Biochimica et Biophysica Acta 852 (1986) 112–118
Elsevier

BBA 42142

A pre-steady-state kinetic study of electron transfer in the isolated cytochrome *bf* complex from spinach

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(Received 22 May 1986)

Key words: Cytochrome *bf* complex; Cytochrome *f*; Cytochrome *b*-563; Rieske iron-sulfur center; Electron transfer; (Kinetics)

The stopped-flow and rapid-freeze-EPR methods were used to study the reaction of the solubilized cytochrome *bf* complex from spinach with duroquinol and plastocyanin. Both cytochrome *f* and cytochrome *b*-563 show biphasic kinetics with duroquinol. The initial phase of reduction of the cytochromes shows second-order kinetics with a common rate constant of $1.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. The rapid phase is followed by further slow reduction of cytochrome *f* and reoxidation of cytochrome *b*-563. The Rieske iron-sulfur center is reduced together with cytochrome *f* but remains at a higher level of oxidation during the reaction. The reaction of the partially reduced complex with plastocyanin indicates that the Rieske center and cytochrome *f* equilibrate rapidly. The quinone antagonists DBMIB and DNP-INT both inhibit the reduction of the cytochromes. The results are consistent with a mechanism where the semiquinone resulting from the reduction of the Rieske center reduces a low-potential *b*-563 as has been suggested for a Q-cycle model for the cytochrome *bf* complex.

Introduction

Photosynthetic electron transport from water to NADP is mediated by three membrane-bound supramolecular electron-transport complexes; the light-driven Photosystems I and II and an interconnecting cytochrome *bf* complex. This complex contains one *c*-type cytochrome (*f*), two *b*-cytochromes (*b*-563 or b_6) and a Rieske-type iron-sulfur center and takes part in the electron transport by accepting electrons from Photosystem II via plastoquinol and delivering these to plastocyanin. The complex is also involved in a cyclic electron transport around Photosystem I (reviewed in Ref. 1).

There are structural analogies between the cytochrome *bf* complex and the cytochrome *bc* complexes in bacterial and mitochondrial electron-transport systems as well as similarities in the reactions of the complexes (reviewed in Refs. 2 and 3), e.g., the observation of an 'oxidant-induced' reduction [4]. This has supported the view that the cytochrome *bf* complex may take part in a vectorial transport of protons across the thylakoid membrane, e.g., in a Q-cycle (reviewed in Ref. 5). In such a mechanism the quinol first reduces the Rieske center/cytochrome *f* and the resulting semiquinone is then able to act as a

Abbreviations: DBMIB, 3,5-dibromomethylisopropyl-*p*-benzoquinone; DNP-INT, 2′,4′-dinitrophenyl ether of 2-iodo-4-nitrothymol; DQH_2, duroquinol; Pipes, piperazinediethanesulfonic acid.

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reductant for the *b* cytochromes which have a lower reduction potential. Although this sequence of events has gained some experimental support [5], it has been contradicted by observations that the reduction of cytochrome *b*-563 may precede that of cytochrome *f* [6,7].

With the development of methods for the isolation of the cytochrome *bf* complex [8,9] new possibilities have opened for the study of the electron transfer catalyzed by the complex. For example, optical studies of the reaction are simplified when the chlorophyll is absent, and characterization of the reduction and oxidation of the complex can easily be made in separate experiments. With the high concentration of the complex now possible, a direct comparison can be made between optical and EPR studies of the reactions, which may shed further light on the mechanism. Also, EPR allows a direct observation of the Rieske iron-sulfur center which is not normally accessible with optical methods.

In this communication we report on the results from rapid-mixing kinetic experiments, where optical and EPR methods were used to investigate the reaction of the isolated cytochrome *bf* complex with duroquinol and plastocyanin. The reduction kinetics were also studied in the presence of the quinone antagonists DBMIB and DNP-INT. The results are in agreement with the proposed Q-cycle.

