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CHARACTERIZATION OF TWO QUENCHERS OF CHLOROPHYLL FLUORESCENCE WITH DIFFERENT MIDPOINT OXIDATION-REDUCTION POTENTIALS IN CHLOROPLASTS

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Summary

The properties of two redox quenchers of chlorophyll fluorescence in chloroplasts at room temperature have been investigated.

(1) Redox titration of the fluorescence yield reveals two $n = 1$ components with $E_{m_{7.8}}$ at —45 and —247 mV, accounting for approx. 70 and 30% of the total yield, respectively.

(2) Neutral red, a redox mediator often used at redox potentials below —300 mV, preferentially quenches the fluorescence controlled by the —247 mV component. Titrations using neutral red artifactually create an $n = 2$ quenching component with $E_{m_{7.8}} = -375$ mV.

(3) Analysis of fluorescence induction curves recorded at different redox potentials indicates that both the —45 and —247 mV components can be photochemically reduced. The reduction of the —247 mV component corresponds to a fast phase of the induction curve whilst the slower reduction of the —45 mV component accounts for the tail phase.

(4) The excitation spectra for the fluorescence controlled by the two quenchers show small differences in the ratio of chlorophyll *a* and *b*.

(5) Whereas the —247 mV component readily shows a 60 mV per pH unit dependency on solution pH, the ability of the —45 mV component to respond to pH change is restricted.

(6) Triton Photosystem II particles contain both quenchers but the

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Abbreviations: E_h = oxidation reduction potential; E_m = midpoint oxidation reduction potential; f_0 = initial fluorescence level; f_v = variable fluorescence; f_{max} = maximum fluorescence level; Q = quencher of chlorophyll fluorescence; Q_L = Q with $E_{m_{7.8}} = -247$ mV; Q_H = Q with $E_{m_{7.8}} = -45$ mV; PS II, Photosystem II; MES, *N*-morpholinoethane sulfonic acid.

—247 mV component accounts for approx. 70% of the fluorescence and the high component has an $E_{m_{7.8}}$ of +48 mV.

The relative merits of sequential and parallel models in explaining the presence of the two quenchers are considered.

Introduction

The fluorescence yield of chlorophyll in chloroplast membranes is determined by the redox state of the primary acceptor of Photosystem II [1], provided that the conditions are such that the primary donor (*P*-680) is not allowed to accumulate in its oxidized form [2,3]. Thus, when the acceptor is oxidized, fluorescence is quenched to a minimal f_0 level (all traps are open) and when the acceptor is fully reduced the fluorescence rises to a maximum level (all traps closed). The designation Q was used to describe this phenomenon, with Q being the primary acceptor and quencher of fluorescence when oxidized [1]. The redox nature of Q is clearly shown by the ability of dithionite to increase the fluorescence yield to a maximum level. Therefore, it should prove possible to potentiometrically titrate chlorophyll fluorescence and obtain the midpoint potential of the primary acceptor. When such an experiment is performed a complex titration curve is obtained showing two components with E_{m_7} at —35 and —270 mV [4]. Recently, Ke et al. [5] obtained similar data with E_{m_7} at +10 and —320 mV. Interpretation of the multicomponent quenching has proved difficult; explanations concerning secondary acceptors [5] have been made but are problematical, since, under the prevailing conditions of low light intensity, addition of diuron only stimulates fluorescence by 10% [4]. For chemical reduction of secondary acceptors to enhance the fluorescence yield to about 50% f_{max} would invoke the presence of an acceptor, prior to the diuron site and having a major responsibility for keeping the primary acceptor oxidized under low light. Alternative explanations, that the two quenchers represent two different redox states of the quinone acceptor or that the low potential quencher is unrelated to the primary acceptor [4] seem inconsistent with the presence of only the low potential quencher in active PS II particles [5]. On the other hand, alternative methods of estimating the E_m of the acceptor such as C-550 reduction [6] or oxidation of cytochrome *b*-559 [7] have yielded values close to that of the high potential quencher, suggesting that it too is the primary acceptor. No evidence for a second component was seen in these experiments. It would seem important to resolve this enigma because the presence of two quenchers appears not to fit currently accepted models for the photochemistry of PS II (e.g., ref. 8—10) suggesting that more complex schemes (e.g., see ref. 11) are required.

Materials and Methods

Chloroplasts were isolated from peas as previously described [12]. PS II fragments were prepared using Triton X-100 according to the procedure of Vernon et al. [13].

