FERREDOXIN-MEDIATED REDUCTION OF CYTOCHROME *b*-563 IN A CHLOROPLAST CYTOCHROME *b*-563/*f* COMPLEX

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1. Introduction

Chloroplasts catalyze a non-cyclic electron transport from water to NADP which is coupled to the synthesis of ATP. A cyclic electron-transport pathway, which produces only ATP is also present. Although the physiological function of the cyclic pathway is not resolved [1], the cyclic pathway may contribute ATP for CO_2 fixation by chloroplasts [2–4]. The mechanism of the cyclic pathway and the relationship of the electron carriers in the cyclic pathway to those of the non-cyclic pathway remain unresolved problems [5]. One chloroplast electron carrier, cytochrome *b*-563, has been viewed as a participant in cyclic electron transport (review [6]); this component has been considered in relation to 'Q-cycle' mechanisms in proton and electron transfer in chloroplasts [7–9].

A protein complex containing cytochromes *b*-563 and *f* as well as the Rieske iron–sulfur center has been isolated from spinach chloroplasts [10]. The complex catalyzes electron transfer from reduced quinones to high-potential electron acceptors, such as plastocyanin and *c*-type cytochromes, and this electron transport is sensitive to inhibitors of non-cyclic electron transport, such as DBMIB [10]. The Rieske iron–sulfur center has been isolated and a Rieske-depleted preparation obtained [11]. However, no reactions of cytochrome *b*-563 in the complex have been reported, although this preparation would appear to have numerous advantages over unfractionated membranes for the study of the function of this carrier.

Here we show that the cytochrome *b*-563 present in the isolated cytochrome complex can be reduced by reduced ferredoxin generated in the presence of NADPH and ferredoxin-NADP reductase. The effects of non-cyclic and cyclic electron transport inhibitors as well as depletion of the Rieske iron–sulfur center on the reactions of cytochrome *b*-563 are also described.

2. Materials and methods

The cytochrome *b*-563/*f* complex from spinach chloroplasts was isolated according to [10]. In some preparations, the ammonium sulfate precipitate (40–60% saturation) was fractionated on a sucrose density gradient containing 30 mM β-octylglucoside and 0.5% sodium cholate instead of 0.2% Triton X-100. This enabled isolation of the intact complex free of Triton. Preparation of the Rieske-depleted cytochrome *b*563/*f* complex was as in [11]. The cytochrome *f* and *b*563 content was determined by redox difference spectrometry using ferricyanide, ascorbate and dithionite. The extinction coefficients of 18 and 21 $mM^{-1} \cdot cm^{-1}$ are assumed for cytochrome *f* (λ_{max} 554 nm) and cytochrome *b*-563 (λ_{max} 563 nm), respectively. Plastoquinone was extracted and its concentration in absolute ethanol determined as in [12].

Conditions for measuring kinetics of cytochrome *b*-563 reduction by reduced ferredoxin are as follows: to 1.2 ml 50 mM Tricine–KOH (pH 8.0) was added 40–50 μl complex (~16 nmol cytochrome *f*/ml). Then, 0.1 ml 100 mM glucose, 20 μl catalase (3 mg/ml) and 20 μl glucose oxidase (10 mg/ml) were added

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazol; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether

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as an oxygen trap to keep the suspension anaerobic. Since cytochrome *b*-563 is auto-oxidizable, this oxygen trap is essential for studying the reduction of the cytochrome. The mixture was flushed with nitrogen gas and then transferred anaerobically to two separate nitrogen-flushed cuvettes (either 1 cm or 1 mm light path) which were sealed with serum caps. Ferredoxin-NADP reductase (10 μl, 40 μM) and spinach ferredoxin (3 μl, 833 μM) were then injected into the cuvette using Hamilton microsyringes. The reaction was started by the addition of 10 μl NADPH (100 mM) to the sample cuvette. Cytochrome *b*-563 reduction was followed either by monitoring the absorption difference ($A_{563}-A_{575}$) or by rapid scanning in the region from 540–580 nm.

Absorption measurements were made with an Aminco DW-2 spectrophotometer at 22°C. β-Octylglucoside, sodium cholate, glucose oxidase, catalase, and antimycin A were obtained from the Sigma Chemical Co. The inhibitors, DBMIB and DNP-INT, were obtained from Dr A. Trebst and UHDBT was obtained from Dr B. Trumpower. Inhibitors were prepared as concentrated stock solutions in DMSO and added to reaction mixtures in μl amounts. Ferredoxin and ferredoxin-NADP reductase were isolated from spinach leaves by R. K. Chain of our laboratory. All other reagents were of the highest grade available.