Materials and Methods

Protein preparation

The cytochrome *bf* complex was prepared from spinach as in Ref. 10 with the following exception: Pipes-NaOH (pH 6.5) was used instead of Tris-succinate buffer. The final solution contained the cytochrome *bf* complex in 0.7 M sucrose/30 mM Pipes-NaOH (pH 6.5)/30 mM octylglucoside/ 12.5 mM sodium cholate. When necessary, the complex was concentrated by ultrafiltration (Amicon Diaflo YM100 filter). The preparation was frozen as small beads (2–5 mm) in liquid nitrogen and stored at 77 K until used. The cytochrome *bf* complex was fully oxidized as prepared. However, if the sucrose was omitted in the final buffer medium the complex had a tendency to autoreduce. Partial reduction of the complex was obtained by adding 5- to 10-fold excess of ascorbate 5–10 min before use. To suppress inhibitor-induced reduction of the complex with DBMIB [11] *Polyporus versicolor* laccase was added (its molar concentration was 1% of the cytochrome *f* concentration). The rate of reoxidation of the complex with laccase was much slower than other reactions studied.

Plastocyanin was prepared from spinach as in Ref. 12 or isolated from the supernatant from the last ammonium sulfate fractionation in the cytochrome *bf* complex preparation [10] by ion-exchange chromatography and gel filtration. The plastocyanin was stored in 0.7 M sucrose and 30 mM Pipes-NaOH (pH 6.5) at −20°C until used.

Chemicals

Plastoquinone-1 was synthesized as in Ref. 13. The purity was judged from its NMR spectrum [14] to be more than 95%. Before use, the quinone was reduced to plastoquinol-1 with dithionite [13]. Duroquinol was purchased from Pfaltz & Bauer Inc., U.S.A. DBMIB (3,5-dibromomethylisopropyl-*p*-benzoquinone) and DNP-INT (the 2′,4′-dinitrophenyl ether of 2-iodo-4-nitrothymol) was a kind gift from Prof. A. Trebst, Bochum. All other chemicals used were of analytical grade.

Stopped-flow and rapid-freeze

Stopped-flow experiments were carried out at 22°C with an apparatus described in Ref. 15 connected to a Data General Nova 3 minicomputer.

Rapid-freeze experiments were performed as in Ref. 16 with an Update Instruments apparatus controlled by the same minicomputer. The temperature of the cold isopentane bath was kept at −130°C. Bubbling with nitrogen gas served to keep the oxygen concentration low.

Spectroscopy

Room-temperature optical spectra were recorded with a Beckman Acta MIV double-beam spectrophotometer. EPR spectra were recorded with a Varian E-9 spectrometer equipped with an Oxford Instruments ESR-9 helium flow cytostat. The EPR spectra of rapidly frozen samples were normalized using the $g = 4.3$ signal as an internal

standard. This signal was unaffected by the reductants used.

Results

Stopped-flow studies of the reaction with duroquinol

The reduction of the oxidized cytochrome *bf* complex was followed in the stopped-flow apparatus at 554 nm (cytochrome *f*) or 563 nm (cytochrome *b*-563). Because of its very low solubility in aqueous media the natural substrate, plastoquinol-9, could not be used. As a reasonable compromise between lipofilicity and solubility duroquinol was chosen as a reductant.

The mixing of duroquinol with the oxidized *bf* complex resulted in biphasic traces at both 554 and 563 nm (Fig. 1). Cytochrome *f* was partially reduced in the first phase which was followed by a much slower further reaction toward the fully reduced state. The rate of the rapid phase was dependent on the concentration of the substrate,

