Sites of cytochrome *b*-563 reduction, and the mode of action of DNP-INT and DBMIB in the chloroplast cytochrome *b*-563/*f* complex

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When ferredoxin is the reductant of cytochrome *b*-563 in the chloroplast cytochrome *b*-563/*f* complex, the reaction rate varies with pH in a manner which permits identification of a plastoquinone/plastosemiquinone couple as an intermediate in the reaction. The requirement for the inhibitor, DNP-INT, in this reaction, suggests that there are two sites in the complex where plastosemiquinone can react with cytochrome(s) *b*-563. The ineffectiveness of DBMIB in promotion of ferredoxin-mediated cytochrome *b*-563 reduction has led to a model where DBMIB and DNP-INT inhibit different electron transfer steps at the same, or overlapping, sites in the complex.

Chloroplast *Cytochrome* b *Electron transfer* *Ferredoxin* *Plastoquinone*

1. INTRODUCTION

Cyclic electron transfer around photosystem I of green plants is vital for the generation of ATP [1,2] and for preventing photochemical damage by the photosystem II herbicides [3,4]. Its role in the photosystem I cycle illustrates that the cytochrome *b*-563/*f* complex can utilize the reducing power of either photosystem I or photosystem II in the generation of an electrochemical gradient. Reducing equivalents from photosystem II enter the complex as plastoquinol during 'linear' electron transfer (plastoquinol: plastocyanin oxidoreductase) [5]. The entry of reducing equivalents from photosystem I during cyclic electron transfer is a much less thoroughly described pathway which involves ferredoxin and perhaps the ferredoxin: NADP oxidoreductase [6-8].

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether

Given the number of components in the cytochrome *b*-563/*f* complex (1 cytochrome *f*, 1 Rieske iron-sulfur cluster, 2 cytochromes *b*-563, and 1 plastoquinone [5]), the number of possible electron transfer reactions would be very high without the restrictions posed by the overall organization of the complex. In particular, those reactions involving plastoquinone, which are difficult to measure directly, are further complicated by the variety of reduction and protonation states possible to a quinone molecule, yet the electron transfer events most central to the generation of an electrochemical gradient by the cytochrome *b*-563/*f* complex are those between quinones and either *b*-cytochromes or iron-sulfur proteins. Here, electron transfer between ferredoxin and the cytochrome *b*-563/*f* complex has been used to characterize two separate sites of plastoquinone-cytochrome *b*-563 interaction and to demonstrate a difference between the mode of action of two well-known inhibitors of chloroplast 'linear' electron transfer, DBMIB and DNP-INT [9,10].

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2. MATERIALS AND METHODS

The cytochrome *b*-563/*f* complex was isolated as in [11]. Spinach plastocyanin, ferredoxin, and ferredoxin:NADP oxidoreductase were prepared as in [12-14]. DNP-INT and plastoquinol-1 were synthesized as in [10,15].

Absorbance changes and spectra were measured on a Johnson Foundation SDB-3A dual wavelength spectrophotometer. The mixing in a rapidly stirred sample was complete in <0.7 s, as determined by ascorbate reduction of cytochrome *f*. The experimental conditions for measuring cytochrome *b*-563 reduction were described in [16] and the procedure for measurement of plastoquinol:plastocyanin oxidoreductase activity was described in [5].

3. RESULTS

Authors in [16] originally observed in the purified cytochrome *b*-563/*f* complex a slow cytochrome *b*-563 reduction dependent on NADPH, ferredoxin, ferredoxin:NADP oxidoreductase, and DNP-INT [16]. Ferredoxin:NADP oxidoreductase also catalyzes a rapid reduction of ferricytochrome *f* by NADPH (fig. 1, 10 s) [17]. Complete in <1 s, unaffected by inhibitors of the cytochrome *b*-563/*f* complex (DBMIB, DNP-INT, and antimycin were tested) and not requiring ferredoxin, the reduction of cytochrome *f* is probably the result of a direct interaction between the cytochrome and ferredoxin:NADP oxidoreductase [17]. The much slower reduction of cytochrome *b*-563 (fig. 1, 3 min, 20 min) only reaches 60-80% completion [16].