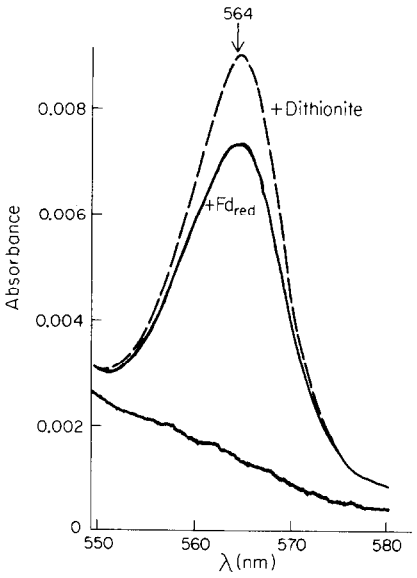

Fig.1. Reduction of cytochrome *b*-563 in the cytochrome *b*-563/*f* complex. The complex used was prepared with a Triton X-100 sucrose density gradient, according to [10]. Conditions were as described in section 2. Cuvettes with a 1 mm light path were used. The reduction was started by the addition of NADPH to the sample cuvette. After 50 min, the absorbance spectrum from 550–580 nm was recorded. The spectrum in the presence of dithionite was then obtained after the addition of a few grains of sodium dithionite to the sample cuvette.

3. Results and discussion

Fig.1 shows that cytochrome *b*-563 in the cytochrome *b*-563/*f* complex can be reduced by reduced ferredoxin which is generated by the presence of NADPH and the ferredoxin-NADP reductase. The difference spectrum, with a peak at 564 nm, confirms the absence of the low-potential form of cytochrome *b*-559 in the preparation. The reduction of several electron carriers, including cytochrome *b*-563, in unfractionated chloroplast membranes by reduced ferredoxin has been reported [5,13,14]. In contrast to [14], Mg^{2+} were not required for reduction of the cytochrome in the isolated cytochrome complex, but there was an absolute requirement for ferredoxin, NADPH and the reductase. One observation of interest is that not all of the cytochrome *b*-563 present in the complex (based on reduction by sodium dithionite) can be reduced by reduced ferredoxin. About 20% of the total cytochrome *b*-563 was not reduced even after prolonged incubation with reduced ferredoxin. This observation suggests the presence of a heterogeneous population of cytochrome *b*-563 in the isolated cytochrome complex.

Fig.2 shows the kinetics of the reduction of cytochrome *b*-563 by reduced ferredoxin. The effects of the electron-transport inhibitors, DNP-INT and antimycin A, are also shown. Cytochrome *b*-563 reduction by reduced ferredoxin is inhibited slightly by antimycin A while DNP-INT shows a dramatic stimulation of the rate of reduction. Neither DNP-INT nor antimycin A alters the absorption spectrum of the cytochrome (not shown). A striking characteristic of the ferredoxin-dependent reduction of cytochrome *b*-563 in the cytochrome complex is its slow rate. This slow rate of reduction was not observed in unfractionated membranes. One possible explanation for this phenomenon might be that the site of interaction between the cytochrome and ferredoxin is protected behind a hydrophobic barrier from the external medium. The sensitivity of the reduction to antimycin A suggests that this inhibitor inhibits the process at

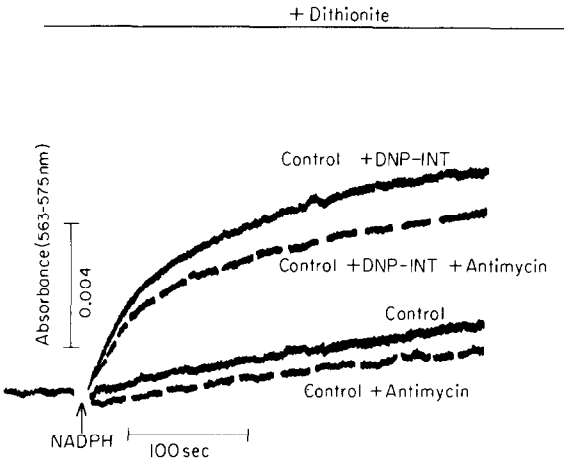

Fig.2. Kinetics of the reduction of cytochrome *b*-563 by reduced ferredoxin and effects of electron transport inhibitors. The complex used was prepared with an octylglucoside-cholate sucrose density gradient. Conditions were as in section 2. Cuvettes with a 1 cm light path were used. Final concentrations of inhibitors were: DNP-INT, 4.2 μM; antimycin A, 46 μM.

the cytochrome *b* level. This mode of action of antimycin A had been proposed in the case of mitochondria [15] and bacterial chromatophores [16]. The stimulation of the reduction by DNP-INT can be explained by one or both of two possibilities:

(1) The midpoint redox potential (E_m) of the cytochrome is increased by the presence of DNP-INT;
(2) The interaction between the cytochrome complex and DNP-INT resulted in exposure of the site of interaction of cytochrome *b*-563 to reduced ferredoxin.

