Biochimica et Biophysica Acta, 767 (1984) 389–395
Elsevier

BBA 41649

CYCLIC ELECTRON TRANSPORT IN CHLOROPLASTS

THE Q-CYCLE AND THE SITE OF ACTION OF ANTIMYCIN

DAVID A. MOSS and DEREK S. BENDALL

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW (U.K.)

(Received June 27th, 1984)

Key words: Electron transport; Q-cycle; Antimycin; Photosystem I; (Pea chloroplast)

Cyclic electron transport systems have been set up in broken chloroplasts, with photochemically reduced ferredoxin or 9,10-anthraquinone-2-sulphonate as cofactor. In good agreement with the literature, only the ferredoxin-catalyzed pathway was found to be inhibited by antimycin; but both pathways were found to have a slow electrogenic reaction, both were inhibited by the cytochrome *b*-563 oxidation inhibitor 2-heptyl-4-hydroxyquinoline *N*-oxide (the inhibition being strongest at limiting light intensity), and the two pathways had the same proton/electron stoichiometry at limiting light intensity. It is concluded that a Q-cycle can occur in cyclic electron transport with either cofactor; and therefore that the site of action of antimycin in chloroplasts is not within the Q-cycle, as it is believed to be in mitochondria and bacteria. Instead, a ferredoxin-quinone reductase is proposed as the site of action of antimycin in the ferredoxin-catalyzed cyclic pathway. It is also concluded that the data presented here are consistent with the suggestion that the Q-cycle in photosynthetic electron transport is a facultative one, its degree of engagement depending on competition between the Rieske centre and cytochrome *b*-563 for reducing equivalents from plastosemiquinone.

Introduction

Cyclic electron transport around Photosystem I can be catalyzed by the endogenous cofactor ferredoxin [1] and is therefore considered to be physiologically important. It is now generally accepted that the cyclic pathway shares with the non-cyclic pathway the following chain of carriers: plastoquinone → Rieske centre → cytochrome *f* → plastocyanin → Photosystem I → ferredoxin (reviewed in Ref. 2). The cyclic chain is presumably completed by the return of reducing equivalents from ferredoxin to plastoquinone, though the mechanism of this step remains to be elucidated.

A closely related area of uncertainty is the interaction of plastoquinol with the cytochrome *bf* complex in both cyclic and non-cyclic pathways, and the role of cytochrome *b*-563. Cytochrome *b*-563 was at one time thought to catalyze the reduction of plastoquinone by ferredoxin. However, the absence of a rapid reduction of cytochrome *b*-563 by ferredoxin [3] renders this possibility unlikely, and most now favour a role for cytochrome *b*-563 in a Mitchellian Q-cycle (reviewed in Ref. 4). There are two lines of evidence that suggest that the concept of a Q-cycle may be particularly relevant to cyclic electron transport. First, ferredoxin-catalyzed cyclic electron flow is

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; AQS, 9,10-anthraquinone-2-sulphonate; PMS, *N*′-methylphenazonium methosulphate.

inhibited by antimycin [5], which in mitochondria and bacteria is believed to inhibit the reoxidation of the *b*-type cytochrome participating in the Q-cycle (reviewed in Ref. 4). Second, the slow phase of the electrochromic bandshift (slow ΔA_{518}) has been shown to occur in intact and broken chloroplasts under conditions that would be expected to favour cyclic electron flow [6,7]; and the kinetics and extent of the slow ΔA_{518} are consistent with its resulting from the electrogenic step of a Q-cycle [8].

A Q-cycle mechanism predicts a proton/electron stoichiometry of 2 for electron transport through the quinone-cytochrome region. Measurements of proton/electron stoichiometries in chloroplasts have resulted in conflicting answers (reviewed in Ref. 9), and there is evidence to suggest that the proton/electron stoichiometry in chloroplasts varies with conditions [10–12]. Rich and Bendall [13,14] have proposed that plastosemiquinone produced by oxidation of plastoquinol by the Rieske centre may reduce either cytochrome *b*-563 or the Rieske centre again (following its reoxidation); in this model, the proton/electron stoichiometry would depend on the relative rate constants for the two reactions, and on the steady-state redox poises of cytochrome *b*-563 and the Rieske centre. A similar model has been proposed by Crowther and Hind [12].

