Kinetics of the electrogenic step and cytochrome b_6 and f redox changes in chloroplasts

Evidence for a Q cycle

M.A. Selak and J. Whitmarsh

Department of Botany, USDA/ARS, University of Illinois, Urbana ILL 61801, USA

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The spectroscopic measurements of the slow phase of the electrochromic effect and the redox kinetics of cytochrome b_6 and f provide strong evidence that a Q cycle operates in chloroplasts under conditions of non-cyclic electron transport. The effect of HQNO and DBMIB on the extent and kinetics of these light-induced changes places several constraints on the mechanism of quinol oxidation by the cyt. b/f–FeS complex: for each electron removed from the cyt. b/f–FeS complex by P700 an additional charge is transferred across the membrane; the cyclic pathway of electrons involved in quinol oxidation by the cyt. b/f–FeS complex includes at least one of the two b_6 cytochromes; the electrogenic step associated with quinol oxidation is subsequent to the reduction of at least one cytochrome b_6 quinol oxidation may proceed in a stepwise manner, with the first electron going to cytochrome b_6 and the second electron going to the FeS center and cytochrome f.

Photosynthesis *Non-cyclic electron transport* *Q cycle* *Cytochrome* b_6 *Cytochrome* f *Electrochromic*

1. INTRODUCTION

The cytochrome b/f–FeS complex isolated from chloroplasts mediates electron transport between PS II and PS I, oxidizing quinol and reducing plastocyanin [1,2]. The complex contains one Rieske FeS protein, one cytochrome f, and two b_6 cytochromes. The mechanism of quinol oxidation by the complex has yet to be determined, although several models that can be generally classified as modified Q cycles [3] have been suggested [4–6]. A Q cycle involves the sequential or concerted two step oxidation of quinol by components in the cyt. b/f–FeS complex and results in the translocation of two protons across the membrane for every electron transferred from the complex to P700. In photosynthetic bacteria and mitochondria a Q cycle mechanism has been better established and more fully characterized than in chloroplasts, due in part to the antimycin A sensitivity of cytochrome b oxidation in these systems [7,8]. In chloroplasts, however, antimycin does not inhibit cytochrome b_6 turnover in non-cyclic electron transport [9]. Furthermore, under many experimental conditions the slow phase of the electrochromic shift, indicative of a Q cycle in chloroplasts, is not observed [10] and the additional proton translocation predicted by the Q cycle has been difficult to demonstrate, with different laboratories reporting different proton to

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PS, photosystem; UHDBT, 5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole

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electron ratios [11–13]. These problems coupled with the fact that, in almost all cases, the ATP/e_2 ratio measured in chloroplasts can be accounted for without invoking a Q cycle have lead to proposals that quinol oxidation does not involve a Q cycle in chloroplasts [13,14].

We have investigated the function of the cyt. *b/f*–FeS complex in chloroplasts by spectroscopic measurements of the electrochromic shift at 515 nm and cytochrome b_6 and *f* redox changes. In order to elucidate the role of a possible Q cycle we have used duroquinol as the electron donor and selected experimental conditions to maximize the slow component of the electrochromic shift. We have found that HQNO, under conditions of non-cyclic electron transport, inhibits the slow electrogenic step, apparently by blocking the oxidation of cytochrome b_6. We have investigated the mechanism of quinol oxidation by measuring the extent and kinetics of the electrochromic shift, and cytochrome b_6 and *f* turnover in the presence and absence of HQNO as well as other inhibitors. Our data are most simply explained by a Q cycle in which quinol oxidation proceeds in a stepwise manner, with one electron going to cytochrome b_6 and the other to the Rieske FeS center and subsequently to cytochrome *f*.

2. MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves purchased from local markets according to the procedure in [15]. The experiments described here were done using class II naked lamellae for which the cofactors for cyclic electron transport have been washed away; consequently, this study deals only with non-cyclic electron transport. Chloroplasts were suspended in a reaction medium containing 30 mM Tricine/KOH (pH 8.0); 0.1 M sorbitol; 20 mM KCl; 3 mM $MgCl_2$; 0.5 mM duroquinol; and 0.1 mM methylviologen. Other additions are indicated in the figure legends. Duroquinol was prepared according to the technique in [16]. All samples were thermostatically controlled (±0.2°C) at 15–18°C.

Light-induced absorbance changes were measured using a laboratory-built single beam spectrophotometer. Actinic flashes were provided by a xenon flash lamp (FX-193 EG&G ElectroOptics). The lamp was filtered by a red blocking filter (Corning CS 2-58) and exhibited a half-peak width of 6 μs. This flash duration was short enough to ensure a single turnover activation of PS I reaction centers (<5% double hits [18]). The intensity of the flashes was saturating inasmuch as changing the intensity by a factor of two did not alter the extent or kinetics of the absorbance change attributed to the electrochromic shift, cytochrome *f*, or cytochrome b_6. Cytochrome content was determined using a millimolar difference extinction coefficient of 20 $mM^{-1}.cm^{-1}$ for cytochrome *f* at the wavelength pair 553–540 nm and 15 mM^{-1} for cytochrome b_6 at the wavelength pair 563–572 nm. All measurements were done using a 1 cm square cuvette.

3. RESULTS AND DISCUSSION

We have investigated the function of the cyt. *b/f*–FeS complex in chloroplasts in which PS II has been inhibited by DCMU. Electron transport was driven solely by PS I in single turnover flashes using duroquinol as the electron donor and methylviologen as the acceptor. The reaction is sensitive to DBMIB [16] and UHDBT [17,18] indicating that the pathway of electrons from duroquinol includes the site of quinol* oxidation by the cyt. *b/f*–FeS complex. The electron-transport reaction is rapid and coupled to the synthesis of ATP in continuous light and single turnover flashes [16,19].

In the presence of duroquinol the electrochromic shift, monitored by the light-induced absorbance change at 515 nm, exhibits two distinct kinetic phases, one fast (515_f) and one slow (515_s) (fig. 1A) The spectrum of each of the two kinetic phases is the same [20]; both are due to an electrochromic shift induced by a transmembrane electric field (not shown). The fast phase is due to charge separation at the PS I reaction center, while the slow phase is most simply accounted for by an electrogenic reaction on the donor side of PS I [20], specifically a Q cycle [3] or modified Q cycle [4–6], in which the oxidation of quinol by the cyt. *b/f*–FeS

* In these experiments we are unable to determine whether the immediate reductant for the cyt. *b/f*–FeS complex is the physiological donor plastoquinol or whether it is duroquinol [3]; hence, we will refer to the donor as quinol

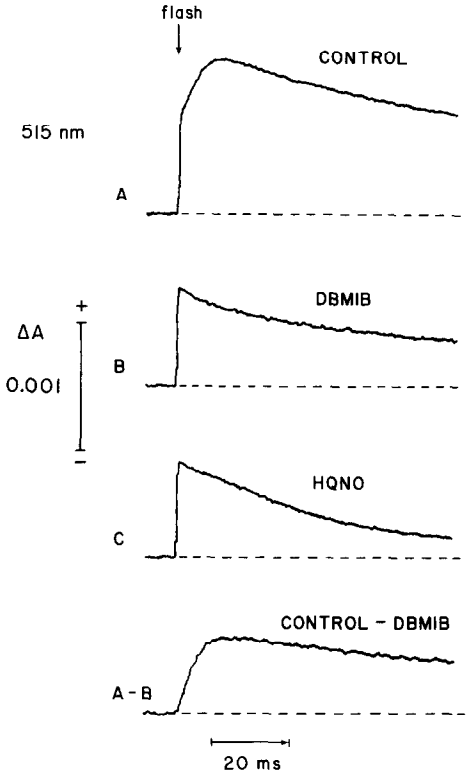

Fig. 1. Kinetics of the absorbance change at 515 nm due to the electrochromic shift induced by a short flash. The traces shown are the average of 64 runs at a repetition rate of 0.1 Hz. The half-bandwidth of the measuring beam was 2 nm. In addition to the reaction medium in section 2 the reaction mixture contained 10 μM DCMU, chloroplasts equivalent to 15 μg chl./ml, and: (A) control, no further additions; (B) 0.5 μM DBMIB; (C) 20 μM HQNO. The bottom trace is the difference between trace A and B.

