In vitro reconstitution of the electron pathway from water to cytochrome *f* of spinach thylakoids

Eric Lam and Richard Malkin +

Department of Biophysics and + *Division of Molecular Plant Biology, University of California, Berkeley, CA 94720, USA*

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Spinach chloroplasts *Electron-transfer pathway* *Cytochrome* f *water* *Redox protein* *Thylakoid-membrane*

1. INTRODUCTION

The photosynthetic apparatus in thylakoid membranes of higher plant chloroplasts contains 3 integral protein complexes that are involved in electron transfer from water to NADP: photosystem II, the cytochrome b_6–*f* complex and photosystem I. Physiologically, electron transfer through these complexes involves light absorption by the 2 photosystems and mediation of electron transfer between the 2 light reactions by the cytochrome complex (review [1]). Electron transfer between the light reactions also results in proton translocation across the thylakoid membrane which ultimately yields ATP [2].

It is known that photosystem II oxidizes water and reduces plastoquinone [3]. The reduced plastoquinone transfers electrons to plastocyanin via the Rieske iron–sulfur center and cytochrome *f* [1,2,4]. Reduced plastocyanin then donates an electron to the reaction center chlorophyll of photosystem I, P700 [5]. Cytochrome b_6, plastoquinone, the Rieske iron–sulfur center, cytochrome *f* and plastocyanin have also been implicated in cyclic electron flow around photosystem I [6,7]. One approach to elucidate the mechanism and relationship of the various components involved in the photosynthetic apparatus involves isolation and characterization of the chloroplast electron transfer complexes. Subsequent reconstitution of the different segments of the electron-transfer pathway may provide a valuable tool in the study of chloroplast electron-transfer events. This approach has been successful with the electron-transfer protein complexes of mitochondria [8] but has not yet been actively pursued in studies with chloroplasts, although recent reports have described the interaction of a chloroplast electron-transfer complex (the cytochrome b_6–*f* complex) with reaction centers from photosynthetic bacteria [9] and a purified photosystem I preparation [10].

Photosystem I complexes have been described which are free from photosystem II, the light-harvesting chlorophyll *a*/*b* protein and the cytochrome b_6–*f* complex [11,12]. Recently, the isolation and characterization of a chloroplast cytochrome complex preparation has been described [13]. This preparation contains cytochrome *f*, cytochrome b_6, and the Rieske iron–sulfur center in the ratio of 1:2:1 and has been shown to catalyze electron transfer from plastohydroquinone to plastocyanin [13]. Photoreduction of cytochrome b_6 and cytochrome *f* photooxidation can be demonstrated when the isolated cytochrome b_6–*f* complex is mixed with a purified photosystem I preparation [10], and photoreduction of both cytochromes has been observed in a reconstructed system involving the bacterial reaction center and the cytochrome complex [9]. The activities of the isolated cytochrome complex have been shown to be sensitive to non-cyclic elec-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DNP–INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitro-diphenyl ether; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

+ To whom correspondence should be addressed

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tron-transport inhibitors such as DBMIB and DNP–INT [13]. A photosystem II preparation devoid of photosystem I as well as the cytochrome complex has been described in [14]. This preparation is distinguished from other photosystem II preparations by its resolution with respect to contamination from photosystem I or the cytochrome complex [15,16] and its ability to evolve oxygen. This report demonstrates the reconstitution of electron transfer from water to the cytochrome complex as measured in the photoreduction of cytochrome *f* in the isolated b_6–*f* complex by electrons from the oxygen-evolving photosystem II complex. This photoreduction process is shown to be inhibited by known inhibitors of non-cyclic electron transport and is probably dependent on the presence of the Rieske iron–sulfur center and/or the bound plastoquinone associated with the cytochrome complex.

2. MATERIALS AND METHODS

The cytochrome b_6–*f* complex and photosystem II particles were isolated from freshly-picked spinach as in [13,14]. During the preparation of the cytochrome complex, the ammonium sulfate precipitate was fractionated on a sucrose density gradient containing 30 mM β-octylglucoside and 0.5% sodium cholate instead of 0.2% Triton X-100 [17]. This Triton-free preparation contains 1 bound plastoquinone molecule/cytochrome *f* as determined by extraction studies (unpublished). The Rieske Fe–S center can be resolved from the cytochrome b_6–*f* complex as in [18]. The depleted complex is found to have lost the bound plastoquinone molecule (E. L., unpublished). Cytochrome *f* in the cytochrome complex was oxidized by incubation with ferricyanide and then dialyzed overnight with 200 mM potassium phosphate (pH 6.8), plus 0.05% Triton X-100. The cytochrome *f* content was determined by redox difference spectrometry using ferricyanide and ascorbate. An extinction coefficient of 18 $mM^{-1} \cdot cm^{-1}$ at 554 nm was used in calculations. Plastoquinone was extracted and its concentration determined as in [19].