The procedure for potentiometric titration of the fluorescence yield was

based on that of Cramer and Butler [4]. Fluorescence was excited by 650 nm irradiation as defined by an Oriel monochromator giving an intensity of less than 0.1 J/m^2 per s, such that at a chlorophyll concentration of 20 μg/ml, addition of 10 μM diuron increased the yield by only 10% or less. The intensity was hence low enough to prevent appreciable steady-state reduction of the primary acceptor. Emission was detected through a 694 nm Balzar interference filter by a EMI 9558 photomultiplier tube. The current was amplified by a Keithley model 521 amplifier and fed to a strip chart recorder. Redox titration was performed in a cuvette similar to that described by Dutton [14], the E_h of the solution being adjusted by additions of microliter quantities of 10 or 50 mM ferricyanide and dithionite solutions for oxidative and reductive titrations, respectively. A Fisher combination redox electrode was used to monitor the solution E_h and precautions were taken to ensure oxygen-free solutions and environment throughout by using Matheson high purity argon. To ensure efficient equilibration between electrode and chloroplast the following mediators (20 μM) were used: 1,4-napthoquinone (+60 mV *), 1,2-napthoquinone (+135 mV), duroquinone (0 mV), 2-hydroxy-1-4-napthoquinone (—137 mV), 2,5-dihydroxybenzoquinone (—60 mV), anthraquinone-2-sulfonate (—225 mV) and neutral red (—325 mV). In addition, the medium contained 0.1 M sucrose, 2 mM $MgCl_2$, 2 mM NaCl and 50 mM Tricine (pH 7.8).

Fluorescence induction curves were determined as previously described [15], except that excitation was at 650 nm. Prior to induction, the suspension E_h was adjusted by dithionite or ferricyanide as described above. Because the response of the fluorescence quencher to changes in redox state is sluggish, the suspension was left to equilibrate for 10 min before the induction curve was determined. Analysis of the kinetics of the growth of area above the curves was performed as described recently by Melis and Homann [16,17].

Fluorescence excitation spectra at different E_h were measured by scanning the low intensity excitation using the wavelength drive on the Oriel monochromator. A Balzer 707 nm interference filter defined the emission. Spectra recorded at different E_h values were stored in a Tracor Northern NS570 Signal Averager and the f_0 (recorded at E_h = +100 mV) spectrum subtracted from each scan. This gave spectra for f_v at different values of E_h and allowed correction for any exciting light transmitted by the 707 nm filter.

Emission spectra were recorded using the same apparatus only with the monochromator placed between the sample holder and the photomultiplier tube. Fluorescence was excited using low intensity blue light (defined using Corning 4-96, 5-68 and 1-75 glass filters). Again, the intensity was such that no steady-state reduction of Q was detected.

Results

Titration of fluorescence yield

Fig. 1 shows the results of oxidative and reductive titration of the variable fluorescence yield in freshly isolated chloroplasts. Overall, the features are the

* E_{m_7} values from refs. 4 and 25.

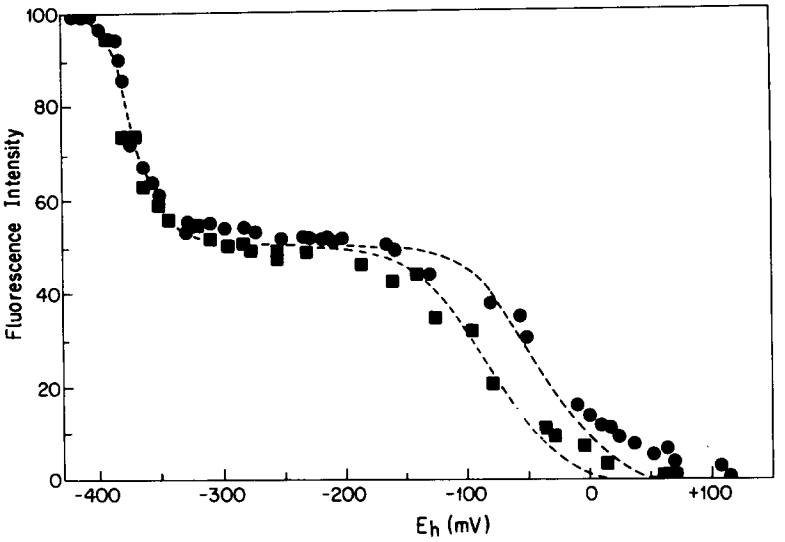

Fig. 1. Redox titration of the fluorescence yield. Conditions exactly as described in the text. Chlorophyll conc. 20 μg/ml. •, reductive titration; ▪, oxidative titration. Dotted line represents Nernst equations with E_m = −375 mV (n = 2) accounting for 50% and −85 and −40 mV (n = 1) accounting for 50%. Data are taken from several different titrations.

same as reported by Cramer and Butler [4] and Ke et al. [5], i.e. two components are seen with $E_{m_{7.8}}$ of −375 and −85 mV (oxidative) or −40 mV (reductive). Although there is some hysteresis in the high potential component, the reversibility is better than in some previous reports [5]. This is probably due to the greater equilibration time allowed in the sluggish reductive titration (up to 10 min, if necessary) in the present experiments. If sufficient equilibration time is not allowed, the plateau in the −150 to −275 mV region can be obscured. One unusual feature of the titration is that, while the high potential component fits a n = 1 Nernst plot, as expected for the single electron quinone acceptor [18,19], the low component is steeper (n = 2). In Fig. 1, this transition is reversible, so any argument based on lack of equilibration to explain the steep slope can be discounted.

