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KINETICS OF THE REDUCTION AND OXIDATION OF CYTOCHROMES b_6 AND f IN ISOLATED CHLOROPLASTS

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SUMMARY

Absorbance changes induced by flash illumination of a chloroplast suspension were monitored kinetically at selected wavelengths between 510 and 575 nm. Digital subtraction of the P518 component from the total transient signal permitted isolation of the responses due to cytochromes *f* and b_6. The half rise time for cytochrome b_6 reduction (1.3 ± 0.1 ms) was much greater than a previously reported value (< 10 μs); the half time for cytochrome b_6 oxidation was 35 ± 4 ms. Cytochrome *f* was oxidized with a half time of about 0.22 ms and the subsequent reduction occurred in two phases with half times of about 7.3 and 83 ms. These kinetic data show that cytochrome b_6 cannot be a primary electron acceptor in Photosystem 1. The rate of oxidation of cytochrome b_6 is consonant with this cytochrome being the source of electrons for the slower phase of cytochrome *f* reduction.

INTRODUCTION

The role of the two chloroplast *b*-type cytochromes in photosynthetic electron transport remains to be established, and is currently at the focus of much research interest. Upon fractionation of chloroplasts by detergent [1–3] or comminution [4, 5], cytochrome *b*-559 and b_6 (*b*-563) are predominantly found in association with the Photosystem 2 and Photosystem 1 complexes, respectively. Spectrophotometric studies with unresolved chloroplasts have so far supported the above distribution, in that cytochrome *b*-559 is oxidized by Photosystem 2 in a photochemical reaction at −196 °C [6], whereas cytochrome b_6 is reduced by light predominantly absorbed by Photosystem 1 [7, 8].

The midpoint potential of cytochrome b_6 (about 0 mV [9, 10]) precludes its mediation in electron flow between the reduced product of Photosystem 1 and $NADP^+$. Numerous cyclic pathways around Photosystem 1 have therefore been suggested in the literature, which differ in the route by which cytochrome b_6 returns electrons to the oxidized photoproduct, P700. In most of these schemes, at least one coupling ("phosphorylation") site is assumed to lie within the cycle [7–9]. Evaluation

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of such pathways awaits identification of the immediate reaction partners of cytochrome b_6 and determination of the effect of coupling, uncoupling and inhibitory treatments on relevant rate parameters.

This report is addressed exclusively to the former issue. It describes kinetic and steady state studies which define the conditions under which cytochrome b_6 responses are reliably detectable, and it gives a revised value for the rate of cytochrome b_6 reduction.

METHODS

Washed spinach leaves were homogenized in a chilled Waring Blendor with 0.3 M sucrose, 2 mM $MgCl_2$ and 30 mM morpholinopropane sulfonic acid adjusted to pH 7.75 with NaOH. The homogenate was filtered through cheesecloth and centrifuged for 5 min at $3000 \times g$. The sedimented chloroplasts were then swollen in a medium containing 0.02 M sucrose, 2 mM NaCl, 0.2 mM $MgCl_2$ and 1 mM morpholinopropane sulfonic acid adjusted to pH 7.5 with NaOH. The suspension was then centrifuged for 5 min at $12\,000 \times g$, followed by resuspension of the swollen thylakoid sediment in a small volume of 10-fold concentrated swelling medium. This resuspension buffer was also used in the preparation of samples (final chlorophyll concentration, 100 μg/ml). No terminal electron acceptor system or mediator of cyclic electron flow was added. The ability of cytochrome b_6 to compete with exogenous oxidants for electrons flowing from the reduced side of Photosystem 1 will be discussed in a subsequent article.

Absorbance changes were monitored by a conventional flash spectrophotometer [11] with a 0.05 Hz–0.85 MHz bandwidth. Following each actinic flash the amplified difference between the sample and reference photomultiplier tube outputs was stored in the memory of a Fabritek (Nicolet) 1052 signal averager. The limited sweep rate of the signal averager (50 μs/address) was overcome when necessary by the use of a Fabritek 952 fast buffer memory (1.0 μs/address). The actinic flash was limited to the spectral region above 640 nm by a Corning 2-58 filter and had a nominal lifetime of 12 μs. The flash repetition rate was about 2 per s and the intensity $2 \cdot 10^{-4}$ J $\cdot$ cm^{-2} per flash.

