Biochimica et Biophysica Acta, 1058 (1991) 312–328

ADONIS 000527289100165F

BBABIO 43433

The interactions of duroquinol, DBMIB and NQNO with the chloroplast cytochrome *bf* complex

Peter R. Rich, Sally A. Madgwick and David A. Moss *

Glynn Research Institute, Bodmin, Cornwall (U.K.)

(Received 27 December 1990)

Key words: Cytochrome *bf* complex; Duroquinol; DBMIB; NQNO; HQNO: H^+/e^- ratio; Electron transfer mechanism

Duroquinol reduces the endogenous plastoquinone pool of chloroplasts only very slowly because of a lack of quinol-quinone transhydrogenase activity of the cytochrome *bf* complex, but is a fast direct donor into the cytochrome *bf* complex via the quinol oxidation site. It also accelerates the rate of cytochrome *b*-563 reoxidation in the presence of NQNO. Results of three independent methods are consistent with a requirement of one DBMIB per *bf* monomer for full inhibition of *bf* complex electron transfer activity, demonstrating that a *bf* complex monomeric unit is capable of electron transfer activity. At low molar ratios, inhibition reverses in the dark but is fully restored after several turnovers of the complex. Inhibition is weakened by high concentrations of duroquinol or *decyl*-plastoquinol. Free DBMIB is rapidly reduced to $DBMIBH_2$ by duroquinol. Addition of $DBMIBH_2$ in the dark does not cause a large inhibition of the first turnover of the *bf* complex. It is proposed that $DBMIBH_2$ does not form a stable inhibitory complex but can be oxidised by the quinol oxidation site of the cytochrome *bf* complex, but at a rate which is significantly less than that of oxidation of plastoquinol or duroquinol. The tight inhibitory complex involves the semiquinone or quinone form of DBMIB and this bound species is only slowly reduced in the dark back to free $DBMIBH_2$. Subsequent flash oxidation leads to a competition between $DBMIBH_2$, duroquinol and plastoquinol for the quinol oxidation site. NQNO and HQNO are effectors of the quinone reduction site of the chloroplast cytochrome *bf* complex. In the analogous mitochondrial bc_1 complex, HQNO inhibits electron transfer through the quinone reduction site and this inhibition is not simply a result of the modulation of the midpoint potential of the cytochrome b_H haem. In the cytochrome *bf* complex, a large increase of the midpoint potentials of the cytochrome *b* haems has been reported, but we were unable to repeat this observation. Although the extent of oxidant-induced reduction of haems *b* is increased by N(H)QNO, inhibition of electron transfer through the quinone reduction site is small, especially in the presence of duroquinol, and the electrogenic reaction which is associated with protonmotive function of the enzyme is not impaired even after multiple turnovers. It is proposed that the Q_i site of the cytochrome *bf* complex can still turn over sufficiently rapidly in the presence of NQNO or HQNO so that there is little inhibition of turnover, even although transient kinetic behaviour can be markedly affected.

* Present address: Institut für Biophysik und Strahlenbiologie, Albert-Ludwigs-Univertsität, Freiburg, F.R.G.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; $DBMIBH_2$, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzohydroquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; H(N)QNO, HQNO and NQNO; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; duroquinol, 2,3,5,6-tetramethyl-*p*-benzohydroquinone; PMS, 5-methylphenazinium methosulphate (phenazine methosulphate); Q_o site, the quinol oxidation site and site of proton output of the cytochrome *bf* complex which is in contact with the positive aqueous intrathylakoid phase (also termed the Q_z or Q_p site); Q_i site, the quinone reduction site and site of proton input of the cytochrome *bf* complex which is in contact with the negative aqueous extrathylakoid stromal phase (also termed the Q_c, Q_r or Q_n site); Q_B (site), the (binding site of the) secondary quinone acceptor of photosystem II; haem b_H, the high potential haem of cytochrome *b*-563, thought to be associated with the quinone reduction site, Q_i, and sometimes termed haem b_n; haem b_H, the high potential haem of cytochrome *b*-563, thought to be associated with the quinol oxidation site, Q_o, and sometimes termed haem b_p; SHE, standard hydrogen electrode; E_h, ambient potential vs. SHE; E_{mx}, midpoint potential at pH x vs. SHE.

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

Introduction

The use of specific inhibitors of the mitochondrial cytochrome bc_1 complex has greatly contributed to the elucidation of its mechanism [1]. The equivalent bacterial enzyme shows the same inhibitor sensitivities [2]. The chloroplast enzyme, however, although superficially similar in electron transfer proteins and general mechanism, is distinctly different in terms of its inhibitor sensitivities [2,3]. Of the known highly specific and naturally-occurring antibiotic inhibitors of the *bc* complexes, only stigmatellin appears to affect the *bf* complex [4–7]. Because of this, much use has been made instead of the synthetic inhibitors DBMIB [8] and H(N)QNO [9,10]. In both the *bc* and *bf* complexes these are generally thought to primarily affect respectively the quinol oxidation and quinone reduction sites, Q_o and Q_i.

Unfortunately, both DBMIB and H(N)QNO have properties which cause complications. In particular, both compounds are themselves redox active (see Fig. 1), and it is likely that the redox state of the inhibitor is critical for its inhibitory action. In addition, both inhibit the Q_B site of Photosystem II [8,11,12]. It has been reported that only one DBMIB molecule per dimer of the cytochrome *bf* complex is required for full inhibition and this has prompted a view that the enzyme works as a dimer [13], a mechanism which is unlikely to operate in the *bc* complexes [14]. Evidence has been presented that HQNO and NQNO primarily affect the quinone reduction site and also inhibit the quinol oxidation site at higher concentrations in both *bc* and *bf* complexes [10,15]. HQNO has been reported to slightly raise the midpoint potential of haem b_H of the mitochondrial enzyme [16,17] and to dramatically raise the potentials of both haems *b* in the cytochrome *bf* complex [18]. In addition, although H(N)QNO at low concentrations cause an increased oxidant-induced reduction of cytochrome *b*-563 in chloroplasts [9,10,19], indicative of inhibition of quinone reduction at the Q_i site, they do not inhibit electron transfer through the enzyme [10] at the same concentrations. This has led to the view that turnover of the quinone reduction site of the cytochrome *bf* complex may not be mandatory for the quinol-plastocyanin oxidoreductase activity. Such a facultative site could form a mechanistic basis for a variable proton stoichiometry [19–21]. Recently, and in contrast to the generally held view that HQNO and NQNO bind to the Q_i site close to haem b_H, it has been suggested that the NQNO inhibition site may be close to haem b_L in the cytochrome *bf* complex [19].

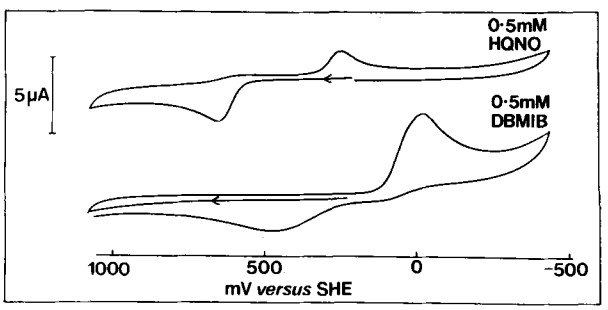

Fig. 1. Electrochemical properties of DBMIB and HQNO at a glassy carbon electrode. Cyclic voltammograms were run in a solvent of 1:1 ethanol/water and containing 10 mM potassium phosphate and 100 mM KCl at pH 7 and 23°C. The mixture was kept anaerobic with oxygen-free nitrogen. The working electrode was the end of a 3 mm diameter, freshly polished, glassy carbon rod and the counter electrode was an approx. 0.5 cm^2 platinum spade. Scans were begun in the oxidative direction at a speed of 20 mV/s.

We report here new observations on the mechanism of interaction of duroquinol, DBMIB and H(N)QNO with the cytochrome *bf* complex and provide an interpretation of the results in terms of the Q-cycle mechanism of the enzyme. Some of this work has appeared in preliminary form [22,23].