Cyclic electron transport can also be catalyzed by a large range of artificial cofactors [15]. Some of these catalyze electron transport pathways whose sensitivities to the plastoquinone antagonists 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) and 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT) are similar to those shown by the ferredoxin-catalyzed pathway, implying a similar route through the plastoquinone/cytochrome *bf* complex region [15,16]. However, no artifical cofactor has yet been found that catalyzes antimycin-sensitive electron transport. This allows a comparison of cyclic electron transport pathways that are similar in every respect except antimycin sensitivity, and thus facilitates investigation of the significance of antimycin sensitivity in the ferredoxin-catalyzed cyclic pathway.

The present study is a comparison of cyclic electron transport pathways catalyzed by ferredoxin and by 9,10-anthraquinone-2-sulphonate (AQS). The technique of photochemical redox poising of the cofactor [17,18] is used to set up cyclic electron transport systems in which antimycin sensitivity, proton/electron stoichiometry, the slow ΔA_{518} and sensitivity to the cytochrome *b*-563 oxidation inhibitor 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) [19] have been investigated.

Part of the work described here has been published previously in preliminary form [20].

Materials and Methods

Chloroplasts were isolated from 10–13-day-old pea seedlings (var. Superb) by a method essentially the same as that of Cerovic et al. [21] except for the inclusion of an osmotic shock in 10 mM $MgCl_2$, followed after 20 s by return to isotonic medium, before the second centrifugation. The broken chloroplasts were stored at 77 K in 5% (v/v) dimethyl sulphoxide.

Cyclic phosphorylation catalyzed by AQS or ferredoxin was measured using a technique based on that of Robinson and Yocum [17,18]. The reaction mixture was as follows: 50 mM Tricine (pH 8.0 with KOH), 5 mM $MgCl_2$, 5 mM KH_2PO_4, 6 mM D-glucose, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 100 μM ADP, approx. 400 U catalase/ml (1 unit (U) decomposes 1 μmol H_2O_2 per min at pH 7.0, 25°C and 10.3 mM H_2O_2), approx. 4 U glucose oxidase/ml (1 unit (U) oxidizes 1 μmol D-glucose per min at pH 5.1 and 35°C, O_2 saturated reaction), and either 100 μM ferredoxin with 20 μM 5-deazaflavin or 100 μM AQS. In some experiments, 5-deazariboflavin was used rather than 5-deazaflavin. 200 μl reaction mixture were placed in a stirred oxygen electrode vessel at 20°C, the space above the reaction mixture being continuously flushed with N_2. When 99% of the oxygen in the air-saturated medium had been consumed, the cofactor was photochemically reduced by a 3 min illumination from a 150 W tungsten/halogen projector lamp. Broken chloroplasts were added to 10 μg chlorophyll/ml, and the reaction mixture illuminated through a Schott RG610 filter, the various intensities shown in the figure legends being achieved using neutral density filters. After 1 min, the lamp

was switched off and the reaction stopped with 20 μl 0.5 M perchloric acid. The reaction mixture was centrifuged for 2 min at 15000 × *g*; the supernatant was neutralized with 0.1 M KOH and assayed for ATP using luciferin/luciferase. The assay for *N'*-methylphenazonium methosulphate (PMS)-catalyzed cyclic phosphorylation differed from the above in that the reaction mixture was aerobic, glucose oxidase was omitted, 1 mM ADP was present, 30 μM PMS replaced the AQS or ferredoxin/deazaflavin, and the cofactor was reduced with 7.5 mM ascorbate rather than photochemically.