complex results in the translocation of an additional proton across the membrane. A general Q cycle mechanism is supported by the observation that inhibitors that block the oxidation of quinol by interacting with the Rieske FeS center also block the 515_s [6]; these include DBMIB (fig. 1B), DNP–INT and UHDBT (not shown).

To separate the two kinetic phases of the electrochromic shift we have subtracted the absorbance at 515 nm in the presence of DBMIB from the 515 nm change observed in the control (bottom trace, fig. 1). The resulting trace reveals the kinetics of the slow electrogenic step. The half-time for the rise of the 515_s is 3.7 ± 0.3 ms. Comparison of the extent of the slow phase to that of the fast phase at a time subsequent to the slow rise kinetics shows their extent to be equal ($515_s/515_f = 1.00 \pm 0.05$). If we assume that the 515_f is due to the transfer of a single charge across the membrane per PS I reaction center, then the stoichiometric relation of the 515_s and the 515_f indicate that under these experimental conditions, for every electron transferred from the cyt. *b/f*–FeS complex to P700, an additional charge is transferred across the membrane. Although in spinach chloroplasts the ratio of P700 to the cyt. *b/f*–FeS complex is ~1 : 1 [21] suggesting that each complex turns over once each flash, the possibility that fewer complexes turnover more frequently cannot be discounted. In order to maximize the extent of the slow phase of the electrochromic shift the actinic flashes were given at a frequency of 0.1 Hz. At higher frequencies the extent of the slow phase decreased.

We have found that the slow phase of the electrochromic shift is abolished by HQNO (fig. 1C) by a mechanism that appears to be different from that of DBMIB, DNP–INT and UHDBT. The difference between the mode of action of HQNO and DBMIB is clearly demonstrated by comparing their effect on the light-induced turnover of cytochrome b_6. In the dark, in the presence of duroquinol, cytochrome b_6 is oxidized while the high potential PS I donor pool, which includes the FeS center, cytochrome *f* and plastocyanin, is reduced. A single turnover flash oxidizes P700. An electron from the high potential donor pool rapidly reduces P700, leaving the FeS center mostly oxidized and cytochrome *f* partially oxidized [18]. It is this light-induced oxidation of the FeS center that instigates the reduction of cytochrome b_6 [2]. Following the actinic flash there is a rapid reduction of cytochrome b_6 followed by a relatively slow reoxidation (fig. 2). These experiments were done under the same experimental conditions that were used in measuring the absorbance change at 515 nm except that gramicidin was added so that cytochrome b_6 could be monitored without interference from the electrochromic shift. The spectrum of the light-induced change indicates the majority of the absorbance change at 563–572 nm is due to cytochrome b_6 (fig. 3). The half-time for reduction in the control sample is ~2 ms, for the

