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Redox-potentiometric titrations of the electrochromic absorption change in chloroplasts

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Two phases of the electrochromic 515 nm absorption change in chloroplasts elicited by microsecond flashes can be resolved kinetically. Redox-potentiometric titrations indicate that the initial amplitude appearing within 0.5 ms, and designated as phase a, has three components in the low-potential region with $E_{m7.5}$ values of +60 mV, −195 mV and less than −400 mV. From the insensitivity to DCMU, we propose that the species with $E_{m7.5}$ values of −195 mV and less than −400 mV are both related to Photosystem I. This conclusion was supported by the loss of both components when the Photosystem I reaction centre (P-700) was chemically oxidised ($E_{m7.5}$ = +370 mV). The species having an $E_{m7.5}$ less than −400 mV is presumed to be the Photosystem I primary acceptor, while the $E_{m7.5}$ = −195 mV wave could be due to a secondary electron acceptor, such as cytochrome b-563$_{LP}$, whose photoreduction is possible owing to the long duration of the excitation flash. The DCMU-sensitive component with an $E_{m7.5}$ of +60 mV is assumed to be the primary quinone acceptor (Q_A) of Photosystem II. Unlike the Photosystem I redox components, the midpoint potential of this species is sensitive to the background ionic level: the $E_{m7.5}$ is shifted to −100 mV when the cation concentration is lowered to facilitate membrane unstacking. The slow phase of the electrochromic signal (phase b) has been estimated by measuring the 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone-sensitive amplitude of the absorption change at 20 ms. The signal appears with an estimated $E_{m7.5}$ = +50 mV, becomes maximal at −50 mV and attenuates with an $E_{m7.5}$ of about −180 mV. These results suggest that phase b occurs when the plastoquinone pool is reduced and cytochrome b-563$_{LP}$ is oxidised.

Introduction

It is well established that the light-induced absorption change at 515 nm (ΔA_{515}) in chloroplasts, and the analogous absorption change which occurs in photosynthetic bacteria, can be used as an empirical indicator of the transmembrane electric field [1]. Two kinetically distinct phases in the ΔA_{515} can be detected when chloroplasts are excited by 'single-turnover' flashes [2,3].

The instantaneous rise (phase a) is attributed to the electric field generated across the thylakoid membrane by the primary charge separation reactions within the photosystems (PS I and PS II). It has been suggested that both PS I and PS II have equal contributions to the extent of phase a, in line

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; ΔA_{515}, the light-induced electrochromic absorption change at 515 nm; Q_A, primary quinone acceptor of Photosystem II; Q_Z, bound quinone; E_h, ambient redox potential; PS I, II, Photosystem I, II; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; $E_{m7.5}$, the redox midpoint potential at pH 7.5.

with their approximately equal stoichiometries in the membrane [4]. From a redox-potential titration study, Malkin [5] has observed that the loss of the PS II contribution at low potentials titrates with two waves consistent with two electron acceptors having midpoint potential values of 0 and -235 mV. These two components could correspond to the quenchers Q_H and Q_L of the chlorophyll *a* fluorecence yield [6,7]. In a recent analysis, Diner and Delosme [8] proposed that, whereas the component with an $E_{m7} = 0$ mV is associated with PS II as demonstrated by its sensitivity to DCMU, the component with an $E_{m7} = -235$ mV is observed at times longer than 50 μs, and this component is probably best assigned to PS I photochemistry.