Measurement of cytochrome *f* reduction by photosystem II particles was performed in an Aminco DW-2 Spectrophotometer equipped with side actinic illumination. A Corning 4-96 filter was placed in front of the photomultiplier tube while actinic light passed through a Corning 2-64 filter before reaching the sample. All studies were done at 22°C. β-Octylglucoside, sodium cholate, sodium ascorbate and Triton X-100 were obtained from Sigma Chem. Co. DNP-INT was a gift from Dr A. Trebst and UHDBT was obtained from Dr B. Trumpower. Inhibitors were prepared in DMSO and added to reaction mixtures in microliter amounts. All other reagents were of the highest grade available.

3. RESULTS AND DISCUSSION

The photosystem II preparation described in [14] catalyzes ferricyanide-dependent oxygen evolution; we have demonstrated the DCMU-sensitivity of this reaction. We have also been able to observe during fluorescence induction studies the photoreduction of Q and the photoreduction of a secondary acceptor pool containing ~7 plastoquinone molecules. The photoreduction of the pool was inhibited by DCMU. These results indicate the photosystem II preparation can reduce endogenous plastoquinone with water as the electron donor. The cytochrome b_6–*f* complex catalyzes the reduction of high-potential electron acceptors, such as plastocyanin, with reduced quinones as electron donors [13]. We therefore were interested in testing the possibility of reconstituting electron transfer into the cytochrome complex using the resolved photosystem II preparation, and we have studied this reconstitution by observing the photoreduction of cytochrome *f* in the complex.

The photoreduction of cytochrome *f* in the cytochrome complex by photosystem II particles from spinach thylakoids in shown in fig.1. It was found that this reduction is stimulated by the presence of Mg^{2+} and an incubation of the complex with photosystem II particles prior to illumination (not shown). Difference spectrum (fig.1, inset) confirms the light-induced absorbance change is due to cytochrome *f*. The level of cytochrome *f* reduced after the addition of ascorbate is also shown. This indicates all the cytochrome *f* in the complex undergoes photoreduction.

The effects of several electron-transfer inhibitors on the photoreduction of cytochrome *f* are shown in fig.2. DCMU and UHDBT, inhibitors known to inhibit the reduction of plastoquinone by the primary acceptor of photosystem II [20,21], both inhibited the photoreduction process completely. The photoreduction was also inhibited by Tris-treat-

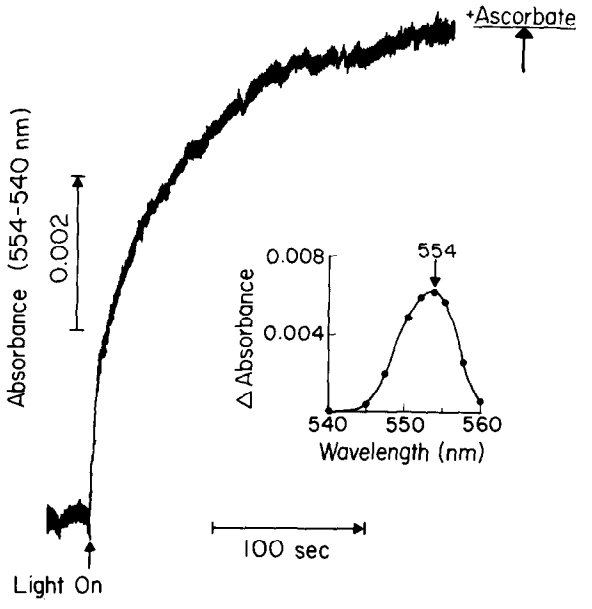

Fig.1. Reconstitution of the photosystem II photoreduction of cytochrome *f* in a cytochrome b_6–*f* complex. The mixture contains 0.64 μM cytochrome *f* and 54 μg chl/ml (reaction center concentration of 0.25 nm based on photoreduction of Q measured at 320 nm) in 15 mM NaCl, 5mM $MgCl_2$ and 20 mM MES at pH 6.0. The mixture is incubated for 4–5 min. at 22°C prior to assay. Light path, 1 cm. Where indicated by the arrow, a few grains of sodium ascorbate is added to the mixture.

ment of the photosystem II preparation. This treatment specifically inactivates water oxidation [22]. The photoreduction of cytochrome *f* could be restored by the addition of diphenylcarbazide to the Tris-treated material. This compound is a specific photosystem II electron donor [23,24]. These results indicate that water is the source of electrons for cytochrome *f* reduction and suggest that plastoquinone is the immediate electron donor to the cytochrome b_6–*f* complex. The inhibitor DNP–INT is observed to inhibit the initial fast phase of cytochrome *f* reduction. Under identical conditions oxygen evolution by the photosystem II particles with potassium ferricyanide as electron acceptor was relatively insensitive to DNP–INT. Thus, the observed inhibition of the photoreduction of cytochrome *f* in our system must be due to interaction of DNP–INT with the cytochrome b_6–*f* complex. DNP–INT is an effective inhibitor of plastoquinone–plastocyanin oxidoreductase activity of the complex [13] and a direct interaction between DNP–INT and the Rieske Fe–S center has also been detected by EPR spectroscopy [17]. This observation strongly suggests that the reduction of cytochrome *f* involves the electron carriers in the complex and is not a direct reduction of the cytochrome by reduced plastoquinone in the photosystem II preparation.