Materials and Methods

Preparation of biochemical materials

Pea plants were grown in a growth chamber at 20–25°C, in an 8/16 h light/dark regime. The illuminance at leaf level was around 100 μmol quanta m^{-2} s^{-1}. Since this illuminance is somewhat low, for comparison purposes chloroplasts were also prepared from pea plants which had been grown in summer daylight. Although this increased the *bf*:chlorophyll and *bf*:*P*-700 ratio as expected [24], no fundamental differences in kinetic behaviour or inhibitor titres were found. Class C chloroplasts were prepared as already described [25] and stored in liquid nitrogen in resuspension medium plus 5% dimethylsulphoxide, or kept on ice for more immediate use. There was little difference in results from fresh or stored chloroplasts.

Cytochrome *bf* complex was prepared from lettuce chloroplasts by a modification [26] of the method of Hurt and Hauska [27]. Plastocyanin was prepared from frozen/thawed spinach chloroplasts. Purified materials were stored in liquid nitrogen until required.

Cytochrome kinetics

Measurements of cytochrome *f* and *b*-563 kinetics were made by analyses of flash-induced absorbance changes at the wavelengths 542, 554, 563 and 575 nm [26]. The individual changes due to cytochrome *b*-563, cytochrome *f*, P700 and plastocyanin were then obtained by matrix deconvolution [26] with the matrix coefficients given in Table I. These values are the most recent refinement of our original values presented in Ref. 26. In practise, the small changes make little difference to the cytochromes *f* and *b* deconvolutions. Data were generally the average of ten recordings at each wavelength, with a response time-constant of 1 ms

TABLE I

Normalised extinction coefficients used for matrix analyses

These values are our most recent refinement of figures orginally given in Table I of Rich et al. [26]

	(ϵ $mM^{-1}cm^{-1}/\epsilon_{max}$ $mM^{-1}cm^{-1}$)			
	542 nm	554 nm	563 nm	575 nm
Cytochrome *b*-563	0.106	0.155	1.0	−0.182
Cytochrome *f*	−0.0905	1.0	−0.132	−0.191
Plastocyanin	0.499	0.683	0.827	1.0
P-700	1.0	0.844	0.692	0.0194

and a measuring beam bandwidth of about 1.5 nm. The results represent the absorbance change at one wavelength of a single component. In all experiments, the measuring beam was switched on only 50 ms before recording commenced and during dark periods the photomultiplier was provided with light from a light-emitting diode of an intensity equal to that of the measuring beam.

Carotenoid bandshift measurements

The absorbance changes due to the flash-induced electrochromic shift of carotenoids [28] were measured at 518 nm. The slow phase of the electrochromic signal was taken as the difference between a control response and that of the same chloroplast suspension to which had been added 2 μM stigmatellin and 1 μM phenazine methosulphate (PMS) (c.f. Refs. 10,29).

Steady state electron transfer measurements

The steady state electron transfer rate from duroquinol to methyl viologen in illuminated chloroplasts was monitored from the associated oxygen consumption. Reaction medium was 330 mM sorbitol, 50 mM Hepes, 10 mM KCl, 1 mM EDTA, 5 mM potassium phosphate, 5 mM $MgCl_2$, 3 mM ammonium chloride, 0.5 mM methyl viologen, 10 μM ATP and 10 μM DCMU at pH 7.8 and 23°C. Chloroplasts were added to 25 μg/ml of chlorophyll ($a + b$), followed by 0.25 mM duroquinol. Oxygen consumption was monitored with a Clark-type oxygen electrode and the saturating actinic beam (300 W/m^2) was filtered with an RG610 Schott glass filter.

Quantitation of cytochrome bf complex

The amount of active cytochrome *bf* complex in chloroplasts was determined from the amount of photo-oxidisable cytochrome *f* in experiments similar to those of Fig. 4B. The absorbance change due to cytochrome *f* rereduction after cessation of actinic illumination at $554 - 0.5 \cdot (545 + 561)$ nm was quantitated with an extinction coefficient of 18 $mM^{-1}cm^{-1}$.

Purified cytochrome *bf* complex was quantitated from the cytochrome *f* in an ascorbate *minus* ferricyanide difference spectrum (ϵ $mM^{-1}cm^{-1}$ of 18 at $554 - 0.5 \cdot (543 + 560)$ nm) and from cytochrome *b*(ϵ $mM^{-1}cm^{-1}$ of 40 at $563 - 0.5 \cdot (552 + 572)$ nm) in a dithionite minus ascorbate difference spectrum. The two values agreed within 6% and the average was used for further calculations.

Redox titrations

Conventional anaerobic redox titrations [30] were carried out under argon gas in a buffer of the potassium salts of 2 mM EDTA and 50 mM tartrate (pH 4.5–5.5), Mes (pH 5.6–6.5), phosphate (pH 6.6–7.5), Taps (pH 7.6–8.5) or glycine (pH > 8.5). Broken pea chloroplasts were used at 100 μg chlorophyll (A + B)/ml and purified lettuce cytochrome *bf* complex at around 1.5 μM. 1 μM gramicidin and 0.3 μM nonactin were present in all cases to ensure full uncoupling. Redox mediators were: anthraquinone-2-sulphonate; anthraquinone-2,6-disulphonate; methyl viologen, benzyl viologen, phenazine ethosulphate, phenazine methosulphate, 2-methyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, trimethyl-1,4-benzoquinone, duroquinone, hexammineruthenium chloride, diaminodurene, 5-hydroxy-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone and 1,4-naphthoquinone-2-sulphonate (all at 20–100 μM) and 5 μM pyocyanine perchlorate. 90 units/ml of catalase were also present. Potential was varied with additions of ferricyanide or dithionite. 5 min were usually allowed for equilibration after each addition and data represent points taken in both oxidative and reductive directions. For measurement of ambient redox potential, we used a glassy carbon and silver/silver chloride electrode system. Cleaning and calibration of this system are described in detail elsewhere [17].

At each ambient potential, spectra were recorded at approx. 1 nm intervals from 540 to 580 nm with a digital single beam scanning instrument which had been constructed in-house. Normally, three spectra were recorded and averaged before storage. Ambient potential was recorded at the start and end of the scans. A programme accessed these accumulated scans to produce the appropriate plots of absorbance change vs. averaged potential during data collection. In this way, there was no user subjectivity in data derivation. Haem *b*-563 was taken to be represented by the absorbance change $564 - 0.5 \cdot (548 + 580)$ nm. This combination gave only a small interference from haem $b\text{-}559_{LP}$ [31], a component which is always present in chloroplasts, but which is absent from these cytochrome *bf* preparations. For data of the isolated cytochrome *bf* complex, computed Nernstian curves for a combination of two non-interacting $n = 1$ components which contributed at a ratio of 0.6 (high E_m

form):0.4(low E_m form) were compared to these plots and a best fit was derived by least squares analysis. Good fits to a single component could not be obtained and fits were also poorer for relative contributions of other than 0.6:0.4. In the case of chloroplasts, such distinction between one and two components by statistical analyses was not easily possible. This arose because of an interference from haem b-559$_{LP}$ at high potentials (see Fig. 9) which could not easily be removed by deconvolution, and from the fact that the splitting of the b haem potentials was somewhat less than in the isolated enzyme, especially at pH values of 7 and above, we estimated that the two b haems were split by up to 100 mV at pH 6, but this dropped to 50 mV or less at pH 8. We therefore only qualitatively estimated a midpoint potential of both haem b-563 groups taken together in order to produce a plot of midpoint potential vs. pH. To obtain this estimate, 10% of the total signal was ascribed to haem b-559$_{LP}$ and the remainder to b_H plus b_L.

Sources and quantitation of reagents

Duroquinol was prepared from the quinone by the method in Ref. 32. The pure white solid was stable for several months at −20°C in the dark. 50–250 mM stock solutions were made up in DMSO plus 10 mM HCl and were stable for at least 1 week at −20°C in the dark.