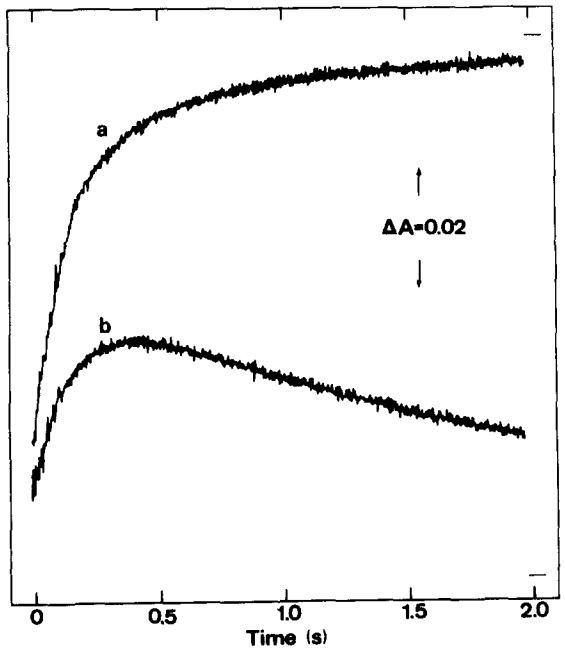

Fig. 1. The reduction of the cytochrome *bf* complex (2.5 μM) with duroquinol (50 μM) as followed in a stopped-flow apparatus at 554 nm (a) and 563 nm (b). The traces have not been corrected for the optical interference between the two cytochromes *f* and *b*-563. The horizontal bars on the right side indicate the end-values for the two wavelengths. Optical pathlength, 2 cm.

but the rate of the slow phase appeared to be concentration independent with a half-time of about 1 s. At 563 nm the reaction of cytochrome *b*-563 was also characterized by a an initial rapid reduction followed by reoxidation of the cytochrome. The reduction showed the same dependence on the concentration of the substrate as that of cytochrome *f*, but the rate of reoxidation was concentration independent with $t_{1/2}$ about 1 s.

The amplitudes of the rapid phases were almost the same for the two cytochromes, and increased slightly with the concentration of duroquinol. At the highest concentrations of quinol used in this study (limited by its solubility) the amplitudes corresponded to 0.65 cytochrome *f* and 0.70 cytochrome *b*-563 per cytochrome *bf* complex.

There is an overlap of the absorption bands of the two cytochromes: the millimolar absorbtivities are 17.5 and −3.8 for cytochrome *f* and 4.5 and 18.0 for cytochrome *b*-563 for the differences between the reduced and oxidized forms at 554 and 563 nm, respectively. Therefore, before calculation of the observed rate of the different reaction phases, a computer program was use to correct for this interference.

The absorbance changes associated with the two cytochromes could each be fitted to two exponentials which gave the observed rate constants and amplitudes of each reaction phase. The dependence of the observed rate constants of the rapid initial phases on the concentration of duroquinol is shown in Fig. 2. The dependence is linear within the concentration interval used with almost the same slope for the two cytochromes. From the slope a second-order rate constant for the reduction of the cytochromes of $1.3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ was found. The second-order rate constants were also calculated from the initial rates of the rapid phases. Again, a linear correlation was found between the rates and the concentration of reductant. The rate constants found with this method were close to those found with the other method.

A few control experiments done with a substrate analogue, plastoquinol-1, gave the same qualitative results as with duroquinol. However, the observed rates were about 25-times higher than with duroquinol with the result that the fast phase of the reaction at high concentrations of substrate was complete within the deadtime of the

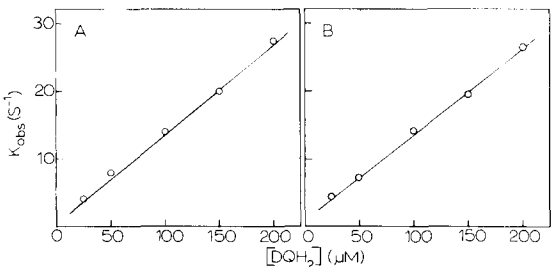

Fig. 2. The effect of duroquinol concentration on the observed first order rate constants (k_{obs}) for the reduction of cytochrome *f* (A) and cytochrome *b*-563 (B) in the isolated cytochrome *bf* complex. The rate constants were calculated from reaction traces corrected for the mutual optical interference of the two cytochromes (see text).

instrument (3–4 ms). The same rate constant (about 1 s^{-1}) for the slow reaction phases of both cytochromes was found with plastoquinol-1 as the reductant.

