# Evidence for a reductant-dependent oxidation of chloroplast cytochrome *b*-563

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## 1. INTRODUCTION

An electron carrier thought to be uniquely associated with the cyclic electron-transport pathway is cytochrome *b*-563 ($b_6$) (reviewed in [1]). This conclusion was based on the early observation that cytochrome *b*-563 underwent both a photosystem I-mediated oxidation and reduction. The function of cytochrome *b*-563 has been explained in terms of a mechanism in which ferredoxin, reduced by photo-070system I, served as the electron donor for cytochrome *b*-563 and that the cytochrome then donated electrons into the plastoquinone pool [1–4]. However, recent findings suggest a reduction which appears to be dependent on the redox state of plastoquinone [3,5]. Such results, as well as those obtained in studies of flash-induced redox changes of the cytochrome [5–8] have led to a general model in which cytochrome *b*-563 functions in a 'Q-cycle' [5,8] similar to that proposed by Mitchell for the cytochrome $b$–$c_1$ region of the mitochondrial electron transport chain [9].

The results reported here support the hypothesis that the role of ferredoxin in cyclic electron flow is to supply reducing equivalents for a 'reductant-dependent oxidation' of cytochrome *b*-563. This reaction may be considered as the converse of the 'oxidant-dependent reduction' which is involved in cytochrome *b*-563 photoreduction [5]. These results are consistent with a 'Q-cycle' mechanism for the redox reactions of cytochrome *b*-563 [9].

*Abbreviations*: DBMIB, 2-5-dibromo-3-methyl-6-isopropyl-benzoquinone; DCMU, 3-(3′, 4′-dichlorophenyl)-1, 1-dimethylurea; Fd, ferredoxin; PQ, plastoquinone; PQH′, plastosemiquinone; $PQH_2$, plastohydroquinone

## 2. MATERIALS AND METHODS

### 2.1. *Chloroplast preparation*

Spinach chloroplast membranes were prepared from freshly harvested greenhouse-grown spinach [10]. These chloroplast membrane fragments were substantially depleted of ferredoxin (some residual $NADP^+$ photoreduction activity remained in the absence of added ferredoxin) and were capable of both cyclic and non-cyclic photophosphorylation with low quantum requirements [11] when used within 30–60 min of preparation and when supplemented with appropriate cofactors.

### 2.2. *Cytochrome determination*

Light-induced cytochrome absorbance changes were followed with an Aminco DW-2 or DW-2A spectrophotometer operated in the dual wavelength mode (slit width 2.0–2.5 nm). A millimolar extinction coefficient of 20 was used for cytochrome *b*-563 (563–575 nm) based on [12]. The reference wavelength for all the cytochrome determinations was 575 nm. Dark reduced-minus-oxidized spectra were recorded in the split-beam mode (slit width 1.0 nm). Illumination was provided by filtering white light through Corning 2–58 filters. The wide band red illumination ($3 \times 10^5$ ergs $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$) was directed onto the front surface of the 2 mm light-path cuvette (at an acute angle to the measuring beam) from a mirror attached inside the sample compartment. The phototube was shielded from the red light by two Corning 4-96 filters. The cuvette was mounted as close as possible to the front surface of the phototube ($\sim$ 10 mm, including filters) to minimize light scattering effects of the chloroplast suspension. The relatively high chlorophyll concen-

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tration (500 μg chl/ml) in 2 mm lightpath cuvettes enhances the absorbance changes arising from cytochrome redox reactions and minimizes absorbance changes due to the reduction and/or oxidation of components (such as ferredoxin) added to the reaction mixture.

The basic reaction mixture (final vol. 0.65 ml) consisted of chloroplast membrane fragments (500 μg chl/ml) and the following (mM): Tricine– KOH (pH 8.35), 100; $MgCl_2$, 5; ADP, 2.5; $K_2HPO_4$, 2.5; and glucose, 10. Glucose oxidase (200 μg/ml) and catalase (30 μg/ml) were added throughout to ensure that, after the reaction mixture had been equilibrated with $N_2$, anaerobic conditions were maintained during the subsequent transfer of the reaction mixture to the anaerobic, capped cuvettes.