DBMIB was a kind gift of Prof. A. Trebst, Bochum and was also purchased from Sigma. An extinction coefficient for the oxidised compound in methanol at 289 nm was taken as 13.7 $mM^{-1}cm^{-1}$. This figure, which we determined gravimetrically, is in agreement with Graan and Ort [13]. Stock solutions were made up fresh in 96% ethanol. Care was taken to keep the samples dark to prevent light-induced reduction or chemical transformation [33,34]. $DBMIBH_2$ was prepared by reduction with potassium borohydride under anaerobic conditions, followed by extraction into diethyl ether. The diethyl ether solution was then treated as in Ref. 32. The quinol was dissolved in acidic ethanol and quantitated with an extinction coefficient in methanol at 300 nm of 4.9 $mM^{-1}cm^{-1}$. Fresh solutions were prepared daily.

NQNO was a kind gift of Prof. G. von Jagow, Münich, F.R.G. and HQNO was purchased from Sigma. Stock solutions were made up in 96% ethanol and were quantitated with an extinction coefficient of 9.5 $mM^{-1}cm^{-1}$at 346 nm. Both compounds induced the characteristic extra oxidant-induced reduction of cytochrome b-563 at expected concentrations.

Simulation of DBMIB inhibition curves

Best fits of an equation for a tight binding inhibitor to the DBMIB inhibition data were determined by least squares analyses. The equation was derived from:

$$E + I \overset{K}{\leftrightarrow} EI$$

Since $K = [EI]/[E]\cdot[I]$, $DBMIB_{added} = I + EI$ and $bf = E + EI$, it follows that

$$EI^2 - EI(1\backslash K + DBMIB_{added} + bf) + DBMIB_{added}\cdot bf = 0$$

This simultaneous equation was conventionally solved to produce curves of EI (i.e., fractional inhibition) vs. $DBMIB_{added}$ at different total concentrations of bf complex.

Electrochemical properties of DBMIB and HQNO

It is well known that DBMIB is electrochemically active, being a typical *para*-benzoquinone species. Its midpoint potential at pH 7 in aqueous solution is around +160 mV with pK values on the quinol at 8.6 and 11.6 [35]. It is not so well known, however, that 4-hydroxyquinoline *N*-oxide derivatives are also redox active. Fig. 1 shows cyclic voltammograms of both DBMIB and HQNO, taken in an aqueous solvent at pH 7. In both cases the anodic and cathodic peaks are widely separated, a behaviour caused by the two-electron nature of the reactions involved, causing the rate determining step to involve an entirely different redox couple for the oxidative and reductive processes [36]. Nevertheless, the voltammograms remain constant for many redox cycles, indicating that there are no dead-end products. Other 4-hydroxyquinoline *N*-oxides gave essentially the same result as HQNO. For all of these 4-hydroxyquinoline *N*-oxides, it was established by changing initial scan direction that the original compound is the reduced form of the redox couple. By using an approximation that the $n = 2$ midpoint potential is close to the average of anodic and cathodic half wave potentials, the voltammograms gave approximate E_{m7} values of +205 mV and +435 mV respectively for DBMIB and HQNO in ethanol/water. The alkyl-4-hydroxyquinoline *N*-oxide redox transitions presumably involve a reversible oxidation to the analogue of a quinone-like structure.

Results

Duroquinol as a reductant of the plastoquinone pool and as a direct donor to the cytochrome bf complex

It has not previously been appreciated (e.g., Ref. 37) that duroquinol reduces the plastoquinone pool only very slowly. In Fig. 2 the rate of reduction of the plastoquinone pool was assessed from the area under a fluorescence induction curve [38]. About 30 s were required for 90% reduction of the pool by 1 mM duroquinol at pH 7.3 and 23°C. Decreasing the concentration of duroquinol below 0.5 mM (the approximate

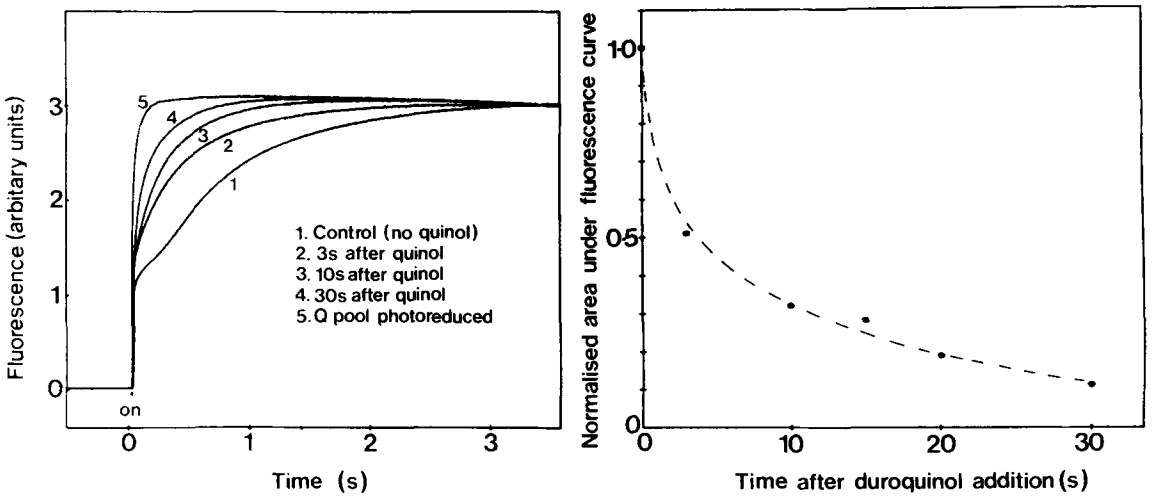

Fig. 2. Rate of reduction of the plastoquinone pool by added duroquinol. Fresh, broken pea chloroplasts were resuspended to 25 μg/ml in 0.4 M sucrose, 50 mM Tricine, 50 mM NaCl and 5 mM $MgCl_2$ at pH 7.3. Red fluorescence of > 635 nm was induced with weak actinic blue light at variable times after the addition of 1 mM duroquinol. The area difference between curves 1 and 5 was taken as a measure of a fully oxidised quinone pool.

solubility limit of duroquinol in these mixtures) caused the timescale of pool reduction to become even longer. This could be demonstrated in an independent method whereby the kinetics of cytochrome *f* rereduction after a series of closely spaced flashes were monitored (Fig. 3). With 0.5 mM duroquinol, cytochrome *f* continued to rereduce with an observed rate constant of around 100 s^{-1}, even after many flashes. However, at low duroquinol concentrations (0.1 mM) with 15 s of dark adaptation between trains of flashes, the observed cytochrome *f* rereduction rate constant diminished from around 50 s^{-1} on the first flash to 20 s^{-1} on the sixth flash. Longer dark adaptation times with 0.1 mM duroquinol resulted in a rate constant on the first flash which approached that of the 0.5 mM duroquinol sample, with a lag in the decrease of this rate constant with flash number. At all dark times with 0.1 mM duroquinol, the observed rate constant eventually decreased to around 20 s^{-1} after many flashes. These effects must have arisen from the very slow reduction of the quinone pool during dark adaptation by 0.1 mM duroquinol, with the level of plastoquinol in the pool diminishing with each flash. The duroquinol at this concentration could only support a cytochrome *f* rereduction rate of 20 s^{-1}. At 0.5 mM duroquinol, however, the direct rate of reduction of the *bf* complex by duroquinol was sufficiently high so that the observed cytochrome *f* rereduction rate did not diminish even after many flashes, regardless of the redox state of the plastoquinone pool.