This unusually steep slope for a transition which represented about 50% of the variable fluorescence seemed to be of sufficient importance to warrant further study. Since the $E_{m_{7.8}}$ of −375 mV is close to that of one of the redox mediators used (neutral red), a titration without this compound was performed. As can be seen in Fig. 2, the major feature of the titration is unchanged (as was stated in a description of a similar control experiment in ref. 4) in that two components are still seen. However, there exists two important quantitative differences upon omitting neutral red. Firstly, the low component now only represents 30% of the total fluorescence. (The total fluorescence yield is virtually unchanged plus/minus neutral red). Secondly, the low component has a higher $E_{m_{7.8}}$ at −247 mV compared to −375 mV in the presence of neutral red. Moreover, this transition at −247 mV perfectly fits an n = 1 Nernst plot, and the problem of the steep slop disappears. The high

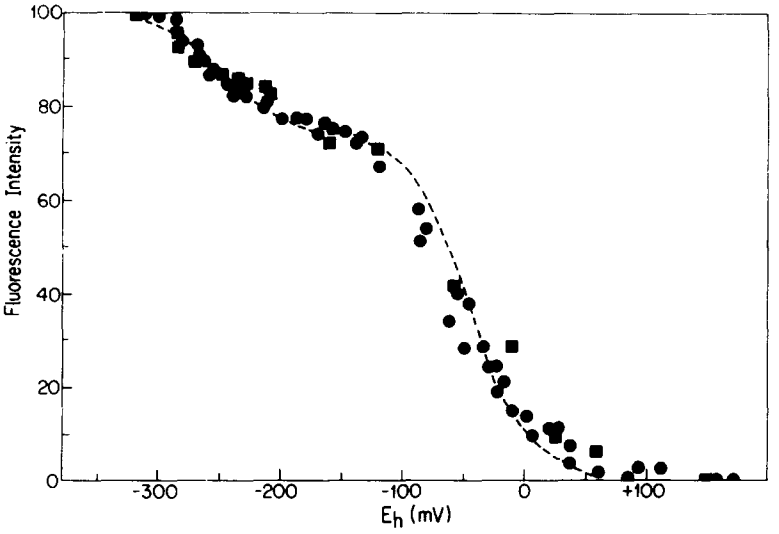

Fig. 2. Redox titration of the fluorescence yield in the absence of neutral red. Conditions as in Fig. 1 except that neutral red was not used. •, reductive titrations; ■, oxidative titrations. Dotted line represents Nernst equations with E_m = −247 mV (n = 1) and −45 mV (n = 1) accounting for 27 and 73% of the fluorescence.

potential component remains unaltered except for its increased proportion of the total, an $E_{m_{7.8}}$ of −45 mV being observed in both oxidative and reductive titration. This titration shows no hysteresis.

The preferential effect on the low component prompted further experimentation. Fig. 3 shows the quenching of fluorescence observed on adding neutral red at −345 mV (A) and −185 mV (B); clearly, neutral red quenches the low potential component completely, and the high potential component by approx. 25%. The f_0 level (i.e. the level in the fully oxidized condition) was quenched

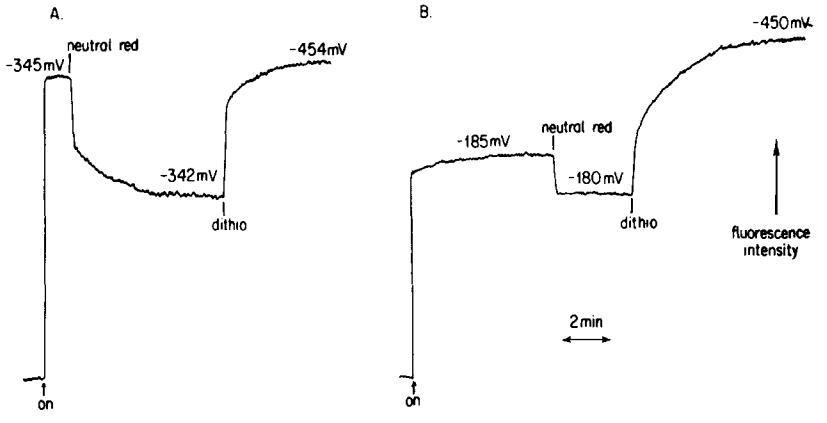

Fig. 3. Quenching of chlorophyll fluorescence by neutral red. (A) E_h poised so that f_{max} is reached immediately upon illumination. (B) E_h poised so that just the −45 mV component in Fig. 2 is reduced. Neutral red concentration 20 μM. Conditions as in Fig. 2.

by less than 10% (data not shown). Upon reduction of neutral red, the fluorescence returns to the maximum level (Fig. 3). Thus neutral red quenches specifically f_v, and preferentially that component of the f_v which titrates at −247 mV. The quenching is reversed when neutral red is reduced and thus a titration of fluorescence in the presence of neutral red gives a component at $E_{m_{7.8}} = -375$ mV, the approximate $E_{m_{7.8}}$ of neutral red ($E_{m_{7.0}}$ is −325 mV and therefore $E_{m_{7.8}} = -373$ mV).

The effect of neutral red is not an artifactual process, caused for example by light absorption. This is demonstrated by saturation of the quenching effect at approx. 20 μM with half maximal quenching at 2 μM. In addition, emission spectra recorded at the start, the plateau region and the end point of the titration in the presence of neutral red show changes in chlorophyll fluorescence are involved (data not shown).