EPR studies of the reduction

Optical absorption measurements allow studies of the redox states of the two cytochromes in the complex, but give only indirect information about the state of the Rieske iron-sulfur center. For this reason, the reaction of the oxidized cytochrome *bf* complex with duroquinol was followed by EPR which allows observation of signals from the oxidized cytochromes and the reduced Rieske center (cf. Ref. 17).

Fig. 3 shows EPR spectra of samples quenched by rapid freezing in cold isopentane. For Fig. 3a the complex was frozen with no addition of reductant, whereas for Fig. 3b–e the sample was mixed with duroquinol and frozen at increasing times after mixing. Due to the high amount of detergent and sucrose in the sample the freezing behaviour was anomalous, and the reaction times given in the legend have a high uncertainty.

The $g = 4.3$ peak comes from an iron impurity. The signals at 3.5 arise from low-spin ferric heme. Earlier work has shown that most of the heme is present in the low-spin form [17]. The relatively sharp g_z-peak at 3.5 of cytochrome *f* is easily observed in the oxidized sample (Fig. 3a), whereas the broader g_z-peak of cytochrome *b*-563 is best observed in the half-reduced sample (Fig. 3e). All features in the right-hand part of Fig. 3 stem from the Rieske center in the reduced form.

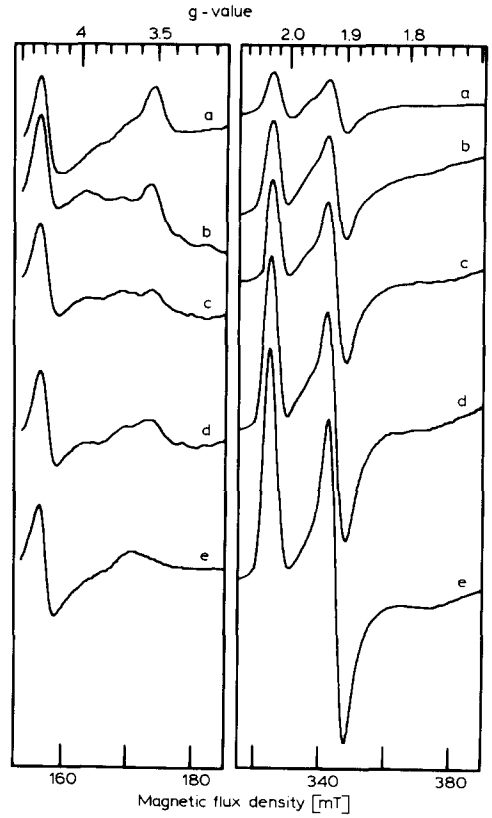

Fig. 3. EPR spectra of the cytochrome *bf* complex (20 μM) reduced with duroquinol (200 μM) and quenched by freezing at different times after mixing. Due to anomalous freezing behavior of the samples the reaction times given are approximate. (a) Without duroquinol added; (b) 50 ms; (c) 100 ms; (d) 250 ms; (e) 2 min. EPR conditions were: Temperature, 15 K; microwave frequency, 9.25 GHz; microwave power, 20 mW; modulation amplitude, 3.2 mT.

A slight autoreduction of cytochrome *f* was frequently observed optically. The EPR spectrum of the cytochrome *bf* complex shows that also the Rieske center is somewhat reduced (about 10%) initially (Fig. 3a). Assuming redox equilibrium, this degree of reduction may seem excessive, considering the usually given reduction potentials of the Rieske center and cytochrome *f* [2] and that cytochrome *f* is almost fully oxidized (more than 80%) in Fig. 3a. However, separate redox measurements (not shown) indicate that the apparent difference in the midpoint potentials between cytochrome *f* and the Rieske center is considerably less as studied by EPR at low temperatures than in room-temperature optical studies. Such shifts in

reduction potentials occurring on cooling from room to cryogenic temperatures have been observed in other heme proteins [18].

