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THE ROLE OF CYTOCHROME b_6 IN CYCLIC ELECTRON TRANSPORT: EVIDENCE FOR AN ENERGY-COUPLING SITE IN THE PATHWAY OF CYTOCHROME b_6 OXIDATION IN SPINACH CHLOROPLASTS

H. BÖHME AND W. A. CRAMER

Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907 (U.S.A.)

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SUMMARY

The problem of selecting reference wavelengths appropriate for the study of reductive and oxidative absorbance changes in cytochromes b_6 and f is discussed.

Action spectra for cytochrome b-563 photoreduction are consistent with this cytochrome functioning in a System I cyclic pathway, but do not exclude the possibility that the cytochrome might also have another function in a DCMU-sensitive pathway connected with Photosystem II.

The dark oxidation of photoreduced cytochrome b-563 is inhibited by the quinone analog 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), and restored by subsequent addition of plastoquinone.

Diaminodurol causes a partial reduction of cytochrome b-563 in the dark. The photooxidation of diaminodurol-reduced cytochrome b-563 by System I is stimulated by addition of NH_4Cl or ADP, and under the same conditions cytochrome f becomes more reduced. The increase in the amplitude of cytochrome b-563 photooxidation caused by addition of NH_4Cl is suppressed by the quinone analog DBMIB. The rate and amplitude of the photooxidation of dithionite-reduced cytochrome b_6 are also stimulated by addition of NH_4Cl, and this photooxidation is also inhibited by DBMIB. It is concluded that in the absence of added cofactors of cyclic electron transport the pathway of cytochrome b-563 oxidation includes plastoquinone and the coupling site between plastoquinone and cytochrome f.

In the presence of DBMIB, phenazine methosulfate (PMS) restores the dark oxidation of photoreduced cytochrome b-563 and also causes a decrease in the amplitude of cytochrome f photooxidation. When DCMU is substituted for DBMIB, PMS has no effect on the amplitude of cytochrome f photooxidation. It is concluded that in cyclic phosphorylation one function of PMS is to transfer electrons from cytochrome b_6 to System I either through the plastoquinone–cytochrome f coupling site or in the presence of DBMIB through a bypass of this site.

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; PMS, phenazine methosulfate.

INTRODUCTION

The chloroplast cytochrome with a reduced α-band at 563 nm was observed to be present in etiolated barley chloroplasts by Hill[1] who defined it as cytochrome b_6. The pathways for the oxidation and reduction of this cytochrome are incompletely understood. The antimycin A sensitivity of ferredoxin-mediated cyclic phosphorylation suggested that a *b*-type cytochrome might participate in this pathway[2]. The observation that a cytochrome with the spectral properties of b_6 could be reduced relatively efficiently by far-red light in spinach chloroplasts[3,4], together with its localization in Photosystem I enriched particle fractions obtained from chloroplasts[5-7], led to the idea that it might be involved in cyclic electron transport and phosphorylation. A role for cytochrome b_6 in cyclic phosphorylation is strongly implied by the correlation of cytochrome *b*-564 photooxidation and CO_2 fixation found in whole cells of electron transport mutants of *Chlamydomonas reinhardii*[8]. The experiments with *C. reinhardii* also demonstrated that without added cofactors, *b*-564 photooxidation by System I requires P700, plastocyanin, cytochrome *f* and at least one electron-transport component between cytochrome *f* and cytochrome *b*-559 in the main electron-transport chain. Rate studies of the photooxidation of cytochrome *b*-564 in the pale green mutant of *C. reinhardii* implied the existence of a slow step in electron transport between cytochrome *b*-564 and cytochrome *f*[9].

There are data in the literature suggesting different or additional functions for cytochrome b_6 in electron transport besides that of the cyclic pathway. Considering at this time only the studies on cytochrome b_6 carried out since it became established that two *b*-type cytochromes are present in chloroplasts: (a) Ikegami *et al.*[10] found that cytochrome *b*-563 in chloroplasts of *Euglena gracilis* becomes reduced in the dark at pH 9.5, photoreduced in the presence of ascorbate or manganese, and photooxidized in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). It was concluded that cytochrome *b*-563 can function in the main electron transport chain connecting Photosystems II and I. It had also been proposed by Hind and Olson[3] that cytochrome *b*-563 might be able to act in a non-phosphorylating basal pathway bypassing the main electron-transport chain as well as in a System I cyclic pathway. Action spectra for cytochrome *b*-563 photoreduction reported below do not exclude the possibility that cytochrome *b*-563 photoreduction might be mediated by Photosystem II as well as Photosystem I. (b) Inhibitor experiments with whole cells of the pale green *Chlamydomonas* mutant suggested the possibility that cytochrome *b*-564 might participate in a respiratory pathway as well as in the photosynthetic cyclic pathway[11]. Our experiments do not bear on this possibility.

The experiments reported below are mainly concerned with establishing the pathway through which cytochrome *b*-563 is oxidized when it is functioning in a cyclic pathway. Studies of the effect on the amplitude and rate of *b*-563 oxidation of the quinone analog 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), which at low concentrations inhibits cyclic phosphorylation mediated by menadione or ferredoxin[12], lead to the conclusion that plastoquinone functions in the pathway of cytochrome *b*-563 oxidation by Photosystem I. Experiments on the effect of uncouplers and phosphorylation cofactors on *b*-563 oxidation indicate that the pathway for cytochrome *b*-563 oxidation includes the energy coupling site between plastoquinone and cytochrome *f*[13].