While these effects of neutral red make interpretation of redox titration in its presence complex, it may provide a tool for probing the reason for the two components in the titration curve, since it preferentially effects one of the components.

The possibility that the low component ($E_{m_{7.8}} = -247$ mV) is an artifact caused by the mediator anthraquinone sulfonate was discounted by titration performed in its absence. The $E_{m_{7.8}}$ is shifted slightly when its mediator is omitted, but two components are clearly still present. In addition, anthraquinone sulfonate is an $n = 2$ component while the fluorescence quencher is $n = 1$.

Fluorescence induction at different redox potentials

Investigation of the nature of the two components in the titrations of Fig. 2 was made by observing the fluorescence induction curve at different E_h values. In these experiments, chloroplasts were poised at measured E_h values and allowed to equilibrate for 10 min before diuron addition which was made 1 min prior to recording the induction curve. This was necessary since the presence of diuron greatly decreased the rate of redox equilibration. Fig. 4A shows fluorescence induction curves recorded at +97 mV, a normal trace is seen with initial sigmoidicity and comprising a fast phase and a slow or tail phase as described many times previously [16,17,20]. At −170 mV a different curve is displayed. First an increase in f_0 is seen. With a shutter opening time of approx. 0.5 ms, the f_0 is a change between two successive address points on the Tracor averager, and thus can be accurately computed. Using this method, the f_0 was shown to increase by a factor of 3.3 as the potential is lowered to −170 mV. In addition, a smaller amplitude of the variable fluorescence is observed and the rise curve appears to lack any slow component. By lowering the potential to −380 mV a fluorescence level close to the maximum is observed instantaneously; this induction curve, with the PS II traps closed by chemical reduction, is now almost completely f_0. A subtraction between the induction curve at −170 mV (which corresponds to the plateau region in Fig. 2) and at +97 mV revealed a slow monophasic curve. Therefore, lowering the potential had preferentially eliminated the tail phase of the rise curve. Induction curves recorded in the presence of neutral red gave different results (Fig. 4B). At +105 mV, a slower and smaller amplitude induction was seem compared to

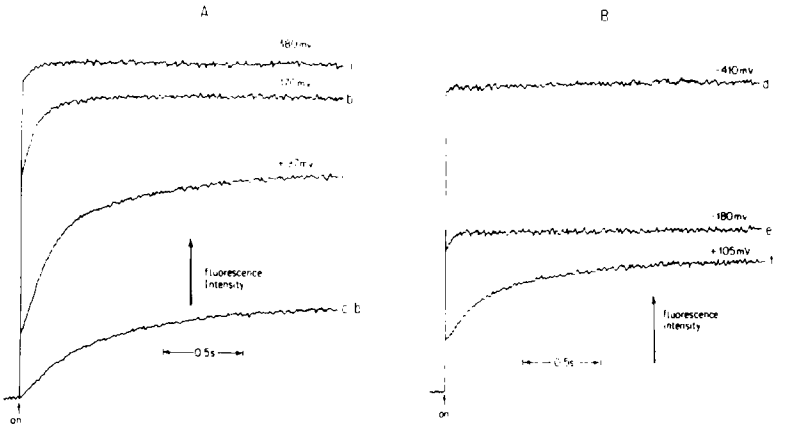

Fig. 4. Fluorescence induction curves at different E_h. Induction curves were recorded following a 10 min dark redox equilibration at appropriate E_h values. Diuron (20 μM) was added 1 min prior to illumination. (A) Control chloroplasts at −380 mV (a), −170 mV (b) and +97 mV (c). c-b is a digital subtraction between curve c and b normalized so as to eliminate the f_0 change. (B) Chloroplasts containing 20 μM neutral red at −410 mV (d), −180 mV (e) and +105 mV (f).

data obtained in the absence of neutral red (see Fig. 4A). Lowering the potential to −180 mV almost completely eliminated the variable fluorescence rise, again different from the observations made in the absence of neutral red. The increase in f_0 on reducing the high potential component is less than in the absence of neutral red, to be expected from the decrease in proportion of this component in the steady-state titration (see Figs. 1 and 2). Decreasing the potential to −410 mV brought the level to the f_{max} level.