The additional slow rise (phase b), observed in the millisecond time domain, is readily detected only under conditions when the plastoquinone pool has been reduced either by preillumination or by chemical reductants [3,9–12]. Since the light-induced absorption spectrum of phase b is similar to that of phase a [13,14], this slow rise in the membrane potential has been attributed to a secondary electrogenic process associated with the cytochrome *b-f* complex (although other mechanisms have been suggested [14,15]). Much of our understanding of this phase b has come from a comparison with the slow phase of the carotenoid band shift observed with bacterial chromatophore membranes. This latter signal has been correlated with the light-induced redox reactions of cytochrome *b*-562 [16–18]. The rise kinetics of the bacterial slow phase are clearly dependent on the ambient redox potential. An increased rate in the rise kinetics of this phase appears on reductive titration below 150 mV (pH 7.0). Such an increase in rate has been ascribed to the reduction of a bound quinone, Q_Z, but is now thought to be associated with an accelerated rate of QH_2 oxidation as the concentration of this substrate increases upon reduction of the pool [19,20]. At low redox potentials, the slow phase in bacterial chromatophore membranes titrates with the loss of photochemical activity as the quinone acceptor of the bacterial reaction centre is chemically reduced [16]. With chloroplasts, however, it is possible to examine phase b at redox potential values which are sufficiently low to block PS II turnovers, but which do not affect PS I. In this way, identification of other components involved in the electrogenic reactions of chloroplasts may be achieved.

In this paper we have reexamined the redox potential dependence of both the fast and slow phases of the ΔA_{515}. An attempt is made to clarify the nature of the -195 mV component of phase a, and to define the E_h range over which phase b can be readily detected. Some of these results have been presented at the 6th International Congress on Photosynthesis (August 1983, Brussels) [21].

Materials and Methods

Intact chloroplasts were freshly isolated from pea leaves by grinding 70 g leaves in 200 ml of an ice-cold grinding medium consisting of 0.33 M sorbitol, 0.2 mM $MgCl_2$, 20 mM Mes adjusted up to pH 6.5 with Tris. After filtering through 10 layers of muslin, the first two layers being separated by a thin layer of cotton wool, the filtrate was subjected to centrifugation at $2200 \times g$ for 45 s at 4°C. The supernatant was removed and the hard and soft pellets were resuspended in a medium containing 0.33 M sorbitol brought to pH 7.5 with Tris before being subjected to centrifugation at $2200 \times g$ for 20 s at 4°C. This time, both the supernatant and soft pellet were removed and the hard pellet was resuspended at high chlorophyll concentration in the above low cation medium supplemented with 3 mM $MgCl_2$ and stored on ice. The chlorophyll concentration was determined by the method of Arnon [22] and was typically 2–3 mg/ml.

Flash-induced absorption changes were monitored using a single-beam flash apparatus as described elsewhere [11]. The measuring beam intensity was less than 0.2 $W \cdot m^{-2}$ and saturating light pulses of duration about 10 μs full width at half maximum were provided by a xenon flash tube at 0.2 Hz. At this flash frequency the PS II reaction centre remains closed in the presence of DCMU. For measurement of 515 nm absorption changes, the actinic flash was passed through a 2 mm Schott RG 665 filter and the photomultiplier was protected by a 4 mm Corning 4-96 filter. The output from the photomultiplier was fed into a signal averager (Nuclear Measurement, model 546C) and could be plotted on an *X–Y* recorder

for further analysis. The time constant of the apparatus was normally 0.3 ms.

Redox titrations of the flash-induced 515 nm absorption change were carried out at 20°C using a thermostated cuvette, similar in design to that described in Ref. 23. The redox potential of the medium was monitored by a combined platinum-calomel electrode (EIL, series no. 1117 combination electrode) connected to a Phillips PW9409 digital pH meter. The electrode was calibrated with respect to quinhydrone at pH 7.0. The following experimental procedure was carried out: intact chloroplasts, equivalent to 50 μM chlorophyll in 3 ml, were osmotically shocked for 30 s in the cuvette using 1.5 ml of ice-cold distilled water containing either 10 mM $MgCl_2$ (stacked membranes) or 20 mM KCl (for unstacked membranes). An equal volume of double strength buffer was then added to give a final concentration of 0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.5) and either 5 mM Mg^{2+} or 10 mM K^+. The suspension was allowed to thermally equilibrate to 20°C. The solution was subsequently flushed for a few minutes with zero grade argon gas (less than 2 ppm O_2) both before and after the addition of similarly deoxygenated redox mediators. This suspension was stirred by a magnetic bar and argon gas was passed over the surface of the solution at all times. Measurements were done by adjusting the potential to a desired value, allowing sufficient time for equilibration, and then switching off the magnetic stirrer before flash-activating the sample. Each titration-point represents the extent of the ΔA_{515} resulting from an average of eight flashes at the indicated potential, the sample being preilluminated with four flashes. Both reductive and oxidative titrations were carried out. The reductive and oxidative titrants were solutions of 50 mM sodium dithionite, freshly prepared before each titration in 0.1 M Tris-HCl (pH 9.0), and 50 mM ferricyanide, respectively. The titration curves presented represent several overlapping titrations, each completed within 1–2 h, and having the titration midpoints reproducible within ±30 mV.