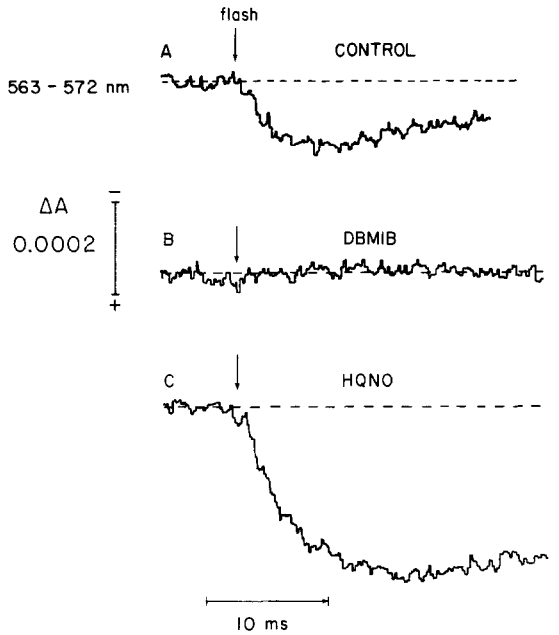

Fig. 2. Kinetics of the flash-induced absorbance change at 563–572 nm due to cytochrome b_6. The traces shown are the average of 128 runs at a repetition rate of 0.1 Hz. The half-bandwidth of the measuring beam was 2 nm. In addition to the reaction medium in section 2 the reaction mixture contained 10 μM DCMU, 5 μM gramicidin, chloroplasts equivalent to 25 μg chl./ml, and: (A) control, no additions; (B) 0.5 μM DBMIB; (C) 50 μM HQNO.

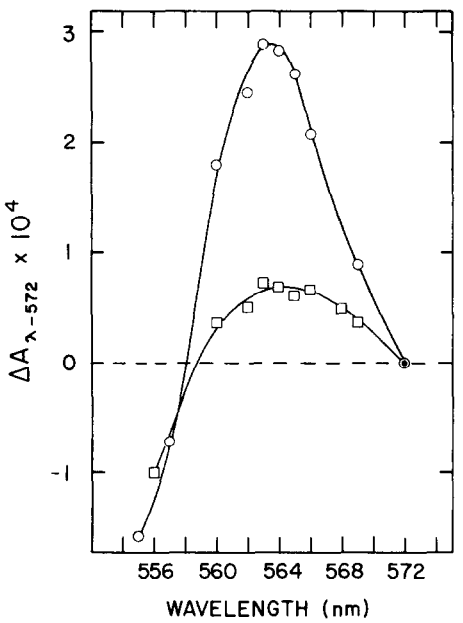

Fig. 3. Wavelength dependence of the flash-induced absorbance change shown in fig. 2. The reference wavelength was 572 nm. The conditions were as in fig. 2 except the repetition rate of the runs was 0.25 Hz: (●) control; (○) 50 μM HQNO.

reoxidation it is 20–30 ms (not shown). However, relatively little cytochrome b_6 is seen to turnover, ~0.2 molecules/cyt. b/f–FeS complex (fig. 2A). The small extent of cytochrome b_6 turnover observed in the control is expected, if in addition to the slow phase of the reoxidation, there is a rapid phase so that the competing reduction and reoxidation reactions result in a limited observable turnover [4]. (This ratio may be even lower since the oxidation of cytochrome f will contribute to the absorbance change observed at 563–572 nm [22]. In the presence of HQNO the flash-induced reduction of cytochrome b_6 is 3-fold larger than the control, typically 0.6 cytochrome b_6 molecules/complex (fig. 2C). The increase in the extent of cytochrome b_6 reduction is most simply accounted for by HQNO inhibiting the reoxidation but not the reduction of the cytochrome [24]. The half-time for the reduction is 2.8 ± 0.2 ms. The slow reoxidation kinetics are biphasic with a half-time of 60 ms (not shown). In contrast, inhibition of quinol oxidation by DBMIB inhibits the reduction of the cytochrome completely (fig. 2B). DNP–INT and UHDBT also inhibit the light-induced reduction of cytochrome b_6 (not shown).

In the presence of HQNO we observe the reduction of a significant fraction of cytochrome b_6 following a flash (fig. 2C), while at the same time the 515_s is inhibited (fig. 1C). These data lead us to conclude that the electrogenic step coupled to quinol oxidation is due to a charge transfer subsequent to the reduction of cytochrome b_6 (at least subsequent to the reduction of one of the two b_6 cytochromes present in the complex). The inhibition of the 515_s by HQNO appears to be a consequence of the inhibition of cytochrome b_6 reoxidation. It is noteworthy that in photosynthetic bacteria the slow electrogenic step has been shown to be associated with the oxidation of cytochrome b-561 [24].

