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Kinetic studies of electron transfer in a hybrid system constructed from the cytochrome *bf* complex and Photosystem I

Peter R. Rich [a], Peter Heathcote [b] and David A. Moss [a]

[a] *The Glynn Research Foundation Ltd., Bodmin, Cornwall and* [b] *School of Biological Sciences, Queen Mary College, University of London, London (U.K.)*

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(1) A hybrid electron-transfer system has been constructed from purified cytochrome *bf* complex and Photosystem I preparations which are active in plastocyanin oxidation. Donor and acceptor characteristics of these purified preparations are described. (2) The use of this simplified system enabled the application of a rigorous method of component deconvolution from spectral kinetic data. This relied on matrix analysis of the extinction coefficients of all components at each wavelength of measurement. (3) Kinetic analyses of flash-induced redox changes of cytochromes *b*-563, cytochrome *f*, plastocyanin and P-700 were made with this method and were compared with computer simulations of predicted behaviour. These studies indicated that cytochrome *b*-563 behaviour was consistent with its being reduced by 'oxidant-induced reduction' at centre o, even although cytochrome *b*-563 reduction was apparently much faster than cytochrome *f* rereduction after a flash. However, in order to obtain good agreement between experiment and simulation, the midpoint potential of the Rieske centre had to be assumed to be +370 mV. (4) Multiple turnover behaviour of the cytochrome *bf* complex at high potentials was also consistent with the Q-cycle feature of oxidant-induced reduction of cytochrome *b*. However, a maximum reduction of only around 25% of total cytochrome *b*-563 could be achieved via this route, and this extent was neither affected by 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (NQNO) nor by added quinone. Similarly, in thylakoids in the presence of NQNO, less than half of the total cytochrome *b*-563 could be reduced by multiple flashes. (5) Continuous illumination experiments at a low potential indicated that cytochrome *b* oxidation occurred on turnover of the cytochrome *bf* complex even when the cytochromes *b* were fully prereduced. The oxidation was sensitive to NQNO.

Abbreviations: DNP-INT, 2-iodo-6-isopropyl-3-methyl-2,4,4-trinitrodiphenyl ether; Mes, 4-morpholinethanesulphonic acid; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; P-700, primary electron donor in the Photosystem I reaction centre; centre o, the site of quinol oxidation on the *bf* complex (the Q_z site in some nomenclatures); centre r, the site of quinone reduction on the *bf* complex (centre i or the Q_c site in some nomenclatures); *b*-563o, the *b*-563 haem associated with centre o; *b*-563r, the *b*-563 haem associated with centre r.

Correspondence: P.R. Rich, The Glynn Research Foundation Ltd., Bodmin, Cornwall, PL30 4AU, U.K.

Introduction

The mechanism of electron and proton transfer through the cytochrome *bf* complex is in general thought to occur by a Q-cycle type of mechanism [1–3]. Electron donation into the complex occurs via centre o [4], a reaction site which is in contact with the positive side of the membrane. The mechanism is a consecutive reaction in which the plastoquinol reduces the Rieske centre and the semiquinone product then reduces cytochromes *b*

by 'oxidant-induced reduction' [5]. This phenomenon has been demonstrated in the *bf* complex [6–8].

A second feature of the Q-cycle is the reoxidation of the cytochromes *b* by a second site, centre r, in contact with the negative side of the membrane [4]. In a Q-cycle, a quinone from the pool acts as the oxidant and is sequentially reduced by two successive one-electron transfers to the quinol [1–4].

Recently, both of these features have been challenged. Firstly, cytochrome *b*-563 has been observed to become reduced more rapidly than cytochrome *f*, indicating that the quinol, rather than the semiquinone, might be the reductant of cytochrome *b* at centre o [9–11]. Secondly, it has been noted that the slow phase of the electrochromic shift can occur even when the cytochromes *b* are fully reduced before a flash [12,13] and that this electrogenic reaction is associated with a rapid cytochrome *b*-563 oxidation [13,14]. Although our own results suggest that this oxidation occurs via centre r [14], Joliot and Joliot [13] suggest that the oxidation is occurring at centre o by oxidation of *b*-563o$^-$ by the o site semiquinone, with a concomitant electrogenic movement of protons from the negative phase. An identical reaction, but with no associated electrogenic proton movement, has been suggested by Hartung and Trebst [15] to be the basis of the ability of the *bf* complex to operate non-electrogenically under some conditions.

We attempt here to investigate these suggestions quantitatively. A major problem with such a quantitative approach is in component deconvolution, since the components have a great deal of spectral overlap. This overlap has made interpretation of some of the literature data difficult. We describe a matrix method of component analysis which allows deconvolution of the overlapping components. It has been applied mainly to a reconstructed well-defined system of quinol, *bf* complex, plastocyanin and Photosystem I. Lam and Malkin [16,17] have demonstrated oxidant-induced reduction of cytochrome *b*-563 in a similar system, as have Prince et al. [8] with a hybrid *bf*/bacterial reaction-centre system. The hybrid system has proved essential in developing the matrix method as even with this system we have required sixteen extinction coefficients in order to resolve the cytochromes *b* and *f*, plastocyanin and P-700. Having obtained kinetic behaviour of the components by this method, we have then attempted to fit computer simulations from our working model of electron transfer.

Materials and Methods

Preparation of chloroplast components

Cytochrome *bf* complex was purified from lettuce by a modification of the procedure of Hurt and Hauska [18]. The final gradient purification step was substituted by the following procedure. The cytochrome *bf* complex was redissolved in a buffer containing 0.67 M sucrose, 1 mM histidine and 10 mM Tris-HCl at pH 8.0 to a final protein concentration of 10 mg/ml. Sodium cholate was added to 0.25 mg cholate/mg protein and after 10 min the mixture was centrifuged at $40\,000 \times g$ for 5 min. The supernatant was fractionated with ammonium sulphate to yield a preparation of equivalent activity and purity to that previously obtained in Ref. 18. This modification has the advantage that it is much faster and allows the preparation of much larger amounts of *bf* complex. Its concentration was estimated from a dithionite-reduced minus oxidised difference spectrum of the cytochromes *b*-563 and assuming 2 *b*-haem/complex and an extinction coefficient at 563 nm (reduced minus oxidised) of 20 $mM^{-1} \cdot cm^{-1}$.

Plastocyanin was prepared from parsley as previously described [19]. Its concentration was estimated by using an extinction coefficient for the monomer of 4.8 $mM^{-1} \cdot cm^{-1}$ at 595 nm for an oxidised minus reduced sample.

A partially purified Photosystem I preparation was obtained from spinach using the method of Mullet et al. [20] to give a final chlorophyll/P-700 ratio in the range of 150–200 : 1, assuming an oxidised minus reduced extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ at 703 nm [21].

