 2), and the glucose oxidase, catalase (final concentration 1.4 and 0.035 mg/ml, respectively), and redox buffers (at the concentrations shown in the legends of Figs. 3 and 4) were added. The buffer concentration of the chloroplast suspension was adequate to restrict pH changes due to the oxygen scrubbing process to less than 0.1 pH unit.

After an additional 5-min dark period, a negative voltage was applied to the working electrode to induce reduction. At the desired potential, the voltage was switched off, and the chloroplasts were allowed to equilibrate for 5 min prior to illumination. The rise curve was then recorded, the system was allowed to reequilibrate in the dark for a further 5 min, and a second rise curve was recorded. The entire process required about 30 min. A fresh sample was used for each determination. Ascorbate and dithionite were as effective as electrochemical reduction in poising the redox system (ascorbate was only able to reduced the system potential to about 50 mV). The results obtained with these agents (not shown) did not differ substantially from those of the electrochemical titrations.

Oxidative titrations were performed similarly, except that the redox system was first taken to −50 mV by electrochemical reduction, allowed to equilibrate for 3 min, and then oxidized by the addition of aliquots of 10 mM potassium ferricyanide. (The ferricyanide was incubated in STN buffer containing the glucose/glucose oxidase scrubber for 10 min prior to use.) After an additional 5-min dark period to achieve equilibrium, the rise curve and potential were recorded. Electrochemical oxidations were not routinely performed due to the length of time required to first reduce and then reoxidize the system to sufficiently high potentials.

The initial level of fluorescence at the onset of illumination is denoted as F_i; its minimum level (observed under oxidizing conditions) is F_0 [7]. The maximum level achieved after a period of continuous illumination is denoted as F_{max}. F_{var}, the amount of variable fluorescence, is the difference between F_{max} and F_0, while $t_{1/2}$ is the time required for the fluorescence to rise halfway from F_i to F_{max} and is a function of light intensity.

Results

Rationale for equilibrating Q and A in the dark

In the present study, we have taken care to exclude all light while equilibrating the chloroplast fragments with the external redox mediators before measuring the fluorescence rise curve. In earlier titrations of Q, an exciting beam was used to continuously monitor the fluorescence yield; presumably its intensity was weak enough not to perturb the system (3—10 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}, Refs. 9, 10). However, in the experiments of Cramer and Butler [9], made in the presence of DCMU, the measuring beam effected 1.6% of the maximal fluorescence increase. Because of energy transfer between PS II units [13], this rise indicates a 4% photoreduction of Q. Since the rate constant of the back reaction of Q^- with the oxidized donor system is about 1 s^{-1} [14], we calculate that this beam induced about one excitation every 25 s per PS II trap.

Later in this paper (Figs. 1, 2), we show that the (half) times needed for dark equilibration of Q and A with the external redox mediators chosen for this study are in this same time range or longer. Evidently, even weak beams can readily cause non-equilibrium conditions. Probably even more important in this connection (as discussed later in this paper), we find that a fraction of Q and A fails to reequilibrate with the redox dyes once the system has been exposed to light under anaerobic conditions. Similar, though undetected, behavior in

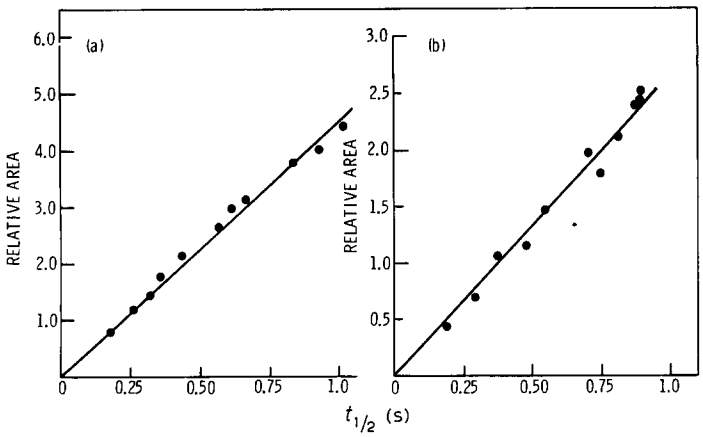

Fig. 1. Relationship between area and half-time ($t_{1/2}$) of the fluorescence rise curve. (a) The recovery of the area and half-time in *Scenedesmus* No. 8 chlorplast fragments using air as oxidant. (b) Same as (a) except reaction mixture included 25 μM 1,2-naphthoquinone and 25 μM 1,4-naphthoquinone.