A second effect of HQNO can be seen in the redox kinetics of cytochrome f. In the control sam-

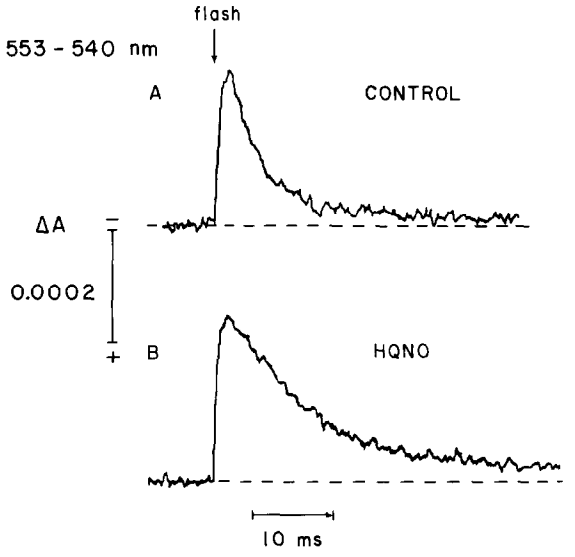

Fig. 4. Kinetics of the flash-induced absorbance change at 553–540 nm due to cytochrome *f* in the absence and presence of HQNO. Conditions as in fig. 2: (A) control; (B) 50 μM HQNO.

ple, monitoring the redox state of cytochrome *f* by the absorbance change at 553–540 nm, a flash of light induces a rapid oxidation (half-time = 220 μs [18]) followed by a much slower re-reduction (fig. 4A). The wavelength dependence of the light-induced absorbance change is as in [18] and is due primarily to cytochrome *f*. The re-reduction kinetics exhibit a delay and a half-time of 5.3 ± 0.4 ms. If the delay is ignored the half-time for re-reduction is ~3 ms. In the presence of HQNO the extent of cytochrome *f* turnover is unchanged while the rate of re-reduction is slowed (fig. 4B). The re-reduction half-time is 10.5 ± 0.7 ms and ~9 ms if the delay is ignored. The disappearance of the 515_s in the presence of HQNO is not due to the decrease in the rate of cytochrome *f* re-reduction, since when the rate of cytochrome *f* re-reduction is slowed $> \frac{1}{2}$ by DBMIB or UHDBT, the extent of the 515_s remains large (not shown). It may be that the re-reduction rate of the FeS center is affected by the redox state of cytochrome b_6 and that the decrease in the re-reduction rate of cytochrome *f* in the presence of HQNO is due to cytochrome b_6 remaining in the reduced state.

In the presence of HQNO the light-induced turnover of 0.6 cytochrome b_6 molecules/complex enables us to determine the rate of cytochrome b_6 reduction following a single turnover flash with minimal interference by the reoxidation kinetics. Comparison of the reduction rate of cytochrome b_6 with that of cytochrome *f* shows cytochrome b_6 to be reduced significantly before cytochrome *f*. Whether we consider half-times or initial rates of reduction, cytochrome b_6 receives an electron 3–4-times faster than does cytochrome *f*. If cytochrome *f* and the FeS center equilibrate rapidly so that the rate of re-reduction of cytochrome *f* reflects the rate of re-reduction of the FeS center*, then this observation would indicate that the first electron leaving quinol goes to cytochrome b_6 and that the resulting semiquinone reduces the FeS center. In this process quinol oxidation is envisioned to occur by a two-step process, whereby the quinol/semiquinone couple reduces cytochrome b_6 and the semiquinone/quinone couple reduces the FeS center at a rate 2–3-times slower than the first step, at least in the presence of HQNO. The fact that we do not observe cytochrome b_6 reduction by quinol in the dark may be accounted for by proposing that quinol binding to the cyt. *b/f*–FeS complex requires an electron hole in the high potential components of the complex. This sequence of quinol oxidation is in marked contrast to most current models in which the quinol/semiquinone couple is envisaged to reduce the FeS center with the resultant semiquinone reducing cytochrome b_6**. The model suggested here would require the quinol/semiquinone couple to be more reducing than the semiquinone/quinone couple; i.e., that the semiquinone is stable. Indeed, according to the kinetics observed here the semiquinone must exist for several milliseconds.