METHODS

Chloroplast preparation

The spinach leaves for these experiments were grown in a controlled climate facility at 22 °C with an 8-h light cycle. The procedure for preparing the chloroplasts has been described previously[14] and involves rapid homogenization of the leaves in hypertonic sorbitol medium, filtering, and one centrifugation step, the whole process requiring less than 5 min. At the time of the experiment the chloroplasts were osmotically disrupted in 25 mM tris(hydroxymethyl)methylglycine (Tricine)–NaOH, pH 7.8, 5 mM $MgCl_2$, and 5 mM K_2HPO_4, the standard reaction medium.

Chloroplast coupling

Oxygen uptake was measured with a YSI 5331 oxygen electrode in the standard reaction medium also containing 0.1 mM methyl viologen and 0.2 mM NaN_3. The incident light intensity was $3 \cdot 10^5$ erg·cm^{-2}·s^{-1} and the chlorophyll concentration was 20 μg/ml. The degree of coupling of the chloroplast preparations was determined through the 2–3-fold stimulation of oxygen uptake by ADP addition as described previously[14].

Action spectra

The action spectrum is defined here as the amplitude of the light-induced cytochrome absorbance change per absorbed quantum as a function of the wavelength of actinic light. The action spectra were obtained by measuring the absorbance changes upon illumination with monochromatic light as defined by interference filters with half bandwidths of 10–13 nm. An auxiliary Corning 2-62 filter was used for short-wavelength blocking. The incident light intensity was attenuated by neutral density filters to $2 \cdot 10^3$ erg·cm^{-2}·s^{-1} to ensure that the amplitude of the absorbance changes was proportional to light intensity. The action spectra were measured with chloroplast suspensions containing 100 μg/ml of chlorophyll. The absorbed light intensity was measured in a chloroplast suspension containing 10 μg/ml chlorophyll with the cathode of a 6255B photomultiplier tube placed 5 mm behind the back face of the cuvette. With this geometry it was found that in the suspension containing 100 μg/ml chlorophyll the actinic light was essentially completely absorbed at all of the wavelengths used for actinic illumination except 702 and 711 nm, where the absorption was 87 and 72 %, respectively.

Measurement of cytochrome-absorbance changes

Cytochrome-absorbance changes were measured with a dual wavelength spectrophotometer, lock-in amplifier, and strip chart recorder or storage oscilloscope (Tektronix 564B). The band width of the measuring light was 1.1 or 1.4 nm and the actinic light intensity of wavelength 713 nm was approximately $1 \cdot 10^5$ erg·cm^{-2}·s^{-1} except where indicated otherwise. Argon was continuously blown over the top of the suspension in experiments in which several cycles of cytochrome *b*-563 oxidation and reduction were measured (Figs 1A, 2, 5 and 11).

Choice of reference wavelength in dual wavelength measurement

In the course of our studies on the cytochrome *b*-563 we encountered some problems which bear on the general use of dual wavelength spectrophotometry and

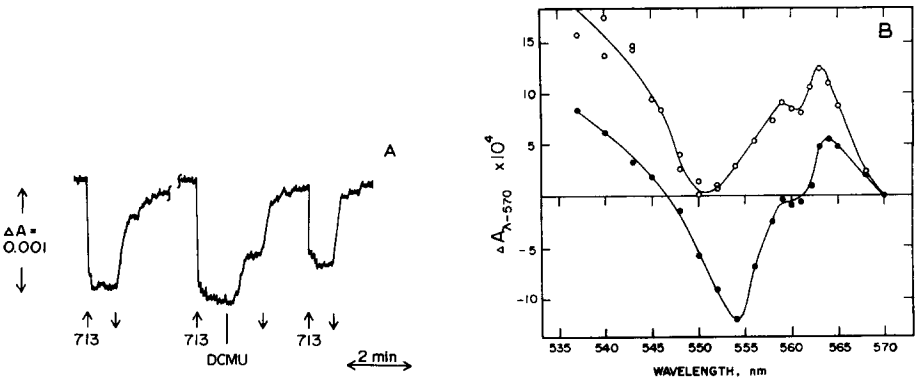


Fig. 1. (A) Light-induced absorbance changes measured at 563 nm in the presence and absence of DCMU. Conditions were as described in Methods. Downward deflection indicates an absorbance increase at 563 nm relative to the 570 nm reference wavelength. Upward arrows, 713 nm light on; downward arrows, light off. DCMU concentration, 1 μM; chlorophyll concentration, 85 μg/ml. The cuvette was continuously flushed with purified argon. (B) Difference spectrum for the far-red light-induced absorbance change. Spinach chloroplasts were prepared as described except that the pH of the homogenizing medium was 6.5, using (*N*-morpholino)ethanesulfonic acid–NaOH, and for resuspension pH 7.5, using *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–NaOH. The reaction mixture at pH 7.5 contained, in mM: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–NaOH, 25; $MgCl_2$, 5; K_2HPO_4, 5; chloroplasts at a concentration of 63 μg chlorophyll per ml. The half band-width of the measuring beam was 1.1 nm. The first illumination with 702-nm light had an intensity of $6 \cdot 10^4$ erg·cm^{-2}·s^{-1} (○—○); a second 702-nm illumination followed after a short dark period (about 1 min) with an intensity of $1.6 \cdot 10^4$ erg·cm^{-2}·s^{-1} (●—●).