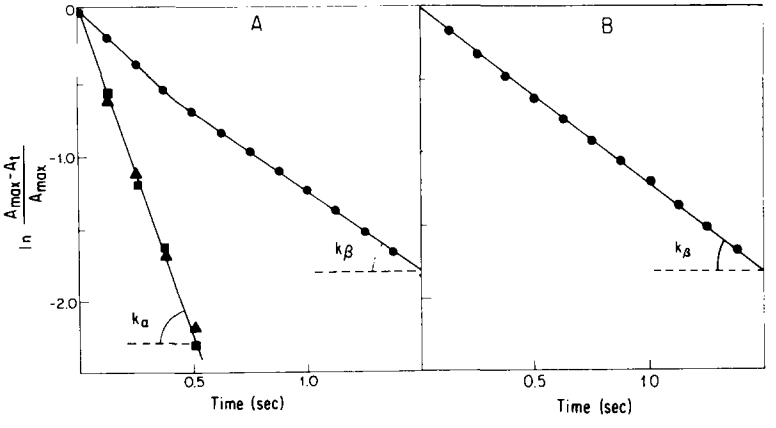

Fig. 5. Analysis of the area growth above the induction curve. First order analysis of the two phases present in the induction curve was performed as described by Melis and Homman [16,17]. A_{max} is the total area above the curve and A_t the area accumulated at any time, t following the onset of illumination. k_α and k_β are the first order rate constants for the fast and slow phases of area growth respectively. (A) Control chloroplasts; first order plot of area above the induction curve (●) showing biphasicity and the resolved fast phase (■) obtained from curve (c) in Fig. 4, when Q is fully oxidized; ▲, plot of induction curve (b) in Fig. 4 when the high potential component is reduced. (B) Chloroplasts with neutral red. Analysis of curve f of Fig. 4, the fully oxidized condition.

The curves were analysed by making first order log plots of the area growth above the rise curve against time. Melis and Homann [16,17] have shown how this analysis defines the fast and slow phases, which were given rate constants k_α and k_β, respectively. At +96 mV, two components are seen, which can be resolved to give $k_\alpha = 4.6\ s^{-1}$ and $k_\beta = 1.1\ s^{-1}$ (Fig. 5). At −170 mV the log plot appears to fit a single curve with a rate constant the same as that of the fast component observed at +97 mV. First order analysis of the rise curve at +105 mV in the presence of neutral red indicated good fit to a single component with a rate constant $1.2\ s^{-1}$ (Fig. 5B). These data suggested that the fast component is associated with reduction of the low potential quencher and the tail with the high potential quencher; reduction of the latter quencher eliminated the tail and quenching of the low component by neutral red eliminates the fast phase. These two phases, observed in separation, are quantitatively identical to the two components seen in control chloroplasts at +97 mV when both quenchers are fully oxidized prior to illumination.

Excitation spectra of variable fluorescence

To further understand the nature of the two fluorescence quenchers, excitation spectra of the variable fluorescence at −408 mV and −180 mV were recorded. (These experiments were done in the presence of neutral red, but similar spectra are seen in its absence). Excitation of the fluorescence increase observed between +100 and −180 mV shows a maximum near 675 nm and a shoulder at 655 nm, corresponding roughly to chlorophyll *a* and *b* (Fig. 6). The ratio of ϕf_{675} to ϕf_{655} was found to be 1.9 ± 0.2 ($n = 4$). On lowering the potential to −408 mV, the excitation spectrum, although generally very similar to that at −180 mV shows a small decrease in the 675/655 ratio. A value of 1.3 ± 0.2 ($n = 4$) was obtained for the excitation of the fluorescence quenched by the low component.

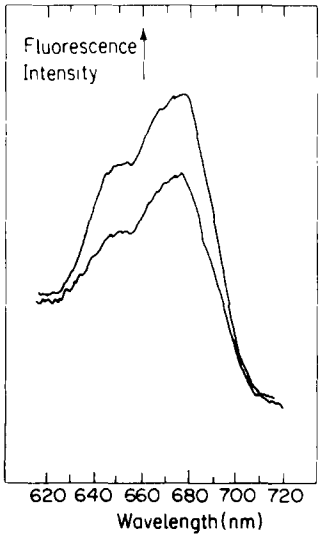

Fig. 6. Excitation spectra of fluorescence at different E_h. Excitation spectra were recorded as described in Methods. The E_h was equilibrated for 10 min prior to recording of spectra. Conditions were as in Fig. 1. Top curve, E_h = −408 mV. Bottom, E_h = −180 mV.

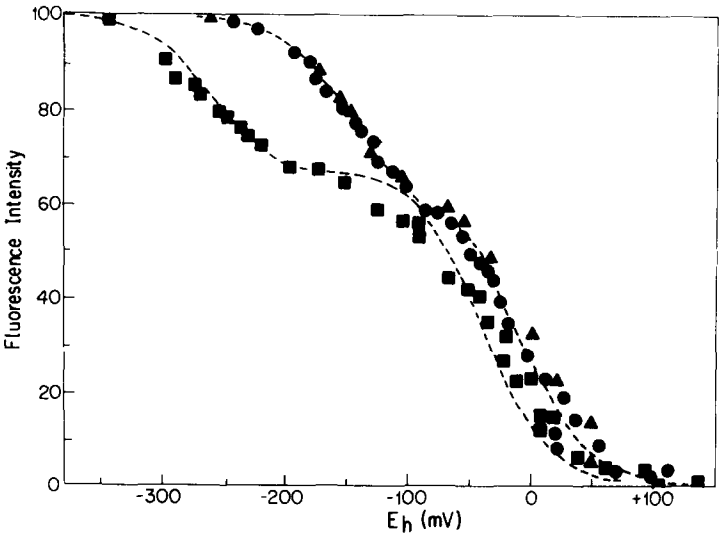

Fig. 7. Effect of pH on redox titration of fluorescence yield. Conditions as in Fig. 2 except that the pH was either buffered at pH 6.0 with 50 mM MES (●, ▲), or at pH 8.0 with 50 mM Tricine (■). ■ and ▲ are reductive, ● oxidative titrations. Nernst equations (n = 1) with E_{m_6} at −5 and −150 mV and E_{m_8} at −40 and −265 mV are shown.