* In the absence of HQNO we have shown that the Rieske FeS center and cyt. *f* equilibrate with one another within 1 ms [18]

** In photosynthetic bacteria the reduction of the low potential *b*-cytochromes prior to cyt. c_1 is explained by the observation that the midpoint potential of the FeS center is higher than that of cyt. c_1 [7]. This is not the case in chloroplasts where the midpoint potential of the FeS center is lower than that of cyt. *f* [26,27] so that we must entertain other possible explanations

Alternatively, the re-reduction of cytochrome *f* by the FeS center may be perturbed by HQNO so that the rate of cytochrome *f* re-reduction does not reflect the rate of re-reduction of the FeS center. This possibility seems unlikely since the extent of cytochrome *f* turnover is not increased by HQNO as would be expected for inhibition at this site [18]. Another possible explanation of these data that avoids assuming cytochrome b_6 reduction by the quinol is to invoke an additional component prior to the FeS center. In this model the first electron leaving quinol could go to the intermediate, enabling the resultant semiquinone to reduce cytochrome b_6. Reduction of the FeS center by the intermediate would follow at a slower rate. For this explanation to account for these data the intermediate must be oxidized in the dark prior to the flash.

4. SUMMARY

The spectroscopic measurements of the slow phase of the electrochromic effect and the redox kinetics of cytochrome b_6 and *f* described above provide strong evidence that a Q cycle operates in chloroplasts under conditions of non-cyclic electron transport (fig. 5). The effect of HQNO and DBMIB on the extent and kinetics of these light-induced changes places several constraints on the mechanism of quinol oxidation by the cyt. *b/f*–FeS complex suggests:

(1) The slow phase of the electrochromic shift is the same magnitude as the fast phase, indicating that under the experimental conditions described here, for each electron removed from the cyt. *b/f*–FeS complex by P700 an additional charge is transferred across the membrane;

(2) The rate and extent of reduction of cytochrome b_6 observed in the presence of HQNO indicates that the cyclic pathway of electrons involved in quinol oxidation by the cyt. *b/f*–FeS complex includes at least one of the two b_6 cytochromes;

(3) In the presence of HQNO the slow phase of the electrochromic shift is completely abolished, whereas the reduction of cytochrome b_6 remains rapid and the extent is enhanced 3-fold. These results demonstrate that the electrogenic step associated with quinol oxidation is subsequent to the reduction of at least one cytochrome b_6;

(4) Comparison of the rate of reduction of cytochrome b_6 with that of cytochrome *f* in the presence of HQNO indicates that quinol oxidation may proceed in a stepwise manner, with the first electron going to cytochrome b_6 and the second electron going to the FeS center and then cytochrome *f*. Alternatively, electron transfer from quinol to the FeS center and cytochrome *f* may be more complex than is shown in fig. 5 (see above).