Refs. 9 and 10 could possibly account for the hysteresis observed in the reported titrations of Q.

Use of redox mediators, quenching by quinones

The purpose of the redox mediators (derivatives of benzoquinone, naphthoquinone and dipyridillium dyes) is to shuttle electrons between the suspension medium, the platinum electrode, and the electron carriers in the chloroplasts.

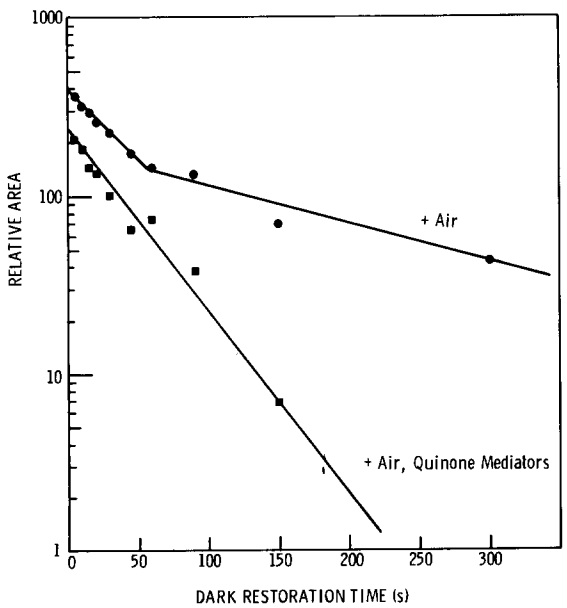

Fig. 2. Recovery kinetics of the secondary electron pool following a long saturating flash. Data are plotted as area under the rise curve vs. dark restoration time using air (●) or an air + 1 : 1 mixture of 1,2- and 1,4-naphthoquinone at 25 μM (■) as oxidant.