pH dependence of fluorescence quenchers

Previous work had demonstrated a pH dependence of approx. 60 mV per pH unit for both quenching components [4,5]. Titration of the chloroplasts used in the above experiments failed to yield a significant pH dependence for the high potential component. Representative titrations are shown in Fig. 7. Over 10 titrations a mean value of −40 ± 15 mV and +5 ± 10 mV are determined at

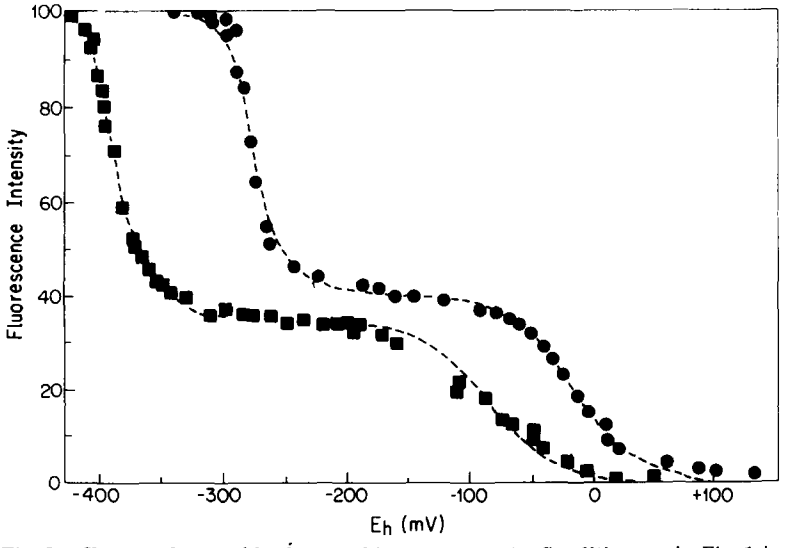

Fig. 8. pH dependence of both quenching components. Conditions as in Fig. 1 including 20 μM neutral red. Chloroplasts were prepared according to the method of Cramer and Butler [4]. pH conditions as in Fig. 7. Nernst equations with E_{m_6} at −15 mV (n = 1) and −280 mV (n = 2) and $E_{m_{7.8}}$ at −85 mV (n = 1) and −390 mV (n = 2) are shown. ■, pH 8, and ●, pH 6 (reductive).

pH 8 and 6, respectively. These titrations are fully reversible. If chloroplasts were prepared, however, exactly as described by Cramer and Butler [4], and titrated under their conditions (plus neutral red) we were also able to demonstrate pH dependence; values of −85 and −15 mV were determined at pH 8 and 6, respectively (Fig. 8). There is indication, therefore, that pH dependence requires the presence of neutral red and more disrupted membranes. In contrast to the high potential quencher, the low potential quencher always showed a pH dependence of approx. 60 mV/pH unit (Fig. 7), with values of −150 and −265 mV recorded at pH 6 and 8, respectively. The precise conditions necessary to observe pH dependence for the high potential component will be the subject of further study.

Titration of Q in Triton PS II particles

Because of the above effects of neutral red on fluorescence it would seem appropriate to re-investigate PS II particles in which the −375 mV component is the only species present [5]. Fig. 9 shows titrations with and without neutral red. Although neutral red has exaggerated the proportion of low potential quencher, it is clear that the ratio is higher in favor of the low component. This component ($E_{m_{7.8}} = -240$ mV) is identical to the low component described earlier. The high potential component has an E_m of +48 mV, somewhat higher than in chloroplasts. Both components fit $n = 1$ Nernst equations. In the presence of neutral red, however, a component with slope $n > 2$ and $E_{m_{7.8}}$ at −355 mV is observed and accounts for over 90% of the fluorescence, as observed by Ke et al. [5]. The more positive E_m for the high component is consistent with positive shifts in the E_m of C-550 in broken chloroplasts [6] and PS II fragments [21].