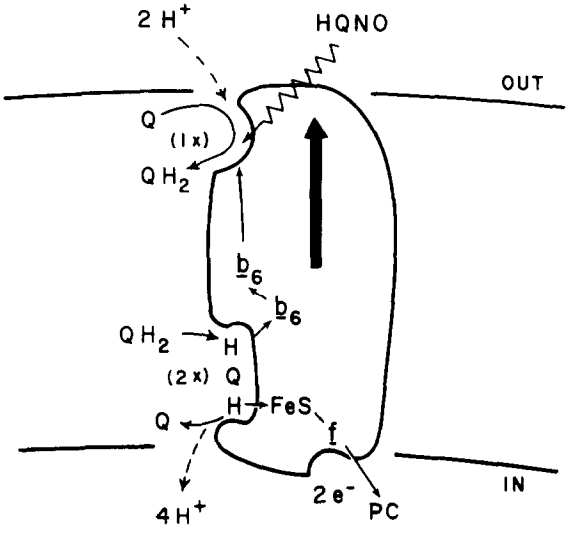

Fig. 5. A modified Q cycle consistent with these data. For kinetic details see text. For a discussion of various modified Q cycles see [24,25]. The solid arrow indicates the electrogenic step and the jagged arrow the site of HQNO inhibition.

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REFERENCES

[1] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.

[2] Prince, R.C., Matsuura, K., Hurt, E., Hauska, G. and Dutton, P.L. (1982), J. Biol. Chem. 257, 3379–3381.

[3] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.

[4] Velthuys, B. (1979) Proc. Natl. Acad. Sci. USA 76, 2765–2769.

[5] Bouges-Bocquet, B. (1980) FEBS Lett. 117, 54–58.

[6] Crowther, D. and Hind, G. (1980) Arch. Biochem. Biophys. 204, 568–577.

[7] Crofts, A.R., Meinhart, S.W., Snozzi, M., Jones, K.R. (1982) 2nd Eur. Bioenerg. Conf. Lyon, abst.

[8] Bowyer, J. and Trumpower, B.L. (1981) J. Biol. Chem. 256, 2245–2251.

[9] Wood, P.M. and Bendall, D.S. (1976) Eur. J. Biochem. 61, 337–344.

[10] Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340.

[11] Velthuys, B.R. (1978) Proc. Natl. Acad. Sci. USA 75, 6031–6034.

[12] Velthuys, R.B. (1980) FEBS Lett. 115, 167–170.

[13] Olsen, L.F., Telfer, A. and Barber, J. (1980) FEBS Lett. 118, 11–17.

[14] Olsen, L.F. and Barber, J. (1981) FEBS Lett. 123, 90–94.

[15] Graan, T. and Ort, D.R. (1981) Biochim. Biophys. Acta 637, 447–456.

[16] Izawa, S. and Pan, R.L. (1978) Biochem. Biophys. Res. Commun. 83, 1171–1177.

[17] Oettmeier, W., Masson, K., and Godde, D. (1981) Z. Naturforsch. 36c, 272–275.

[18] Whitmarsh, J., Bowyer, J.R. and Crofts, A.R. (1982) Biochim. Biophys. Acta in press.

[19] Whitmarsh, J., and Graan, T. (1982) Biophys. J. 37, 224a.

[20] Joliot, P. and Delsome, R. (1974) Biochim. Biophys. Acta 357, 267–284.

[21] Whitmarsh, J. and Cramer, W.A. (1979) Biophys. J. 26, 223–234.

[22] Davenport, H.E. (1972) Proc. 2nd Int. Congr. Photosynth. Res., (Forti, G. et al. eds) pp. 1593–1601, Junk, The Hague.

[23] Hind, G. and Olson, J.M. (1966) Brookhaven Symp. Biol. 19, 188–194.

[24] Cramer, W.A. and Crofts, A.R. (1982) in: Photosynthesis: Energy Conversion by Plants and Bacteria (Govindjee, ed.) pp. 387–467, Academic Press, New York.

[25] Wraight, C. (1982) in: Photosynthesis: Energy Conversion by Plants and Bacteria (Govindjee, ed.) pp. 17–84, Academic Press, New York.

[26] Malkin, R. (1982) in: Electron Transport and Photophosphorylation (Barber, J. ed) Top. Photosynth. vol. 4, pp. 1–47, Elsevier Biomedical, Amsterdam, New York.

[27] Rich, P. and Bendall, D. (1980) FEBS Lett. 116, 51–46.