the selection of reference wavelengths. Fig. 1A shows the absorbance increase observed at 563 nm with 570 nm as a reference wavelength upon illumination with far-red light of a chloroplast suspension without an electron acceptor and initially without DCMU. Light *minus* dark difference spectra for the absorbance changes elicited by far-red light between 537 nm and a 570-nm reference in the absence of DCMU are shown in Fig. 1B. The two spectra are for a first (upper curve) and second (lower curve) illumination received by the sample, the two illuminations being separated by a dark interval of about 1 min. The two spectra have a similar shape although the baseline appears to be offset in the second spectrum. The cause of the baseline shift is not known at present, but may be related to changes in the 520-nm region. The spectra show a positive absorbance peak at 563 nm (first) and at 564 nm (second) which can be identified with the reduced α-band of cytochrome b_6. However, the amplitude and the shape of the band relative to 570 nm, as well as its half band width, depend upon the position of the baseline and on the amplitude of the negative trough associated with cytochrome *f* oxidation. This trough will tend to make the b_6 spectrum sharper on its short wavelength side. It is also possible that there is some contribution to the 563-nm peak from cytochrome *f*, whose α-band spectrum is known to change sign approximately 7 nm above the peak. Any such contribution must be small because it would increase in amplitude monotonically whereas the b_6 peak is well defined. An additional feature of the spectra is that they both contain a shoulder at 559 nm.

Fig. 1B raises the general questions of the selection of a reference wavelength, the complexity of dual wavelength spectrophotometry, and the restrictions which apply to its use. The reference wavelengths which are generally used in the cytochrome

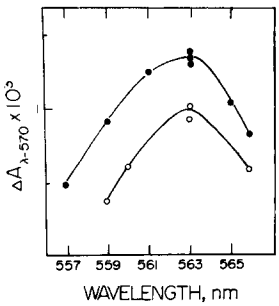
α-band region, but which are not necessarily isosbestic, are 540 nm and 570 or 575 nm. It can be seen from Fig. 1B that absorbance decreases at 554 nm associated with cytochrome *f* oxidation are larger and more reproducible with a 540-nm reference. The absorbance increase at 563 nm associated with cytochrome b_6 reduction is seen better with a 570-nm reference. Indeed, with 570 nm as reference little or no absorbance change is observed at 554 nm in the first illumination in Fig. 1B though the complete spectrum shows the *f* participation. Similarly, the net absorbance changes at 563 nm are very small in both spectra with 540 nm as reference, though again the two complete spectra show about the same absorbance difference between the 563-nm peak and the shoulder at 559 nm. Spectra measured with 540 nm as a reference have the same shape as those in Fig. 1B, as expected, though the zero is naturally at 540 nm instead of 570 nm. The slope of the baseline of the spectra in Fig. 1B shows that an absorbance decrease associated with an oxidative change in the α-band region will generally tend to be enhanced with a 540-nm reference relative to a 570-nm reference. An absorbance increase associated with reduction will appear with a larger amplitude with a 570-nm than with a 540-nm reference. What must be determined from a difference spectrum in each case is whether an apparent absorbance change is associated with the particular cytochrome component or nonspecifically with absorbance changes at the reference wavelength.

In all of the following it is our assumption that absorbance measurements at a peak (*e.g.* 563 nm) selected from Fig. 1B provide a qualitative measure of the effect on the particular component of an inhibitor, uncoupler, or phosphorylation cofactor, though any quantitative inferences about stoichiometry are difficult and will not be attempted here. We have used 570 nm as a reference for studying reduction of cytochrome b_6 at 563 nm and as well when studying reductive changes of small amplitude associated with cytochrome *f* at 554 nm (Fig. 9B). We have used 540 nm as the reference when studying photooxidation of cytochrome *f* and small amplitude oxidative absorbance changes associated with cytochrome b_6 upon addition of uncoupler or ADP (Fig. 9A). In all cases when measuring the perturbation of the absorbance change by an added inhibitor, uncoupler, or cofactor, we have measured difference spectra over a limited region to be sure that most of the absorbance change is specific for the electron transport component in question.

RESULTS

The effect of DCMU on the reversible light-induced changes observed at 563 nm with a 570-nm reference is shown in Figs 1A and 2. DCMU causes a decrease in the amplitude of the reversible change which is tentatively ascribed to its blocking reduction by Photosystem II-absorbed far-red light of cytochrome *b*-559, oxidized in the dark. Cytochrome *b*-559 reduction by far-red light in the absence of DCMU is seen in the difference spectra of Fig. 1B and in the difference spectra of Fig. 4 (below).

Action spectra for reduction of cytochrome *b*-563 in the presence and absence of DCMU, as well as for cytochrome *f* oxidation in the absence of DCMU for purposes of comparison, are shown in Fig. 3. The spectrum for cytochrome *f* oxidation (open circles) and for cytochrome *b*-563 reduction (triangles) in the presence of DCMU both show an increased response caused by illumination with longer wavelengths of light, the *b*-563 spectrum having a smaller amplitude at all actinic wavelengths, and es-



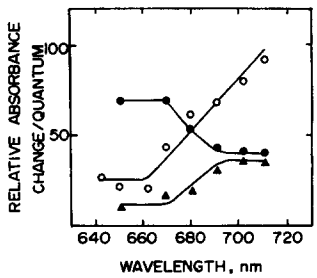


Fig. 2. Difference spectra for the light-induced absorbance changes in the absence and presence of DCMU. Conditions as in Fig. 1A. 713-nm light-induced absorbance change in the presence (○—○) and absence (●—●) of 1 μM DCMU.