The reaction mixture for assaying the slow ΔA_{518} in the AQS- and ferredoxin-catalyzed systems was prepared as described above, except that ADP was omitted, the chlorophyll concentration was 25 μg/ml, the final reaction volume was 3.5 ml, and a 5 min preillumination was used to reduce the cofactor. Saturating flashes came from an EG&G FX-139C-3.5 lamp, through a Schott RG630 filter; pulse width at half-height was less than 6 μs. Each trace shown is the average of 50 traces, taken at a flash frequency of 0.1 Hz; traces were further smoothed by averaging blocks of 8 adjacent digitized signal points at 20-μs intervals, to produce single points at 160-μs intervals.

5-Deazaflavin and 5-deazariboflavin were gifts from Professor V. Massey, and were kept as a 1 mM stock solution in dimethyl formamide at 4°C. Ferredoxin was prepared from 14-day-old pea seedlings following the method of Plesnicar and Bendall [22] as far as the first ion exchange column, followed by gel filtration on Sephadex G-75 and a further ion exchange chromatography step on Whatman DE-52; fractions with A_{422}/A_{277} greater than 0.55 were pooled and stored as an 0.5 mM aqueous solution at 77 K. Antimycin was added where indicated in the figure legends as a solution in 0.1% bovine serum albumin.

Results

Fig. 1 shows the effect of antimycin on cyclic phosphorylation systems catalyzed by ferredoxin and AQS. In good agreement with the literature [5,15], the ferredoxin system is found to be inhibited by submicromolar levels of antimycin that neither inhibit nor uncouple the AQS system. It

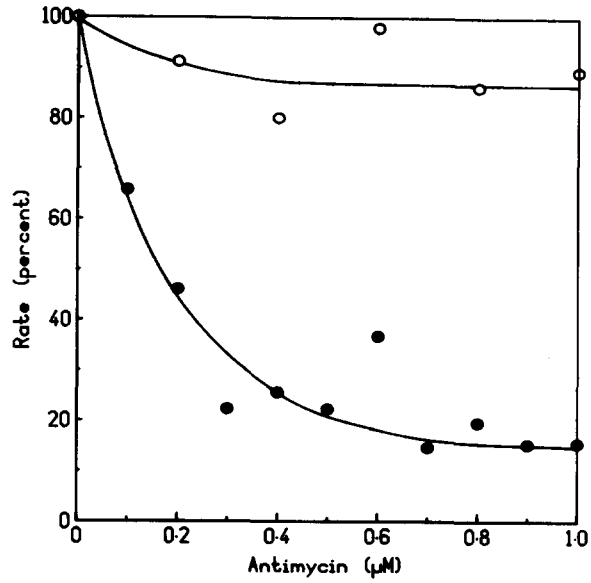

Fig. 1. Effect of antimycin on cyclic phosphorylation catalyzed by ferredoxin (●) and AQS (○). Rates of phosphorylation were measured as described in Materials and Methods. 100% rates were 254 μmol ATP/h per mg chlorophyll with AQS as cofactor, and 120 μmol ATP/h per mg chlorophyll with ferredoxin as cofactor.

should be noted that ferredoxin-catalyzed phosphorylation is not completely sensitive to antimycin; maximal inhibition is about 80%. 0.5 μM was taken as the antimycin concentration to be used in subsequent experiments.

If the site of action of antimycin in chloroplasts were the equivalent of its site of action in mitochondria and bacteria, Fig. 1 would indicate that the ferredoxin-catalyzed pathway includes a Q-cycle while the AQS-catalyzed pathway does not: and this can be tested by comparing the proton/electron stoichiometries of the two pathways. Furthermore, given that at limiting light intensity the rate of turnover of the photosystem is independent of the cofactor used, and that the ATP/proton stoichiometry of the ATP synthetase is constant, then the proton/electron stoichiometries of the two pathways can be compared by comparing the slopes of rate of phosphorylation against light intensity with each cofactor.