## 3. RESULTS

In dark-adapted spinach chloroplast membranes, cytochrome *f* and *b*-559 were predominantly reduced while cytochrome *b*-563 was fully oxidized. The addition of the electron donor couple ferrodoxin-NADPH reduced cytochrome *b*-563 under a nitrogen atmosphere (which prevents the oxidation of reduced ferredoxin and the cytochrome by oxygen). Fig.1 illustrates the absorbance difference spectrum induced by ferredoxin-NADPH with the reduction of 2.4 nmol cytochrome *b*-563/mg chl in the reaction mixture.

With the cytochrome prereduced in the dark, the photooxidation and following dark rereduction reactions were studied. In the absence of added inhibitor (fig.2A) cytochrome *b*-563 was partially photooxidized (~ 1.0 nmol/ml chl) in the light and slowly rereduced in the following dark period. The addition of either DCMU or DBMIB (fig.2B,C) stimulated both the rate and extent of cytochrome *b*-563 photooxidation. The dark rereduction of the cytochrome was accelerated in the presence of DCMU (fig.2B), but was not significantly changed in the presence of DBMIB (fig.2C). The addition of DBMIB to the DCMU-treated system (fig.2D) inhibited the accelerated dark rereduction induced by DCMU alone.

The absorbance difference spectrum of the various light-induced changes are shown in fig.3. In fig.3A (which corresponds to the conditions of fig.2A) the main absorbance change is attributed to cytochrome *b*-563 oxidation.

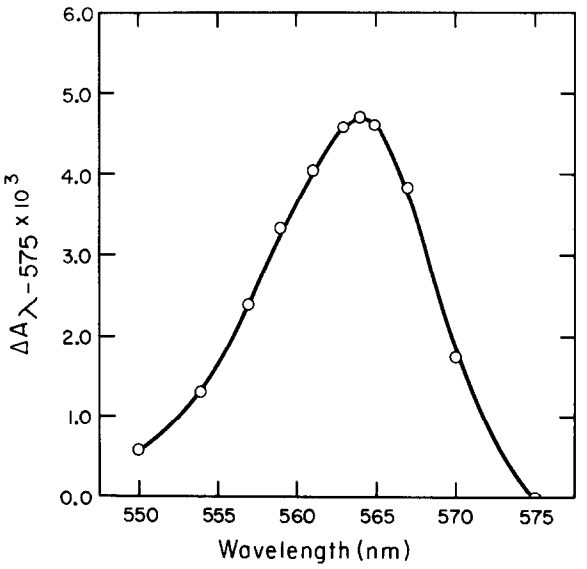


Fig.1. Reduced-minus-oxidized difference spectrum induced by the addition of ferredoxin-NADPH. The reaction mixture and experimental conditions were as in section 2 with spinach ferredoxin (10 μM) added throughout and NADPH (1.7 mM) added to the sample cuvette.

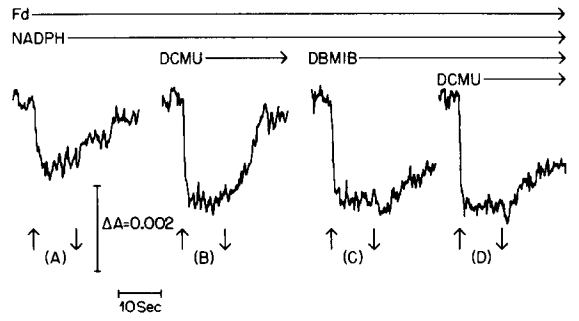


Fig.2. Stimulation of cytochrome *b*-563 photooxidation by DCMU and DBMIB after initial reduction by ferredoxin-NADPH. The reaction mixture and experimental conditions were as in section 2. Spinach ferredoxin (10 μM), NADPH (1.7 mM) were added throughout and 5 μM DCMU (B), 5 μM DBMIB (C) or both (D) were added as indicated: (↑) light on; (↓) light off; a downward deflection indicates a decrease in absorbance where a $\Delta A$ of 0.002 at 563–575 nm represents ~ 1.0 nmol cytochrome *b*-563 oxidized/mg chlorophyll.