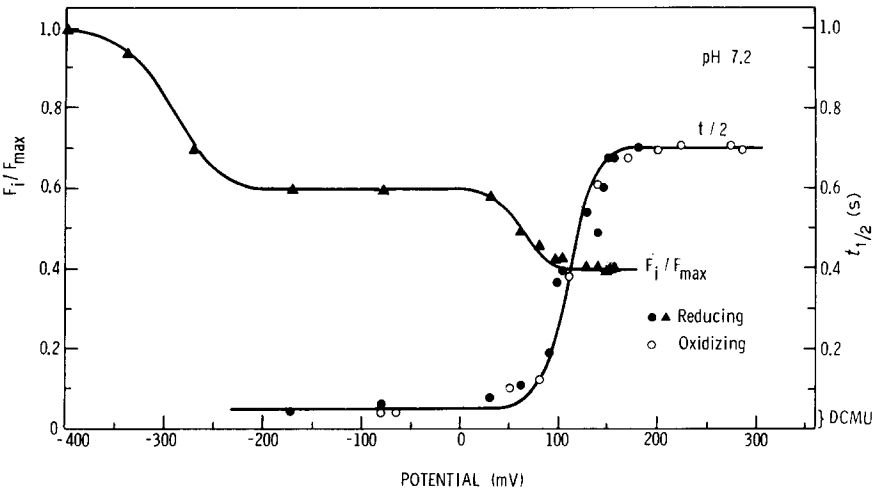

Fig. 3. Redox titration of the half-time ($t_{1/2}$) and F_i in the reductive and oxidative directions at pH 7.2. The redox mediators were: methylviologen (20 μM), benzylviologen (20 μM), 2-hydroxy-1,4-naphthoquinone (10 μM), 2,5-dihydroxy-1,4-benzoquinone (10 μM), 2-methyl-1,4-naphthoquinone (10 μM), 1,4-naphthoquinone (10 μM), 1,2-naphthoquinone (10 μM), in Mops buffer (0.05 M) containing 0.4 M sucrose, 1% glucose, 1 mM NaCl and 5 mM $MgCl_2$. The half-times for the reductive direction are shown as closed circles (●) and for the oxidative direction as open circles (○). The parameter F_i/F_{max} is shown in the reductive direction only as closed triangles (▲). The half-time of the fluorescence rise in the presence of DCMU is depicted by the bracket near the abscissa.

Several of the high-potential quinone mediators were chosen because of their demonstrated ability to interact with the plastoquinone pool [11]. However, this ability to withdraw electrons from the pool may also cause the fluorescence rise curve to be distorted.

One has to strike a balance between the light intensity (the rate of photoreduction of Q and A) and the concentration of mediating quinones which tend to counterbalance (buffer away) the effect of the light. In the presence of the selected quinone mediators in the concentrations used, the dark half-time for the oxidation of the pool was about 25 s (see Fig. 2). Since the half-time for the photoreduction of the pool was about 0.7 s (see Fig. 3), there will be an error of less than 5% for the $t_{1/2}$ of the fluorescence rise curve.

The use of quinones as redox mediators introduces yet another problem: the direct quenching of chlorophyll fluorescence induced by their oxidized forms [15]. Under the conditions of our measurements, as the redox state and therefore the quenching by quinone mediators changes with redox potential, there is no longer a simple relation between the degree of oxidation of the pool and the area under the rise curve.

Consequently, rather than the encompassed area, we used the half-time of the fluorescence rise curve as an indicator of the redox state of the plastoquinone pool. The validity of this approach is shown in Figs. 1 and 2. In one experiment, *Scenedesmus* No. 8 chloroplast fragments were suspended in air-saturated STN buffer (pH 7.4) containing 5 mM $MgCl_2$ (Fig. 1a) and, in a separate experiment, in a 1 : 1 mixture of 1,2-naphthoquinone and 1,4-naphthoquinone in the same buffer (Fig. 1b). The samples were allowed to

equilibrate in the dark for 10 min, and a rise curve was recorded. A fresh sample was introduced and the same protocol was followed except that the dark equilibration time was halved. The process was repeated until only 5 s of dark reequilibration time remained. As shown in Fig. 1, the area under the rise curve and $t_{1/2}$ are linearly related, i.e., follow identical recoveries for either oxygen (Fig. 1a) or oxidized quinone mediators (Fig. 1b) as the pool recovery agent.

We found that while these mediators quenched up to 50% of F_{var}, the $t_{1/2}$ of the fluorescence rise was not significantly affected. The sample containing the quinones showed a $t_{1/2}$ of 0.55 vs. 0.63 s for the control. We can therefore conclude that the half-time of the fluorescence rise curve provides a valid measure for the degree of pool reduction regardless of the degree of quenching. We confirmed this conclusion in additional experiments where the addition of increasing amounts of quinones strongly decreased the area but had little effect on half-time.

In Fig. 2, the dark restoration curve of Fig. 1 is shown as a plot of the area vs. dark restoration time. The data demonstrate: (1) a typical biphasic dark restoration of the pool with oxygen as the electron acceptor, with half-times of 158 s and 2 min, and (2) a monophasic recovery ($t_{1/2}$ = 25 s) of the plastoquinone pool in the presence of the oxidized redox mediators. Note that the half-time of the A_2 sub-pool ($t_{1/2}$ = 18 s) closely corresponds to that of the entire pool in the presence of the mediators ($t_{1/2}$ = 25 s). Although this close correspondence may be fortuitous, the data clearly show that the kinetics of recovery is dependent on the nature of the oxidant. Since the time course fails to reveal the more slowly restored fraction of the pool (previously denoted A_1; Ref. 3), this fraction may not be chemically different from the A_2 subpool, only less accessible to oxygen.

