Construction of noncyclic electron flow from chloroplast photosystem II and mitochondrial cytochrome b–c_1 complex to cytochrome c

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Photosystem II *Cytochrome* c_1 *Cytochrome* b *Reconstitution*

1. INTRODUCTION

The cytochrome b–c_1 complex of the mitochondrial inner membrane is a site of energy transduction in oxidative phosphorylation. It contains two b-type cytochromes, a c-type cytochrome, the Rieske iron–sulfur center, and ubiquinone. In addition, a number of 'core-proteins' are present in active preparations of this complex [1]. Despite numerous laboratories' intensive efforts, a generally accepted model of electron transfer through this complex is still lacking. Recent studies by Trumpower and coworkers [2,3] and other laboratories [4–6] have supported a 'Q-cycle' mechanism for electron flow, although the exact sequence of events is yet unknown.

In order to study the kinetics of redox reactions of the various components in the cytochrome b–c_1 complex, a light-triggered electron donation system would be valuable since this donor system could bypass the inherent difficulties encountered in substrate addition methods (i.e., time resolution, mixing of substrate, etc.). In recent years the development of such a system by Dutton and coworkers [7,8] has been described. In these studies, reaction center preparations from the photosynthetic bacterium *R. sphaeroides* were mixed with the isolated cytochrome complex from beef heart in the presence of cytochrome c. Cyclic electron flow could then be demonstrated after flash illumination. This system has recently been extended to studies of the cytochrome b_6–f complex from spinach thylakoids [9]. One of the disadvantages of this system is that more than one type of c cytochrome is present, making a clear assignment of absorbance changes in the 550 nm region somewhat difficult. We have recently reconstructed noncyclic electron flow from photosystem (PS) II to cytochrome f by mixing an oxygen-evolving PSII preparation with the purified b_6–f complex [10]. No soluble cofactors were required in this system, and the only c type cytochrome present was the bound cytochrome f. In the present report, we present results which demonstrate electron flow from PSII through the mitochondrial cytochrome b–c_1 complex. Inhibitor sensitivities of this system and the interaction between cytochrome c and the cytochrome b–c_1 complex were studied.

2. MATERIALS AND METHODS

The PSII preparation was isolated by the method of Berthold et al. [11] with the following modification: the solubilization step with Triton X-100 was carried out in pH 7.2 instead of pH 7.5. This method yields a preparation which evolves oxygen at a rate of 180 μmol/mg chlorophyll (Chl) per hour. This preparation, characterized by a sensitive spectrophotometric method [12], contained

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about 1 PSII reaction center/250 Chl (based on measurements of reduction of Q, the PSII electron acceptor) and negligible Photosystem I (1 P_{700}/20000 Chl based on measurements of P_{700} oxidation). No cytochrome b_6 or f could be observed by chemical redox difference spectrometry and the cyt. b_{559}/Chl ratio was about 1/130 (E. Lam, unpublished data). No light-induced absorbance change of the cytochrome b_{559} could be detected in the absence or presence of ferricyanide under our present conditions. From fluorescence studies and extraction with petroleum ether [13], it was determined that the PSII preparation contained a photoreducible plastoquinone pool whose reduction could be effectively inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

The cytochrome b–c_1 complex was obtained by the method in [14] and was provided by Dr B. Trumpower. Essentially, the succinate-cytochrome c oxidoreductase was first isolated and subsequently washed with alkaline buffer to remove succinic dehydrogenase. Although SDS–PAGE analysis of the complex showed that some large subunit of succinate dehydrogenase was still present, no succinate-cytochrome c reductase activity could be observed with this preparation. Duroquinol, however, was an effective electron donor for cytochrome c reduction catalyzed by the cytochrome complex. This preparation was stored at −20°C until use. The concentration of cytochrome c was estimated from redox difference spectrum using an extinction coefficient of 20 mM^{-1} cm^{-1} at 550 nm.

For light-induced absorbance studies, a 1-ml cuvette with 1 cm lightpath and polished sides was used. An Aminco DW-2a spectrophotometer equipped with side illumination was employed. 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. Cytochrome c (horse heart), Triton X-100 and antimycin A were obtained from Sigma Chem. Co. All other reagents were of the highest grade available. Myxothiazol was a generous gift from Dr Reichenbach of Germany.

3. RESULTS AND DISCUSSION

Photoreduction of horse heart cytochrome c by PSII under various conditions is shown in fig.1. It