In mitochondria, duroquinol-ubiquinone transhydrogenase activity is in contrast rapid. The reaction is catalysed by a quinol-quinone transhydrogenase activity of the Q_i site of the cytochrome bc_1 complex and is inhibited by antimycin A [39] and by low concentrations of H(N)QNO (Table II). In confirmation of the

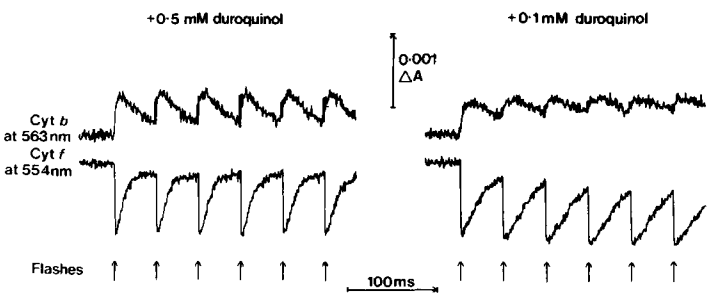

Fig. 3. Rate of rereduction of cytochrome *f* during multiple flashes in the presence of varying duroquinol concentrations. Broken pea chloroplasts were resuspended to 75 μg/ml in a medium of 330 mM glucose, 50 mM Tricine, 10 μM DCMU, 0.3 μM nonactin, 1 μM gramicidin and 100 μM methyl viologen at pH 8 and 23°C. Duroquinol was added at the concentration indicated. Trains of six saturating flashes were provided with a xenon flashlamp filtered with an RG635 Schott glass filter and 15 s dark adaptation was given between each train. Data were deconvoluted as described in Materials and Methods and are the average of eight recordings at each wavelength with a time constant of 2.5 ms.

TABLE II

Quinol-quinone transhydrogenase activities of the isolated beef heart mitochondrial succinate-cytochrome c oxidoreductase and the isolated lettuce chloroplast cytochrome bf complex

Transhydrogenase activity was measured spectrophotometrically at 285 nm with ϵ $mM^{-1}cm^{-1} = 8.5$ (duroquinol → ubiquinone) or at 262 nm with ϵ $mM^{-1}cm^{-1} = 3.5$ (duroquinol → plastoquinone) in a medium of 50 mM potassium phosphate, 2 mM EDTA and 0.05% Tween-80 at pH 7 and 23° C.

Conditions	Beef heart SCR (turnover number quinols s^{-1})	Lettuce *bf* complex (turnover number quinols s^{-1})
50 μM duroquinol → 50 μM *decyl*-ubiquinone	6–7 s^{-1}	< 1 s^{-1}
50 μM duroquinol → 50 μM *decyl*-ubiquinone +2 μM HQNO	< 2 s^{-1}	not tested
50 μM duroquinol → 50 μM *decyl*-plastoquinone	2–3 s^{-1}	< 1 s^{-1}

results described above, however, the cytochrome *bf* complex lacked any detectable quinol-quinone transhydrogenase activity (Table II).

Mechanism of inhibition by DBMIB

(i) Stoichiometry of inhibition. The stoichiometry of inhibition of the cytochrome *bf* complex by DBMIB was assessed by three independent means. The effects of addition of 0.5 DBMIB per *bf* complex on the raw data obtained from each of these assays is shown in Fig. 4. In Fig. 4A, its effect on steady state electron transfer from duroquinol to methyl viologen in broken pea chloroplasts is shown; in Fig. 4B, its effect on the rereduction kinetics of cytochrome *f* by duroquinol in pea chloroplasts after 2 s of far-red-induced cy-

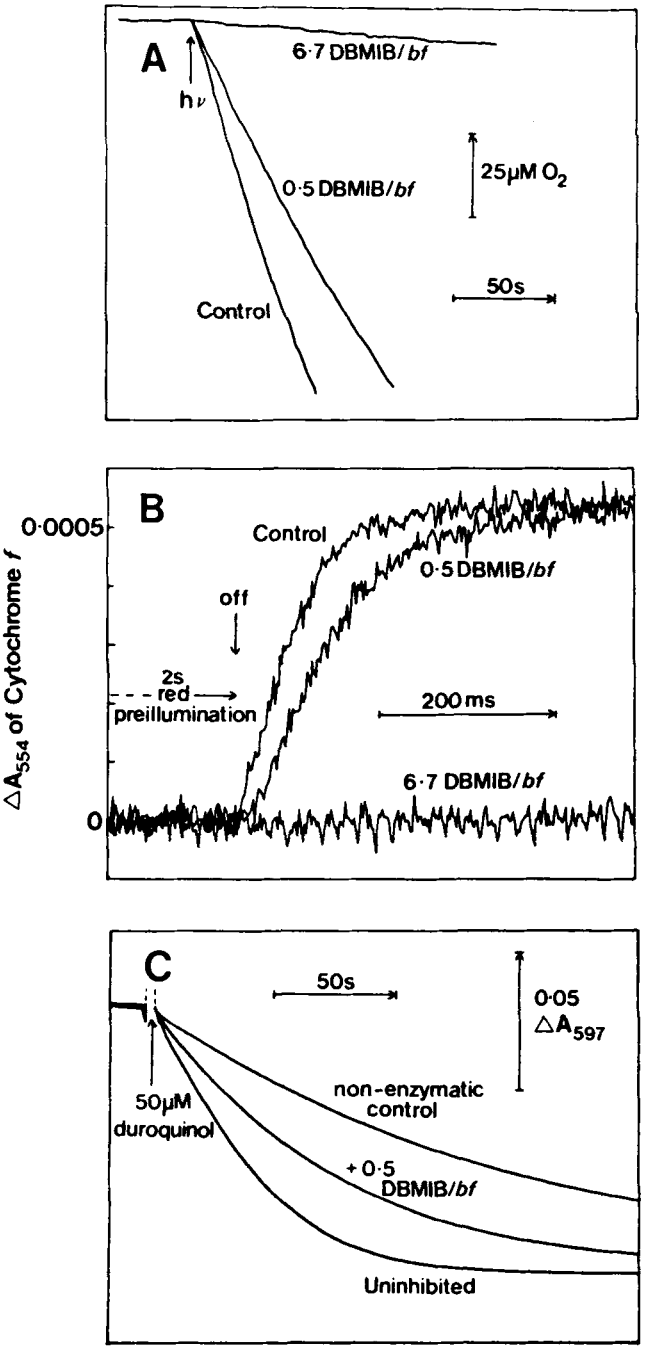

Fig. 4. The effects of 0.5 DBMIB/*bf* complex on electron transfer through the enzyme. Identical samples were used for experiments A and B and consisted of 330 mM sorbitol, 50 mM Hepes, 10 mM KCl, 1 mM EDTA, 5 mM potassium phosphate, 5 mM $MgCl_2$, 3 mM ammonium chloride, 0.5 mM methyl viologen, 10 μM ATP and 10 μM DCMU at pH 7.8 and 23°C. Chloroplasts were added to 25 μg/ml of chlorophyll A+B (31.3 nM *bf* complex), followed by 0.25 mM duroquinol. In A, oxygen consumption was monitored with a Clark-type oxygen electrode and the saturating actinic beam (300 W/m^2) was filtered with an RG610 Schott glass filter. The control rate was equivalent to 306 μmol O_2 consumed/h per mg chlorophyll (273 electrons/s turnover of the *bf* complex). In B, 2 s of preillumination was provided by a saturating actinic beam filtered with an RG645 Schott glass filter and optical changes on switching off the light were followed at 545, 554 and 561 nm. Cytochrome *f* was approximated to be represented by the optical change at 554 nm minus the weighted average of 545 and 561 nm. For each condition, data are the average of 25 recordings at each wavelength, taken with a 5 ms time constant. Wavelengths were rotated between each measurement to remove possible operational errors from long term changes of the sample. Dark adaptation was for 7 s before each measurement and a new sample was used after each batch of 15 cycles. In C, the duroquinol-plastocyanin oxidoreductase activity of purified lettuce cytochrome *bf* complex was assayed. The buffer was 50 mM Mes and 2 mM EDTA at pH 6.2 and 23°C. To this were added sequentially: 100 nM *bf* complex; 20 μM plastocyanin and 50 μM duroquinol. The extent of inhibition by 50 nM DBMIB was calculated after subtraction of the control (no *bf* complex) non-enzymatic rate. Turnover number of the uninhibited enzyme under this condition of low duroquinol concentration was 2.6 electrons/s (c.f. Ref. 26).

tochrome *f* photo-oxidation; in Fig. 4C, its effect on the duroquinol-plastocyanin oxidoreductase activity of isolated lettuce cytochrome *bf* complex.