Increasing the concentration of ferredoxin ac-

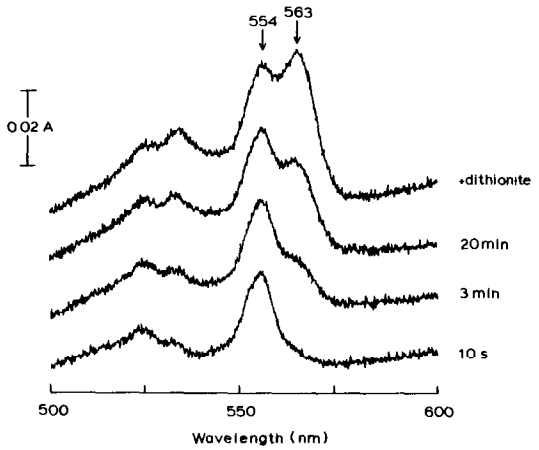

Fig.1. Spectra of cytochrome *b*-563/*f* complex at various times after the addition of NADPH. The nitrogen-flushed cuvette contained 1.5 ml of 0.1 M HEPES (pH 7.5), 1.2 μM cytochrome *f* as *b*-563/*f* complex, 225 nM ferredoxin:NADP oxidoreductase, 1.8 μM ferredoxin, 13 μM DNP-INT, 10 μM ferricyanide, 10 mM glucose, 40 μg/ml catalase and 120 μg/ml glucose oxidase. The reference spectrum was scanned, 600 μM NADPH was added, and subsequent spectra obtained at the times indicated. The total scanning time for one spectrum was 17 s.

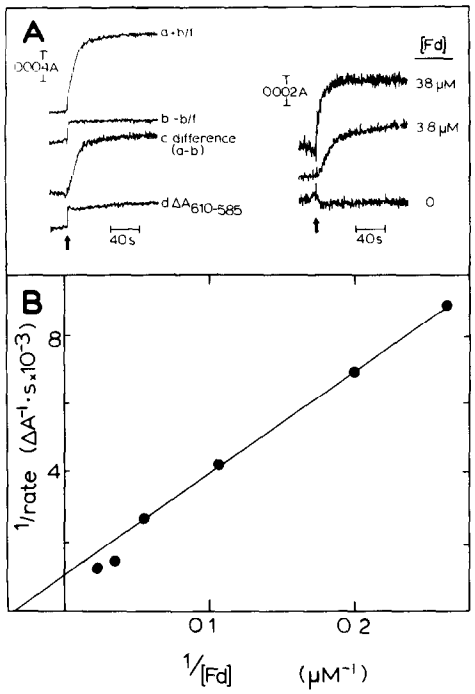

Fig.2. Corrections to cytochrome *b* reduction kinetics, and ferredoxin concentration dependence. Reaction conditions as in fig.1 except for 0.1 M Tricine (pH 8.37), 414 nM cytochrome *f* as *b*-563/*f* complex, and the ferredoxin concentration as indicated. (A) The traces on the right show the absorbance changes at 563-540 nm (a-c) in the presence of 19 μM ferredoxin both with (a) and without (b) the cytochrome *b*-563/*f* complex. NADPH was added as indicated by the arrow. Trace d shows the absorbance change at 610-585 nm in the presence of the *b*-563/*f* complex. The traces on the left show the ferredoxin concentration dependence of the corrected cytochrome *b* kinetics. (B) A double reciprocal plot of the initial rate of cytochrome *b*-563 reduction.

celerates the rate of cytochrome *b*-563 reduction, but also increases the spectral contribution of NADPH-reduced ferredoxin at 563–540 nm, as shown in fig. 2A, traces a,b. By subtracting the contribution from ferredoxin, the kinetics of cytochrome *b*-563 reduction alone are obtained (trace c). The absorption change at 610–585 nm (trace d), due primarily to ferredoxin reduction, shows that the presence of the cytochrome *b*–563/*f* complex has little effect on the ferredoxin reduction kinetics and that this correction procedure is valid. Notably, the concentration of reduced ferredoxin remains essentially constant throughout the time-course of cytochrome *b*-563 reduction.