RESULTS AND DISCUSSION

Upon subjection of a thylakoid suspension (without added electron acceptors) to a brief flash of broad band red light, we observe an absorbance change at 563.5 nm (Fig. 1a) having a fast initial rise ($t_{0.5} < 10$ μs), similar to the response described by Witt's group [12]. However, we detect in addition a slow component with an estimated half rise time of 1 ms (Fig. 1b). The wavelength dependence of the fast rise component is presented in Fig. 2. For comparison, Fig. 2 also shows a continuous spectrum of the electrochromic shift known as P518, which was obtained by subtracting a dark from a light steady state spectrum as described elsewhere [13]. Except for having a wider bandwidth around the peak at 518 nm, the spectral dependence of the fast kinetic component agrees with the spectrum of P518.

Fig. 3 shows a continuous spectrum of the difference between the illuminated and dark steady states in a chloroplast sample prepared as for Fig. 1. The dissimilarity

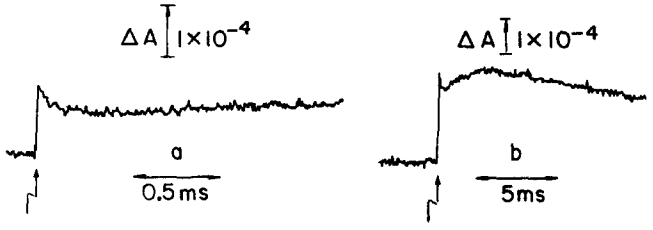
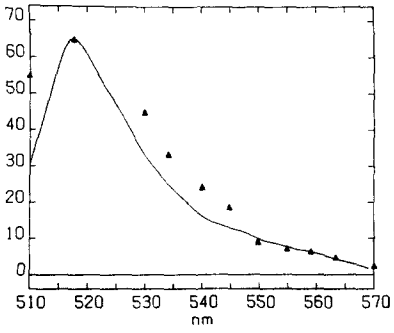


Fig. 1. Reversible absorbance change at 563.5 nm induced by flash illumination of spinach chloroplasts in suspension. The flash (at arrow) promotes a rise in absorbance which is complete in 4 μs (one address in Fig. 1a). The extents of the fast and slow phases were not increased by raising the flash intensity. Number of sweeps: (a) 512; (b) 256. Other details as given in Methods.

between this spectrum and that of the fast rise component (Fig. 2) in the cytochrome α-band region (550–570 nm) indicates that kinetically slow components contribute to later stages of the response to illumination. These slow components are demonstrated upon plotting the signal amplitude 3.75 ms after a flash (isolated points in Fig. 3). P518 still contributes as a slow component by reason of incomplete relaxation, and other slow components centered near 554 and 563 nm are in evidence. The dissimilarities between the two plots in Fig. 3 are not understood for the range below 540 nm, but above 540 nm may be accountable to differing contributions from C550 and cytochrome *b*-559.

In order to observe the kinetics of the slow components it is clearly necessary that the contribution of P518 be removed from the absorbance changes, and this has been accomplished as follows. At 531 nm the extinction of P518 is relatively high while the reduced and oxidized spectra of cytochromes *f* and b_6 are almost isosbestic. After accumulation of a number of sweeps (N_λ) in the signal averager at the wave-



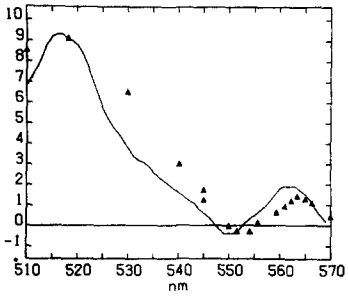


Fig. 2. Identification of the fast rise component seen at 563 nm as the electrochromic band shift, P518. Triangles: extent of the fast phase of the flash induced absorbance increase (8 μs/address). Continuous trace: spectrum of P518 obtained by illuminating pea chloroplasts with continuous weak light at 710 nm, in the presence of 25 μM ferricyanide, and subtracting a dark spectrum of the same sample. Absorbance values (arbitrary units) normalized at 518 nm.