Preparation of quinones and quinols

All plastoquinone derivatives were synthesised from 2,3-dimethyl-1,4-benzoquinone. This was most conveniently prepared [22] from 4-amino-2,3-dimethylphenol hydrochloride (Aldrich). 5 g were added to 0.16 g of stannous chloride dihydrate in 21 ml concentrated HCl and 80 ml water,

and the whole was heated to 70 °C until the aminophenol had dissolved and then filtered through a sintered glass funnel. This solution was added in subdued light dropwise to a boiling solution of 65 g ferric chloride dihydrate in 400 ml water. The quinone product can be distilled off as the solution is added. This distillate was collected in diethyl ether, washed, dried over anhydrous sodium sulphate and then evaporated to dryness.

Undecanoyl peroxide was prepared by refluxing 100 g undecanoic acid with 127.8 g thionyl chloride at 30–40 °C for 3 h. Excess thionyl chloride was removed by steam bath heating at reduced pressure and the undecanoyl chloride was distilled off with an oil bath under reduced pressure at around 130 °C. 41 g were dissolved in 350 ml diethyl ether at 0 °C. 8.5 g of 60% hydrogen peroxide and 19 g pyridine were added dropwise and the diacyl peroxide precipitated as it formed. The ice bath was removed and the slurry stirred for 1 h. A homogeneous solution was obtained at room temperature by addition of more diethyl ether. This was washed with dilute HCl, then 5% potassium bicarbonate and dried over anhydrous sodium sulphate and the diundecanoyl peroxide was precipitated by cooling to 0 °C. Decyl-plastoquinone was synthesised from 2,3-dimethyl-1,4-benzoquinone and diundecanoyl peroxide [23], essentially following the method of Wan et al. [24] for the analogous ubiquinone derivative. 1.36 g of 2,3-dimethyl-1,4-benzoquinone was dissolved in 50 ml glacial acetic acid and the mixture was refluxed at 90–95 °C under nitrogen. A mixture of 7.4 g of diundecanoyl peroxide in 5 ml glacial acetic acid was added dropwise over a 2 hour period. The whole was stirred and refluxed at 90–95 °C for a further 20 h. The products were evaporated to dryness in vacuo and fractionated in toluene on a silica gel column. Further purification was achieved with silica TLC plates in a solvent of chloroform/hexane/diethyl ether at 10 : 10 : 1 and structures were confirmed by NMR. It was stored under nitrogen at −20 °C. The method was also applicable to the synthesis of 2-decyl-1,4-naphthoquinone and 2-decyl-3-methyl-1,4-naphthoquinone (decyl-menaquinone) by substituting an equimolar quantity of 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone for the 2,3-dimethyl-1,4-benzoquinone.

Plastoquinone-2 was prepared by condensation of geraniol with 2,3-dimethylquinol by refluxing in the presence of fused zinc chloride (cf. plastoquinone-1 synthesis [25]). The product was fractionated in toluene on a silica gel column and stored in the same way as decyl-plastoquinone. Its structure was confirmed by NMR.

Electron-transfer assays

Cytochrome *bf* complex activity was assayed in a buffer of 50 mM Mes plus 2 mM EDTA at pH 6.2 or 7 which contained either 5.2 μM plastocyanin or 1 mM potassium ferricyanide as acceptor. 60 μM plastoquinol-1 or plastoquinol-2 or 20 μM decyl-plastoquinol was added to start the reaction and the reduction of plastocyanin (ε $mM^{-1} \cdot cm^{-1}$ of 4.8 at 595 nm) or of ferricyanide (ε $mM^{-1} \cdot cm^{-1}$ of 1.0 at 420 nm) was monitored spectrophotometrically. The blank rate in the absence of enzyme was subtracted from the rate on addition of cytochrome *bf* complex (generally 10–20 nM complex added) to give the enzymatic rate.

Electrochemical poising

Electrochemical poising of ambient potential was achieved with a platinum working electrode (surface area, 2 cm^2) inserted into the 0.7 ml sample. A platinum spade counter electrode and silver/silver chloride reference were connected to the sample via an 0.1 M KCl salt bridge. A fourth electrode was also present in the sample for direct voltammetric measurement of E_h. Potential of the solution was altered by switching potential of the working electrode to −1.0 V or +0.8 V vs. the silver/silver chloride reference. This technique will be described in detail elsewhere.

Kinetic analyses and component deconvolution

Flash-induced changes of P-700 alone were monitored at 703 nm where there were no spectral contributions from other components in the system. The flash was filtered with a 620 nm short pass filter and the photomultiplier was protected with infra-red and 695 nm long pass filters.

For all wavelengths, the measuring beam was shuttered and the shutter opened less than 100 ms before the actinic beam was activated. This was necessary to avoid any actinic effects of the mea-

suring beam. Electron-transfer components were monitored with a computer-controlled single beam instrument. Traces were taken at 575, 563, 554 and 542 nm and stored independently. Continuous illumination was provided by a source filtered with an RG635 Schott glass filter and an infra-red filter and provided around 8 W/m^2 at the cuvette surface. Actinic flashes were provided with a xenon lamp operating at 10 J per flash and with a half peak width of less than 5 μs, filtered with an RG635 filter. The photomultiplier was protected with a 1 cm solution of saturated copper sulphate and a 580 nm short pass filter.

All four components (cytochromes *b*-563 and *f*, plastocyanin, P-700) have spectral contributions in the 540–580 nm region. In order to deconvolute the spectral changes of each component, it was necessary to produce four simultaneous equations of the form:

observed ΔA at y nm = Σ (conc. change × extinction coefficient at y nm)

By using for each component relative, rather than absolute, extinction coefficients (i.e., ε $mM^{-1} \cdot cm^{-1}$ at y nm / ε $mM^{-1} \cdot cm^{-1}$ at a measuring wavelength), the equations become:

observed ΔA at y nm

= Σ(ΔA of a component at measuring wavelength × relative extinction coefficient at y nm)

We have used measuring wavelengths of: 563 nm for cytochrome *b*-563; 554 nm for cytochrome *f*; 575 nm for plastocyanin; 542 nm for P-700. 16 absolute extinction coefficients were measured experimentally with the purified components. For each component, its four values were normalised and the results are presented in Table I. By matrix inversion, these values were used to provide the following solution to the original four simultaneous equations:

$$\Delta A \text{ of } b\text{-563 at 563 nm} = 0.958\Delta A_{563} - 0.015\Delta A_{554} - 0.519\Delta A_{575} - 0.576\Delta A_{542}$$

$$\Delta A \text{ of } f \text{ at 554 nm} = 0.889\Delta A_{554} - 0.099\Delta A_{563} - 0.208\Delta A_{575} - 0.667\Delta A_{542}$$

$$\Delta A \text{ of PC at 575 nm} = 0.928\Delta A_{575} + 0.200\Delta A_{554} + 0.084\Delta A_{563} - 0.297\Delta A_{542}$$

$$\Delta A \text{ of P-700 at 542 nm} = 1.083\Delta A_{542} - 0.016\Delta A_{554} - 0.385\Delta A_{575} - 0.060\Delta A_{563}$$

The final results are in terms of absorbance changes of each component at its measuring wavelength and are independent of the absolute values of the extinction coefficients. It might also be noted that the solution is independent of the molar ratios of reactants.