It is clear that there was significant, but partial, inhibition of all three reactions. It should be noted that conditions for these assays were chosen to give the maximum possible inhibitory effect of DBMIB. Excessive concentrations of duroquinol in any assay weakened the inhibition by DBMIB, presumably because of competition between them [40]. In assays of the isolated *bf* complex, equivalent inhibition of *decyl*-plastoquinol-plastocyanin oxidoreductase or *decyl*-plastoquinol-ferricyanide oxidoreductase activities required far more DBMIB, again presumably because of competition for the Q_o site [8,40].

Quantitation of the degree of inhibition of steady state electron transfer in the assays of Fig. 4A and C was relatively easy since tangents to the curves could be accurately drawn. This gave an inhibition at 0.5 DBMIB/*bf* of $51 \pm 3\%$ (five assays) and $51 \pm 12\%$ (three assays), respectively, for duroquinol to methyl viologen activity of chloroplasts and duroquinol-plastocyanin oxidoreductase activity of isolated *bf* complex.

Quantitation of the degree of inhibition of cytochrome *f* rereduction in chloroplasts (Fig. 4B) was more subjective, however. This arose because the shape of the rereduction curve was complicated by having an initial lag phase (caused by electron flow through to *P-700*$^+$ and plastocyanin). We chose to obtain a rough estimate of inhibition both from the extent of decrease

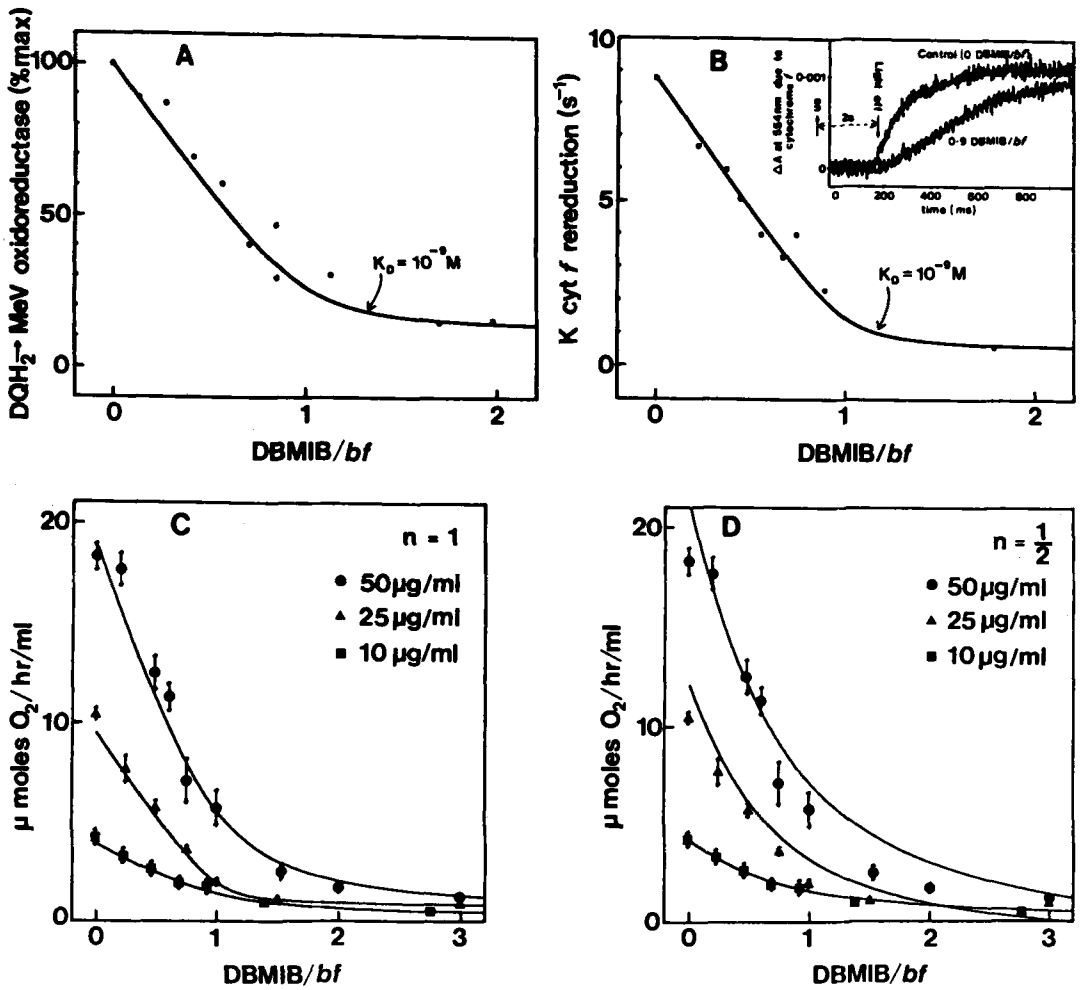

Fig. 5. Estimation of the DBMIB/*bf* ratio in pea chloroplasts. In A, the activity of duroquinol → MeV was titrated with DBMIB. The reaction was monitored by light-induced oxygen consumption of broken chloroplasts (50 μg/ml; 33 nM *bf*) in 330 mM sorbitol, 50 mM Hepes, 10 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$ and 5 mM KPO_4 at pH 7.8 and 23°C and containing 0.5 mM MeV, 3 mM NH_4Cl, 10 μM DCMU, 10 μM ATP and 0.25 mM duroquinol. In B, the rate constant of cytochrome *f* rereduction after 2 s red preillumination was titrated with DBMIB. Reaction mixture was broken chloroplasts (75 μg/ml; 67 nM *bf*) in 160 mM sucrose, 40 mM KCl, 0.8 mM EDTA and 10 mM potassium phosphate at pH 7.25 and containing 0.5 mM MeV, 0.5 mM duroquinol, 10 μM DCMU, 1 μM gramicidin and 0.1 μM nonactin. The cytochrome *f* rereduction profile was approximated, after a lag phase, to a first order process for rate constant fitting. In both cases a theoretical curve for 1DBMIB/monomer with $K_D = 10^{-9}$ M is plotted for comparison. In C and D, the activity of duroquinol → MeV was titrated with DBMIB as in 5A, but at a variety of chlorophyll concentrations. The computer produced best fits by least squares analyses with the constraint that either 1DBMIB/monomer (Fig. 5C) or 0.5 DBMIB/monomer (Fig. 5D) was required for inhibition.

of a first-order rate constant which was fitted to the latter part of the curve after the lag phase and also from an estimate of the time between switching off the actinic beam and the point at which half of the cytochrome *f* had become rereduced. For 0.5 DBMIB/*bf* these gave respectively 47 ± 11% (five assays) and 45 ± 9% (five assays) inhibition of cytochrome *f* rereduction.

In Fig. 5A and B, the effects of varying amounts of DBMIB on the inhibition of the activities of Fig. 4A and B are shown. The solid line represents the simulated behaviour for a requirement of one DBMIB binding site per monomer with a dissociation constant of 10^{-9} M. Sets of data such as those in Fig. 5 were analysed statistically by finding the least squares best fit for a tight binding inhibitor of unknown number of binding sites and dissociation constant, assuming that data at 2DBMIB/*bf* complex represented maximal inhibition. Three sets of data for the duroquinol → methyl viologen assay gave best fits with DBMIB/*bf* and K_D values of 0.89 and $10^{-8.8}$ M, 1.00 and $10^{-8.8}$ M and 1.32 and $10^{-8.5}$ M. Two sets of data for cytochrome *f* rereduction gave best fits with DBMIB/*bf* and K_D values of 1.03 and $10^{-8.72}$ M and 0.90 and $10^{-8.45}$ M. In all cases, the fits of the data were better with 1, rather than with 0.5, DBMIB binding site/*bf* complex. This is clearly illustrated in a comparison of Fig. 5C and D, where the duroquinol → methyl viologen activity of thylakoids has been titrated with DBMIB at a variety of chlorophyll concentrations. Again using the standard equation for a tight binding inhibitor, best fits have been produced by least squares analyses with the