Fig. 3. Wavelength dependence of slow components in the response to flash illumination. Triangles: extent of the absorbance change 3.75 ms after a flash. Continuous trace: illuminated minus dark steady-state difference spectrum of chloroplasts, obtained as described in ref. 12 and Methods, with a continuous light source substituted for the flash but with no change in sample composition. Absorbance values (arbitrary units) normalized at 518 nm.

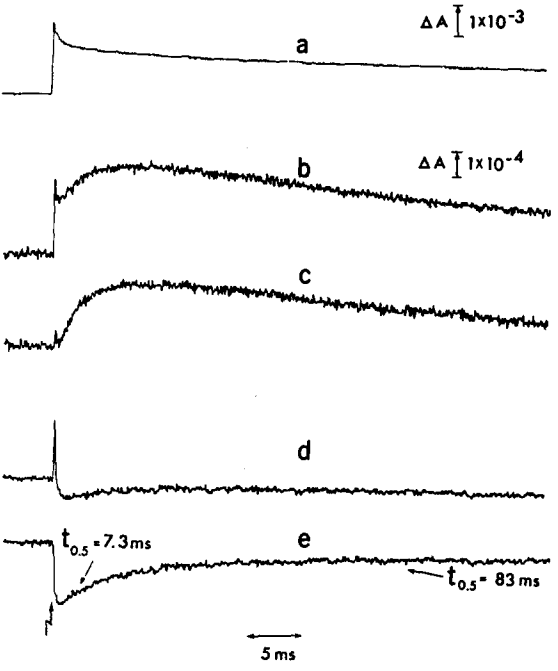


Fig. 4. Removal of the P518 component from kinetic traces by means of a digital subtraction technique. Trace (a), flash induced response at 531 nm having the kinetic characteristics of P518. Trace (b), flash-induced response at 563.5 nm before subtraction of trace (a), as described in the text; and trace (c), the result of digital subtraction. Traces (d) and (e) are the responses at 554.5 nm before and after subtraction of trace (a), respectively. The initial rise in trace (d) is truncated compared to trace (b) owing to the opposing absorbance change of cytochrome *f* within one address (50 μs) of the signal averager. Half times for the cytochrome *f* oxidation were determined by a computer routine which optimally fitted the data to be a biexponential form, with the fast component contributing 63 % to the overall relaxation. Number of sweeps: (a) 64, (b, c, d, e) 256. N_{531} = (c) 32, (e) 42 (see text).

length of interest (λ), the wavelength was changed to 531 nm and the averager mode was inverted. A number of sweeps, N_{531}, (Fig. 4a) was then accumulated (and hence subtracted) such that N_{531} equals $(\Delta A_\lambda/\Delta A_{531})N_\lambda$. The ratio $\Delta A_\lambda/\Delta A_{531}$ was obtained from the P518 spectrum (Fig. 2).

Fig. 4 illustrates kinetic traces before (Fig. 4b, d) and after (Fig. 4c, e) the above stripping procedure. At 563.5 nm (Fig. 4c) the half rise and decay times after stripping are 1.3 ± 0.1 and 35 ± 4 ms, respectively. The maximum absorbance occurs approximately 7.5 ms after the flash and its wavelength dependence (Fig. 5) agrees closely with the reduced minus oxidized difference spectrum for the α-band of cytochrome b_6 [3]. Chance et al. [14] have reported half rise and decay times at 563 nm of about 1 ms and 6.5 ms, respectively, upon flash illumination of a pale green mutant of *Chlamydomonas*. Larkum and Bonner [15] obtained 4 and 500 ms for these same kinetic parameters in high salt pea chloroplasts.