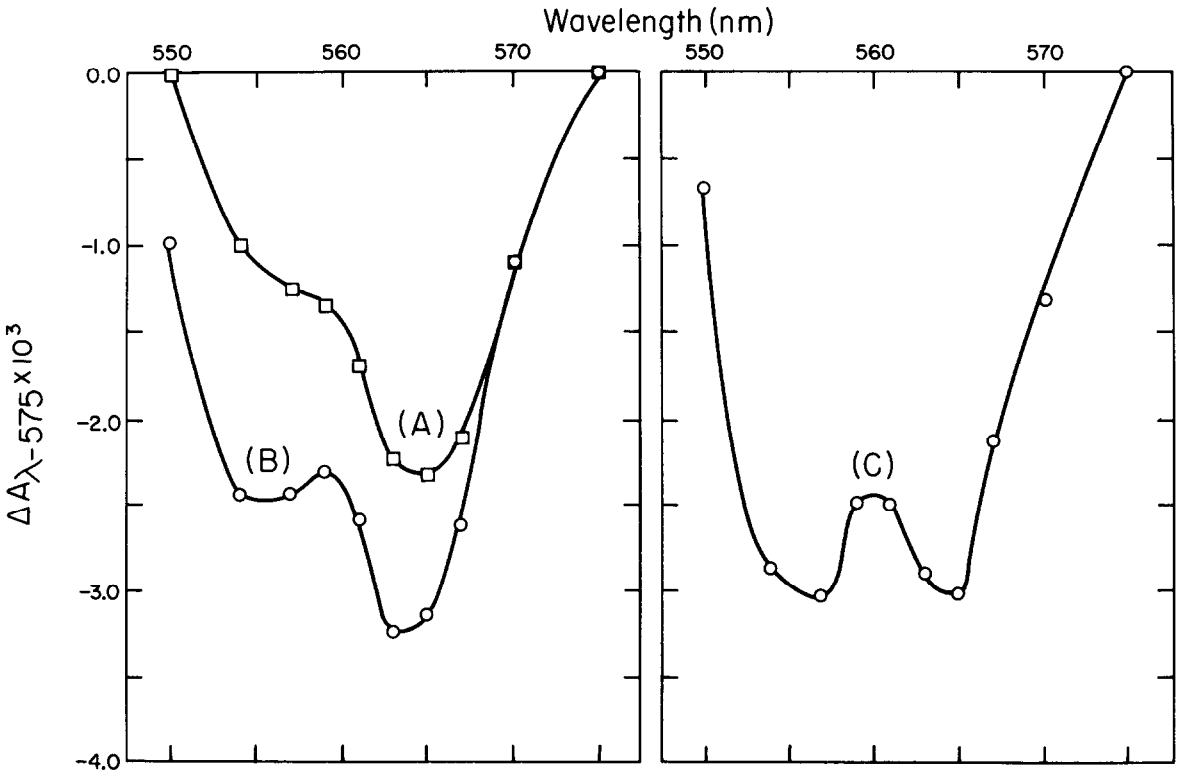


Fig.3. Light-minus-dark difference spectrum of the absorbance decrease in fig. 2A–C. The experimental conditions were the same as in fig.2 with ferredoxin-NADPH added throughout and either DCMU (B) or DBMIB (C) added as in fig.2. A fresh dark-adapted sample was used for each point with the maximum deflection after 10 s illumination plotted as a function of wavelength (550–575 nm).

Both DCMU (fig.3B) and DBMIB (fig.3C) increase the extent of steady state photooxidation of cytochrome *b*-563. In addition, both inhibitors stimulate the photooxidation of cytochrome *f* in the 554–556 nm range (fig.3B,C). The effect of DCMU and DBMIB on cytochrome *f* (fig.3B,C) clearly indicate that these two inhibitors are acting in the expected manner under our experimental conditions as discussed in [1]. Although spectral changes from components other than cytochromes *b*-563 and *f* are known to occur in the 550–575 nm region (e.g., $C_{550}$ [13] and the electrochromic band shift [14]), clearly the predominant feature of fig.3 were the light-induced redox reactions of *b* and *c* type cytochromes.

## 4. DISCUSSION

It is generally accepted that oxidizing equivalents generated in the light by P700 are required to photooxidize cytochrome *b*-563 by way of PQ [1] and the more positive electron carriers located between PQ and the photosystem I reaction center. The site of inhibition of electron transport by the plastoquinone antagonist DBMIB is generally considered to be on the oxidizing side of the PQ pool [15], probably at the Rieske iron–sulfur center [16–20]. Assuming that under these experimental conditions, the function of the Rieske iron–sulfur center is inhibited by DBMIB, than a pathway must exist which can account for the DBMIB-insensitive photooxidation of cytochrome *b*-563. A model, serving as a working hypothesis, which can explain the effect of DCMU and DBMIB on the redox reactions of cytochrome *b*-563, is presented in fig.4. In this model the redox reactions of plastosemiquinone ($PQH^{\cdot}$) drive the contrary reactions of cytochrome *b*-563.