The corrected absorption changes shown on the right of fig.2A demonstrate the ferredoxin concentration dependence of the rate of cytochrome *b*-563 reduction. The initial rates from data such as these were used to construct the double reciprocal plot shown in fig.2B. From this graph, values of 29 μM for the K_m and 0.9 μequiv. (nmol cyt. *f*)$^{-1}$.h^{-1} for the V_{max} are calculated (using a reduced-minus-oxidized extinction coefficient for cytochrome *b*-563 of 15.3 mM^{-1}.cm^{-1} calculated from [18]. This K_m-value may be inflated because only 24% of ferredoxin is reduced by NADPH at the pH used in fig.2, and oxidized ferredoxin may inhibit competitively. (The large excess of NADPH insured that this percentage remained constant over the concentration range of ferredoxin used.) The V_{max} of cytochrome *b*-563 reduction is ~15-times greater than the rate measured in [16], yet is 10–30-times slower than the published rates of plastoquinol:plastocyanin oxidoreductase [5,19].

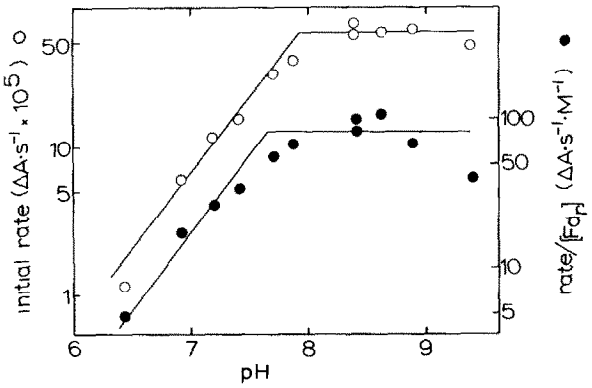

Fig.3. pH Dependence of the initial rate of cytochrome *b*-563 reduction; Reaction conditions as in fig. 1 except that the buffer contained 20 mM MES, 20 mM Tricine, 20 mM Bis-Tris Propane at the pH indicated, 430 nM cytochrome *f* as *b*–563/*f* complex, and 19 μM ferredoxin. The concentration of NADPH-reduced ferredoxin at each pH was calculated from the NADPH-induced reduction compared to the dithionite-induced reduction. Initial rate of cytochrome *b*-563 reduction (○); and the rate divided by the concentration of NADPH-reduced ferredoxin (●).

Fig.3 shows the pH dependence of the initial reduction rate of cytochrome *b*-563 (○). The reduction of ferredoxin by NADPH is pH-dependent with experimentally determined values that range from 8–41% reduced as the pH is increased from 6.4 to 9.4. In order to maintain initial conditions which were pseudo first order with respect to cytochrome *b*–563/*f* concentration, the rate data in fig.3 were normalized to the concentration of NADPH reduced ferredoxin (●). When this correction is made, the rate of cytochrome *b*-563 reduction increases 10-fold with each unit in-