At 554.5 nm the stripped response (Fig. 4e) is a light-induced bleaching, with a half rise time of about 0.22 ms (Fig. 6), followed by a biphasic relaxation with half decay times of 7.3 ± 0.6 and 83 ± 10 ms. The wavelength dependence of the absor-

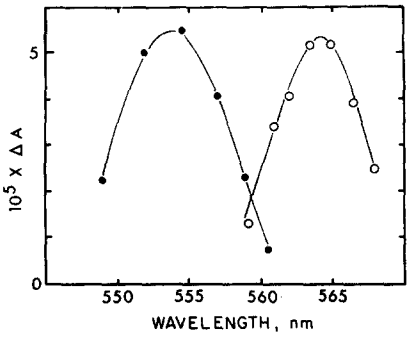


Fig. 5. Wavelength dependence of slow phases of the flash induced absorbance change, revealed by digital subtraction of the P518 component. ●–●, extent of the absorbance decrease, 0.5 ms after a flash; ○–○, extent of the absorbance increase, 7.5 ms after a flash.

bance decrease (Fig. 5) peaks near 554.5 nm and is indicative of cytochrome *f* oxidation, although a small contribution from C550 cannot be ruled out. The above rate of cytochrome *f* oxidation falls between the range of values obtained by Hildreth [16] using whole leaf (60–150 μs) and chloroplast material (0.30–9.0 ms). It corresponds closely with the slow phase ($t_{0.5}$ = 0.2 ms) of P700 reduction following a brief flash, which Haehnel et al. [17] ascribed to electron transfer from cytochrome *f*.

Kok [18] briefly reported a cytochrome *f* half oxidation time of 60 μs for chloroplasts in the presence of 10 μM *N*-methylphenazonium methosulfate. The subsequent reduction had a half time of 7 ms in accord with the faster of the components ($t_{0.5}$ = 7.3 ms) reported in this work. Hildreth [16] obtained a half time for cytochrome reduction of about 6 ms in intact spinach leaf and 50 ms in isolated chloroplasts. The above grouping of cytochrome *f* reduction half times into values around 7 or 50–83 ms and sometimes both, suggests the participation of two sources of electrons. Intactness of the thylakoid membrane could determine the relative contribution from each source. Biggins [19] has shown that following activation of both photosystems, cytochrome *f* in *Porphyridium* is reduced with half times of 25 and 150 ms,

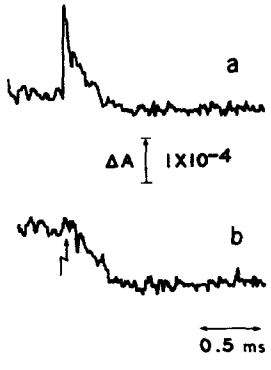


Fig. 6. Kinetics of cytochrome *f* oxidation. Trace (a), flash-induced response at 554.5 nm and trace (b), the result of digital subtraction of the P518 component. Number of sweeps: 256. N_{531} = 42 in trace (b) (see text).

the fast phase being ascribed to electron flow from Photosystem 2 and the slow phase to a cyclic return. In higher plants, biphasic kinetics could also result from two physically distinct pools of cytochrome *f* as, for example, in grana lamellae and stroma lamellae [5]. On the basis of our values for the half times for oxidation of cytochrome b_6 (35 ms) and reduction of cytochrome *f*, it follows that only the slower phase of cytochrome *f* reduction might be ascribed to an (indirect) interaction with cytochrome b_6.

Ke [20] has demonstrated that P430 is the primary electron acceptor of Photosystem 1, and is photoreduced in < 100 ns. The relative slowness of cytochrome b_6 reduction ($t_{0.5} = 1.3$ ms) shows that b_6 cannot be an alternative primary acceptor, as suggested [12]. In digitonin Photosystem 1 particles (without added terminal electron acceptor) P430 is reoxidized very slowly ($t_{0.5} = 55$ ms) [20] and the cytochromes are inactive [21]. Presumably digitonin disturbs the physical interaction of P430 and cytochrome b_6, or releases an as yet unknown intermediate electron carrier.

Though our kinetic data for cytochrome *f* are in accord with previous work, the data for b_6 are not. This probably results from past failure to appreciate the interference at 563.5 nm due to the P518 response [8, 12]. Kinetic or steady state studies of chloroplast cytochromes must be questioned where this interference was not recognized.

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