A list of the redox mediators used is given below. They were all used at a concentration of 20 μM, except phenazine methosulphate which was used at 5 μM. The number in parenthesis is the E_{m7} of the mediator. Ferricyanide (+430 mV), diaminodurene (+220 mV), 1,2-naphthoquinone (+143 mV), phenazine methosulphate (+80 mV), 1,4-naphthoquinone (+36 mV), duroquinone (+5 mV), 2,5-dihydroxybenzoquinone (−60 mV), 2-hydroxy-1,4-naphthoquinone (−130 mV), anthroquinone-2,6-disulphate (−185 mV) and anthroquinone-2-sulphate (−225 mV).

Results

Fig. 1a shows the distinct biphasic kinetics in the generation of the 515 nm absorption change, ΔA_{515}, resulting from flash activation of DCMU-treated chloroplasts supplemented with 0.5 mM duroquinol. Although the addition of 15 μM DBMIB had little effect on the amplitude of the fast absorption change (phase a), the slow absorption change (phase b) was completely suppressed (Fig. 1b). Thus the isolation of phase b from the

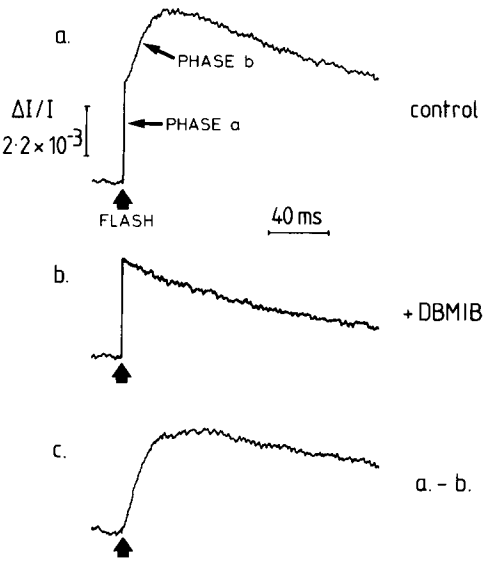

Fig. 1. Flash-induced absorption changes at 515 nm. Chloroplasts were osmotically shocked in the presence of 10 mM $MgCl_2$ (see the Materials and Methods section) and then double-strength buffer was added to give a final concentration of 0.33 M sorbitol/10 mM tricine-KOH (pH 8.3)/5 mM $MgCl_2$/50 μM chlorophyll. The addition of 0.5 mM duroquinol ensured that the plastoquinone pool was reduced chemically. (a), Control, containing 25 μM DCMU and 0.5 mM duroquinol; (b), as (a), but +15 μM DBMIB; (c), subtraction: a − b. Phase a is defined as the instantaneous absorption change (complete within less than 0.5 ms). Phase b is defined as the slow absorption change, which is fully sensitive to DBMIB.