The time course of Fig. 2 indicates that the 5 min of dark incubation used in this study are sufficient for equilibration of the A pool with the (oxidized form of) the redox dyes.

Titration of the plastoquinone pool

Fig. 3 shows the results of a typical redox titration of the half-time of the fluorescence rise curve in both the reductive and oxidative directions at pH 7.2. Several features are immediately apparent: (1) the oxidative titration (open circles) closely matches the reductive titration (closed circles), a good indication that chemical equilibrium has been established. Chemical reduction with ascorbate or dithionite yielded a very similar titration curve; (2) the redox titration shows a midpoint potential of 106 mV at pH 7.2 and (3) is consistent with a theoretical n = 2 electron reduction. (Linear regression of the Nernst plot showed n = 2.15 in the reductive direction and n = 2.13 in the oxidative direction.); (4) the whole plastoquinone pool appears to titrate in a single wave.

The area under the rise curve indicates approx. 14 electron equivalents reside in the secondary pool, assuming the DCMU area corresponds to one equivalent (however, see Ref. 4 for a discussion of this assumption). This calculation depends on a high quantum efficiency; a damaged water-splitting system will cause an increase in the area under the normal rise curve but not necessarily in the DCMU area. To check this point, we have compared the relative quantum

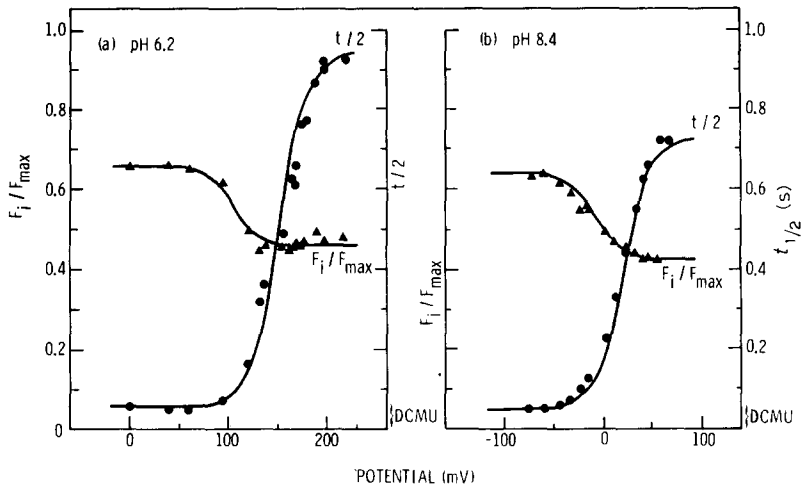

Fig. 4. Redox titration of the half-time ($t_{1/2}$) and F_i at pH 6.2 and 8.4. Conditions identical fo Fig. 4 except 50 mM Mes buffer was used at pH 6.2 (a) and 50 mM Tricine buffer was used at pH 8.4 (b).

efficiency of our mutant No. 8 preparations with that of freshly prepared spinach chlorolasts. With benzoquinone as acceptor, we found equivalent low light rates; with 2,5-dimethylquinone, we observed 82% of the rate with spinach. Since the absolute quantum efficiency of carefully prepared spinach chlorplasts is high [16], we concluded that our preparation of mutant No. 8 had an equally high quantum efficiency.

Further oxidation (with ferricyanide and a potential up to +360 mV) did not appreciably change the half-time of the rise curve. The number of equivalents, the lack of further increase in the half-time of the rise curve at potentials beyond 160 mV, and the homogeneous dark restoration curve in the presence of mediators (Fig. 2) all indicate that the titration curve in Fig. 3 reflects the entire plastoquinone pool. (Complications arise when the system recovers from illumination and is exposed to light a second time; these will be discussed in the next section).