Data simulation

The simulation procedure was an iterative one, with the simplifying assumption that the three components FeS/*f*/PC were always in redox equilibrium, i.e., redox reactions within this unit are extremely fast compared to the rate of electron transfer into and out of the unit. The initial condition (immediately following the actinic flash) was

TABLE I

NORMALISED EXTINCTION COEFFICIENTS USED FOR MATRIX ANALYSIS

All of these are for reduced minus oxidised difference spectra. Cytochrome *f* figures were measured from a methylhydroquinone reduced vs. ferricyanide oxidised difference spectrum of purified cytochrome *bf* complex and cytochrome *b*-563 from a dithionite-reduced minus methylhydroquinone-reduced difference spectrum of the same material. Plastocyanin figures were obtained from a pure sample of parsley plastocyanin. P-700 figures were measured as flash-induced optical changes of the dark-adapted, ascorbate-reduced Photosystem I preparation in the presence of methyl viologen as acceptor. For each component, the extinction coefficients were normalised to the highest of its four values. These maxima (ε $mM^{-1} \cdot cm^{-1}$) were: cytochrome *b*-563, 20 at 563 nm; cytochrome *f*, 18 at 554 nm; plastocyanin, −4.16 at 575 nm (assuming a value of −4.8 for the monomeric form at 595 nm); P-700, −4.235 at 542 nm (assuming a value of 64 at 700 nm [21]).

	(ε $mM^{-1} \cdot cm^{-1}/\varepsilon_{max}$ $mM^{-1} \cdot cm^{-1}$)			
	542 nm	554 nm	563 nm	575 nm
Cytochrome *b*-563 [a]	0.019	0.101	1.0	−0.016
Cytochrome *f*	0.074	1.0	−0.151	−0.226
Plastocyanin	0.240	0.630	0.798	1.0
P-700	1.0	0.843	0.659	0.078

[a] Average values for the two cytochrome *b*-563 haems.

taken as full reduction of plastoquinol and FeS/*f*/PC, and full oxidation of P-700. From these initial concentrations and the appropriate second-order rate constants, the number of electrons transferred into and out of FeS/*f*/PC in a small unit of time dt were calculated as follows:

$$N_{in} = [PQH_2] \times [FeS_{ox}] \cdot k_1 \cdot dt$$

$$N_{out} = [PC_{red}] \times [P\text{-}700_{ox}] \cdot k_2 \cdot dt - [PC_{ox}] \cdot [P\text{-}700_{red}] \cdot k_{-2} \cdot dt$$

N_{in} and N_{out} were then used to calculate the new redox poise of PQ, P-700 and FeS/*f*/PC. Finally, the thermodynamic equilibrium distribution of the total number of electrons in FeS/*f*/PC amongst the three components was calculated. This yielded new concentrations for all components, so that the process could be repeated. It was found that setting dt such that the simulation consisted of 200 such cycles yielded traces that were sufficiently free of distortion.

In order to model accurately the reduction kinetics of P-700, a biphasic process was used. To achieve this, a fraction, f, of the P-700 was considered to react as shown in the above equations with a rapid rate constant; the remainder, $1-f$, reacted with a smaller rate constant.

Simulations were fitted to specific sets of experimental data by setting the total concentrations of *bf*, PS I, plastoquinone/ol and plastocyanin, and then manipulating the rate constants, midpoint potentials and the fast-reacting proportion of P-700 until a good fit was obtained between the experimental and simulated data (with appropriate extinction coefficients).

The end product of the simulation was a rate of plastoquinol oxidation which was taken as the rate of production of reductant for cytochrome *b*-563, assuming a Q-cycle model for electron transfer through the *bf* complex. This was multiplied by the extinction coefficient for the cytochrome to allow comparison of the theoretical and observed rates of cytochrome *b*-563 reduction.

Results

Turnover numbers of the cytochrome bf complex

Table II compares turnover numbers of the purified cytochrome *bf* complex when assayed with the variety of plastoquinol analogues synthesised. As emphasised previously, such turnover numbers do not usually represent a saturated rate [3]. In general the reaction may be represented by two second-order reactions up to any reasonable limits for solubility or ideal behaviour of the plastoquinol or plastocyanin. Turnover numbers are therefore defined at specific concentrations of these reagents and a change in these concentrations will alter the turnover number of the enzyme accordingly.

TABLE II

QUINOL-PLASTOCYANIN AND QUINOL-FERRICYANIDE OXIDOREDUCTASE ACTIVITIES OF SOLUBILISED CYTOCHROME *bf* COMPLEX

The buffer was 50 mM Mes and 2 mM EDTA at 25 °C. The acceptor was either plastocyanin or 1 mM potassium ferricyanide. Reaction was initiated by addition of quinol, followed by enzyme. The blank rate in the absence of enzyme was subtracted for turnover number calculations. At pH 6.2, the non-enzymatic rate constants for quinol reduction of plastocyanin were 201 $M^{-1} \cdot s^{-1}$, 82 $M^{-1} \cdot s^{-1}$ and less than 20 $M^{-1} \cdot s^{-1}$ for plastoquinol-1, plastoquinol-2 and decyl-plastoquinol, respectively.

Donor	Acceptor	pH	Turnover number (s^{-1})
60 μM plastoquinol-1	8 μM plastocyanin	6.2	6
60 μM plastoquinol-2	8 μM plastocyanin	6.2	12
20 μM decyl-plastoquinol	8 μM plastocyanin	6.2	14
20 μM decyl-plastoquinol	8 μM plastocyanin	7.0	25
20 μM decyl-plastoquinol	1 mM ferricyanide	6.2	48
20 μM decyl-plastoquinol	1 mM ferricyanide	7.0	75

The following points may be noted.

(1) Both plastoquinol-2 and decyl-plastoquinol are better substrates for the cytochrome *bf* complex than the more generally used plastoquinol-1. This is consistent with the properties of analogous ubiquinol derivatives with the cytochrome bc_1 complex [24] and presumably reflects a better partitioning of these more hydrophobic quinols into the hydrophobic centre o.

(2) Both plastoquinol-2 and especially decyl-plastoquinol are superior in that the non-en-

zymatic rates of plastocyanin reduction are lower than those of plastoquinol-1. The non-enzymatic rates of plastocyanin or ferricyanide reduction increase with pH (cf. Ref. 26). This precludes the use of plastoquinol-1 or plastoquinol-2 as substrates at pH 7 when plastocyanin is the acceptor and neither are suitable at either pH 6.2 or 7 when ferricyanide is the acceptor. However, the non-enzymatic rate of decyl-plastoquinol reduction of plastocyanin or ferricyanide is extremely small, even at pH 7. This quinol is therefore recommended for assay of quinol-plastocyanin or quinol-ferricyanide oxidoreductase activities of the cytochrome *bf* complex.