Fig. 3. Action spectra for the photooxidation of cytochrome *f* and photoreduction of cytochrome *b*-563. General conditions are described in Methods. Cytochrome *f* photooxidation (○—○) in the presence of 0.1 mM methylviologen as electron acceptor measured at 554 nm with 540 nm as reference wavelength. Cytochrome *b* photoreduction in the absence of an electron acceptor and in the absence (●—●) and presence (▲—▲) of 1 μM DCMU measured at 563 nm *versus* a 570-nm reference.

pecially in the far-red region of the spectrum. The low amplitude of cytochrome *b*-563 photoreduction could be caused partly by its participation in a cyclic reaction in which it is oxidized efficiently as it becomes reduced, and as well by oxidative absorbance changes in cytochromes *f* and *b*-559 which would subtract from the positive absorbance change measured for *b*-563 reduction. An action spectrum for the absorbance changes measured at 563 nm in the absence of DCMU (closed circles) is also shown. The amplitude of the absorbance change at 702 nm is approximately equal to that with DCMU. The amplitude at 652 nm, however, is about six times as large as that observed in the absence of DCMU. The increase in absorbance at 563 nm caused by short wavelength actinic light is partly due to reduction of cytochrome *b*-559, a small part of which is present initially in the oxidized form. This is indicated by the difference spectra of Fig. 4 for the absorbance changes caused by 652- and 702-nm light under the conditions of Fig. 3. The peak for the 702-nm light-induced absorbance change is at approximately 563 nm, and that for the changes caused by 652 nm light at 561 nm, centered between the reduced α-band peaks of cytochromes *b*-559 and *b*-563. Thus, though the data do not allow a precise estimate, a significant part of the absorbance change at 563 nm caused by short wavelength red light seems to be caused by cytochrome *b*-559 reduction.

To avoid any possible effects of Photosystem II in the experiments reported below, the measurements were all done in the presence of DCMU except where indicated. Figs 3 and 4 also imply that the net oxidative effect of DCMU seen upon adding DCMU in the light in Fig. 1A is very likely due to inhibition of the contribution of cytochrome *b*-559 photoreduction to the initial absorbance increase.

Effects of inhibitors and cofactors of cyclic phosphorylation

Inhibition by DBMIB. DBMIB at a concentration of about 10 nmoles/mg chlorophyll will completely inhibit cyclic phosphorylation catalyzed by ferredoxin or menadione, and partially inhibit with diaminodurol as cofactor[12]. Cyclic phosphory-

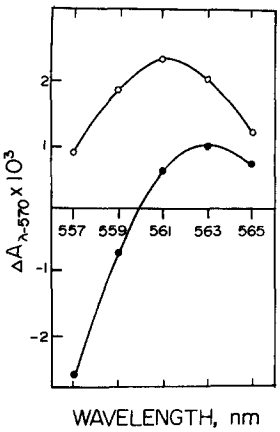


Fig. 4. Difference spectra for the light-induced absorbance changes obtained in the absence of DCMU with low light intensities as applied in the action spectra. Conditions are those described for the action spectra in Fig. 2: The absorbance changes induced by either 652 nm (○—○) or 702 nm (●—●) light were plotted as λ *versus* 570 nm reference.

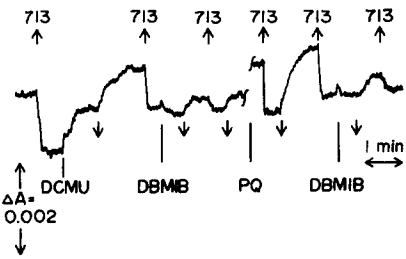


Fig. 5. Inhibition of the light-induced absorbance changes of cytochrome *b*-563 by DBMIB and its reversal by plastoquinone. Conditions as in Methods. The following additions were made: DCMU, 1 μM, and DBMIB, 5 μM, both additions during 713 nm illumination; plastoquinone (PQ), 100 μM. The cuvette was continuously flushed with argon. Chlorophyll concentration, 70 μg/ml.

lation mediated by phenazine methosulfate (PMS) is insensitive to relatively high concentrations (10^{-5} M) of DBMIB[12]. In its inhibition of non-cyclic as well as cyclic phosphorylation this quinone analog appears to be a competitive inhibitor at the functional site of plastoquinone[12]. At concentrations very similar to those which inhibit phosphorylation DBMIB blocks System II reduction of cytochrome *f* and System I oxidation of a low potential form of cytochrome *b*-559[15,16]. When DBMIB (5 μM) is added to chloroplasts illuminated by far-red light in the presence of DCMU the dark reoxidation of cytochrome *b*-563 is blocked and there is no further reduction by the far-red light (Fig. 5). Addition of plastoquinone in the dark restores both the photoreduction and the dark oxidation of cytochrome *b*-563. A second addition of DBMIB again inhibits the reversible absorbance change at 563 nm. The inhibition of the dark oxidation of cytochrome *b*-563 by DBMIB and its restoration by plastoquinone implies that in the absence of added cofactors, *b*-563 oxidation may be mediated by a specific pathway containing plastoquinone.

As noted above, PMS-mediated cyclic phosphorylation is insensitive to DBMIB[12]. Fig. 6 shows that addition of PMS to the chloroplast suspension subsequent to DBMIB addition restores the oxidation of cytochrome *b*-563. In this experiment DBMIB itself causes a decrease in absorbance at 563 nm relative to the reference wavelength. PMS seems to allow a bypass of the inhibition site of DBMIB, whereas menadione does not (data not shown). Besides providing a pathway from cytochrome *b*-563 to an oxidant alternate to that blocked by DBMIB, PMS appears to function in Fig. 6 as a source of electrons for photoreduction of cytochrome *b*-563.