Fig. 4a and b shows the results of similar redox titrations performed at pH 8.4 and 6.2. In Fig. 4a, the midpoint potential of the plastoquinone pool is 15 mV (n = 1.80); in Fig. 4b, the midpoint is 151 mV (n = 1.65). Relative to the DCMU area observed at the respective pH values, the fully oxidized pool represents 13.2 equivalents at pH 6.2 and 12.1 equivalents at pH 8.4, i.e., the pool size does not vary beyond experimental uncertainty. The midpoint potentials in these figures and in Fig. 3 show an average pH sensitivity of nearly —60 mV/pH unit between pH 6.2 and 8.4. These data indicate that the 2-electron reduction of the plastoquinone pool coincide with the uptake of 2 protons (1 H^+/e).

Irreversible changes induced by light under anaerobic conditions

In the absence of oxygen, the equilibrium between the acceptors of PS II and the external redox mediators (as demonstrated in Fig. 3) occurs only if the chloroplasts are incubated in complete darkness prior to measurement. Upon a

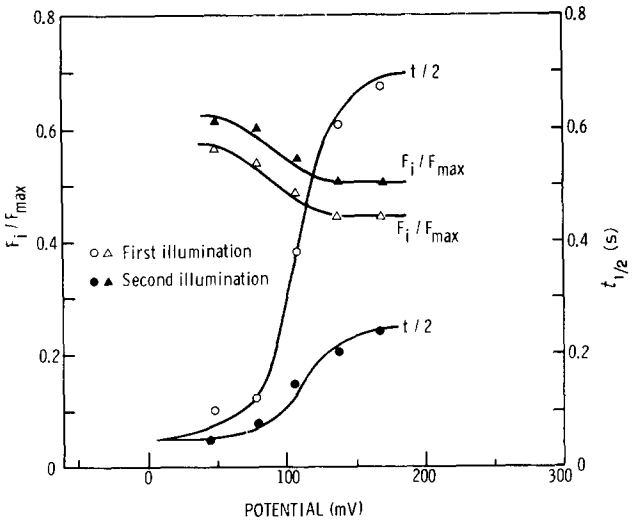

Fig. 5. Recovery of Q and A upon a second illumination following a 5-min dark reequilibration period. The reaction mixture (containing chloroplast fragments and redox buffers as in Fig. 3) was first taken to −50 mV by electrochemical reduction and then reoxidized by the addition of anaerobic potassium ferricyanide solution to achieve the desired potential. After the first rise curve was determined (○ for $t_{1/2}$ and △ for F_i/F_{max}), the system was allowed to reequilibrate in the dark for 5 min and a second rise curve was recorded. The $t_{1/2}$ and F_i/F_{max} upon the second illumination are shown as closed circles (●) and triangles (▲), respectively.

second illumination, separated from the first by a 10-min dark period, only a portion of Q and the plastoquinone pool recover at a given potential. This effect is shown in Fig. 5 where the half-time of the fluoresencence rise and the rise in F_i is plotted during the first and second illumination for the oxidative titration shown in Fig. 3. Clearly, 60% of the plastoquinone pool and 10% of the variable fluorescence, which corresponds to 40% of Q (transfer probability $p = 0.7$, Ref. 13, 17), fails to reequilibrate with the external redox buffers during a dark period at potentials near the end point of the titration.

It is noteworthy that the fraction of the pool which does recover readily equilibrates with the external mediators: as shown in Fig. 5, the midpoint potential of the oxidative titration observed upon a second illumination closely matches that on the first illumination. Increasing the dark period between illuminations to 1 h or increasing the concentrations of redox buffers by a factor of 5 did not significantly increase the recovery of Q or the plastoquinone pool (at potentials near 160 mV). Upon a third illumination, separated from the second by a 10 min dark interval, even less of the plastoquinone pool was seen to recover. The addition of uncouplers (methylamine, gramicidin) did not affect this curious behaviour. We found that the only effective way to obtain complete reoxidation of the pool was by introducing some molecular oxygen into the vessel. After such an addition, Q and the plastoquinone pool are totally reoxidized (at 160 mV, pH 7.2). However, in a subsequent (anaerobic) illumination, the system reverted to its previous partially irreversible behavior. Details of this peculiar non-equilibrium phenomenon are under investigation.