(3) At high levels of decyl-plastoquinol and acceptor, turnover numbers of the complex in excess of 70 s^{-1} are possible. Even under these conditions, turnover is limited only by the amounts of substrates which can be added. Such turnover numbers suggest that the purified complex has retianed a large part of its catalytic activity during purification.

Properties of the purified Photosystem I preparation

Some heterogeneity was evident on the acceptor side of the photochemical reaction centre as shown in Fig. 1A. In the absence of an added electron acceptor or a rapid donor to P-700$^+$, only aroud 70% of the P-700$^+$ rapidly rereduced with a half-time of around 30 ms as electrons back-reacted from the bound iron-sulphur centres [27]. The fate of the electrons in those photosystems which do not back-react is presumably to react with oxygen. When methyl viologen was added as an acceptor of electrons from the acceptor-side components, the rapid back reaction was mostly prevented (Fig. 1B). However, even in the presence of this acceptor, a fraction of the P-700$^+$ still apparently became rereduced rapidly. It is suggested that this is caused by a fraction of the photosystems having lost or damaged acceptors. These photosystems would then be incapable of forward electron transfer to an added acceptor and so would be inactive in multiple turnover electron transfer. The amount of active P-700 was taken to be the amount which could be stably photooxidised by a saturating flash in the presence of methyl viologen. The Photosystem I preparations were also tested for their ability to accept

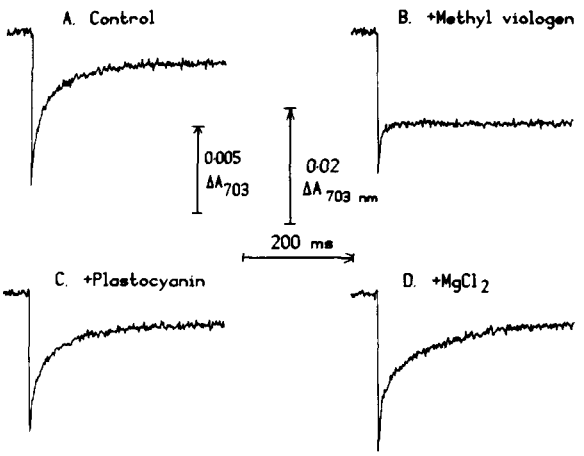

Fig. 1. Electron donors and acceptors for Photosystem I. Isolated Photosystem I particles were dissolved to 90 nM in 0.16 M sucrose, 40 mM KCl, 0.5 mM EDTA and 10 mM potassium phosphate at pH 7.25. 8 mM sodium ascorbate and 500 Sigma Units of catalase were then added. The sample was dark-adapted for 120 s between flashes. Other additions were made sequentially to the same sample of 1 mM methyl viologen, 0.5 μM plastocyanin and 20 mM magnesium chloride. P-700 changes were followed at 703 nm. A saturating flash was provided by a xenon lamp filtered with a 620 nm short pass filter and the photomultiplier was protected by a 695 nm long pass and infra-red filters. Traces are each the average of two flashes.

electrons from reduced plastocyanin in solution, as indicated in Figs. 1C and D. In Fig. 1B, rereduction of the majority of the P-700$^+$ is very slow, since methyl viologen has been added as an acceptor of electrons from the acceptor-side components. On addition of 500 nM plastocyanin the P-700$^+$ exhibited biphasic rereduction. Semilog plots indicated that 65% had a second order rate constant of $6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ and the remainder one of around $2.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. Both of these processes were second order in that the rates increased linearly with increasing plastocyanin concentrations, at least up to 10 μM. Bottin and Mathis [28] have reported similar data, but have also described a faster phase which could be observed at higher concentrations of plastocyanin than those used here. Addition of 20 mM $MgCl_2$ inhibited the rapid phase (Fig. 1D) but stimulated the slow phase (not shown), so that rereduction approached more monophasic behaviour.

Electron transfer through the cytochrome bf complex

A linear electron transfer system in solution was constructed with the following components: decyl-plastoquinol, cytochrome *bf* complex, plastocyanin, Photosystem I and methyl viologen. Ascorbate was also added before the quinol to prereduce the high potential components. A series of continuous illumination cycles were given whilst monitoring either 575, 563, 554 or 542 nm. From these data it was possible to deconvolute the redox behaviour of plastocyanin, cytochromes *f* and *b* and P-700, as described in the Methods section. The behaviour of cytochromes *b*-563 and *f* are illustrated in Fig. 2. On light activation in the absence of added quinol, a rapid photooxidation of P-700 occurred, followed by photooxidation of plastocyanin and cytochrome *f*. Very little change in redox state of cytochrome *b*-563 was observed. Cytochrome *f* and plastocyanin rereduced slowly on switching off the actinic beam, presumably by non-enzymatic rereduction by the added ascorbate. When decyl-plastoquinol was present, the behaviour was quite different because of the electron flow which now occurred from quinol to plastocyanin via the cytochrome *bf* complex. Only a small steady-state oxidation of plastocyanin and cytochrome *f* were seen which rapidly reversed when the light was switched off. Furthermore, cytochrome *b*-563 became reduced during the course of the illumination. The extent of this cytochrome *b*-563 reduction was around one quarter of the total cytochrome *b*-563 haem present in the sample, i.e., approx. 0.5 *b*-563 haems/complex. We were unable to increase this extent by altered ratios of components or by anaerobiosis. Addition of the r site inhibitor NQNO [9,29] only slightly decreased the cytochrome *b*-563 reoxidation rate when the light was switched off, as observed for HQNO in Refs. 16 and 29. At this high (1 μM) concentration of *bf* complex, 2 μM NQNO had no effects on the cytochrome *f* and plastocyanin kinetics or extent, indicating that it was not inhibiting the o site reaction. In chloroplast experiments where *bf* concentration is generally below 0.1 μM, even 1 μM NQNO has some effect on the o site reaction (Ref. 30 and Fig. 5). That the majority of the cytochrome *b*-563 reduction during illumination, and of cytochrome *f* and plastocyanin rereduction in the dark, was occurring enzymatically was confirmed by the addition of the o site inhibitor stigmatellin [31]. This caused the cytochrome *f* and plastocyanin to exhibit behaviour similar to that in the absence of quinol and abolished the reduction of cytochrome *b*-563. DNP-INT [32,33] at much higher concentrations (30 μM) had an effect essentially identical to that of stigmatellin.