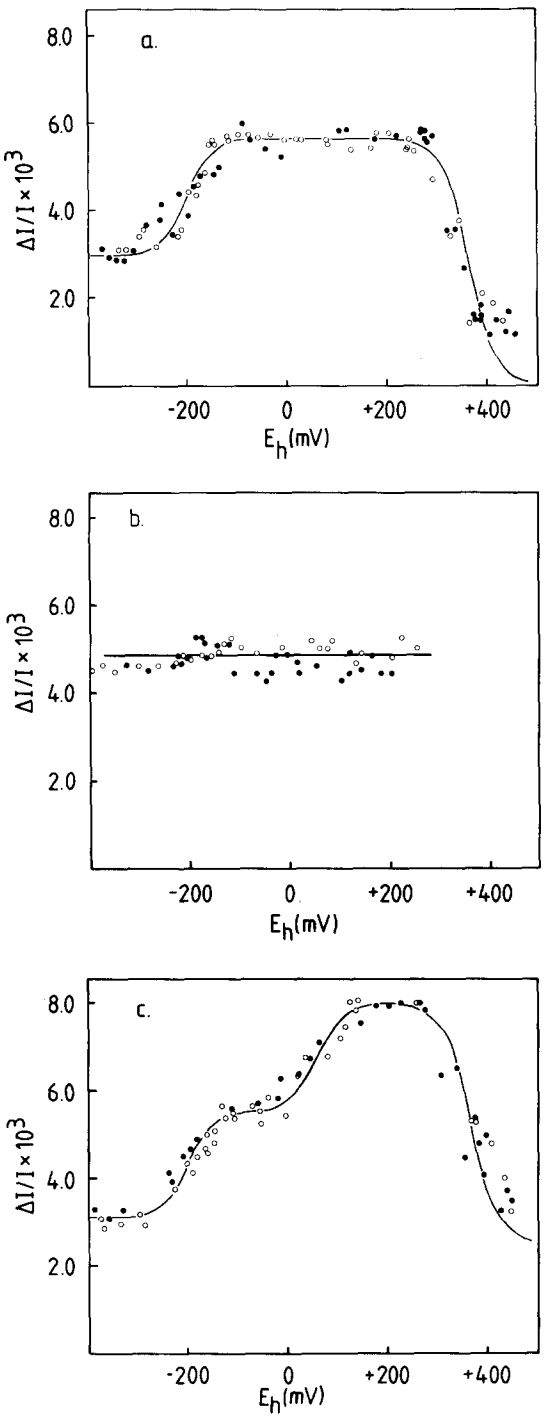

Fig. 2. Redox-potential titrations of the amplitude of phase a using stacked membranes. (a), +25 μM DCMU; (b), +25 μM DCMU + 50 μM methyl viologen; (c), no added inhibitors. Experimental conditions were as described in the Materials and Methods section. Both reductive titrations (○) and oxidative titrations (●) were undertaken. The titration waves have been fitted to $n = 1$ Nernst curves. In Fig. 2c the curve at high redox potentials (over +200 mV) was drawn assuming that a non-titratable portion of ΔA_{515} was derived from PS II and had the same amplitude as the DCMU-sensitive low-potential wave ($E_{m7.5} = +60$ mV).

overall absorption change can be achieved by the subtraction of the ±DBMIB signals (see Fig. 1c). We note, however, that by comparison with the trace in Fig. 1a, the decay of the absorption change in Fig. 1b is slightly accelerated, indicating that DBMIB caused an increase in the conductance of the membrane. For this reason the isolated phase b signal is probably affected both in its amplitude and decay kinetics. In agreement with the work of Selak and Whitmarsh [12], we have observed that the extent of phase b is not affected by the addition of 50 μM methyl viologen (data not shown), indicating that cyclic electron flow around PS I is not an essential requirement for its generation.

Under optimal conditions (see below), the amplitude of phase b in DCMU-treated samples can approximately match the extent of phase a. However, as demonstrated by Olsen and Barber [14], the relative extent of the two phases can be altered by manipulation of the cation levels of the chloroplast suspending medium. With low levels of cations, in which the thylakoids unstack, phase a is approx. 20% larger than its amplitude in the presence of high cation levels when normal membrane stacking exists. Phase b shows an equal but opposite change of amplitude in response to alterations in cation concentrations [14].