Fig. 7 indicates that cytochrome *f* may be an acceptor for electrons transferred from cytochrome *b*-563 by PMS in the presence of DBMIB. The amplitude of

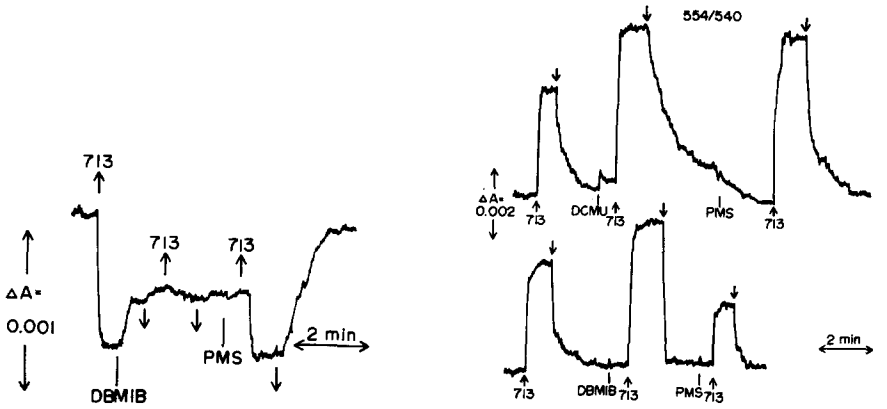


Fig. 6. Inhibition of the far-red light induced absorbance change of cytochrome *b*-563 by DBMIB and its reversal by PMS. Cytochrome *b*-563 was measured at 563 nm with a 570 nm reference. DBMIB, 5 μM; PMS, 30 μM. Chlorophyll concentration, 60 μg/ml.

Fig. 7. The effect of PMS on DCMU- or DBMIB-stimulated photooxidation of cytochrome *f*. Conditions as described in Methods. Cytochrome *f* was measured at 554 nm with 540 nm as reference wavelength. Additions: DCMU, 1 μM (upper trace); DBMIB, 5 μM (lower trace); PMS, 30 μM. Chlorophyll concentration, 85 μg/ml.

the cytochrome *f* photooxidation is increased by DCMU (upper trace), or DBMIB (lower trace). Subsequent addition of PMS markedly decreases the amplitude of photooxidation in the presence of DBMIB, but not with DCMU. A tentative explanation of the data in Figs 6 and 7 is that in the presence of DBMIB, PMS-mediated electron transfer from cytochrome *b*-563 to cytochrome *f* is fast enough to keep cytochrome *f* appreciably reduced in illuminated chloroplasts. However, when the inhibitor is DCMU, electrons transferred by PMS from cytochrome *b*-563 to the main chain tend to go through plastoquinone and a relatively slow electron transfer step before reaching cytochrome *f*. A more complete discussion of this pathway including Figs 8–13 will be given in the Discussion.

Reduction of cytochrome b-563 by diaminodurol. Diaminodurol supports DCMU-insensitive electron transport to System I acceptors which is at least partially coupled to phosphorylation and dependent upon plastocyanin[17–20]. As noted above, cyclic phosphorylation mediated by diaminodurol is partly inhibited by DBMIB[11]. We wished to determine the site(s) of electron donation by diaminodurol for coupled electron transport, and found that reduced diaminodurol (10^{-4} M) added to the chloroplast suspension in the dark causes reduction of cytochrome *b*-563, though this reduction is only about 25 % of the total cytochrome *b*-563. The ability of diaminodurol to reduce cytochrome *b*-563 is inferred from the difference spectrum for diaminodurol added in the dark (Fig. 8) and from the uncoupler and ADP-stimulated photooxidation of cytochrome *b*-563 observed in the presence of diaminodurol (Fig. 9A). The difference spectrum for diaminodurol addition (Fig. 8) shows that diaminodurol probably causes the reduction of an oxidized cytochrome *b*-559 component as well.

Photooxidation of cytochrome b-563 coupled to phosphorylation and its inhibition by DBMIB. Electrons donated by diaminodurol might enter the electron transport chain at several points[17–19], and the question about donation to cytochrome *b*-563 is

whether transfer of these electrons to Photosystem I is coupled to phosphorylation. The existence of a coupling site in the pathway of *b*-563 oxidation can be inferred from the photooxidation of diaminodurol- (Fig. 9A) or dithionite-reduced (Fig. 11) cytochrome *b*-563 upon addition of NH_4Cl. The measurements of the oxidative changes are seen more clearly with a 540-nm than with a 570-nm reference. As explained in Methods in connection with Fig. 1B, spectral measurements over a range of wavelengths with either reference would show a similar though offset spectrum. The initial oxidative change in Fig. 9A does not have a spectrum specific for cytochrome *b*-563 (data not shown). The spectrum for the additional oxidative absorbance change obtained upon addition of NH_4Cl in Fig. 9A identifies the oxidation as belonging to cytochrome *b*-563 (Fig. 10A). The oxidative absorbance change at 563 nm obtained upon ADP addition in the presence of orthophosphate also appears to be associated with cytochrome *b* 563 from the three points shown in the preliminary