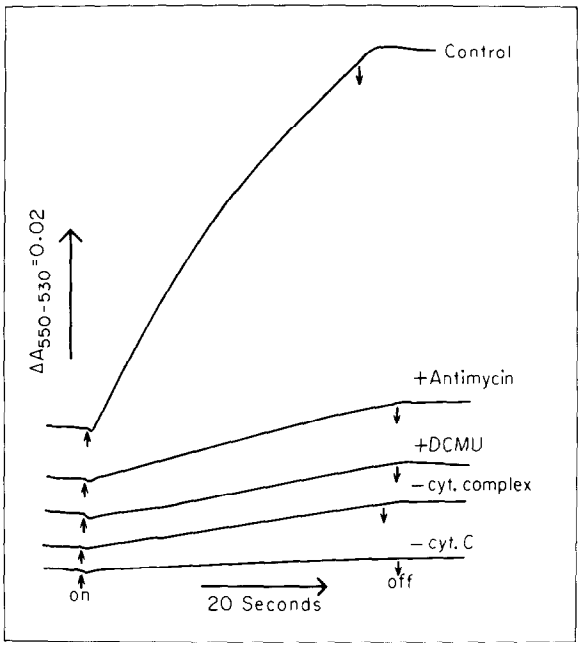

Fig.1. Photoreduction of cytochrome c by PSII with the cytochrome b–c_1 complex from bovine heart mitochondria. The assay medium contained 20 mM Mes (4-morpholineethane–sulfonic acid), buffer (pH 6.0), 5 mM $MgCl_2$ and 15 mM NaCl. The concentrations of the various additions were: PSII preparation (66 μg Chl per ml), cytochrome b–c_1 complex (0.8 μM cytochrome c_1) and 15 μM cytochrome c. When present, 12 μM DCMU and 3.2 μM antimycin A were added in small aliquots of ethanolic solution.

is obvious that the presence of the cytochrome complex dramatically enhanced the rate of photoreduction of cytochrome c. In the absence of cytochrome c, no change in absorbance at 550 nm was observed, while in the presence of antimycin A or DCMU, the b–c_1 catalyzed photoreduction process was drastically reduced. Myxothiazol, another specific inhibitor of the b–c_1 region of mitochondrial respiration [15], also completely inhibited the b–c_1-catalyzed reduction of cytochrome c and, in the absence of PSII, no light-induced absorbance changes were observed (data not shown). These observations establish that PSII can photoreduce the electron carriers in the b–c_1 complex which then transfer these electrons to soluble cytochrome c.

In order to demonstrate that the light-induced absorbance changes are due to cytochrome c reduction, we obtained a light minus dark spectrum

of the system described in fig.1. As shown in fig.2, an absorbance peak of 550 nm, characteristic of reduced cytochrome *c*, was observed. A point worth noting is that no oxidation of the reduced cytochrome *c* was observed after illumination. This observation leads us to conclude that there are no dark oxidation reactions involving the reduced cytochrome *c* and this provides evidence that our system does not contain a cyclic pathway involving the cytochrome.

The sensitivity of the reconstructed system to antimycin A was also investigated. As shown in fig.3, at an antimycin/cytochrome c_1 ratio of about 1, 70% of the activity was inhibited. This is slightly less inhibition than that observed with duroquinol or succinate as reductant for the b–c_1 complex [16] which gave $\gtrsim 85\%$ inhibition at inhibitor/c_1 ratio of 1. The slightly lower inhibitor sensitivity in the reconstructed system might be due to nonspecific binding of antimycin A to the PSII preparation in our system.

We have applied the present system to study the kinetic properties for the cytochrome b–c_1-catalyzed reduction of cytochrome *c* in the reconstructed system. An advantage of the present light-induced reduction system is the ability to resolve the initial rates quite easily. As the Lineweaver–Burk plot in fig.4 shows, a clear biphasic behavior was observed for the interaction of cytochrome *c* with its reductant, the b–c_1 complex. The low affinity binding site, shown in fig.4A has a K_m of about 10.2 μM at a c_1 concentration of 0.8 μM. The high affinity binding site, under identical conditions, showed a K_m of 0.47 μM (cf. fig.4B). The V_{max} of the reduction process, as extrapolated in fig.4, has the value of about 0.17 nmol cytochrome *c* reduced per second. Under our conditions, this corresponds to a turnover rate of 0.2 s^{-1} for cytochrome c_1. The turnover rate of cytochrome c_1 might be limited by the accessibility of the cytochrome complex to the plastoquinone pool of the PSII preparation. No stimulation by preincubation of concentrated complexes and no requirement for cations were observed in the present system, in contrast to our earlier report on the interaction between PSII and the b_6–f complex from spinach [10]. Thus, the interactions of the two cytochrome complexes with PSII is probably quite different. However, the fact that the cytochrome b–c_1 complex is required for the mediati-

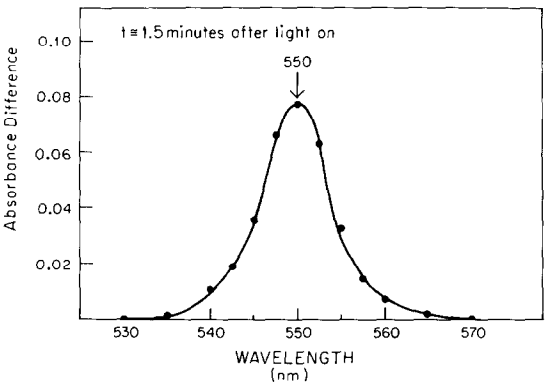

Fig.2. Difference spectrum of cytochrome *c* photoreduction by PSII and the cytochrome b–c_1 complex. The conditions were the same as those in the *Control* of fig.1 except that split beam mode was used instead of dual beam mode. The reference cuvette contained 12 μM DCMU. After 1.5 min of illumination, the spectrum was recorded from 530 to 570 nm. The difference between the light and dark spectrum was then calculated manually. The absorbance at 570 nm was used as a reference wavelength.