With the Rieske center as the initial acceptor of electrons from the quinol, one could expect a transient reduction of this component to occur followed by a reoxidation as the electron is transferred to cytochrome *f*. However, the reduction appeared to be largely monophasic and occurred in conjunction with that of cytochrome *f* (Fig. 3). This and the observation that the Rieske center was less reduced than cytochrome *f* are consistent with a rapid equilibrium situation with the midpoint potential of the Rieske center somewhat lower than that of cytochrome *f*.

The cytochrome *b*-563 kinetics are more difficult to assess, due to the low peak amplitude of the cytochrome and the overlap of the low-spin heme signals, but it is consistent with the reduction and reoxidation observed in the optical experiments.

At the final equilibrium, Fig. 3e, cytochrome *f* and the Rieske center were fully reduced and cytochrome *b*-563 fully oxidized as expected from their midpoint potentials.

Stopped-flow studies of the reaction with oxidized plastocyanin

To investigate further possibility that cytochrome *f* and the Rieske iron-sulfur center are equilibrating rapidly in the isolated cytochrome *bf* complex, studies were performed on the reaction of oxidized plastocyanin with the half-reduced complex, i.e., with cytochrome *f* and the Rieske center reduced. Because of its high rate [19], most of this reaction occurred within the deadtime of the stopped-flow apparatus (Fig. 4). However, the extent of the reaction could be measured by observing the total amount of plastocyanin reduced in the rapid phase. Before calculating the amplitudes of the reactions the traces were corrected for the mutual optical interference of cytochrome *f* and plastocyanin using the millimolar absorbtivities 17.5 and −3.9 for cytochrome *f* and −2.7 and −4.5 for plastocyanin for the differences between the reduced and oxidized forms at 554 and 600 nm, respectively. When cytochrome *f* was fully reoxidized, 1.7 plastocyanin molecules had been reduced which indicated rapid electron trans-

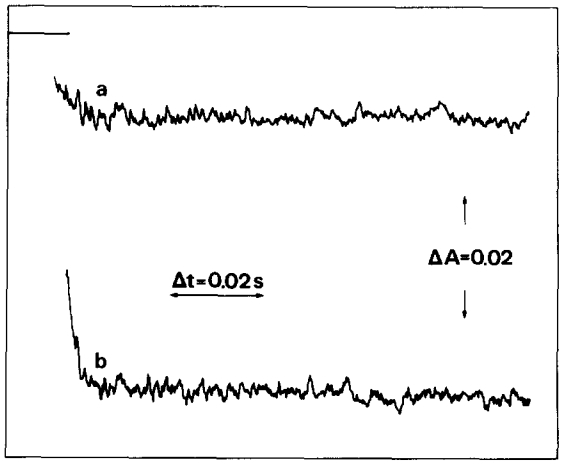

Fig. 4. Stopped-flow traces of the oxidation of the half-reduced cytochrome *bf* complex (2.5 μM) with oxidized platocyanin (12.5 μM) as followed at (a) 600 nm (plastocyanin) and at (b) 554 nm (cytochrome *f*). The traces have not been corrected for the optical interference between cytochrome *f* and plastocyanin. Because of small variations (1–2 ms) in the dead-time of the instrument the traces are not perfectly aligned along the time axis, but they have been adjusted vertically to a common start level time (horizontal bar in the upper left corner). Optical path-length, 2 cm.