The redox-potential titration of phase a of DCMU-treated chloroplasts, suspended in a sufficiently high level of cations (5 mM Mg^{2+}) to induce membrane stacking, is shown in Fig. 2a. The low-potential titration of the amplitude exhibits a single wave with a redox midpoint potential ($E_{m7.5}$) value of −195 mV, and having a −60 mV/pH unit dependence (data not shown).

This titration wave has an approximate 50% contribution to the maximal extent of phase a. The residual component at a redox potential of −300 mV was not titrated. At high redox potentials, the amplitude of phase a decreases with an $E_{m7.5} = +360$ mV, presumably due to the chemical oxidation of the P-700 chlorophyll donor of PS I.

In order to determine the possible influence of the redox mediators on the titrations of phase a, we have performed experiments (over the whole redox-potential range) in which the mediator concentrations were reduced by half: this was observed to have no effect. It was also established that the presence of phenazine methosulphate, 1,4-naphthoquinone, 2,5-dihydroxybenzoquinone, anthroquinone-2,6-disulphate and anthroquinone-2-sulphate had no deleterious action.

An interesting observation was that the addition of 50 μM methyl viologen to DCMU-treated chloroplasts was found to abolish the titration of phase a at low redox potentials (over the range tested) (Fig. 2b).

In the absence of DCMU, the redox-potential titration of phase a will contain a contribution from PS II. The observation of an additional titration wave at low redox potentials with an $E_{m7.5} = +60$ mV (Fig. 2c) is thought to represent the chemical reduction of the primary plastoquinone acceptor of PS II [24]. The extent of this titration wave indicates that the maximal contribution of PS II is one-half that of PS I. Since the P-680 chlorophyll donor of PS II has a higher midpoint potential than that of P-700 [24], an $n = 1$ Nernst curve ($E_{m7.5} = +360$ mV) has been fitted to the data points at high redox potential with an amplitude which matches that observed in Fig. 2a.

Fig. 3 shows similar redox potential titrations of phase a, but carried out at a sufficiently low cation concentration to induce unstacking of the thylakoids. In the presence of DCMU, a single wave in the titration at low redox potentials could be observed with a comparable $E_{m7.5}$ to that measured in Fig. 2a. Although the extent of this titration wave was not affected, a comparison between Fig. 2a and 3a indicates that the amplitude of the non-titratable low-potential component is increased by 15%. The DCMU-sensitive low redox-potential wave (see Fig. 3b) is, however, significantly modified by low salt conditions. The $E_{m7.5}$ of this component is lowered to -100 mV, and its amplitude is decreased by about 25% as compared with the plus Mg^{2+} condition.

Fig. 4 shows the flash-induced ΔA_{515} at three redox potentials (pH 7.5). This figure demonstrates that the DBMIB-sensitive slow phase, which can be detected at an $E_h = +20$ mV, is not clearly