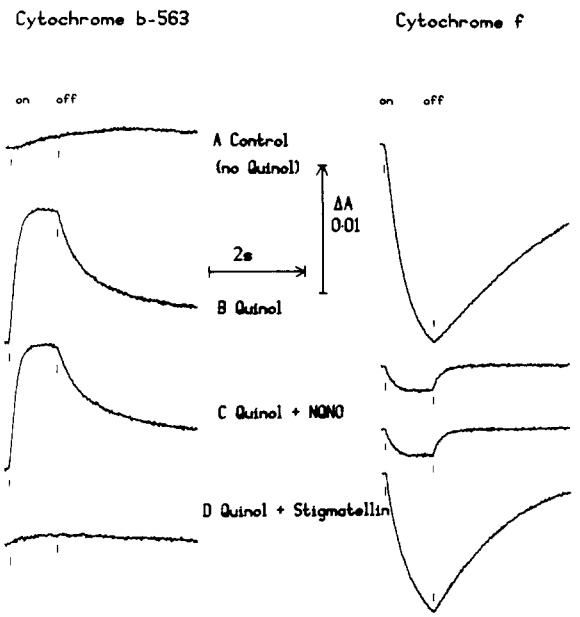

Fig. 2. Cytochrome changes during steady-state illumination at high potentials. Reaction buffer was 0.16 M sucrose, 40 mM KCl, 0.5 mM EDTA, 20 mM $MgCl_2$ and 10 mM potassium phosphate at pH 7.25 and contained 100 nM P-700, 1 μM cytochrome *bf* complex, 5 μM plastocyanin, 2 mM sodium ascorbate, 1 mM methyl viologen and 500 Sigma units of catalase. Experiments A–D were performed on fresh samples to avoid problems of loss of quinol during illumination. Other additions were: B, 60 μM decyl-plastoquinol; C, 60 μM decyl-plastoquinol + 2 μM NQNO; D, 60 μM decyl-plastoquinol + 5 μM stigmatellin. Actinic illumination (8 W/m^2) after 10 s dark adaptation was with light filtered with a Schott RG635 long pass filter and infra-red filters. The photomultiplier was protected with a 600 nm short pass filter and a copper sulphate solution. Components were deconvoluted as described in Materials and Methods for four components but plastocyanin and P-700 changes are not shown.

These inhibitor effects appear to be only partly consistent with a Q-cycle mechanism. In this model, electron donation into the complex from quinol occurs through the stigmatellin-sensitive

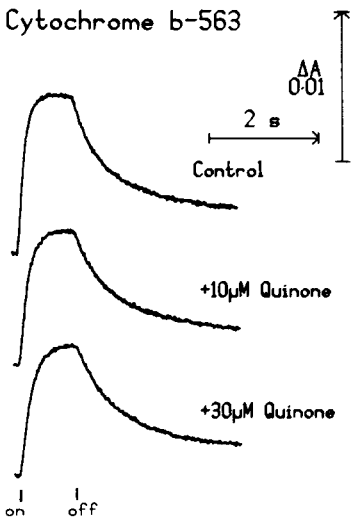

Fig. 3. The effects of added decyl-plastoquinone on cytochrome *b*-563 behaviour during continuous illumination at high potentials. The initial reaction mixture was the same as in Fig. 2B, i.e., containing 60 μM decyl-plastoquinol. New samples were used for each experiment. Cytochrome *b*-563 was deconvoluted from a four-component analysis as described in Materials and Methods.

centre o. One electron from quinol is donated to Rieske centre/cytochrome *f* and the semiquinone product reduces cytochrome *b*-563o. The cytochrome *b*-563o would be reoxidised by cytochrome *b*-563r which in turn would be reoxidised by a quinone species at the NQNO-sensitive centre r. The model predicts that addition of NQNO, if it is a potent inhibitor of the r site, should significantly increase the amount of cytochrome *b*-563 which could be 'oxidant-induced reduced' and would also inhibit the cytochrome *f* rereduction which occurs when the actinic beam is switched off. Neither of these predictions is observed (Fig. 2).

A further experiment was performed to test the efficiency of added decyl-plastoquinone as an oxidant for cytochromes *b*-563 (Fig. 3). The experiment was carried out in the same way as that of Fig. 2, but with a range of decyl-plastoquinone concentrations added in addition to the quinol before the illumination cycle was begun. Again, and apparently in contrast to expected behaviour (but see Discussion), added quinone had little effect on the extent of cytochrome *b*-563 reduction in the steady state, or on its subsequent rate of reoxidation on switching off the light. A rather similar observation was made by Lam [29] and implications of such behaviour are considered further in the Discussion section.

Neither antimycin A, myxothiazol nor strobilurin A had any effects on the redox changes observed in these experiments.

Flash activation of single electron turnover

Flash activation of the reconstructed system as detailed in Fig. 4 caused P-700 photooxidation and the resulting $P\text{-}700^+$ was biphasically rereduced with $t_{1/2}$ values of around 5 ms and 120 ms, with a concurrent oxidation and subsequent rereduction of plastocyanin and cytochrome *f* (Fig. 4A). Both 10 μM stigmatellin and 20 μM DNP-INT severely inhibited the rate of cytochrome *f* and plastocyanin rereduction and also prevented observable cytochrome *b*-563 reduction, consistent with their site of inhibition at centre o [31–33]. 10 μM NQNO increased the extent of cytochrome *b* reduction after the flash by a factor of 2–3, but slowed cytochrome *f* rereduction only a little. In a separate experiment, the behaviour of all four components in the presence of NQNO was simulated (Fig. 4B) assuming the model described in Materials and Methods. Several points can be noted.

(1) Cytochrome *b*-563 reduction ($t_{1/2}$ of 9 ms) appears to be faster than cytochrome *f* and plastocyanin rereduction ($t_{1/2}$ of 15 ms). However, the rate of cytochrome *b*-563 reduction is the same as the calculated rate at which electrons are donated back into the cytochrome *bf* complex from quinol at centre o. This is seen in Fig. 4B as a close match between the observed initial rate of cytochrome *b*-563 reduction and that simulated on the basis that one cytochrome *b*-563 will become reduced for each electron which passes from quinol to Photosystem I acceptors. This is consistent with a Q-cycle mechanism in which cytochrome *b*-563 is reduced by the semiquinone produced when quinol reduces the iron-sulphur centre.