Titration of the fluorescence quencher, the low potential component

We found that the titration of F_i occurred in two waves, one with a midpoint potential at 68 mV and the other with a potential near −300 mV (pH 7.2) (Fig. 3). The low potential component, operationally defined as Q_l, accounted for 60—70% of the variable fluorescence yield. Similar low potential rises have been observed in studies of Q and flash-induced field changes at 518 nm [9,10,18].

In our present experiments we have given only cursory attention to this rise; the reasons for this will be given in the Discussion.

The high potential component

The high potential component, Q_h, consistently (i.e., at all three pH values studied) accounted for 30—40% of the total variable fluorescence. For the presumed energy transfer between units with a probability $p = 0.7$ [17], this implies that 60—70% of acceptor Q is involved in the high-potential transition. As shown in Figs. 3, 4a and 4b, the midpoint potential of this fluorescence rise is 25—45 mV (average: 36 mV), more negative than the midpoint of the plastoquinone pool. Again, $p = 0.7$ implies that the midpoint potential of Q_h is, on the average, 30 mV lower than that of the pool.

While our data firmly support these approximate numbers, they do not readily yield more precise ones: the experimental accuracy is limited, and it is difficult to design a rigorous correction for F_0, F_i, and F_{max} for the quenching by external quinone(s) (that changes with potential along with the quenching by Q). Thus, to compute E_m values, we used the simple procedure shown in Fig. 6 (data from Fig. 4a). For reference, the values of F_0 and F_{max} observed in the absence of redox mediators are shown as horizontal lines. In the presence of the mediators, at potentials where the pool is oxidized (>60 mV), F_0 is quenched 15%, and F_{max} 26%. Between +60 and −25 mV, the potential range where the pool becomes reduced, F_{max} remains constant, but F_i begins to increase and surpasses the reference value of F_0. In this range, the photochemical quencher Q is becoming reduced. From −25 to −75 mV, the rise in F_i continues, but F_{max} also increases, indicating that in this region the quenching form of a redox mediator is disappearing (2-methyl-1,4-naphthoquinone). To account for this latter change, we assumed that a change in F_{max} would correspond to a similar but proportionally smaller change in F_0 (the correction is small anyway). These corrected values of F_0 are plotted in Fig. 6 as triangles and subtracted from the corresponding values of F_{max} and F_i to calculate $f = F_i - F_0/F_{max} - F_0$, the normalized increment of fluorescence yield. Since $[Q^-] = f/[1 - p(1 - f)]$, we computed that 67% of Q was involved in the high potential titration for $p = 0.7$. If we used a lower value for p (for a system under the influence of the quinone), this fraction would be less, e.g., 60% if $p = 0.6$.

Half of this fraction of Q (Q_h) was reduced at a potential $E_m = -3$ mV (pH = 8.4). This can be seen more clearly in Fig. 7 (left) where the log of the ratio $Q_h(red)/Q_h(ox)$ is plotted vs. potential. The slope of the pH 8.4 plot corresponds to $n = 1.56$. The right-hand plot of Fig. 7 shows similar data obtained at pH 6.2. In this case, $E_m = 115$ mV and the slope yields $n = 2.03$. We are uncertain whether or not the decreasing n values at higher pH values have