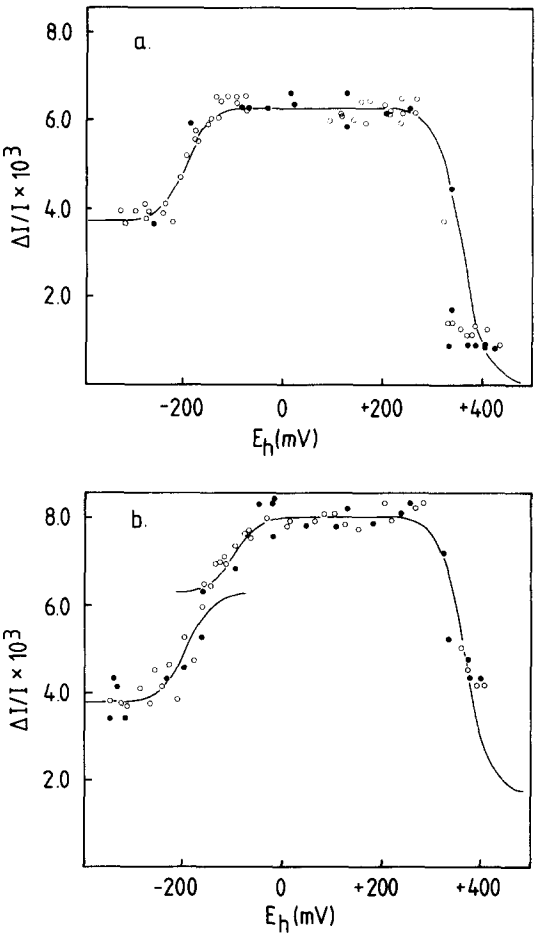

Fig. 3. Oxidative (●) and reductive (○) redox potential titrations of the amplitude of phase a using unstacked membranes. (a), +25 μM DCMU; (b), no added inhibitors. Experimental conditions were as described in the Materials and Methods section. $n = 1$ Nernst curves were fitted to the data making the same assumptions as in Fig. 2 with the low-potential wave of Fig. 3b being divided into a DCMU-sensitive and -insensitive component.

discernible at either high ($E_h = +130$ mV) or low ($E_h = -230$ mV) redox potentials.

Fig. 5 shows the redox potential dependence of phase b obtained by plotting the amplitude of the ΔA_{515} at 20 ms in DCMU-treated and DCMU-plus-DBMIB-treated chloroplasts. The difference between the two titrations gives the redox properties of phase b. It can be seen that the amplitude of phase b increases as the redox potential is lowered from +150 mV. The maximal extent of phase b at 20 ms occurs at about -50 mV where

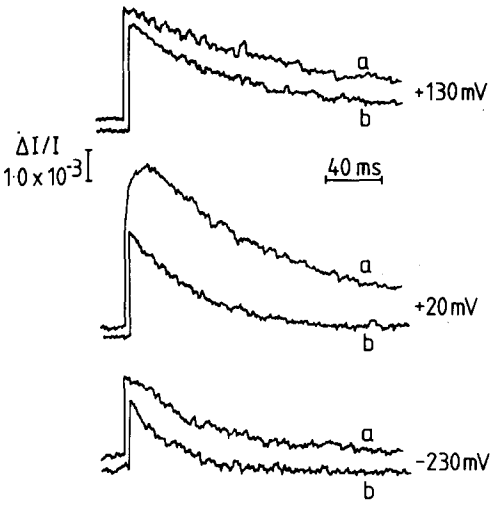

Fig. 4. The flash-induced 515 nm absorption change at three E_h values in the absence (a) or presence (b) of 15 μM DBMIB. Experimental conditions as for Fig. 2a.

the chemical reduction of the plastoquinone pool would be complete [25]. At more reducing redox potentials, phase b is attenuated with an $E_{m7.5}$ of about −180 mV. Both the high- and low-potential attenuations of phase b had a −60 mV/pH unit dependency (data not shown). In chloroplasts suspended in low cation concentrations, the redox

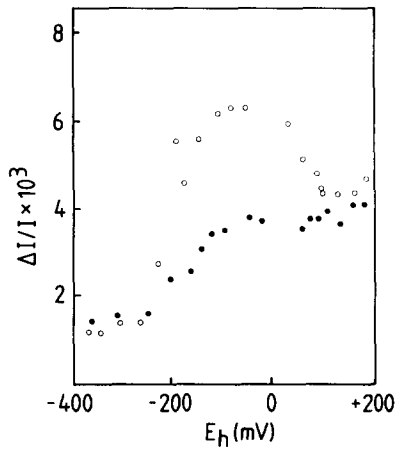

Fig. 5. Redox-potential titration of the amplitude of the absorption change at 20 ms in DCMU-treated (○) and DCMU- and DBMIB-treated (●) chloroplasts. Experimental conditions as for Fig. 2, except that only reductive titrations were undertaken. The concentration of DBMIB used was 15 μM.

properties of phase b were unaffected (unpublished results).