(2) A 1–2 ms lag in cytochrome *b*-563 reduction is observed experimentally and in the simulation, and is caused by the delay in the arrival of the electron holes on the iron-sulphur centre. A similar lag was observed in chromatophores when

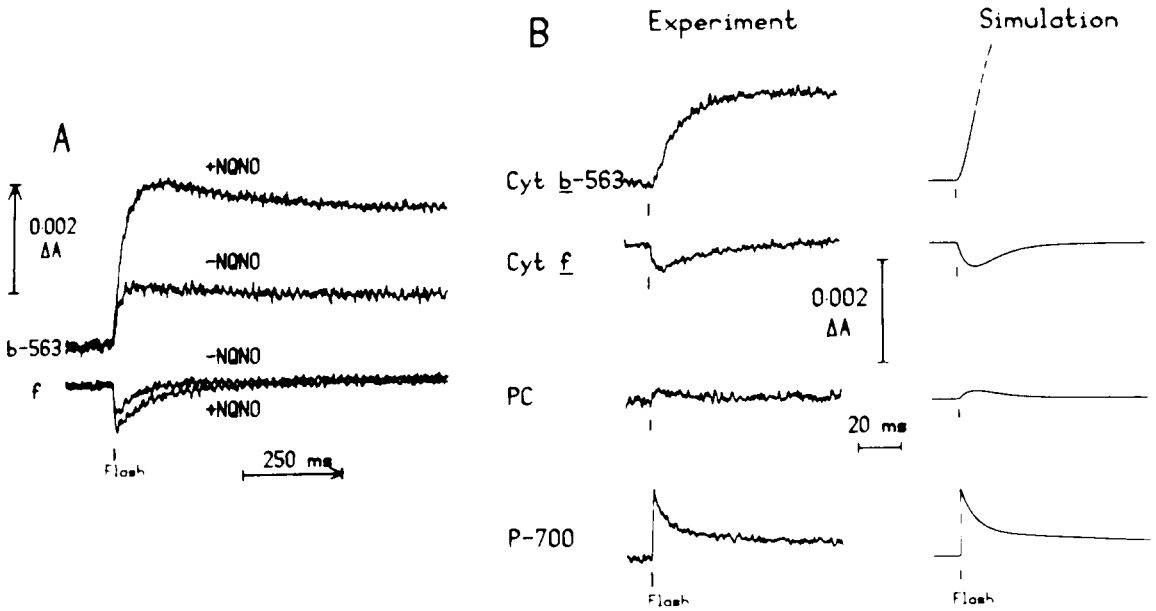

Fig. 4. Flash-activated redox changes in the presence of added quinol. Reaction buffer was 0.16 M sucrose, 40 mM KCl, 0.5 mM EDTA, 20 mM $MgCl_2$ and 10 mM potassium phosphate at pH 7.25 and contained 300 nM P-700, 5 μM cytochrome *bf* complex, 5 μM plastocyanin (PC), 4 mM sodium ascorbate, 1.5 μM anthraquinone sulphonate and 60 μM plastoquinol-2. Component analyses of A and B and simulations of B are described in Materials and Methods. Data are the averages of 25 flashes at each of the four wavelengths used. 10 s dark adaptation was given between each flash.

the quinone pool is oxidised and has been attributed to slow diffusion of quinol from the reaction centre to centre o [34].

(3) The rate and extent of cytochrome *b*-563 reduction soon becomes slower than expected from the simulation, even in the presence of NQNO to inhibit cytochrome *b*-563 reoxidation. This is probably caused by the incomplete blocking of cytochrome *b* reoxidation even in the presence of NQNO. The simulation is for a system where no cytochrome *b*-563 reoxidation occurs.

(4) In order to fit the observed extents of component redox change with the known amount of P-700 turnover, it was necessary to use an operative midpoint potential for the iron-sulphur centre of around +370 mV, rather higher than the equilibrium midpoint potential [35,36].

Both shortfall in cytochrome *b*-563 reduction and the question concerning the operating midpoint potential of the iron sulphur centre arise in thylakoid studies also (Fig. 5). An 80%-saturating flash given to thylakoids in the presence of duroquinol caused the production of 80 nM holes and 40 nM of these holes appeared transiently on cytochrome *f*. We have measured a ratio of FeS/*f*/plastocyanin/P-700 of 1:1:4:1.25 in these

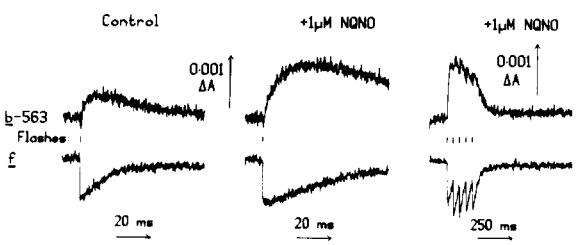

Fig. 5. Flash-induced changes in cytochromes *f* and *b*-563 in broken thylakoids. Broken thylakoids were prepared from pea seedlings as in Ref. 46. The assay medium contained 0.16 M sucrose, 40 mM KCl, 0.5 mM EDTA and 10 mM potassium phosphate at pH 7.25. Also present were 1 μg/ml valinomycin, 1 μg/ml gramicidin, 0.5 mM duroquinol, 100 μM anthraquinone sulphonate and broken thylakoids at 75 μg/ml. [*bf*] and [P-700] in the cuvette were estimated to be 80 nM and 100 nM, respectively, and the flash was 80% saturating. Data are the average of 20 recordings at each of 563, 554, 575 and 542 nm and were deconvoluted by the method used for the purified system.

thylakoids. Using equilibrium midpoint potentials for these components of 300, 370, 360 and 430 mV, respectively, less than 7% of these holes might have been expected to have been observed on cytochrome *f*. This therefore suggests a higher potential for the iron-sulphur centre than measured at equilibrium. In the presence of NQNO, only 50 nM electrons appear on cytochrome *b*-563 when these 80 nM holes are refilled, so illustrating the shortfall in cytochrome *b*-563 reduction. Furthermore, this represents somewhat less than one *b*-563 haem per *bf* complex. Subsequent flashes actually caused a decrease in this amount of *b*-563 reduced, making it rather analogous to the d.c. light experiments on the reconstructed system described above.

Cytochrome b-563 reoxidation at low potentials

In order to explain the apparent observations that the *bf* complex can still turn over in the presence of an r site inhibitor [15], that it may be able to operate with an H^+/e^- ratio of 1 [37–39], and the lack of NQNO-sensitivity of cytochrome *b*-563 oxidation at low potentials [13], a number of models have been proposed in which a semiquinone at centre o can act as both a reductant and an oxidant of the *b*-563o haem [13,15]. The experiments of Fig. 6 were designed to test this. The cytochrome *bf* complex was poised at low potential electrochemically in the presence of 25 μM anthraquinone sulphonate and 10 μM methyl viologen, added both for redox mediation between electrodes and proteins and as electron acceptors for Photosystem I. Only with this electrochemical method of redox poising could we get stable potentials at such low mediator concentrations. Addition of higher concentrations of the mediators caused a 'short circuit' of the system at these low potentials by their direct chemical reduction of photooxidised plastocyanin and/or cytochrome *f*.