We have examined the E_m of the cytochrome in the isolated complex in the presence and absence of DNP-INT (in collaboration with E. Hurt, Regensburg) and have obtained an E_m-value of -120 mV ± 20 mV at pH 6.5 in both cases. Thus, alteration of the E_m of cytochrome *b*-563 by DNP-INT does not appear to be a factor in the increased rate of reduction in the presence of the inhibitor. The cytochrome *f* present in the isolated complex is in the reduced state, and the addition of ascorbate to the reaction medium did not affect the rate of reduction of cytochrome *b*-563.

The effects of quinone analogues (DBMIB and UHDBT) which act as electron-transfer inhibitors have also been studied. DBMIB causes only a marginal stimulation of the rate of cytochrome *b*-563 reduction in the same concentration range as DNP-INT. UHDBT shows a stimulation of cytochrome *b*-563 reduction comparable to that of DNP-INT. It is also noted that in all cases, the final level of cytochrome *b*-563 reduction was 20% less than the amount reducible by dithionite. Thus, the heterogeneity of the cytochrome population was not affected by any of the inhibitors studied. The same extent of cytochrome reduction was observed in a preparation made as in [10]. Since the original preparation is in Triton X-100 while the present material is in octylglucoside plus cholate, the extent of reduction is independent of these detergents.

Fig.3 shows that cytochrome *b*-563 in the Rieske-depleted cytochrome complex can be reduced by reduced ferredoxin much more readily than in the intact complex (cf. fig.2). However, no E_m difference was detected for the cytochrome in the Rieske-depleted complex as compared with the intact complex. As with the intact complex, the rate of reduction was slightly inhibited by antimycin A and markedly stimulated by DNP-INT. It is interesting to note that all (98%) of the dithionite-reducible cytochrome *b*-563 in the depleted complex is now accessible to reduced ferredoxin. Thus, depletion of the Rieske Fe–S protein seems to abolish the heterogeneity present in the cytochrome *b*-563 population of the native complex. Again, antimycin A and DNP-INT

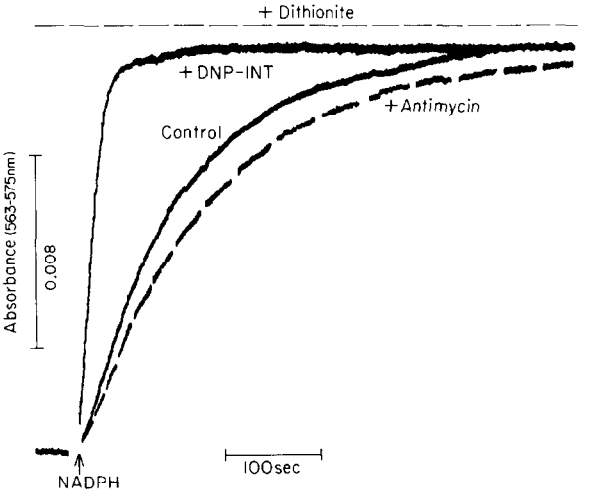

Fig.3. Kinetics of the reduction of cytochrome *b*-563 in the Rieske-depleted cytochrome *b*-563/*f* complex. Conditions were as in fig.2 except that the cytochrome complex depleted of the Rieske iron–sulfur center was used.

had no effect on the E_m of the cytochrome or on its absorption spectrum in the depleted complex, and only the rate of reduction was affected.

Here, we have primarily used a preparation of the cytochrome *b*-563/*f* complex which is isolated without the use of Triton X-100. In contrast to the original preparation [10], which contains 0.2 mol plastoquinone/mol cytochrome *f*, the present preparation contains plastoquinone and cytochrome *f* in a 1:1 ratio. However, when the cytochrome complex is depleted of the Rieske iron–sulfur center as in [11], we find that plastoquinone is also removed and that the only electron carriers in the depleted complex are cytochromes *b*-563 and *f*.

Although previous EPR studies have obtained evidence for an interaction of the inhibitor DNP-INT with the Rieske center in the cytochrome complex (unpublished), the present results indicate DNP-INT can still affect cytochrome *b*-563 reduction in a Rieske-depleted complex. It can also be concluded that plastoquinone is not an obligatory component in the binding of DNP-INT to the complex. This could be interpreted as indicating a close association between cytochrome *b*-563 and the Rieske center in the intact complex, such that inhibitor binding at or near cytochrome affects the EPR properties of the Rieske iron–sulfur center.

The results with the cytochrome complex depleted of both plastoquinone and the Rieske center indicate that neither of these components is required for reduction of cytochrome *b*-563 in the complex by reduced ferredoxin. On the basis of studies of the inhibition by DBMIB of cytochrome *b*-563 oxidation and reduction in intact chloroplasts, a role for plastoquinone was suggested in both reduction and oxidation of the cytochrome [8]. The mechanism of plastoquinone involvement in cytochrome *b*-563 reduction has not been clarified, but our findings suggest an alternative pathway for reduction of the cytochrome exists which does not require plastocyanin. Because of the relatively simple composition of the depleted cytochrome complex, it is likely this involves a direct interaction between reduced ferredoxin and cytochrome *b*-563.

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