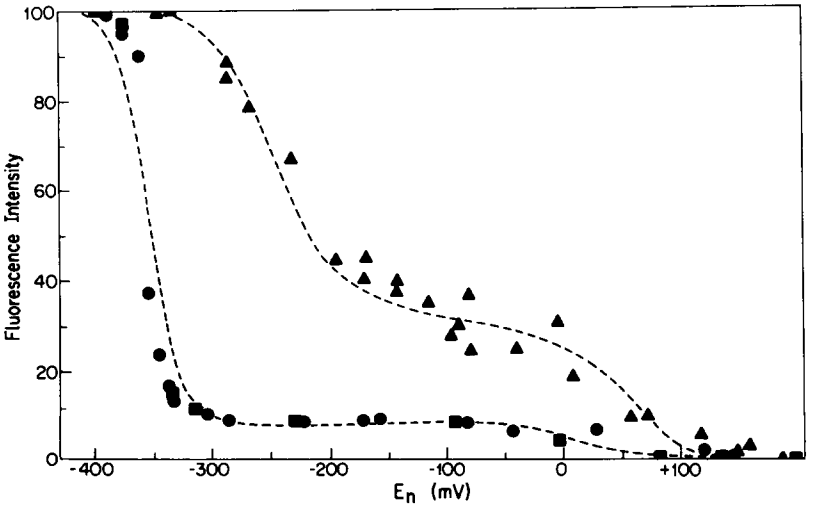

Fig. 9. Redox titration of fluorescence yield on PS II particles. Titration conditions as in Figs. 1 and 2 in the absence of neutral red (▲, reductive titration) and in the presence of 20 μM neutral red (■, oxidative; ●, reductive). Nernst equations with $E_{m_{7.8}}$ of −355 mV ($n = 2$), −240 mV ($n = 1$) and +48 mV ($n = 1$) are shown.

Discussion

The fluorescence yield of chloroplasts under the conditions used here is determined by the redox state of the primary acceptor, Q [1]. The fluorescence quenching is seen to be controlled by two single electron redox components, with $E_{m_{7,8}}$ at −247 mV (Q_L) and −45 mV (Q_H), accounting for 20—30 and 70—80% of the fluorescence yield, respectively. These data are qualitatively but not quantitatively the same as those of Cramer and Butler [4] and Ke et al. [5]. Both these groups used neutral red to mediate potentials less than −300 mV, but clearly neutral red does not fulfill the requirement of lack of interference with the system necessary for the successful use of redox mediators in potentiometric titrations [22]. The mechanism of effect of neutral red is not elucidated as yet but appears to differentially quench Q_L and Q_H; neutral red eliminated the Q_L component completely and prevents its reduction by light. On the other hand the properties of Q_H are only slightly affected. The primary acceptor of PS II shows spectral properties of a semiquinone anion when in its reduced form [18,19,23] and appears therefore to be a single electron carrier. Both Q_L and Q_H are n = 1 transitions and hence both could be the primary acceptor. The problem of the steep n = 2—3 slope for Q_L has now been resolved [5]. Q_L accounts for approximately 20—30% of the total fluorescence but of course this does not indicate the relative concentrations of Q_L and Q_H, merely their quenching efficiency. Nevertheless, it would seem likely that Q_L is an important component of the photosynthetic apparatus, a notion supported by its even greater presence in PS II particles. Besides the quenchers characterized potentiometrically in this paper and in previous reports [4,5], other evidence has suggested two Q or primary acceptors. Joliot's laboratory has accumulated considerable evidence for more than one PS II primary acceptor [24—27]. Fast and slow quenchers have been recognized by many laboratories [16,17,20,28,29] and the decay kinetics of X-320 which monitors the reoxidation of Q are biphasic [30].

Two general types of schemes have been proposed (Fig. 10) to account for the function of these two quenchers. First, they may represent electron acceptors operating in series (Fig. 10A); Q_L would be the primary acceptor and

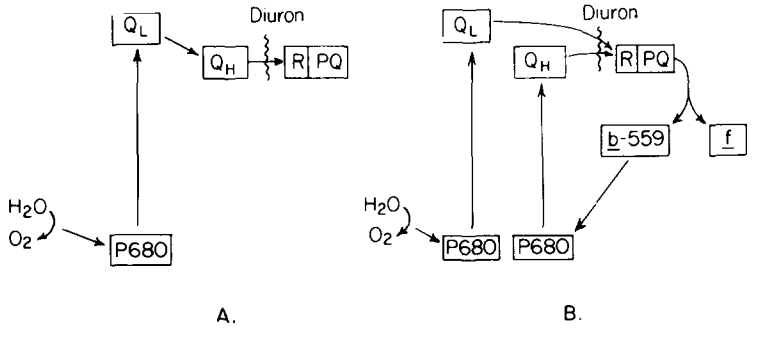

Fig. 10. Involvement of two quenchers in photosynthetic electron transport. (A) Sequential model invoking Q_L and Q_H as primary and secondary acceptors prior to the site of diuron inhibition. (B) Parallel model with Q_L and Q_H as primary acceptors. For details see text.

Q_H a secondary acceptor. Since both Q_L and Q_H are reduced by light in the presence of diuron, the secondary acceptor would be prior to the inhibition site of diuron. This would make Q_H distinct from postulated intermediates such as R [31] or B [32] which occur after the site of diuron inhibition. In addition, B or R would titrate as two-electron carriers whereas Q_H is a single-electron carrier. Thus Q_L and Q_H would be considered as tightly coupled carriers within the reaction centre itself. It is tempting to speculate that Q_L is equivalent to the transient phaeophytin acceptor I, that has been identified as the primary acceptor in bacteria [33—36]. Thermodynamically it would be predicted that an I intermediate in PS II would have an E_m close to the value of Q_L assuming the existence of the 70% energy conversion in bacteria [33]. Further study is required to probe the relationship between Q_L and I.