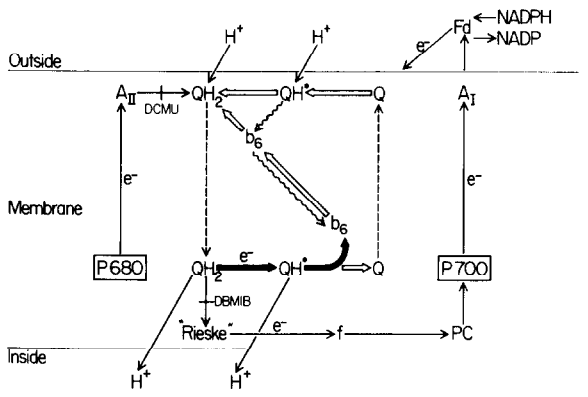


Fig.4. Proposed model for the oxidation-reduction reactions of cytochrome *b*-563: Q, plastoquinone; QH·, plastosemiquinone; $QH_2$, plastohydroquinone; 'Rieske', Rieske iron–sulfur center; $b_6$, cytochrome *b*-563; *f*, cytochrome *f*; PC, plastocyanin; P680, reaction center chlorophyll for photosystem II; $A_{II}$, primary acceptor of photosystem II; P700, reaction center chlorophyll for photosystem I; $A_I$, primary acceptor for photosystem I; (⟶) pathway of cytochrome *b*-563 photoreduction; (⟹) pathway of cytochrome *b*-563 photooxidation; (⇝) pathway of cytochrome *b*-563 dark reduction; (- - -) translocation of plastoquinone or plastohydroquinone across membrane.

The model presented here is consistent with the following sequence of reactions for the light and dark redox reactions of cytochrome *b*-563 and are similar in part to those proposed to function in the cytochrome $b$–$c_1$ region of mitochondria [9,21,22], photosynthetic bacteria [23,24], and chloroplasts [5,7,8]. This model consists of 3 different reactions involving cytochrome *b*-563 and are illustrated in the following equations:

(A)*Dark ferredoxin-mediated reduction of cytochrome* b-563

$$Fd^{red} + PQ + H^+ \rightarrow Fd^{ox} + PQH^{\cdot} \quad (1)$$

$$PQH^{\cdot} + b\text{-}563^{ox} \rightarrow PQ + H^+ + b\text{-}563^{red} \quad (2)$$

(B)*Reducant-dependent oxidation of cytochrome* b-563

$$Fd^{ox} \xrightarrow{h\nu} Fd^{red} \quad (3)$$

$$Fd^{red} + PQ + H^+ \rightarrow Fd^{ox} + PQH^{\cdot} \quad (4)$$

$$PQH^{\cdot} + H^+ + b\text{-}563^{red} \rightarrow PQH_2 + b\text{-}563^{ox} \quad (5)$$

(C)*Oxidant-dependent reduction of cytochrome* b-563

$$PQH_2 + Rieske^{ox} \rightarrow PQH^{\cdot} + Rieske^{red} + H^+ \quad (6)$$

$$PQH^{\cdot} + b\text{-}563^{ox} \rightarrow PQ + b\text{-}563^{red} + H^+ \quad (7)$$

The initial dark reduction of cytochrome *b*-563 by ferredoxin-NADPH is thought to occur by the mechanism in scheme (A). These reactions are thought not to occur under normal conditions of electron transport. The reduction of cytochrome *b*-563 by this mechanism does not result in proton translocation across the chloroplast membrane because this reaction would occur on the stromal side of the membrane. The proton taken up from the external medium would be released to the same medium upon the reduction of cytochrome *b*-563.

Upon illumination, in the absence of inhibitors a steady state of cytochrome *b*-563 oxidation/reduction was established with the cytochrome ~60% reduced. It is proposed that, as the name 'reductant-dependent oxidation' implies, reducing equivalents generated in the light by photosystem I drive the oxidation of cytochrome *b*-563 as illustrated in scheme (B). If reduced ferredoxin functions as an electron donor to PQ rather than to cytochrome *b*-563 as proposed in [3,8,25,26], then illumination might be expected to initiate this oxidation. As electrons enter PQ from ferredoxin and cytochrome *b*-563, the requirement for ferredoxin in cyclic photophosphorylation is fulfilled [10] along with ATP formation under long wavelength illumination [10]. Electrons from reduced ferredoxin are thought to enter the PQ pool as discussed in [26].