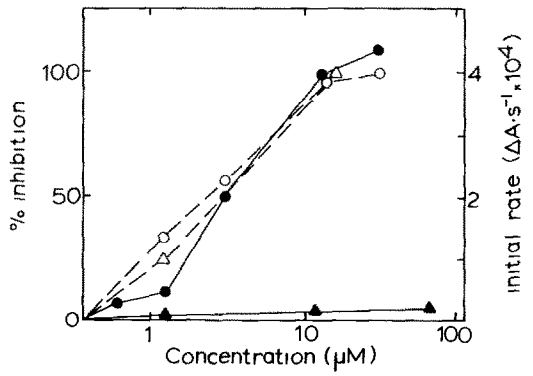

Fig.4. Comparison of the inhibition of plastoquinol:plastocyanin oxidoreductase activity with the stimulation of the rate of ferredoxin-mediated cytochrome *b*-563 reduction. For measurement of the plastoquinol:plastocyanin oxidoreductase activity, the sample contained 0.1 M Tricine-NaOH (pH 8.0), 418 nM cytochrome *f* as *b*–563/*f* complex, 2 μM plastocyanin, and inhibitor as indicated. The reaction was initiated by the addition of 18 μM plastoquinol-1. The absorption change at 597–500 nm was subtracted from the rate observed in the absence of the cytochrome *b*–563/*f* complex. The uninhibited rate was 25 μmol.(nmol cyt *f*)$^{-1}$·h^{-1}. Measurement of the rate of cytochrome *b*-563 reduction was as in fig.2A. Plastoquinol:plastocyanin oxidoreductase inhibition in the presence of DNP-INT (○) or in the presence of DBMIB (△); initial rate of cytochrome *b* reduction plus DNP-INT (●), or plus DBMIB (▲).

crease in pH, until the pH is $\gtrsim$ 7.7. Above this value, the rate is relatively constant. If the correction is not made, there is still a 10-fold increase in rate with pH, but this increase continues to about pH 8.0. This behavior is consistent with a rate-limiting step in the reduction of cytochrome *b*-563 that is dependent on the concentration of an unprotonated reductant with a pK_a of 7.7. Given the relatively pH-independent midpoint potentials of cytochrome *b*-563 [20,11] and ferredoxin [21] over pH 6.5–8.0, at least one component in addition to these must be operational and rate-limiting in this reaction. The low pK_a of this component is consistent with its identification as a plastosemiquinone [22–24]. Furthermore, fig.3 demonstrates that the plastosemiquinone anion is required for the reduction of cytochrome *b*-563 [24–27].

Although DNP-INT and DBMIB have been described as equivalent inhibitors of electron transfer between photosystems II and I [9,10], fig.4 (solid symbols) shows that only DNP-INT is effective in stimulating the reduction of cytochrome *b*-563. In contrast, under nearly identical conditions, both DBMIB and DNP-INT, exhibit very similar inhibitions of the plastoquinol : plastocyanin oxidoreductase activity of the complex.

4. DISCUSSION

As an inhibitor of 'linear' electron transport through the *b*-563/*f* complex, DNP-INT is recognized for blocking the reduction of cytochrome *b*-563 by a semiquinone in an oxidant-induced reduction reaction [28,29]. This report contains evidence which suggests that a plastosemiquinone anion is also an intermediate in the reduction of cytochrome *b*-563 by ferredoxin, yet this reaction is stimulated by DNP-INT. The concentration curve in fig.4 provides no evidence that more than one DNP-INT binding site is necessary to inhibit linear electron transfer or stimulate the ferredoxin-mediated reduction of cytochrome *b*-563. Thus, it would appear that the reduction of cytochrome *b*-563 by a semiquinone can occur at two distinct sites: the oxidant-induced reduction, or inner site, where DNP-INT functions, and the site described here, designated as the outer site. With the plastoquinone sites and only one plastoquinone/cytochrome *f* isolated with the complex, the availability of quinone at each site may present a significant limitation. Experiments with up to 70 μM added plastoquinone-1 had no effect on the rate of cytochrome *b*-563 reduction, with or without DNP-INT (not shown), demonstrating that the endogenous plastoquinone must be either equally distributed, or freely exchangeable between the sites.

Recent experimental findings have demonstrated that both the Rieske iron-sulfur protein and cytochrome *b*-563 polypeptides contribute to the binding site(s) of plastoquinone in the cytochrome *b*-563/*f* complex, [30], and that the binding site of DBMIB and plastoquinol on the Rieske iron-sulfur protein overlaps a second binding site for DNP-INT [31]. The results presented here further suggest that there are completely separate electron transfer steps inhibited by DNP-INT and DBMIB. The differences in the inhibition by DBMIB and DNP-INT do not preclude a common binding site, provided that the inhibition is exerted on different reactions at the same site: DBMIB could exhibit its activity by competing with the binding of plastoquinol, but not plastoquinone, at the inner site, thereby blocking oxidation of plastoquinol. By competing primarily with plastoquinone or plastosemiquinone at the same site, DNP-INT could inhibit electron transfer between the quinone or semiquinone and cytochrome *b*-563.