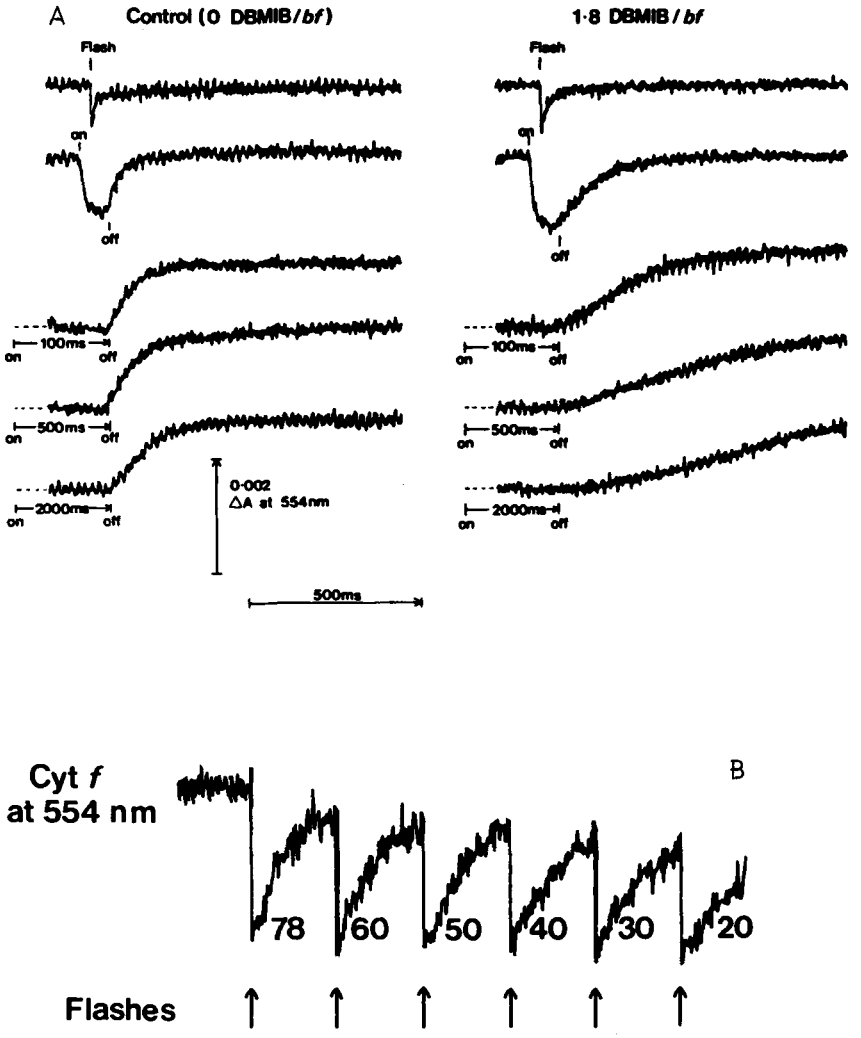

Fig. 6. Increase in extent of inhibition of cytochrome *f* rereduction by DBMIB during multiple turnovers. In A, experiments were carried out as in Fig. 4B except that the time of red (RG635) preillumination of dark-adapted chloroplasts was varied. Cytochrome *f* rereduction on cessation of this illumination was then assayed in control chloroplasts (left) or chloroplasts which contained 1.8 DBMIB/*bf* monomer (right). In B, conditions were the same as Fig. 3, except that data were the average of four recordings at each wavelength with a 1 ms time constant and 40 s of dark adaptation was given between each train of flashes. Duroquinol concentration was 0.5 mM and 1DBMIB/*bf* complex was also present. The estimated rate constants of cytochrome *f* rereduction as a function of flash number are listed.

assumption that either 1DBMIB/monomer (Fig. 5C) or 0.5DBMIB/monomer (Fig. 5D) are required for inhibition of activity. Essentially identical titrations were obtained with chloroplasts grown under daylight conditions.

(ii) Dark reversal of DBMIB inhibition. Experiments were also performed to determine the inhibitory action of low amounts of DBMIB on the rate of cytochrome *f* rereduction after a single turnover flash. Such experiments are normally performed with a long dark adaptation time between flashes. To our surprise we found that there was little inhibition even with 1.8 DBMIB/*bf* complex (see top traces of Fig. 6A). Only at frequencies of flash activation in excess of 1 Hz and after several turnovers did inhibition become apparent.

We investigated this further by testing the effects of time of illumination on the potency of DBMIB inhibition of cytochrome *f* rereduction rate (Fig. 6A). Full inhibition did not occur until after several seconds of illumination, suggesting that the *bf* complex has to turn over several times before maximal inhibition by low concentrations of DBMIB could be observed. The effect of a progressive increase of the extent of inhibition by DBMIB after a dark adaptation was also clearly seen in experiments with multiple rapid actinic flashes (Fig. 6B) given to dark-adapted chloroplasts in the presence of low concentrations of DBMIB. It may be seen that the rate of cytochrome *f* rereduction decreased with each successive flash. Maximal inhibition can only be attained in this experiment with many closely-spaced flashes.

We tested the timescale of dark recovery from inhibition by DBMIB by firstly giving a 2 s preillumination in the presence of 2 DBMIB/*bf* monomer, followed by a single saturating flash given at various times after the preillumination had been stopped. Recovery from inhibition could be assessed from the rate of cytochrome *f* rereduction after this flash. Fig. 7 shows that, at 2 DBMIB/*bf* monomer under these conditions, the half-time of recovery from inhibition was around 10 s. This was considerably slower than the rate at which cytochrome *f* became rereduced under the same conditions, so that recovery was not synchronous with rereduction of the cytochrome *f*.

As the stoichiometric ratio of DBMIB/*bf* was increased, recovery in the dark became less marked and progressive inhibition after each flash in the flash train became more severe. At 10 DBMIB/*bf* complex, reversal of inhibition was maximally only around 50%. However, the time constant of the recovery to this level remained approximately constant.

(iii) Reduced DBMIB as a substrate for the cytochrome bf complex? Since it appeared likely that the inhibitory action of DBMIB was critically dependent upon its redox state, we investigated whether the fully reduced form, $DBMIBH_2$, could rapidly donate electrons into the *bf* complex via the quinol oxidation site. The results are shown in Fig. 8. $DBMIBH_2$ or duroquinol was added to isolated enzyme at pH 6.2 and the rates of reduction of cytochrome *f* were compared. The low quinol concentration (5 μM) and low pH ensured that the rate was sufficiently slow to be measurable with this protocol. It may be seen that the rate of cytochrome *f* reduction was roughly comparable with both quinols. However, the stigmatellin-sensitivity of the duroquinol-induced rate was much greater, presumably because the $DBMIBH_2$ reduced cytochrome *f* both via the quinol oxidation site and via the plastocyanin binding site. At pH 7, both quinol-induced rates were much faster as expected [36], and although the duroquinol-induced rate remained fairly sensitive to stigmatellin, the $DBMIBH_2$-induced rate was not inhibited at all. The results overall suggested that $DBMIBH_2$ could be oxidised by the solubilised cy-

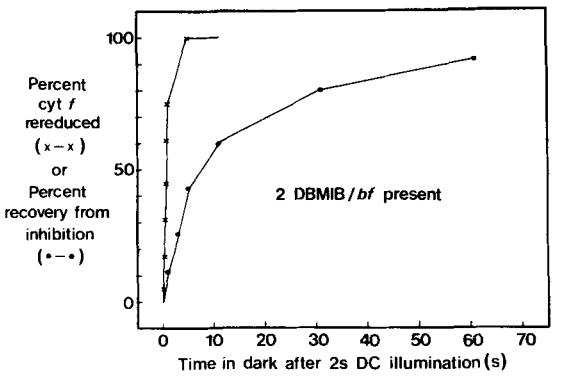

Fig. 7. Kinetics of recovery from inhibition by DBMIB in the dark. The experimental conditions were as for Fig. 4B, with 2 s of red preillumination. This was sufficient to give maximal inhibition. In order to determine the extent of recovery from inhibition in the dark, a single saturating flash was given at various dark times after the preillumination had been switched off and cytochrome *f* rereduction rate constant was estimated. The extent of cytochrome *f* rereduction at various times after switching off the actinic beam was measured from the decay curve of cytochrome *f* of the type shown in Fig. 4B and with a measuring beam sufficiently weak so as to not interfere with this decay.