The result of this experiment is shown in Fig. 2. It can be seen from Fig. 2a and c that the ferredoxin- and AQS-catalyzed pathways do not dif-

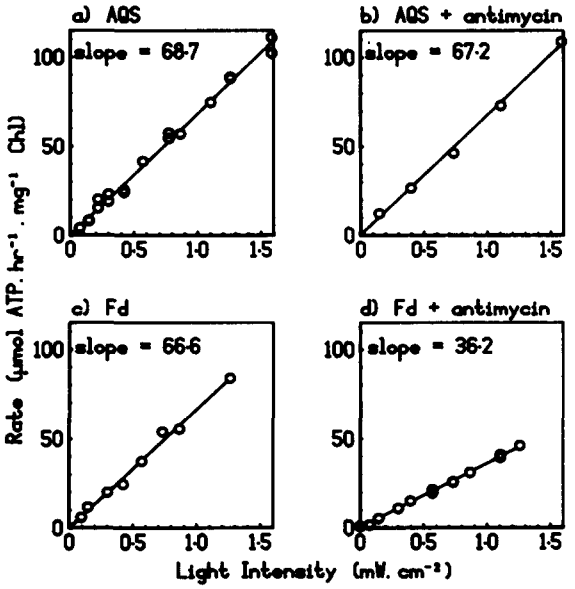

Fig. 2. Effect of light intensity on cyclic phosphorylation catalyzed by AQS and ferredoxin (Fd) in the absence and presence of 0.5 μM antimycin. Rates of phosphorylation were measured as described in Materials and Methods. The figures are the slopes of the regression line for each set of data.

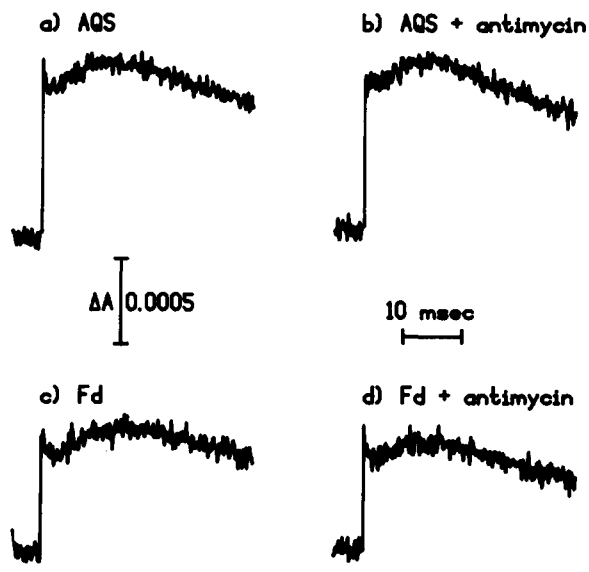

Fig. 3. Flash-induced absorbance changes at 518 nm in cyclic electron transport systems catalyzed by AQS and ferredoxin (Fd), in the presence and absence of 0.5 μM antimycin. Conditions as described in Materials and Methods.

fer in proton/electron stoichiometry. Fig. 2b and d confirm that the differential effect of antimycin remains at the low light intensities used in this experiment. It will also been seen from Fig. 2d that antimycin almost halves the rate vs. light intensity slope of the ferredoxin-catalyzed pathway; this could be taken as implying that the proton/electron stoichiometry has been halved, presumably from 2 to 1.

The possibility of a difference between the two pathways with respect to the presence of a Q-cycle can also be tested by measuring the slow ΔA_{518}. Fig. 3 shows the ΔA_{518} in the two cyclic systems; the spectra of the fast and slow phases were similar, and both were in good agreement with the published spectra [23] (not shown). It is clear from Fig. 3 that the electrogenic step is present with either cofactor; in contrast to an earlier report [20], a low AQS concentration was not found to be necessary in order to see the slow phase. Moreover, 0.5 μM antimycin has no effect on the slow ΔA_{518} in either case, and little if any inhibition was seen at 1.25 μM antimycin (not shown). By contrast, Hind and his co-workers [6,23] have found the slow ΔA_{518} to be inhibited by antimycin. However, higher concentrations of antimycin were used than here; in the present study, care has been taken to ensure that the concentration used is no higher than that needed to maximally inhibit the rate of ferredoxin-catalyzed cyclic phosphorylation. It can be concluded that inhibition of the slow ΔA_{518} by antimycin is a secondary or indirect effect, the primary effect being elsewhere in the ferredoxin-catalyzed pathway.