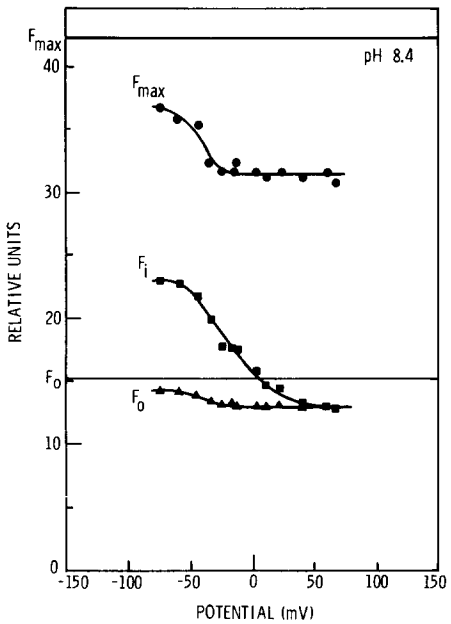

Fig. 6. F_i and F_{max} values from the pH 8.4 titration shown in Fig. 4a. F_0 and F_{max} of mutant No. 8 chloroplast fragments in the absence of redox mediators are shown as straight lines. F_i and F_{max} values for the titration in the presence of the redox mediators are shown as squares and circles, respectively. Values of F_0 shown as triangles, were estimated by assuming that a fractional change in F_{max} due to the change in quenching properties of the redox mediators corresponded to a similar fractional change in the quenching of F_0. The normalized increment of fluorescence, f, was determined from the formula $f = (F_i - F_0)/(F_{max} - F_0)$. We assumed $F_0 = F_i$ when the plastoquinone pool was fully oxidized, i.e., at 70 mV.

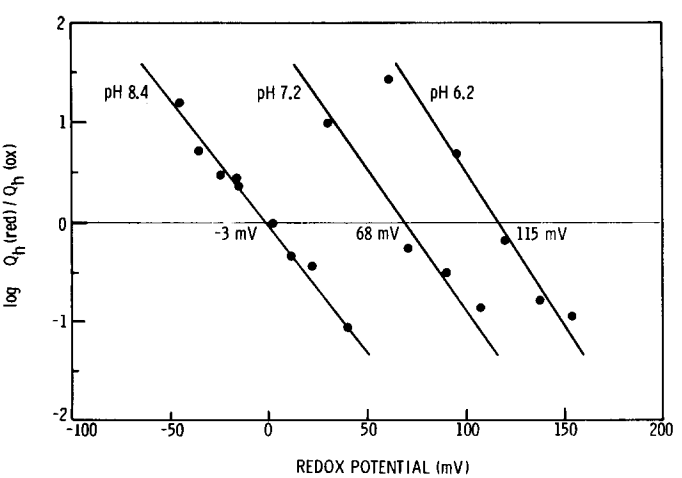

Fig. 7. The midpoint potential of Q, as determined by analysis of the Nernst plot. The relative amount of Q_h(red) was determined from the data of Fig. 6 using the formula $Q^- = f/[1 - p(1 - f)]$ (assuming p = 0.7) to account for energy transfer between PS II units. The log of Q_h(red)/Q_h(ox) was plotted vs. redox potential and the midpoint and slope determined by least squares analysis.

physiological significance. However, even though the precision is limited (and in the absence of a convincing model), these and additional experiments point to (1) a 2-electron reduction of Q and (2) a change in E_m of −60 mV per pH unit, suggesting that Q becomes protonated upon chemical reduction.

Discussion

The plastoquinone pool

We have shown that, with adequate dark equilibration, the redox titration of the fluorescence rise curve yields significant information about the electron acceptor system of PS II. Using the parameter $t_{1/2}$ as the indicator, we found that the plastoquinone pool titrates as a 2-electron component with a midpoint potential of 106 mV (pH 7.2). The pH sensitivity, −60 mV/pH unit between pH 6.2 and 8.4, indicates the involvement of 1 H^+/e during the reduction. This titration involves the entire A pool: (1) it accounts for 12—14 electron equivalents, and (2) the addition of ferricyanide causes no further increase in the fluorescence rise half-time. We should point out that the observed midpoint potential of the plastoquinone pool is quite similar to the polarographically-determined potential of plastoquinone A dissolved in a mixture of ethanol and light petroleum (E'_0 = +113 mV at pH 7.0) [19].

Contrary to our actual results, we had expected to find two distinct components in the A pool with different midpoint potentials: several earlier studies of the kinetics of DPIP reduction [2] and fluorescence kinetics [2,3] clearly differentiated two secondary electron acceptor pools in PS II (A_2 and A_1, Refs. 2, 3). Presumably, one pool (A_2) would show a low equilibrium constant with Q; the second pool (A_1) would exist at about the same concentration in the chloroplast but would show a higher equilibrium constant. If true, the midpoint potential of A_2 should be nearly equal to that of Q, while the A_1 pool should have a somewhat higher potential.