A model, shown in fig. 5, which describes the reactions of cyclic electron transfer, has ferredoxin and the components of the cytochrome *b*-563/*f* complex arranged in a 'Q-cycle' [32], similar to that in [23]. Two unique features are proposed here:

(i) Both plastoquinone sites are divided into two partial sites formed by an iron-sulfur protein and a *b*-cytochrome, and these partial sites can exhibit different inhibitor sensitivity but are not necessarily structurally distinct;

(ii) Although the solid arrows in fig.5 show the predominant direction of the reactions during cyclic electron flow, any of the thermodynamically possible quinone–cytochrome or quinone–iron-sulfur center reactions may occur at the site, but are limited by other factors.

This second feature encourages consideration of electron transfer mechanisms as a function of the redox states of the components at each site. Production of a plastosemiquinone at the outer site

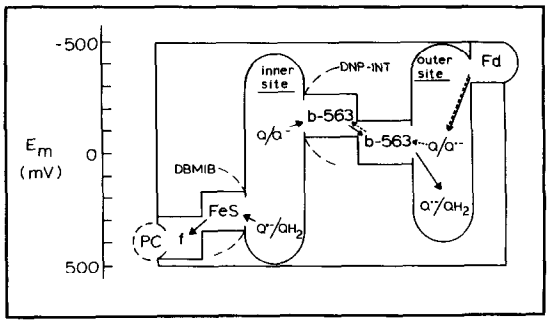

Fig.5. A model of electron transfer in the cytochrome *b*-563/*f* complex. The components are arranged vertically on a midpoint potential scale, and horizontally according to an approximate position in the membrane. The midpoint potentials for the Q/QH_2 couples are arbitrarily assigned to be 100 mV, with semiquinone stability constants of 10^{-7} and 10^{-4} at the inner and outer site, respectively. The E_{m7}-values for two cytochromes *b*-563 measured in [11] are used. The reactions designated by the solid arrows are those which could occur during cycle electron transfer, provided that a quinol was initially available at the 'inner' site and a quinone at the 'outer' site. 'Linear' electron transfer could take place by a similar set of reactions, where photochemistry makes available plastoquinol at the inner site, and oxidizing equivalents to cytochrome *f*. In this case however, cytochrome *b*-563 at the outer site must reduce the quinone to quinol in two steps (not shown) without the aid of ferredoxin. The dotted lines show the pathway of electron transfer during ferredoxin-mediated reduction of cytochrome *b*-563 in the presence of DNP-INT.

can result in reduction of cytochrome *b*-563, as shown here (fig.5, dashed arrows). However, if the cytochrome *b*-563 is already reduced, as in cyclic electron transfer by oxidant-induced reduction of plastoquinone to plastoquinol by ferrocytochrome *b*-563 and reduced ferredoxin at the outer site can occur (solid arrows).

Many of the prerequisites necessary for the use of fig.5 as a model of ferredoxin-mediated cytochrome *b*-563 reduction are consistent with the results reported here and in [16]:

(i) The rate-limiting step (plastosemiquinone reduction of ferricytochrome *b*-563) is probably not normally involved in cyclic electron transfer, hence the slow observed rate;

(ii) The rate of ferredoxin reduction of the plastosemiquinone/plastoquinol couple is slower, but competitive with the rate of plastosemiquinone reduction of ferricytochrome *b*-563, resulting in the incomplete reduction of the cytochrome in spite of the low midpoint potential of ferredoxin;

(iii) The oxidation of cytochrome *b*-563 at the inner site (a 2-step reduction of plastoquinone?) is faster than its reduction at the outer site, so that no net reduction of cytochrome *b*-563 occurs unless inhibitor (DNP-INT, but not DBMIB) is present at the inner site to prevent the reaction between ferrocytochrome *b*-563 and plastoquinone.

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