If antimycin does not inhibit the electrogenic step of the Q-cycle, which presumably is the oxidation of cytochrome *b*-563, then another inhibitor must be found to test for the involvement of this component in the cyclic pathways. Selak and Whitmarsh [19] have presented evidence that HQNO inhibits at this site, though Houchins and Hind [24] have shown that at higher concentrations, HQNO also inhibits the reduction of cytochromes *b*-563 and *f*. Fig. 4 shows the effect of HQNO on AQS-catalyzed cyclic phosphorylation; the data are consistent with the biphasic effect of HQNO predicted by the data of Houchins and Hind [24]. 10 μM was taken as a suitable con-

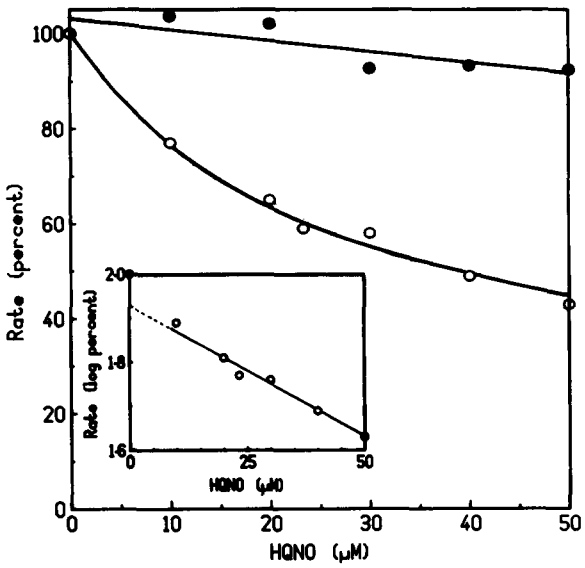

Fig. 4. Effect of HQNO on cyclic phosphorylation catalyzed by PMS (●) and AQS (○). Rates of phosphorylation were measured as described in Materials and Methods. 100% rates were 268 μmol ATP/h per mg chlorophyll with AQS as cofactor, and 1380 μmol ATP/h per mg chlorophyll with PMS as cofactor.

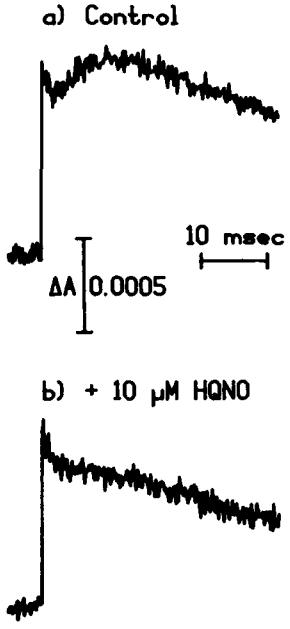

Fig. 5. Effect of 10 μM HQNO on flash-induced absorbance changes at 518 nm in an AQS-catalyzed cyclic electron transport system. Conditions as described in Materials and Methods.

centration of HQNO to test for the involvement of cytochrome *b*-563 oxidation in cyclic phosphorylation; Fig. 5 shows that this concentration was sufficient to markedly inhibit the slow ΔA_{518} in the AQS-catalyzed system. Fig. 4 also shows that PMS-catalyzed cyclic phosphorylation, which bypasses the inter-photosystem electron transport chain and the native coupling site [15], is not affected by HQNO up to 50 μM; thus, HQNO does not uncouple or inhibit ATP synthesis.