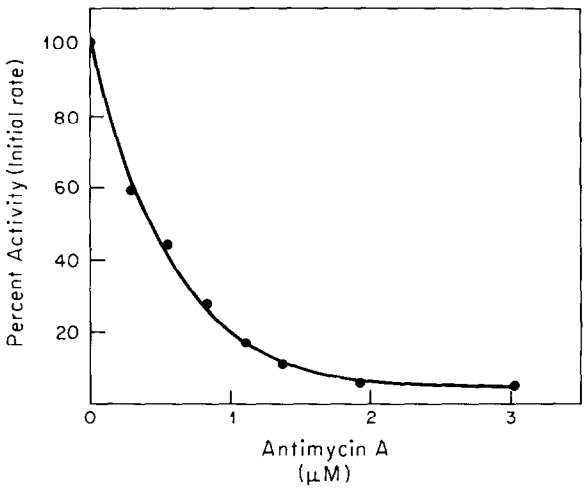

Fig.3. Effect of antimycin A on cytochrome b–c_1 complex-dependent photoreduction of cytochrome *c*. The conditions were identical to that of the control in fig.1. Antimycin A is added in microlitre aliquots to identical samples and the initial rate of cytochrome *c* reduction was then calculated. The rate of cytochrome *c* reduction in the absence of the cytochrome complex was subtracted from each of these rates to obtain the cytochrome complex-catalyzed rate.

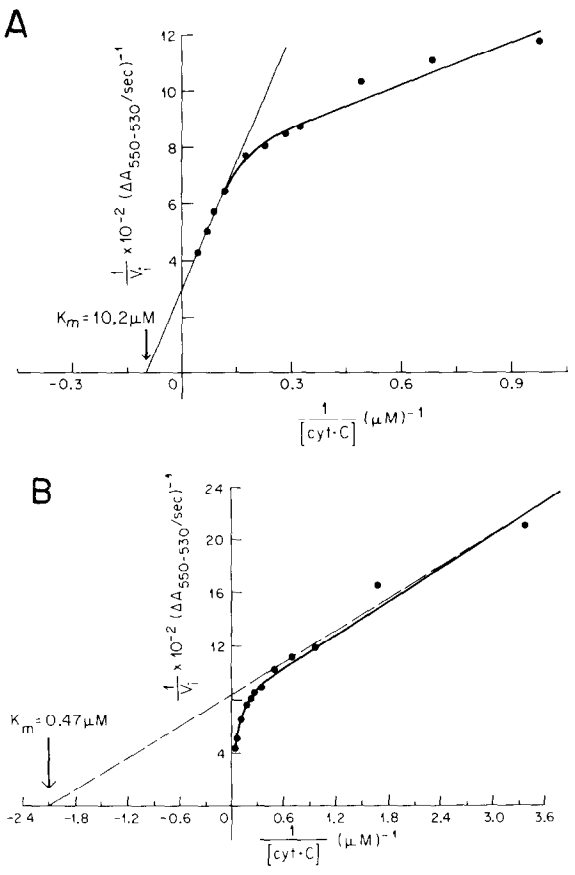

Fig.4. Lineweaver–Burk plot of the reduction of cytochrome *c* by the cytochrome *b*–c_1 complex. The assay medium, similar to that of fig.1, contained cytochrome *b*–c_1 complex (0.8 μM cytochrome c_1) and PSII (66 μg Chl per ml). The initial rate of photoreduction of cytochrome *c* was titrated by the amount of cytochrome *c* (~90% oxidized) added. The difference in A and B is the scale of the coordinates.

on of electron flow between the PSII complex and soluble cytochrome *c* suggests that reduction of electron carrier(s) in the cytochrome complex by the quinone pool of PSII is considerably more facile than the direct reduction of cytochrome *c*. This might arise from higher hydrophobicity of the electron carrier(s) (e.g., ubiquinone) in the cytochrome complex which probably enhances their accessibility to the plastoquinone pool.

In the present study we have introduced a novel system to generate reducing equivalents from water by a PSII preparation isolated from spinach. When illuminated, this system can donate electrons to cytochrome *b*–*c* complexes. No stable oxidant is generated that might induce a cyclic flow of electrons as in the system described by Dutton and coworkers [7,8]. Thus, our present system provides a means by which one can control the rate of electron donation to the cytochrome complex by either light intensity or PSII concentration in addition to having the advantage of being able to measure initial reaction rates more easily and accurately. One finding of interest in the present report is the observation of two apparent binding sites for cytochrome *c* on the *b*–c_1 complex. As reviewed in [17], previous studies have defined only one binding site for cytochrome *c* on the reductase. Other studies, however, have shown the possibility of one or more binding sites depending on conditions of the system [18,19]. At present, we cannot explain this discrepancy. The accuracy of the determination of initial rates of the reduction process might be a factor, especially at low concentrations of cytochrome *c*. Alternatively, a heterogeneity in the cytochrome *b*–c_1 complex preparation used might also give rise to our observation.

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