At a potential of −230 mV and at a pH 7.25, an absorption difference spectrum of the sample vs. the same sample at +100 mV indicated that both cytochrome *b*-563 haems were fully reduced. On light activation, cytochrome *b*-563 rapidly oxidises in an NQNO-sensitive (Fig. 6) or stigmatellin-sensitive manner. The amount of NQNO added in this experiment has little effect on centre

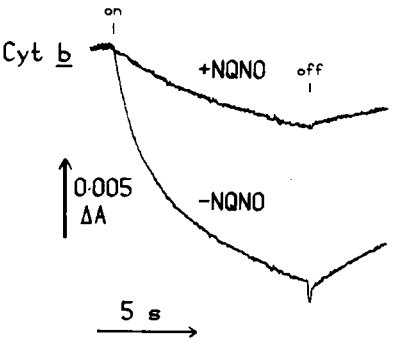

Fig. 6. Cytochrome *b*-563 oxidation at low potential induced by continuous illumination. Reaction buffer was 0.16 M sucrose, 40 mM KCl, 0.5 mM EDTA, 20 mM $MgCl_2$ and 10 mM potassium phosphate at pH 7.25 and contained 400 nM P-700, 5 μM cytochrome *bf* complex, 10 μM plastocyanin, 250 μM sodium ascorbate, 10 μM methyl viologen, 25 μM anthraquinone sulphonate, 6 μM decyl-plastoquinol, 10 mM glucose, 150 μg/ml glucose oxidase and 500 Sigma units of catalase. It was kept anaerobic with nitrogen above the surface and the potential was maintained electrochemically at −232 to −258 mV. Actinic illumination was the same as for Fig. 2. 30 s dark adaptation was given before each illumination cycle.

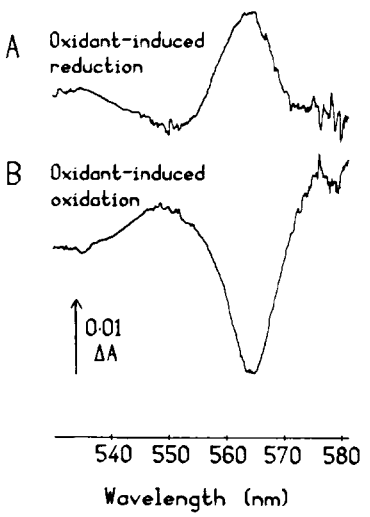

Fig. 7. Light minus dark difference spectra of cytochrome *b*-563. In A, the sample was identical to that of the legend to Fig. 3B and the spectrum is that of the cytochrome *b*-563 which is reduced by oxidant-induced reduction during d.c. illumination. In B, a sample identical to that of Fig. 6 was electrochemically poised in the dark at −230 mV, and the spectrum is that of the cytochrome *b*-563 which is oxidised by oxidant-induced oxidation during d.c. illumination.

o and so it is concluded that the NQNO inhibition is caused through its effect on centre r.

Fig. 7 is a comparison of the spectrum of the oxidant-induced reducible cytochrome *b*, presumably the higher potential *b*-563o, with the spectrum of oxidant-induced oxidisable cytochrome *b*, presumably the lower potential *b*-563o. There is no significant difference in the peak positions of these two components, in contrast to their spectra in whole *Chlorella* cells [13].

Discussion

We describe here some further properties of purified cytochrome *bf* and Photosystem I multiprotein complexes. It has been shown that our purified cytochrome *bf* complex is able to turnover at rates in excess of 70 s^{-1} with appropriate types and amounts of substrates. The figures obtained with decyl-plastoquinol are the highest so far reported and this substrate is also far superior to other plastoquinol derivatives in that the nonenzymatic reactions are extremely slow (Table II). The data of Fig. 2 may be used to deduce that the preparation behaves homogeneously: in Fig. 2A essentially all of the cytochrome *f* present is oxidised by plastocyanin when the light is switched on, indicating that all complexes have an active plastocyanin reduction site. Also, all of this cytochrome *f* can be rapidly reduced by added decylplastoquinol (Fig. 2B), indicating that all complexes have an active quinol oxidation site also.

The Photosystem I preparations are much more heterogeneous (Fig. 1). Generally, around 20% are inactive in forward electron transfer on their acceptor side, since they back-react rapidly even in the presence of methyl viologen. Of the remainder, around 30% appear to have a mechanism in which an electron entering the acceptor side on flash activation becomes unavailable for back reaction with P-700$^+$, whereas the remainder behave like the in vivo system. In addition, around 30% of the photosystems capable of stable charge separation appear to have lost the ability for rapid P-700$^+$ rereduction by reduced plastocyanin, with a second-order rate constant under the conditions of Fig. 1 of only $5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. The remainder is rapidly reduced by plastocyanin with a second-order rate constant approaching 10^8 $M^{-1} \cdot s^{-1}$. This biphasic second order reduction of P-700$^+$ by plastocyanin has been reported in more detail by Bottin and Mathis [28]. Who also demonstrated a third phase at even higher plastocyanin concentrations than used here, and suggested that it corresponded to saturation of the plastocyanin binding site.

A number of mechanistic points have arisen. The first concerns the finding that cytochrome *b*-563 reduction might be too fast to be accounted for by 'oxidant-induced reduction' by the semiquinone product of the reaction between quinol and the iron sulphur centre at centre o [9–11]. We show here that although cytochrome *b*-563 reduction is observed to be faster than cytochrome *f* rereduction, its behaviour is still consistent with the conventional 'oxidant-induced reduction' mechanism. Its initial rise is fast because it corresponds to the total electron-transfer flux rate from quinol into all oxidised components (iron sulphur centre, cytochrome *f*, plastocyanin and P-700$^+$). The apparent half-time of cytochrome *b*-563 reduction is small both because of this and because the eventual level of cytochrome *b*-563 reduction falls short of the amount expected, even when NQNO is present (Figs. 4 and 5). This shortfall in the amount of cytochrome *b* reduced appears in the results of other groups [9–11] as judged by the relative absorbance changes observed for cytochromes *f* and *b* and considering that at least twice as many holes are produced by Photosystem I oxidation than appear on cytochrome *f*.

The simulations did draw attention to one unexpected feature in the reconstructed system – the fact that the number of electron holes appearing on cytochromes *f* and plastocyanin corresponded closely to the number of holes produced by P-700$^+$ rereduction after the flash, suggesting that very few holes actually appeared on the iron-sulphur centre. Fig. 5 highlights this problem in thylakoids also. Since we are assuming that the iron-sulphur centre, cytochrome *f* and plastocyanin are remaining in thermodynamic equilibrium with each other during turnover we had to use a midpoint potential for the iron-sulphur centre of +370 mV in the simulation, a value much higher than the measured equilibrium midpoint potential of around +280 – +320 mV [35,36].

This discrepancy might mean that our assumption of equilibrium between these components is invalid, or that there is an operative midpoint potential of the iron sulphur centre which is different from the values measured in equilibrium redox titrations. Whitmarsh et al. [40] have reported a similar discrepancy in thylakoids and suggested that the added duroquinol might be responsible. We have observed the discrepancy under a variety of conditions both with the purified and thylakoid-bound complex, making this explanation unlikely. At present we suggest that the midpoint potential is probably much closer to that of cytochrome *f* than equilibrium titrations might suggest. This would make sense from the point of view of energy conservation, where there is no evidence for electrogenic electron transfer between the iron-sulphur centre and cytochrome *f*.