Table I shows the effect of 10 μM HQNO on cyclic phosphorylation catalyzed by ferredoxin and AQS at high and low light intensities. With either cofactor, a reduction in the light intensity results in an increase in the proportion of the phosphorylation that is attributable to HQNO-sensitive electron transport. The results would be difficult to account for in terms of a site of action of HQNO within a simple unbranched pathway, since there is no simple relationship between the overall rate of phosphorylation and the proportion sensitive to HQNO inhibition. However, the data are consistent with an increased flux through the Q-cycle at low light intensities; possible mechanisms for such an effect are considered in Discussion. At high light intensity, it would seem that the Q-cycle is partially engaged with AQS as cofactor; while in the case of ferredoxin, the low level of inhibition observed may be interpreted as inhibition of a

TABLE I

EFFECT OF LIGHT INTENSITY ON THE HQNO SENSITIVITY OF CYCLIC PHOSPHORYLATION CATALYZED BY FERREDOXIN AND AQS

Rates of phosphorylation were measured as described in Materials and Methods. Data represent means, with the range and number of individual replicates shown in brackets. *P* values represent the probability that light intensity has no effect on the HQNO sensitivity of cyclic phosphorylation with each cofactor, and were calculated using a one-tailed Student's *t*-test.

Cofactor	Light intensity ($mw \cdot cm^{-2}$)	Percent of control rate inhibited by 10 μM HQNO	*P*
AQS	200	28.3 (range = 18.4 $n = 4$)	< 0.025
AQS	1	50.8 (range = 1.6 $n = 2$)	
Ferredoxin	200	16.5 (range = 23.3 $n = 4$)	< 0.025
Ferredoxin	1	44.8 (range = 24.6 $n = 3$)	

small flux through cytochrome *b*-563, or as simply a partial inhibition of the reduction of the Rieske centre by plastoquinol. However, the data do not show conclusively that the HQNO sensitivity of the ferredoxin-catalyzed pathway is less than that of the AQS-catalyzed pathway at high light intensity (P between 0.1 and 0.05, one-tailed Student's t-test).

The different HQNO sensitivities of the two pathways at high light intensity might result from binding of HQNO to ferredoxin, which is present at high concentration in the ferredoxin-catalyzed system. However, this possibility seems unlikely since addition of ferredoxin to an aqueous solution of HQNO has no effect on HQNO fluorescence, other than the expected absorption by ferredoxin at the excitation and emission peaks (not shown).

Discussion

The data presented here are clearly not compatible with the suggestion that the antimycin sensitivity of the ferredoxin-catalyzed pathway results from the presence of a Q-cycle that is not present in antimycin-insensitive pathways; moreover, direct observation of cytochrome *b*-563 turnover has generally failed to show any evidence of its being inhibited by antimycin (reviewed in Ref. 4). In considering possible sites of action of antimycin, it should be noted that the chain of carriers from plastoquinol to Photosystem I is common to antimycin-sensitive and insensitive pathways, as is the reduction of ferredoxin by Photosystem I. The only remaining step in the ferredoxin-catalyzed pathway is the return of reducing equivalents from ferredoxin to the inter-photosystem electron transport chain. Accordingly, we suggest that antimycin inhibits the reduction of plastoquinone by ferredoxin. This is different to the action of antimycin in mitochondria and bacteria, but some similarity would remain if antimycin were seen as attacking the plastoquinone-binding site of a ferredoxin-quinone reductase. Such a component would clearly not play a role in non-cyclic electron transport and would presumably be bypassed by artificial cofactors that reduce plastoquinone directly [25,15,16]. The residual antimycin-insensitive activity in ferredoxin-catalyzed phosphorylation (Fig. 1) may result from incomplete inhibition of ferredoxin-quinone reductase by antimycin, or it may represent a slow uncatalyzed reduction of plastoquinone by ferredoxin. A role for the ferredoxin-NADP reductase in ferredoxin-quinone reductase activity [26] would remain a possibility.