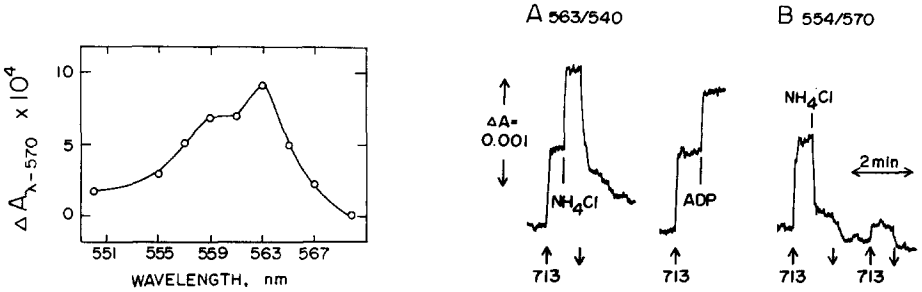


Fig. 8. Difference spectrum for the reduction of cytochrome *b*-563 by diaminodurol. The conditions were as described in Fig. 1B. Reduced diaminodurol was dissolved and kept under anaerobic conditions in 20% methanol; it was added at a final concentration of 0.1 mM; chlorophyll concentration 65 μg/ml. The spectrum is corrected for the dilution factor introduced by diaminodurol addition.

Fig. 9. Effect of ADP and NH_4Cl on the light-induced absorbance changes of cytochrome *b*-563 prereduced by diaminodurol, compared to those of cytochrome *f* under the same conditions. The standard reaction mixture contained the additional substances: methyl viologen, 0.1 mM; DCMU, 2 μM; diaminodurol, 0.1 mM. During far-red illumination the following additions were made: ADP, 9 μM; NH_4Cl, 1 mM; chlorophyll concentration, 30 μg/ml. Cytochrome *b*-563 was measured at 563 nm with 540 nm as reference wavelength (A), and cytochrome *f* at 554 nm with 570 nm as reference wavelength (B).

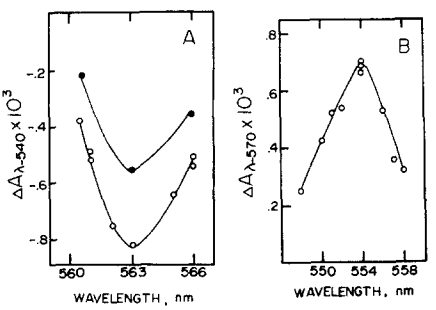


Fig. 10. Difference spectra of the ADP or NH_4Cl induced changes on cytochromes *b*-563 and *f* in the presence of diaminodurol. Conditions are those of Fig. 8. (A) Absorbance change induced by NH_4Cl (○—○) or ADP (●—●) measured at variable wavelength with 540 nm as reference. (B) Absorbance change induced by NH_4Cl (○—○) measured at variable wavelength with 570 nm as reference wavelength.

spectrum of Fig. 10A. These points are the average of several experiments. The spectra were not extended to shorter wavelength because of noise and drift at this sensitivity.

The effect of NH_4Cl on the rate and amplitude of cytochrome *b*-563 photooxidation can also be clearly seen when cytochrome *b*-563 is initially reduced by dithionite under anaerobic conditions. Under these conditions the amplitude of the cytochrome *b*-563 photooxidation is larger than in the experiments with diaminodurol as reductant. The rate and amplitude of the absorbance decrease at 563 nm are increased by a factor of 1.5 and 2.5, respectively, by addition of NH_4Cl (Fig. 11). The difference spectra for the light-induced absorbance decrease measured in the absence and presence of NH_4Cl have a peak at 563 nm (Fig. 12). The data of Figs 9A, 10A, 11 and 12 lead to the conclusion that the pathway of cytochrome *b*-563 photooxidation by System I includes a rate-limiting energy-coupling site.

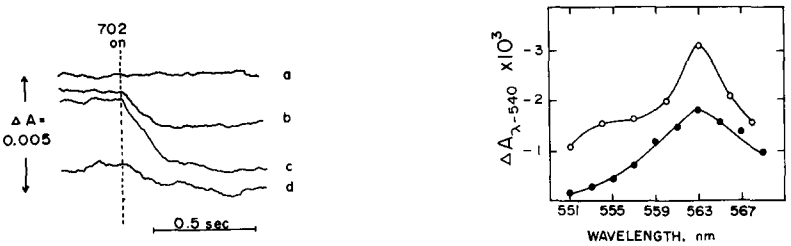


Fig. 11. Photooxidation of reduced cytochrome b_6 by Photosystem I. The experimental conditions were as described with the modifications of Fig. 1B. The output of the lock-in amplifier was displayed on a storage oscilloscope. Time constant of the amplifier, 100 ms; chloroplasts at a chlorophyll concentration of 65 μg/ml were reduced with a minimum amount of dithionite, approximately $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M, under anaerobic conditions. A platinum electrode was used to monitor the approximate redox potential of the mixture, assuring that all of the cytochrome *b*- was reduced. Trace a is the baseline of this recording; Trace b shows the absorbance change at 563 nm with 540 nm as reference wavelength induced upon 702-nm illumination ($6 \cdot 10^4$ erg$\cdot$cm$^{-2}\cdot$s^{-1}); Trace c shows the light-induced change of the same sample, after 1.5 mM NH_4Cl was added, and Trace d the light-induced change in the presence of 1.5 mM NH_4Cl after preincubation of the chloroplasts with 8 μM DBMIB for 4 min before addition of dithionite.

Fig. 12. Difference spectrum for cytochrome *b*-563 photooxidation in the absence and presence of NH_4Cl. The conditions are described in Fig. 11 where cytochrome *b*-563 was prereduced with dithionite under anaerobic conditions; chlorophyll concentration, 50 μg/ml. 702-nm light-induced absorbance change in the absence (●—●) and presence (○—○) of 1.5 mM NH_4Cl.