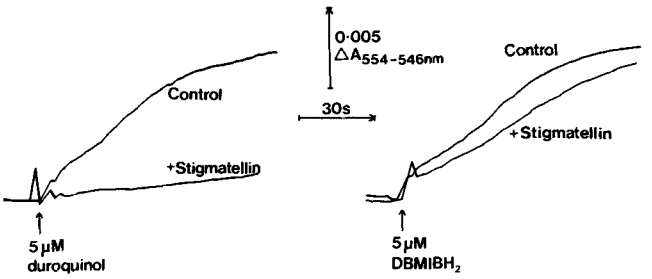

Fig. 8. Direct reduction of isolated cytochrome *bf* complex by duroquinol and $DBMIBH_2$. Cytochrome *bf* complex was suspended to around 0.5 μM in 50 mM Mes and 2 mM EDTA at pH 6.2 and 23°C and with or without 5 μM stigmatellin present. Cytochrome *f* reduction on addition of 5 μM duroquinol or $DBMIBH_2$ was monitored at 554–546 nm.

tochrome *bf* complex both via the quinol oxidation site and via the plastocyanin site, with the latter route becoming dominant as the pH was raised above 6.

(iv) Inhibition in the dark by DBMIBH$_2$. In order to test whether DBMIBH$_2$ itself caused a large inhibition of cytochrome *f* rereduction, experiments were performed as in Fig. 6B with a train of six actinic flashes given at various times after 2 μM DBMIBH$_2$ had been added in the dark. A new sample had to be used for every flash train since recovery from inhibition was so slow at this concentration. It was found with this very high concentration that, although inhibition was complete by the fourth flash, inhibition on the first flash was only around 50% at all times after addition, a result confirming the poor inhibitory effects of the quinol form of the inhibitor. Unfortunately, we were unable to repeat the experiment with oxidised DBMIB since we determined separately that it became reduced in less than 1 s at pH 8 by the added duroquinol, and so gave the same result as addition of DBMIBH$_2$.

Further experiments which involved the redox poising of samples in the presence of redox mediators at potentials low enough to reduce added DBMIB corroborated the notion of a lack of inhibitory effect of 2 μM DBMIBH$_2$. For example, in samples with 100 μM anthraquinone-2-sulphonate poised at -280 mV and at pH 8 (c.f. experiments in Ref. 41) it was found that inhibition of cytochrome *f* rereduction by 2 μM DBMIB was maximally reversed after only 30 s of dark adaptation.

Mechanism of inhibition by H(N)QNO

(i) Effects of HQNO on the midpoint potentials of the haems of cytochrome b-563. HQNO raises the midpoint potential of haem b_H of the mitochondrial bc_1 complex by around 25 mV over a wide pH range [16,17]. Its effects on the haems *b* of the cytochrome *bf* complex have been reported to be more dramatic, raising the E_m values of both haems *b* by around 100 mV [18]. We reinvestigated this effect using both isolated enzyme and whole chloroplasts (Fig. 9). Redox titrations of the isolated enzyme clearly indicated two distinguishable components separated by around 100 mV as already reported [42], with both exhibiting a weak pH-dependency. In chloroplasts their behaviour could also be fitted by two components although, because of interference from cytochrome *b*-559$_{LP}$ at high potentials [31] and from an unidentified non-haem interference at low potentials, we were unable by quantitative statistical analyses to definitively assign two different midpoint potentials to these two *b*-563 haems. Instead, we qualitatively assigned a single midpoint potential to the total haem *b*-563 waves, after ascribing 10% of the total haem change to *b*-559$_{LP}$ at high potential. The weak pH-dependency of this combined midpoint potential of the two haems is plotted in the inset to Fig. 9B.

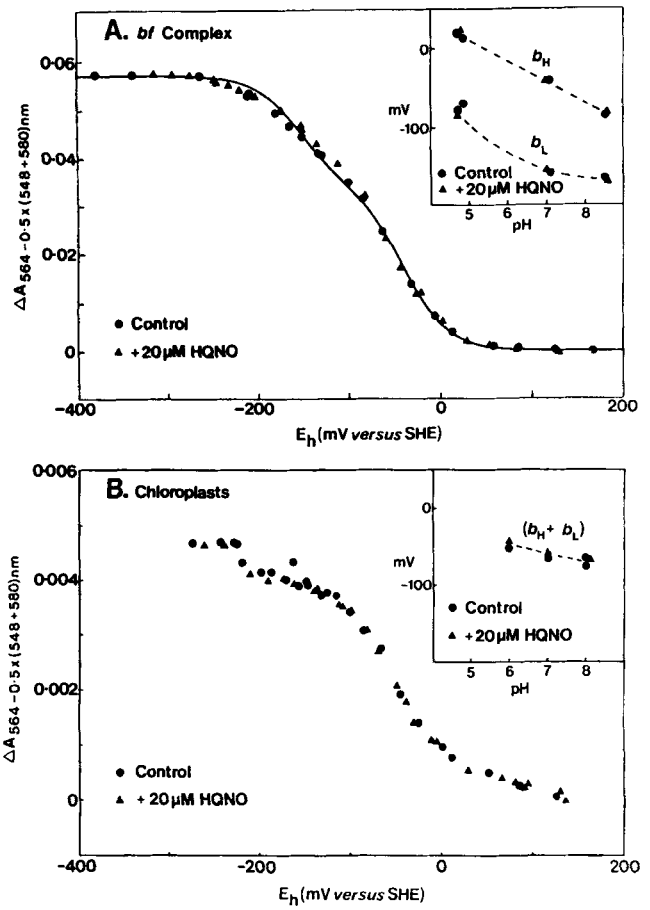

Fig. 9. Redox titrations of the haems of cytochrome *b*-563 in purified cytochrome *bf* complex and in chloroplasts. Cytochrome *bf* complex (A) or broken chloroplasts (B) were redox titrated as described in Materials and Methods in the absence (•) or presence (▲) of 20 μM HQNO. Representative data at pH 7 are shown. In the case of isolated enzyme, a least squares best fit for two components which contribute 60% and 40% of the $564-0.5\cdot(548+580)$ nm signal has been overlaid. The inset in 9A show the dependence on pH of the midpoint potentials of such fits for control (•) and +20 μM HQNO (▲) samples. In the case of chloroplasts, 10% of the total haem signal is caused by cytochrome *b*-559$_{LP}$ and occurs on the high potential end and there is a small spectral interference from dyes at very low potentials. A value has been qualitatively ascribed to the midpoint potential of the combined haems *b*-563 and the pH-dependency of this value is plotted in the inset.

All of these data are roughly consistent with the majority of previous reports (Refs. 19,42,43 and see Discussion) on the redox properties of the *b*-563 haems. Most importantly in the present context, however, we were unable to detect a significant effect of 20 μM HQNO on the midpoint potentials of the haem *b*-563 (or *b*-559$_{LP}$) in isolated enzyme or in chloroplasts at any pH, in contrast to findings of Clark and Hind [18].