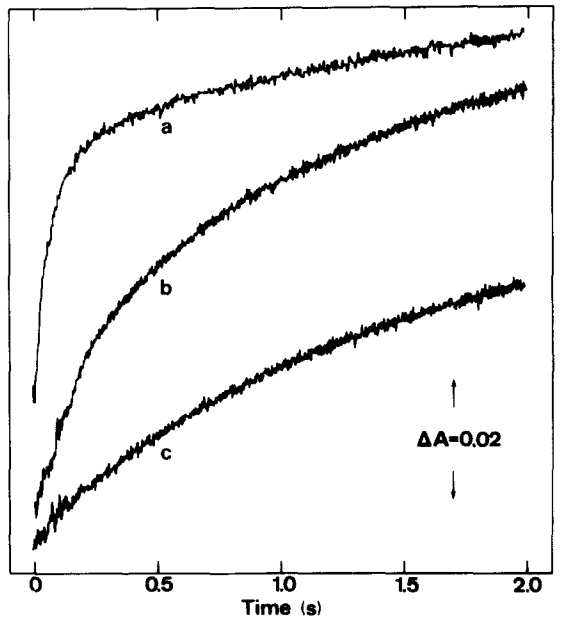

Fig. 5. The reduction of cytochrome *f* in the cytochrome *bf* complex (2.5 μM) with duroquinol (100 μM) in the presence of inhibitors as followed at 554 nm. (a), no inhibitor added; (b) 25 μM DBMIB; (c), 25 μM DNP-INT. The traces have been corrected for the optical interference from cytochrome *b*-563. Optical path-length, 2 cm.

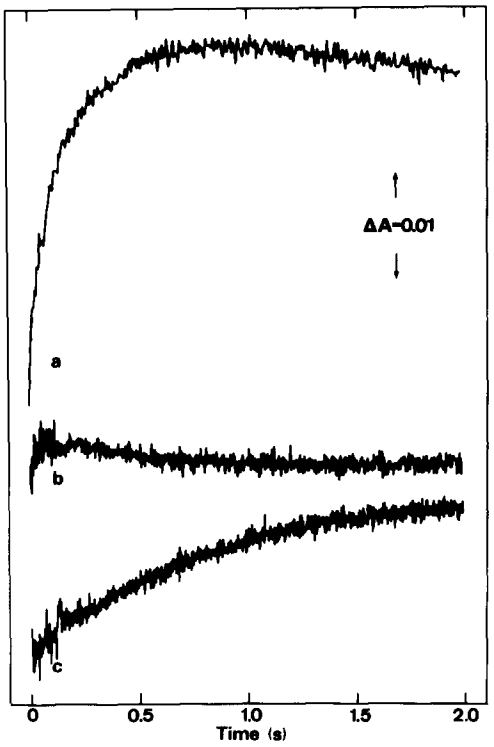

Fig. 6. The reduction of cytochrome *b*-563 in the cytochrome *bf* complex (2.5 μM) with duroquinol (100 μM) in the presence of inhibitors as followed at 563 nm. (a) No inhibitor added; (b) 25 μM DBMIB; (c) 25 μM DNP-INT. The traces have been corrected for the optical interference from cytochrome *f*. Optical path-length, 2 cm.

fer also from the Rieske center. Further reduction of plastocyanin occurred over several tens of seconds and represented the reaction with the excess reductant used to set the initial conditions of the cytochrome *bf* complex.

Rapid reoxidation of the Rieske center and cytochrome *f* was confirmed by the EPR spectrum (not shown) from a sample frozen about 5 s after manual mixing of the half-reduced complex (10 μM) with a 5-fold excess of plastocyanin. The EPR signal from cytochrome *f* had reappeared, while that of the Rieske center was absent. A quantitation of the amount of oxidized plastocyanin remaining after the reaction showed that 1.8 electrons had been transferred from the complex.

The effect of inhibitors on the reduction of the complex

Figs. 5 and 6 show the effect of inhibitors on the reduction of the cytochromes by duroquinol. Both DBMIB and DNP-INT were effective in slowing the rate of reduction of cytochrome *f*, the latter to the extent that only a slow phase remained, similar to that observed in the absence of inhibitor. DBMIB completely inhibited the reduction of cytochrome *b*-563, whereas in the presence of DNP-INT a small amount of this cytochrome less than 1/3 of that reduced in the absence of inhibitor) was slowly reduced to be reoxidized later as equilibrium was approached.