In the presence of DCMU and diaminodurol as reductant, an absorbance increase is seen at 554 nm with a 570-nm reference upon addition of NH_4Cl during far-red illumination (Fig. 9B). The absorbance changes observed under these conditions with a 540-nm reference are much smaller. A spectrum taken over a limited wavelength region indicates that addition of NH_4Cl under these conditions causes reduction of cytochrome *f* (Fig. 10B). These experiments imply that the energy coupling site located on the pathway of cytochrome b_6 oxidation precedes cytochrome *f*.

It was shown in Fig. 4 that the plastoquinone analog DBMIB inhibited dark reoxidation of cytochrome *b*-563 and that the inhibition was relieved by plastoquinone, the inference being that oxidation of cytochrome *b*-563 is mediated by plastoquinone. Fig. 11 shows that DBMIB inhibits the uncoupler stimulated oxidation observed at 563 nm using dithionite-reduced cytochrome *b*-563. Figs 13B and C show

that DBMIB also inhibits the uncoupler-stimulated oxidation of smaller amplitude observed in the presence of diaminodurol. In the absence of diaminodurol an initial absorbance decrease is observed at 563 nm, but there is no stimulation by NH_4Cl (Fig. 13A). Thus, it is considered very likely that plastoquinone mediates electron flow from diaminodurol- or dithionite-reduced cytochrome *b*-563 to cytochrome *f* through a pathway containing a site of DBMIB inhibition, and an energy coupling site. It has been found previously that an energy coupling site in non-cyclic electron transport lies between plastoquinone and cytochrome *f*[13]. The tentative inference is that this coupling site is common to non-cyclic electron transport and to cyclic electron flow involving cytochrome *b*-563 (Fig. 14).

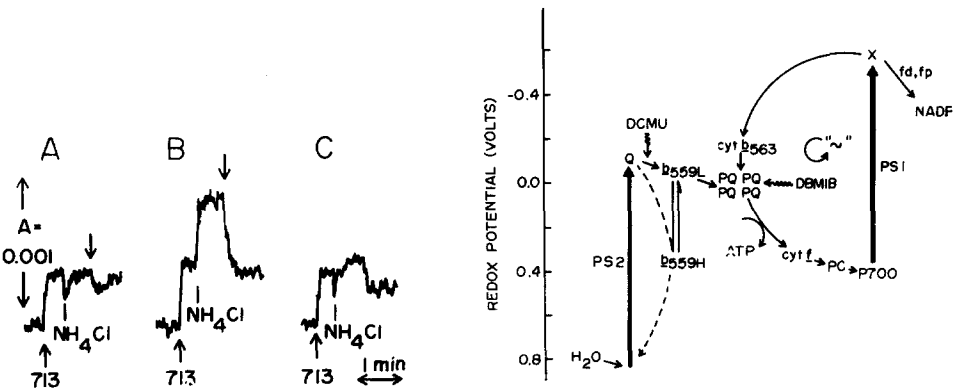


Fig. 13. Inhibition of cytochrome *b*-563 photooxidation by DBMIB. The standard reaction mixture adjusted to pH 7.5 contained: Methyl viologen, 0.1 mM, DCMU, 1 μM. (A) Control without diaminodurol or DBMIB; (B) Cytochrome *b*-563 prereduced with 0.1 mM diaminodurol; (C) 0.1 mM diaminodurol and 5 μM DBMIB were added prior to illumination. 1 mM NH_4Cl was added as indicated. Reference wavelength, 540 nm; chlorophyll concentration, 30 μg/ml.

Fig. 14. Formulation of the 'Z' scheme for photosynthetic electron transport including the pathway for System I cyclic electron transport involving cytochrome b_6.

DISCUSSION

Localization of an energy coupling site between plastoquinone and cytochrome *f* in the pathway responsible for oxidation of cytochrome *b*-563 helps to explain the effect of PMS in bypassing the site of DBMIB inhibition and a slow step in electron transport between cytochrome *b*-563 and cytochrome *f* (Figs 5 and 6). Using the data supplied by Figs 8–13, the ability of PMS to keep cytochrome *f* at a more reduced level under actinic illumination in Fig. 6B is explained by a faster rate of electron flow to cytochrome *f* in the PMS pathway bypassing the coupling site. When electron flow through the plastoquinone–cytochrome *f* coupling site is not inhibited in the presence of DCMU (Fig. 6A), addition of PMS has no effect on the amplitude of cytochrome *f* photooxidation. In this case it is assumed that the preferred PMS pathway is through the plastoquinone–cytochrome *f* coupling site.

The lack of inhibition by DBMIB of PMS-mediated cyclic phosphorylation[12] implies the existence of at least one more phosphorylation site in the PMS cyclic system, as indicated by the squiggle in Fig. 11. The heptane-extraction experiments of Black[21] and Laber and Black[22] also indicate that there is more than one site of

phosphorylation in PMS cyclic electron transport, with one of the sites very likely common to non-cyclic electron transport. The differences in sensitivity to uncouplers of PMS cyclic and non-cyclic phosphorylation[23–25] according to these ideas would be due to different properties of the second site of cyclic phosphorylation. Although DBMIB does not inhibit the overall rate of PMS cyclic phosphorylation[12], the model we have proposed for DBMIB action says that one of the two sites is blocked by DBMIB. The lack of net inhibition by DBMIB implies that the increase in electron transport rate accompanying the PMS bypass of the blocked site, discussed in the preceding paragraph, compensates for the loss in phosphorylation efficiency. The lack of saturation of PMS cyclic phosphorylation at light intensities large enough to saturate non-cyclic electron transport and phosphorylation[24] implies that phosphorylation at the non-overlapping site in the cyclic chain is not rate-limiting, and that PMS can bypass the rate limiting site between plastoquinone and cytochrome *f* at high light intensities. The pathways of PMS and diaminodurol cyclic phosphorylation may be similar[18] except that (a) reduced diaminodurol can donate electrons to cytochrome b_6; and (b) the diaminodurol pathway may not be able to bypass the plastoquinone–cytochrome *f* coupling site as easily as PMS, and so can be partly inhibited by DBMIB[12].