The experimental conditions used in fig.2A similar to those required to partially poise a ferredoxin cyclic system [4]. This suggests that cytochrome *b*-563 is actively undergoing oxidation/reduction. The reduction is thought to proceed as in scheme (C). The interaction of schemes (B) and (C) results in the steady-state redox level of the cytochrome. After illumination, the dark rereduction of cytochrome *b*-563 is by the pathway in scheme (A). The slow rate of this reduction suggests that the PQ pool is predominantly reduced by electrons from $H_2O$ and ferredoxin (scheme (B)) and that little oxidized PQ remains for the reactions in scheme (A).

The addition of DCMU stimulates the oxidation of cytochrome *b*-563 and *f*. This stimulation is thought to occur because electrons from $H_2O$ can no longer form $PQH_2$, which is the reductant for

both cytochromes. Some $PQH_2$ is formed by the oxidation of cytochrome *b*-563 (scheme(B)) which results in the two cytochromes not being fully oxidized in the light. The accelerated rate of cytochrome *b*-563 dark rereduction is considered to be the result of the increased amount of oxidized PQ available for the reactions in scheme (A) due to the presence of DCMU.

Up to this point there is no compelling reason to accept the mechanisms in schemes (A)–(C) for the redox reactions of cytochrome *b*-563 over those proposed in the conventional 'Z' scheme. However, the total lack of inhibition of cytochrome *b*-563 oxidation by DBMIB clearly illustrates that oxidizing equivalents generated by photosystem I do not drive the oxidation of this cytochrome. With the cytochrome reduced before illumination and DBMIB present to inhibit the oxidation of $PQH_2$ by photosystem I, no change in the redox state of cytochrome *b*-563 could occur if the 'Z' scheme, in its present form, were correct. In contrast, the mechanism described in scheme (B) would be insensitive to DBMIB because the Rieske protein is not involved in the oxidation of cytochrome *b*-563.

The inhibitor DBMIB is clearly functioning by blocking electron transport between the Rieske protein and photosystem I as indicated by the increased photooxidation of cytochrome *f*. Under these conditions neither electrons from $H_2O$ nor reduced ferredoxin can reach cytochrome *f*, and maximum cytochrome *f* oxidation was observed.

The addition of DBMIB inhibits not only the photoreduction of cytochrome *b*-563 [8] but also the dark ferredoxin-NADPH mediated reduction. The dark rereduction of cytochrome *b*-563 after photooxidation in the presence of DCMU was substantially inhibited by DBMIB, suggesting a role for PQ even in the dark reduction. In the presence of DBMIB no oxidized PQ would be formed in the light, while the oxidation of cytochrome *b*-563 would consume more PQ, resulting in an inhibited rate of dark reduction by the mechanism in scheme A.

The effect of DBMIB on the dark rereduction of cytochrome *b*-563 also argues against the direct reduction of the cytochrome by reduced ferredoxin as indicated below:

$$Fd^{red} + b\text{-}563^{ox} \not\rightarrow Fd^{ox} + b\text{-}563^{red} \quad (8)$$

The scheme in fig.4 also illustrates two sites of competition for oxidized PQ. One such site is reduced ferredoxin where the electron goes to the PQ as in scheme B. The successful competition of the cyclic pathway for electrons from reduced ferredoxin under conditions of non-cyclic electron flow from $H_2O$ to $NADP^+$ is discussed in detail in [4,10].

The second site where competition takes place is in the reduction of PQ by photosystem II. The oxidation of both cytochrome *b*-563 and the primary electron acceptor of photosystem II ($A_{II}$) depend on PQ. The effect of DCMU and NADPH on ATP formation [4,10,11] strongly support the existence of such a site. Either of these sites would be a good candidate for a regulatory site to manipulate the ATP/NADPH ratio in vivo.

No direct observations of PQ have been made in this study, so its involvement in schemes (A)–(C) is highly speculative. The proposed function of $PQH^*$ in both the reduction and oxidation of cytochrome *b*-563 is based on the 'Q-cycle' [eq.(24) in [9]], observations in photosynthetic bacteria [23,24], and the proposed role of ubiquinone in mitochondria [27].

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