The Rieske iron–sulfur center can be resolved from the isolated complex by hydroxyapatite chromatography [18]. After extraction of the Rieske center, the reduction of cytochrome *f* by photosystem II particles is inhibited drastically (fig.3). The cytochrome *f* in the depleted complex remains ascorbate-reducible and it is reasonable to conclude that its E_m in the depleted complex is not significantly decreased during the resolution of the Rieske center. This observation again suggests that the cytochrome *f* in our system is not directly reduced by photosystem II but requires specific complex-bound electron carriers. The Rieske-depleted b_6–*f* complex was also depleted of a bound plastoquinone molecule found to be associated with the intact cytochrome b_6–*f* complex. Our observations

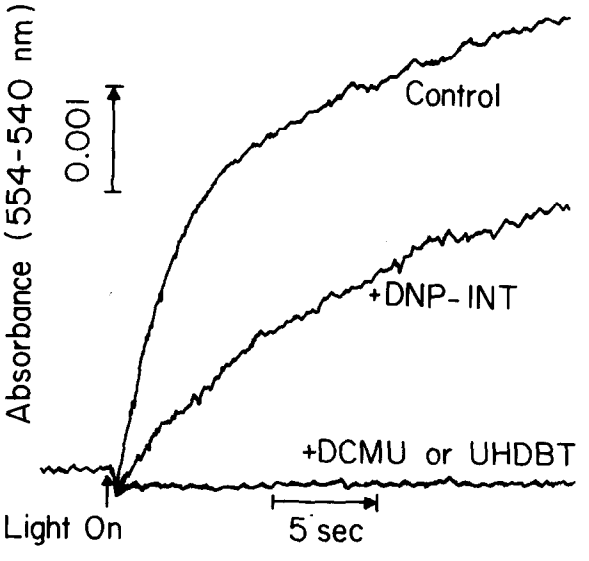

Fig.2. Effects of inhibitors on the photoreduction of cytochrome *f* in a reconstituted system. Conditions are as in fig.1. Where indicated, DNP–INT (1 μM), DCMU (10 μM) or UHDBT (1.3 μM) were added 2 min before assay.

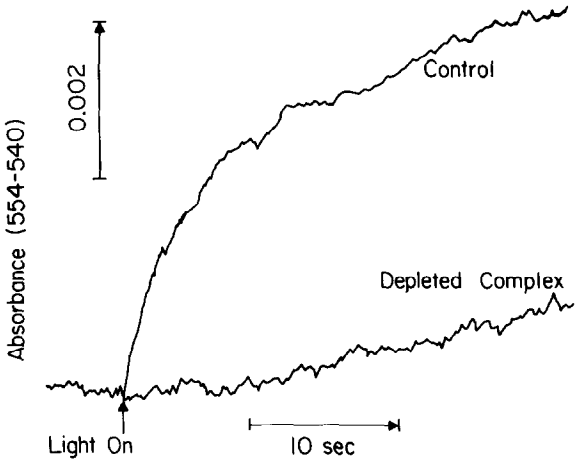

Fig.3. Effects of Rieske and bound plastoquinone-depletion on photoreduction of cytochrome *f* in a reconstituted system. The assay system is identical to that of fig.1. The depleted complex contains the same amount of ascorbate-reducible cytochrome *f* as that of the native complex.

thus indicate a possible requirement for these electron transfer carriers in mediating electron transfer from photosystem II to cytochrome *f*, but studies involving reconstitution of these carriers are required to define their respective roles in greater detail.

By monitoring cytochrome b_6 absorbance changes (563–575 nm), it has also been possible to observe transient photoreduction of this carrier in the reconstituted electron transport system described above. The properties of this photoreduction, which will be described subsequently in greater detail, indicate cytochrome b_6 is being reduced via an 'oxidant-induced reduction' pathway, similar to that in [9].

We demonstrate for the first time the reconstitution of electron flow from water to cytochrome *f* with isolated integral protein complexes from thylakoid membranes. The sensitivity to inhibitors and the requirement for specific electron carriers in the cytochrome complex show that the observed reduction proceeds via a similar route to that of the in vivo system. No soluble cofactors or proteins were required to mediate electron transfer from the photosystem II complex to the cytochrome complex here and a lipid requirement was not observed. It is anticipated that the present system will be useful in the elucidation of the physiological mechanism of the transfer of electrons between photosystem II and the proton-translocating chloroplast cytochrome complex.

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