We found, instead, that the heterogeneity of the plastoquinone pool, apparent from recovery kinetics, disappeared when quinone mediators were used as oxidants in lieu of molecular oxygen. Likewise, the redox titration (in particular, Fig. 3 at pH 7.2) showed the plastoquinone pool to be thermodynamically homogeneous. The n values somewhat below 2 (1.8 at pH 8.4 and 1.65 at pH 6.2) could be due to experimental inaccuracy or, alternatively, could reflect the participation of two acceptor pools that only differ by a few tens of millivolts in their midpoint potentials. Two pools of 6 electron equivalents each, separated by 20—30 mV in midpoint potential, might appear as one 'wave', depending on the degree of scatter in the original data. However, we have surmised, instead, that the heterogeneity of the plastoquinone pool is a kinetic rather than a thermodynamic property, e.g., the biphasic kinetics of O_2-dependent pool recovery could be due to a greatly different accessibility of this oxidant to the subpools A_1 and A_2.

The high- and low-potential fluorescence quencher

Earlier in this laboratory, H. Hardt (personal communication), working with isolated chloroplasts, had observed that the fluorescence yield in the dark rises to 30—40% of F_{var} if the conditions are anaerobic and mildly reducing (e.g., in

the presence of ascorbate). In this study, we found that 30--40% of F_{var} correlated well with the redox potential of the medium. After corrections for quenching and energy transfer, 67% of Q (Q_h) consistently showed a midpoint potential only slightly (average: 30 mV) more negative than that of the plastoquinone pool: −3 vs. +15 mV at pH 8.4, 68 vs. 106 mV at pH 7.2, and 115 vs. 151 mV at pH 6.2. Interestingly, the titrations suggest that 2 electrons and 2 protons are involved in the reduction of Q.

The simplest and most straightforward interpretation of the data is to view the reaction as:

$$Q_h \overset{2e^-,2H^+}{\rightleftharpoons} Q_hH_2 \tag{1}$$

However, this hypothesis does not account for the second (low-potential) rise. Rather than assuming the existence of two types of Q (Q_h and Q_l), we ascribe the apparent presence of fraction Q_l to an inaccessibility problem, similar to the one encountered in our data concerning the pool (Fig. 5) on second and third illuminations. It is possible that these two phenomenon are ultimately related, although the mechanism remains obscure.

At very low potentials, this inaccessibility of Q_l apparently disappears. This might possibly be due to a reductive change in the environment, so that we may view this rise as 'pseudo-titration' of the 'box' that encloses Q_l.

Another difficulty with the interpretation of Eqn. 1 is that, under normal conditions, i.e., when photosynthesis proceeds, Q functions as a single, non-protonated electron acceptor [20,21]. However, all earlier titrations of Q that monitored the fluorescence yield [9,10], cytochrome *b*-559 [22], or the absorption change at 518 nm [18] showed a pH -dependent E_m value. We should not forget that the conditions used for these titrations were unusual: exposures of several minutes to an anaerobic, reducing medium and e.g., they do not allow O_2 evolution.

Eqns. 2 and 3 present a more coherent, but also more complicated hypotheses:

$$QR \underset{68\ mV}{\overset{2e^-,2H^+}{\rightleftharpoons}} QRH_2 \overset{K=2}{\rightleftharpoons} QHRH, \text{ at pH } 7.2 \tag{2}$$

$$QRH_2 \underset{-300\ mV}{\overset{e^-,\ H^+}{\rightleftharpoons}} QHRH_2 \tag{3}$$

Presumably Eqn. 2 describes the high-potential event, and Eqn. 3 the low-potential event. The additional assumption of $K = 2$ for the equilibrium constant of Eqn. 2 would explain why only 67% of Q is reduced in the high-potential wave. Previously, Diner [6] had computed a much lower value, $K \leqslant 0.02$, for the $Q \rightleftharpoons R$ equilibrium. However, his conditions and his assay were different; they concerned Q^- active in a back reaction with the S states and not QH.

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