The experiments reported here also fit very well with the observations of Levine[8], if it is assumed that component 'M' defined by *Chlamydomonas* mutant *ac*-21 is analogous to the plastoquinone component inhibited by DBMIB. It has been shown that *ac*-21 has only about half the plastoquinone/chlorophyll ratio of the wild type[16]. This deficiency is actually larger than can be accounted for by our model, since we tentatively identify the plastoquinone component inhibited by DBMIB with a small pool of plastoquinone observed to be associated with an energy coupling site of non-cyclic electron transport[13]. Levine's data indicated that the PMS bypass of the electron transport defect in the 'M' mutant most likely involved plastocyanin and not cytochrome *f*. We have not attempted any measurements of plastocyanin in these experiments.

The ability of diaminodurol to partially reduce cytochrome *b*-563 does not prove that the *b*-563 is the principal site of electron donation in coupled open-chain electron transport mediated by this compound. Diaminodurol could also donate electrons before a coupling site by reducing plastoquinone. The reduction of cytochrome *b*-563 by diaminodurol is, in any case, an unexpected phenomenon. The oxidation–reduction potential of cytochrome b_6 has been titrated to be —180 mV in spinach chloroplasts[27], and as about 0.0 V in etiolated barley chloroplasts[28], with the latter measurements not distinguishing cytochromes *b*-559 and *b*-563. Titration of the 480-nm adsorption band of diaminodurol, which belongs to the diaminodurol free radical[29], shows a midpoint potential of approximately +230 mV for 10^{-3} M diaminodurol in 0.1 M phosphate buffer at pH 7 (W. A. Cramer, unpublished data). These titrations of diaminodurol were reversible with a slope intermediate between a one- and two-electron transition. Since there are no reports of a cytochrome *b*-563 species with a midpoint above 0.0 V*, the partial reduction of cytochrome *b*-563 by diaminodurol is either driven by an energy-linked process or the potential of diaminodurol in the environment of the chloroplast membrane is appreciably lower than the value measured in

* A recent report[34] suggests that the midpoint potential of cytochrome b_6 is near 0.0 V, though a titration is not shown.

aqueous solution. The latter possibility is also suggested by diaminodurol-stimulated oxygen uptake in chloroplasts[20].

Other suggestions in the literature for the pathway of cytochrome *b*-563 oxidation involve direct transfer to cytochrome *f*[11,30] or plastocyanin[3]. We would agree that these pathways are the preferred ones in the presence of PMS and DBMIB. A cytochrome b_6–cytochrome *f* complex has recently been purified from System I particles of lettuce chloroplasts[31]. The particle is devoid of chlorophyll, but contains non-heme iron, phospholipid, and carotenes. The existence of this complex implies that cytochromes b_6 and *f* are bound together tightly enough so that the possibility of electron transfer between them is suggested. Our experiments imply that this intermolecular electron transfer *in situ* proceeds more efficiently through plastoquinone mediating between the heme groups of the two proteins. Our experiments further imply that the plastoquinone–cytochrome *f* interaction in the complex is coupled to an energy-transfer mechanism. The antimycin A sensitivity of ferredoxin-stimulated phosphorylation[2] might imply that this agent would be useful in defining the position of cytochrome b_6 in cyclic electron transport. We have tried to study the effect of antimycin A on cytochrome *b*-563 turnover, but our results have been ambiguous, perhaps because of antimycin A effects on cytochrome *b*-559 turnover[14,32].

The uncorrected action spectrum for the absorbance increase at 563 nm (Fig. 3) would suggest that Photosystem II might reduce cytochrome *b*-563 more efficiently than Photosystem I. The actual efficiency of Photosystem II reduction of cytochrome *b*-563 will depend on the correction for reduction of cytochrome *b*-559 (Fig. 4), the amount of light energy transfer or spillover from Photosystem II to I, and the accuracy of the light absorption measurements made in the far-red region of the spectrum. We have not yet tried to quantitatively correct the action spectrum for these effects. Because of the action spectrum we are not able to exclude the possibility that cytochrome *b*-563 might have a function in the main chain of the photosynthetic electron transport scheme[3,10] as well as in the cyclic chain, though we would not expect this if the midpoint potential of cytochrome *b*-563 is —180 mV[27].

Finally, it can be observed that as a consequence of these experiments and those on the cytochrome *b*-559 and plastoquinone function[13–16], the 'Z' scheme model which we propose contains a symmetry between the two photosystems. Each contains a tightly bound *b*-type cytochrome which can cycle (though the cyclic reaction in Photosystem II is believed to be inefficient[14]) and transfer electrons to a common plastoquinone pool. This suggests the possibility of evolution of the two photosystems in higher plant and algal photosynthesis from an ancestor containing two cyclic systems connected by a common plastoquinone pool, as suggested by Olson[33].

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