Discussion

In a Q-cycle type of mechanism for the cytochrome *bf* complex in chloroplasts the reductant for cytochrome *b*-563 is the semiquinone resulting from the oxidation of plastoquinol by the Rieske iron-sulfur center. One would, therefore, expect to see this component being reduced before or at least as fast as cytochrome *b*-563. In the stopped-flow experiments with duroquinol as a reductant cytochromes *f* and *b*-563 are reduced simultaneously. No signs of saturation in the rates are observed at any concentration of reductant chosen. Therefore, the rate-limiting reaction in the sequence leading to the reduction of the cytochromes appears to be the association of the reductant with the cytochrome *bf* complex (also see Ref. 3). This model is in line with the observed high and equal rate for the reduction of the two cytochromes with plastoquinol-1.

Our finding of equal rates of reduction of the cytochromes are seemingly in contrast with those reported in Refs. 6 and 7. However, as discussed by Rich [5], rapid electron transfer to plastocyanin and P-700 in chloroplasts and concomitant reoxidation of cytochrome *f* may explain why the reduction of cytochrome *b*-563 appears to be faster than that of cytochrome *f* in chloroplasts.

The reduction of cytochrome *b*-563 is analogous with the 'oxidant-induced reduction' observed in the cytochrome *bf* complex [4]. In the present case, the complex obviously is able to supply its own oxidant, presumably the Rieske center. A similar conclusion was reached in a

study where isolated bacterial photosynthetic reaction centers were used to reduce the cytochrome *bf* complex [20].

The EPR samples taken during the reduction process show that the Rieske center appears to be less reduced than cytochrome *f* (and consequently also less reduced than cytochrome *b*-563). With the reduction potential for the Rieske center lower than that of cytochrome *f*, this is expected if the equilibration between these two components is significantly faster than the initial rate-limiting step. These redox centers were found to equilibrate in less than 1 ms in chloroplasts [21]. The transfer of about two electrons from the complex to plastocyanin in a comparable short time, with the Rieske center and cytochrome *f* reduced initially, shows that the equilibration is rapid also in the isolated complex. Thus, the apparent smaller reduction of the Rieske iron sulfur center does not exclude this as a primary acceptor of electrons from duroquinol.

The experiments with the inhibitors DBMIB and DNP-INT also favour the iron-sulfur center as the initial acceptor of electrons. These compounds have been shown to interact closely with the Rieske center [22] and inhibit photoreduction of the cytochromes in the cytochrome *bf* complex [23]. Both inhibitors eliminate the rapid phase in the reduction of the cytochromes. The reactions observed, mainly the reduction of cytochrome *f*, are slow and possibly rate-limited by the dissociation of the inhibitors from the complex. The slow accumulation of a small amount of reduced cytochrome *b*-563, which is seen with DNP-INT present, probably reflects the ability of this compound to block also the oxidation of this cytochrome [24].

The slow reoxidation of cytochrome *b*-563 appears to be an intramolecular reaction ($t_{1/2}$ about 1 s). The acceptor of the electron is not known but could possibly be a bound quinone [25] or even cytochrome *f*, since the rate of its slow reduction corresponds to the rate of reoxidation of cytochrome *b*-563.

Summarizing, the use of pre-steady-state methods permits the kinetics of the cytochrome *bf* complex to be studied at high time resolution. The reduction kinetics of the redox components in the complex are fully consistent with the Rieske iron-sulfur center being the initial acceptor of electrons which supports a Q-cycle mechanism for the cytochrome *bf* complex. The application of rapid mixing/rapid-quenching techniques promises to be valuable in the study of the electron transfer in solubilized photosynthetic complexes.

Acknowledgements

This work has been supported by a grant from the Swedish Natural Science Research Council.

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