Discussion

The redox potentiometric titration of phase a of the ΔA_{515} electrochromic shift indicates that under high salt conditions it is the light-induced reduction of three low-potential acceptors that generates the full transmembrane electric field in less than 0.5 ms. These three acceptors have $E_{m7.5}$ of +60 mV, −195 mV and less than −400 mV. Only the titration wave with an $E_{m7.5}$ = +60 mV was inhibited by DCMU, and presumably this corresponds to the PS II quinone acceptor Q_A. The observed shift of the $E_{m7.5}$ of this component to −100 mV at low cation concentration could reflect a change in surface potential in the vicinity of Q_A [26]. Because the acceptors with an $E_{m7.5}$ of −195 mV and of below −400 mV were insensitive to DCMU they are assumed to arise from PS I, a conclusion supported by the fact that they were totally removed when P-700 was chemically oxidised ($E_{m7.5}$ = +360 mV). The precise chemical identity of the component with an $E_{m7.5}$ less than −400 mV is not known, but is presumably a PS I reaction centre acceptor.

However, the origin of the −195 mV wave is less clear. Malkin [5] has attributed a −235 mV (pH 7.6) component of the ΔA_{515} as an acceptor in PS II, although Diner and Delosme [8] have recently suggested that such a low potential wave was not due to a primary electrogenic reaction, since they were unable to detect this component at 50 μs. The action spectrum of this titration wave suggested that its origin was in PS I. Our results support their conclusion and it is highly probable that the duration of our flash (10 μs width at half maximum amplitude) was sufficiently long for the PS I reaction centres to turnover twice, a conclusion also reached by Delosme et al. [27] from their flash studies. The −195 mV component is thus suggested to represent a relatively high-potential secondary acceptor. Although the identity of this acceptor is not known, its redox midpoint potential is similar to that of the low-potential form of cytochrome *b*-563 ($E_{m7.5}$ = −170 mV) [28]. The loss of the −195 mV wave in the presence of methyl viologen (Fig. 2b) is presumably due to the

ability of this dye to act as a terminal electron acceptor for PS I, and thus apparently by-pass the secondary stable acceptor. Hence, under the conditions used (long-duration pulse) it seems that the PS I contribution to the transmembrane electric field is twice that of PS II.

The millisecond rise of the ΔA_{515} (phase b) is apparent over a redox span between +100 mV and −230 mV at pH 7.5 with the maximal amplitude occuring at −50 mV. The high-potential value corresponds to the chemical reduction of the plastoquinone pool ($E_{m7} = +110$ mV [25]). A similar result has been obtained by Girvin and Cramer [29], and is consistent with the analogous studies of the slow phase of the electrochromic shift in bacterial chromatophores [16–20]. The attenuation of phase b at lower potentials ($E_{m7.5} = -180$ mV) possibly correlates with the chemical reduction of cytochrome b-563_{LP} [28]. In contrast, Girvin and Cramer detect the full extent of phase b at a redox potential of −200 mV (pH 7.0) [29]. Moss and Bendall have recently been able to repeat both the observations of ourselves [21] and those of Girvin and Cramer [29], by the simple expedient of altering the redox mediator composition [30]. The differential effect of mediators may reflect their varying capacities to facilitate the equilibration of the cytochrome *b-f* complex with the ambient redox-potential poise.

Our results are consistent with the inhibitor studies of Selak and Whitmarsh [12] who suggest that phase b arises from the oxidation of cytochrome *b*-563. The oxidant of cytochrome b-563_{HP} is a quinone at the Q_C site [31]. Under conditions where the plastoquinone pool is reduced, Rich [31] has proposed that the oxidation of cytochrome b-563_{HP} can occur only when quinone is generated by the oxidation of quinol at the Q_Z site. Our redox potential titration of phase *b* is thus consistent with this model, since once cytochrome b-563_{LP} is chemically reduced the full oxidation of plastoquinol at the Q_Z site is prevented, and hence the oxidant of cytochrome b-563_{HP} is no longer generated.

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