It might be pointed out that the reaction in the mitochondrial [41] and bacterial [42] *bc* complexes of quinol reduction of cytochrome *b* directly via centre r does not occur with the chloroplast complex. In the experiments of Fig. 2B, for example, the cytochrome *b* is fully oxidised before illumination, even although quinol has been added. This is consistent with the much lower redox potentials of the chloroplast cytochrome *b* haems.

The continuous illumination and flash experiments (Figs. 2–5) were consistent with a mechanism of cytochrome *b*-563 reduction by oxidant-induced reduction at centre o. The fact, however, that only 25% of the total *b*-563 was reducible in this way and that this extent was relatively unaffected by either NQNO or added quinone, was unexpected. Calculations from data of others [6–8,29] also show that around 0.5 *b*-563 haem/complex is the maximum to have been observed in steady-state uncoupled conditions. There are several mechanisms which could explain these observations. One type of mechanism incorporates the suggestion that the semiquinone at centre o can both reduce and oxidise cytochrome *b*-563o [13,15,43]. The mechanism would have the additional feature of either a shift in the relative midpoint potentials of the two cytochrome *b*-563 haems in these preparations so that the one closest to centre o is of higher potential or that the two cytochrome *b*-563 haems might be unable to transfer electrons between each other, as recently suggested by Cramer et al. [43]. None of these possibilities are features of the conventional Q-cycle [4]. In both cases, the reaction sequence commences with the usual Q-cycle reaction of

$$QH_2 + FeS/b\text{-}563o \rightarrow Q + 2H^+ + FeS^-/b\text{-}563o^-$$

However, the electron on *b*-563o will not be transferred to the second cytochrome *b*-563 at centre r because of one of the reasons above. Once the iron-sulphur centre has been reoxidised, a second quinol would have to react with the complex and this time the semiquinone, Q_0^-, would reoxidise *b*-563o$^-$:

$$Q_0^- + b\text{-}563o^- + 2H^+ \rightarrow QH_2 + b\text{-}563o$$

The overall result of this series of reactions during steady state illumination would be that 50% of *b*-563o (i.e., 25% of total *b*-563) would become reduced during steady-state electron transfer, as we observe here. Such a model has been invoked recently as a possible mechanism for functioning of the complex even in the presence of an r site inhibitor [13], and for its functioning with an H^+/e^- ratio of only one under some conditions [15].

A second possible explanation of the extent of cytochrome *b*-563 reduction which would be more in accord with conventional models, however, is that NQNO is only a rather weak inhibitor when bound at the r site. Assuming that complexes with one electron in the cytochrome *b*-563 must await a further turnover of centre o and that this causes reduction of the second *b*-563 haem, followed by very rapid oxidation of both haems by quinone, then during steady-state turnover the population of complexes would tend towards a 1 : 1 mixture in the states (*bobr*) and (*bobr*)$^-$ if cytochrome *b* reoxidation were still not rate limiting even with inhibitor present. Our own data (Fig. 6) tend to rule out the existence of a rapid oxidation of *b*-563o$^-$ by o site semiquinone, since cytochrome *b* oxidation at low potentials is NQNO-sensitive. This observation argues against the two alternative possibilities described above which both require such a reaction.

The finding that added quinone was unable to

promote rapid cytochrome *b*-563 oxidation (Fig. 3 and Ref. 29) is only superficially at odds with a Q-cycle. When the cytochrome *b*-563 has become reduced to an extent of 0.5 haem/complex, this means that half of the complexes have one electron in their cytochrome *b*-563. Quinone is not able to oxidise this state, since the quinone would have to be reduced to a semiquinone and this semiquinone would then have to leave the r site, a reaction which is unlikely, since the semiquinone will probably be tightly bound. For normal turnover, a second reduction by quinol at centre o would have to occur and the cytochrome *b*-563 with both haems reduced could then reduce quinone to quinol, which could leave the site. We postulate then that pathways of oxidation of half-reduced cytochrome *b*-563 are non-physiological. It might be noted that stability of reduced cytochrome *b* in the presence of quinone has been invoked as evidence for the 'semiquinone cycle' [44] – the above reasoning suggests that such stability could be entirely consistent with the Q-cycle.

In the experiments described here, we have generally used *bf* complex at a concentration of 1–5 μM and used NQNO in a 2-fold molar excess. For these conditions, NQNO has very little effect on centre o (e.g., Figs. 2 and 4). The effects on centre r at high potential can only clearly be seen in single turnover experiments (Fig. 4) where the extent of cytochrome *b*-563 reduction is increased 2–3 fold. In chloroplasts, experiments are generally performed with much lower [*bf*] concentrations (30–100 nM) and NQNO is added at 1–2 μM. These conditions cause inhibition of centre r, although centre o is significantly affected also (Fig. 5 and Ref. 30). One difference in cytochrome *b*-563 behaviour without NQNO present is that the small amount of cytochrome *b*-563 reduced remains stable in the purified system for several hundreds of milliseconds, whereas in chloroplasts it is reoxidised quickly (cf. Figs. 4 and 5), even although the majority of complexes which are activated presumably turn over only once in both cases. One might have expected with thylakoids that this reduced cytochrome *b*-563 would also remain stable, since complexes with only one electron in their cytochrome *b*-563 haems must await a second turnover for reoxidation of *b* haem to occur. This anomaly will be addressed in a future report.

The above proposal of a weak effect of NQNO may partly explain the recent data of Joliot and Joliot [13] who were able to observe NQNO-insensitive cytochrome *b*-563 oxidation provided that the cytocromes *b* were fully prereduced. They proposed an oxidation of *b*-563o by the o site semiquinone with a concomitant electrogenic uptake of protons through a proton channel from the opposite side of the membrane. Our experiments tend to argue against such a reaction, since cytochrome *b*-563 oxidation at low potentials was sensitive to both r site (Fig. 7) and o site inhibitors, as is the case in the analogous experiments with the mitochondrial *bc* complex [45]. Our interpretation is that production of the oxidant for cytochrome *b*-563 requires turnover of the o site, but that it acts at the r site. In line with this notion, we have recently suggested that cytochrome *b*-563 oxidation observed at low potentials in thylakoids also occurs via centre r, but that the NQNO effect is weak [14]. Furthermore, on the basis of concurrent measurements of the electrochromic changes and proton movements, it is most likely that the oxidant is a neutral semiquinone, $QH^{\bullet}$, which moves from centre o to centre r under these reducing conditions (Ref. 14, see also Moss, D.A. and Rich, P.R., unpublished results). It might be pointed out that the known reaction of reversed electron transfer through the o site of the *bf* or the bc_1 complexes should require quinone, not semiquinone, as an oxidant for cytochrome *b*-563o at centre o; a postulated reaction whereby semiquinone oxidises cytochrome *b* at the o site is not simply a reversal of a physiological reaction.

Acknowledgements

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