The data of Table I imply that at low light intensities, a greater proportion of the ATP formed results from the HQNO-sensitive cytochrome *b*-563 oxidation step of a Q-cycle. This is in good agreement with the published observations of an elevated proton/electron stoichiometry at low light intensity [10,11]. Both observations are consistent with the model proposed by Rich and Bendall [13,14] in that a high rate of reoxidation of the Rieske centre would be expected to favour the oxidation of plastoquinol by two successive turnovers of the Rieske centre rather than by the concerted action of the Rieske centre and cytochrome *b*-563. On the other hand, Bouges-Bocquet [27] has emphasised the dependence of the electrogenic oxidation of cytochrome *b*-563 on the transmembrane electric field while noting that electron transfer to cytochrome *f* is still possible when the electrogenic reaction is impeded. These two hypotheses are not mutually exclusive, and it is possible that both factors play a role in determining the distribution of reducing equivalents between cytochrome *b*-563 and the Rieske centre.

Acknowledgements

The authors would like to thank Dr. P.R. Rich for his help and advice on the use of his spectrophotometer, and the Science and Engineering Research Council for financial support.

References

1 Tagawa, K., Tsujimoto, H.Y. and Arnon, D.I. (1963) Nature 199, 1247–1252
2 Bendall, D.S. (1982) Biochim. Biophys. Acta 683, 119–151
3 Cox, R.P. (1979) Biochem. J. 184, 39–44
4 Rich, P.R.(1984) Biochim. Biophys. Acta 768, 53–79
5 Tagawa, K., Tsujimoto, H.Y. and Arnon, D.I. (1963) Proc. Natl. Acad. Sci. USA 49, 567–572
6 Crowther, D., Mills, J.D. and Hind, G. (1979) FEBS Lett. 98, 386–390
7 Shahak, Y., Crowther, D. and Hind, G. (1980) FEBS Lett. 114, 73–78
8 Velthuys, B.R. (1978) Proc. Natl. Acad. Sci. USA 75, 6031–6034

9 Cox, R.P. and Olsen, L.F. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 49–79, Elsevier Biomedical Press, Amsterdam
10 Fowler, C.F. and Kok, B. (1976) Biochim. Biophys. Acta 423, 510–523
11 Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062
12 Crowther, D. and Hind, G. (1982) A.R.C. Research Group on Photosynthesis (Sheffield) Annual Report, pp. 43–45
13 Rich, P.R. and Bendall, D.S. (1980) Biochim. Biophys. Acta 592, 506–518
14 Rich, P.R. and Bendall, D.S. (1982) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 2, pp. 551–558, Balaban International Science Services, Philadelphia
15 Hauska, G., Reimer, S. and Trebst, A. (1974) Biochim. Biophys. Acta 357, 1–13
16 Binder, R.G. and Selman, B.R. (1980) Biochim. Biophys. Acta 592, 314–322
17 Robinson, H.H. and Yocum, C.F. (1979) Photochem. Photobiol. 29, 135–140
18 Robinson, H.H. and Yocum, C.F. (1980) Biochim. Biophys. Acta 590, 97–106
19 Selak, M.A. and Whitmarsh, J. (1982) FEBS Lett. 150, 286–292
20 Moss, D.A. and Bendall, D.S. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.), Vol. 1, pp. 577–580, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
21 Cerovic, Z.G., Kalezic, R. and Plesnicar, M. (1982) Planta 156, 249–254
22 Plesnicar, M. and Bendall, D.S. (1970) Biochim. Biophys. Acta 216, 192–199
23 Slovacek, R.E., Crowther, D. and Hind, G. (1979) Biochim. Biophys. Acta 547, 138–148
24 Houchins, J.P. and Hind, G. (1983) Biochim. Biophys. Acta 725, 138–145
25 Bohme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352
26 Shahak, Y., Crowther, D. and Hind, G. (1981) Biochim. Biophys. Acta 636, 234–243
27 Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340