The second model to explain Q_L and Q_H is that they are primary acceptors operating in parallel. Redox titration of the area above the induction curve indicates that the fast and slow phases correspond to the reduction of Q_L and Q_H respectively. It has been concluded previously that both these phases are photochemically limited and are not considered to be due to reduction of a primary and a secondary acceptor [16,17,28]. Reduction of Q_H is seen to result in an increase in f_0 as observed both in the induction curves and under low intensity illumination. In a series model, the only way to close PS II traps would be to reduce Q_L. However, with a ΔE_m of 200 mV between Q_L and Q_H, reduction of Q_H would cause negligible indirect reduction of Q_L. Indirect reduction of Q as a result of reducing the A pool [37] would be explained in terms of indirect reduction of Q_H. Influence of the A pool on the induction curve is, however, unimportant when considering curves recorded in the presence of diuron. Moreover, in the presence of redox mediators, redox equilibrium will exist, so that indirect reductions of this type can generally be ignored. In terms of Q_H being a secondary acceptor which can determine the turnover capacity or dynamic acceptor pool size for Q_L, one can point out that reduction of Q_H increases the fluorescence level to 70% f_{max}, yet addition of diuron, which drastically reduces the quenching pool size [38] enhances fluorescence by only 10—20%. Titration in the presence of diuron furthermore does not significantly alter the results obtained (data not shown and ref. 4).

The data described in this paper therefore tend to support a parallel rather than series model. The two primary acceptors could exist in two different types of reaction centres [16,17], perhaps in different parts of the chloroplast membrane system. For example Q_H could be present in stroma lamellae and Q_L in grana. The difference in excitation spectra for Q_L and Q_H would support a spatial separation, with chlorophyll *b* being enriched in grana [39,40]. Alternatively the two acceptors could represent a heterogeneity within each reaction centre. The existence of two quenchers per centre has been proposed by Joliot and coworkers [11,24—27]. Joliot and Joliot originally proposed a cooperation in achieving the S_4 state, with Q_L operating in S_0 and S_1 states and Q_H in S_2 and S_3 states [11,26]. More recently a second primary acceptor has invoked to explain 'double hitting' [27], but its involvement in photosynthesis remains obscure.

The scheme presented in Fig. 10B is a different interpretation of the presence of two PS II quenchers. In this model only Q_L and associated donors

are involved in oxygen evolution. Q_H is involved in a PS II cycle and does not contribute to linear electron transport perhaps explaining its inefficient reduction. It is proposed that Q_L and Q_H cooperate at the level of the special plastoquinone R in agreement with the ideas of Witt [23] although the chains could instead join at the plastoquinone pool. As essential feature is that high potential cytochrome *b*-559 is functioning as a donor to the Q_H system and is reduced by plastoquinone, as recently shown by Whitmarsh and Cramer [41, 42]. This model provides an association between high potential cytochrome *b*-559 and Q_H, which is consistent with (a) the reduction of C-550 ($\equiv Q_H$) and oxidation of cytochrome *b*-559 at low temperature [43], (b) the titration of the primary acceptor at 77 K by monitoring cytochrome *b*-559 oxidation giving an E_{m_8} of −90 mV [7]; (c) the decrease in Q_H quenching in PS II particles which lack high potential cytochrome *b*-559 (ref. 44, Horton, P., unpublished data), and (d) the correlation between the slow fluorescence quenching and cytochrome *b*-559 oxidation at −50°C [24]. At 77 K (when Q_L is not observed) reactions leading to water oxidation are inoperative and so the Q_L system could become 'invisible' due either to a fast and stable reduction of Q_L or due to an absence of stabilization of charge separation by electron donation. Oscillation of fast and slow fluorescence rise at −50°C as a function of preillumination flash number [24] could be explained by low temperature inhibition of electron transfer from Z in the S_2/S_3 state reached after two flashes. Under such conditions the Q_H would then become the major reducible quencher of fluorescence.

It should be added that these are not the only possible schemes. In particular a more complex model combining features of both A and B in Fig. 10, involving interaction at both donor and acceptor sides of the two *P*-680 is possible. Thus, in some circumstances Q_L could reduce Q_H and H_2O could donate to both acceptors although these pathways may not be the preferential ones. Finally, mention should be made of the fact that, with an $E_{m_{7.8}}$ of −247 mV for the primary acceptor, free energy will be available to do work as reducing equivalents flow to the plastoquinone pool, irrespective of which model applies. This energy may be important in driving site II photophosphorylation which has been reported to require Q to plastoquinone electron flow [45].

Clearly more information is required before such schemes can be proven or invalidated. Particularly needed are more precise details of the molecular properties of PS II, especially with respect to membrane organization. Experiments in this paper show that Q_L and Q_H may be in different environments; Q_L freely equilibrates with the solution pH whereas Q_H appears to be in a shielded environment. A surface shield inhibiting proton binding by plastoquinone has been described [46] and trypsin treatment of membranes increases the accessibility of the primary acceptor to ferricyanide [47]. In addition, the differential effects of neutral red on Q_L and Q_H indicate a different membrane environment.

In conclusion, the data in this paper demonstrate the involvement of Q_L and Q_H in photosynthesis. This is supported by recent data of Malkin [48] who demonstrated the existence of two redox components equivalent to Q_L and Q_H as being involved in the flash-induced 518 nm change. Thus it is clear that any complete description of PS II now has to include the presence of Q_L and Q_H.

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