(ii) Flash intensity dependency of the extent of P-518$_{slow}$ in the absence and presence of NQNO. It is well known that a slow phase of the carotenoid band-shift, approximately equal in extent to the fast phase caused by Photosystem I, is caused by turnover of the

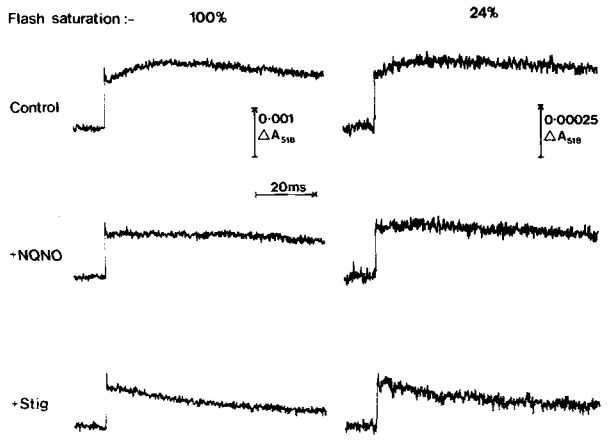

Fig. 10. Effects of flash intensity on the slow carotenoid bandshift in the absence and presence of NQNO. Fresh intact chloroplasts were resuspended to 10 μg/ml in 160 mM sucrose, 40 mM KCl, 0.8 mM EDTA and 10 mM potassium phosphate at pH 7.25 and containing 10 μM DCMU and 1 mM duroquinol. A, control; B, +500 nM NQNO; C, +2 μM stigmatellin. The flashes were directed with light pipes at both sides of the 0.4 cm wide sample to ensure an even actinic illumination. Flash saturation was determined from extent of the fast bandshift. Each trace is the average of fifteen (100% flash) or 80 (24% flash) recordings at 0.5 ms time constant. The transient in the presence of stigmatellin/PMS has no slow carotenoid bandshift and so can be used to determine the extent of slow phase in other traces.

cytochrome *bf* complex [44,45] and that the extent of this slow phase is decreased by around 60% by HQNO or NQNO [9,10]. This has most reasonably been interpreted as indicating a block in the partially electrogenic reaction of haem *b* reoxidation by quinone at the Q_i site. The results on the left side of Fig. 10 with a fully saturating flash are typical.

There is, however, a complication in the interpretation of such an experiment. If the plastocyanin is rapidly mobile between different cytochrome *bf* complexes, then subpopulations of *bf* complexes will turn over a different number of times. In our chloroplasts, the cytochrome *bf*:photosystem I ratio was around 1:1.5. If collisions of plastocyanins and *bf* complexes were completely random, then the Poisson distribution dictates that 48% of the quinol oxidations would have resulted in single turnovers of the complex, with the enzyme ending up in the metastable intermediate one-electron state $(b_L b_H Q_i)^-$. It is possible, therefore, that only double turnovers of enzyme produced an electrogenic reaction and that the effect of NQNO was to increase the number of single turnovers (for example, by preventing a second electron transfer into the enzyme).

The experiment was therefore repeated at a very low flash intensity (Fig. 10) so that the majority of turnovers resulted in a single turnover only (with 24% flash saturation, completely random behaviour would result in 72% of turnovers not being accompanied by a second turnover to complete the reaction cycle). Remarkably, we found little or no difference in the relative extent or risetime of the slow phase in the absence or presence of NQNO. This simple observation leads to two important deductions. In the first instance, a single turnover of the cytochrome *bf* complex must produce a full charge separation under these conditions. Secondly, NQNO must really have an effect on the extent of electrogenic charge separation of the single turnover reaction, as originally proposed by Selak and Whitmarsh [46].

(iii) Effects of NQNO on P-518 changes during multiple flashes. The effects of NQNO and HQNO on the partial inhibition of the extent of the slow rise of the carotenoid bandshift after a single saturating flash have been taken to indicate an inhibition of cytochrome *b* reoxidation, and hence of one of the components of the electrogenic charge separation, by the quinone reduction site. If this explanation is correct, then although the first flash in the presence of NQNO shows a partial electrogenic reaction associated with haem b_H reduction, turnovers of the enzyme on subsequent rapidly spaced flashes would be expected to have no electrogenic component, or even a negative one if the reduced haem *b* could be reoxidised by a semiquinone at the quinol oxidation site, as proposed in some models of the catalytic cycle [20,21]. The experiment of Fig. 11, taken on a time scale slower than that of Fig. 10, was designed with this test in mind. The $P\text{-}518_{slow}$ of control chloroplasts is represented by the difference between traces (A − C); the NQNO-insensitive $P\text{-}518_{slow}$ is given by the difference between traces (B − C). It can be clearly seen that the NQNO-insensitive $P\text{-}518_{slow}$ is at least as large, and possibly faster, on the third flash as compared to the first flash.

(iv) Effects of NQNO on cytochrome b reduction and oxidation via the quinone reactive Q_i site. The quinol-

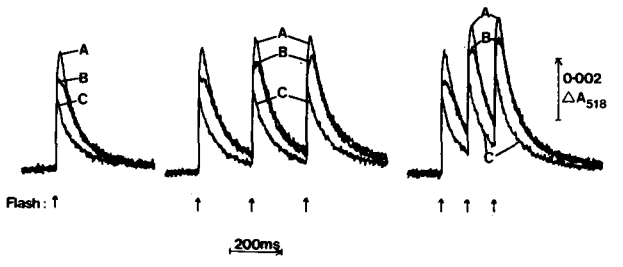

Fig. 11. The extent of $P\text{-}518_{slow}$ during multiple turnover flashes in the presence of NQNO. Intact chloroplasts were resuspended to 25 μg/ml in 160 mM sucrose, 40 mM KCl, 0.8 mM EDTA and 10 mM potassium phosphate at pH 7.8 and containing 10 μM DCMU, 50 μM methyl viologen, 3 mM NH_4Cl and 1 mM duroquinol. Saturating red (RG635) flash(es) were given after 10 s dark adaptation and each trace is the average of five recordings. A, control: B, +500 nM NQNO: C, +2 μM stigmatellin and 1 μM PMS.

quinone transhydrogenase reaction described in Table II is an activity specifically associated with the Q_i site of the *bc* complexes, and is sensitive to Q_i-site inhibitors. Unfortunately, the activity is absent from the cytochrome *bf* complex and so cannot be used as a direct test of whether H(Q)NO inhibits electron transfer through the site. However, one further activity associated with the Q_i site of the *bc* complexes, when the Q_o site has been inhibited, is ferrohaem *b* reoxidation by quinone or ferrihaem *b* reduction by quinol. This latter activity is not easily testable with the *bf* complex because the low potentials of the haems *b* precludes any significant extent of their reduction by plastoquinol or duroquinol via the Q_i site. The only way in which we could test this was to inject oxidised cytochrome *bf* complex into a prereduced anaerobic solution of anthraquinol sulphonate. This quinol is of sufficiently low potential to reduce the *b*-563 haems [47], but does so slowly because it is hydrophilic. Addition of NQNO to the stigmatellin-inhibited enzyme actually stimulated the rate of haem *b* reduction in this assay (Fig. 12A). Although the reduction is slow, it is still likely to proceed through the Q_i site with stigmatellin present. In equivalent experiments with mitochondrial bc_1 complex in which haem *b* reduction through the Q_i site was initiated by the addition of succinate or *decyl*-ubiquinol in the presence of myxothiazol to inhibit the Q_o site, full inhibition of the process occurred with HQNO or NQNO (data not shown).

Oxidation of prereduced haem *b* by added quinone is observable both in *bc* and *bf* complexes. Fig. 12B illustrates the effects of H(N)QNO on this activity in the chloroplast *bf* system. The haems *b* were approx. 70% prereduced by photoreduction under anaerobic conditions. 10 μM duroquinone was then added anaerobically. This caused a rapid and full oxidation of the haems *b* which was stigmatellin-insensitive, and so presumably occurred through the Q_i site. Addition of NQNO (with or without stigmatellin, not shown) did not decrease this oxidation rate. In analogous experiments with the mitochondrial bc_1 complex, the oxidation of haem *b* by added quinones is also insensitive to Q_o site inhibitors, but is severely inhibited by HQNO (data not shown), clearly demonstrating that HQNO is a potent inhibitor of the Q_i site.

(v) The stimulation of cytochrome b-563 reoxidation by duroquinol. One further observation has been relevant to our deductions concerning the mechanism of action of H(N)QNO on the cytochrome *bf* complex. The experiment involved testing whether duroquinol itself could accelerate the rate reoxidation of transiently-generated ferrohaem *b*. This was tested by comparing the rate of haem *b* reoxidation after it had been transiently reduced by oxidant-induced reduction during a series of saturating single turnover flashes with

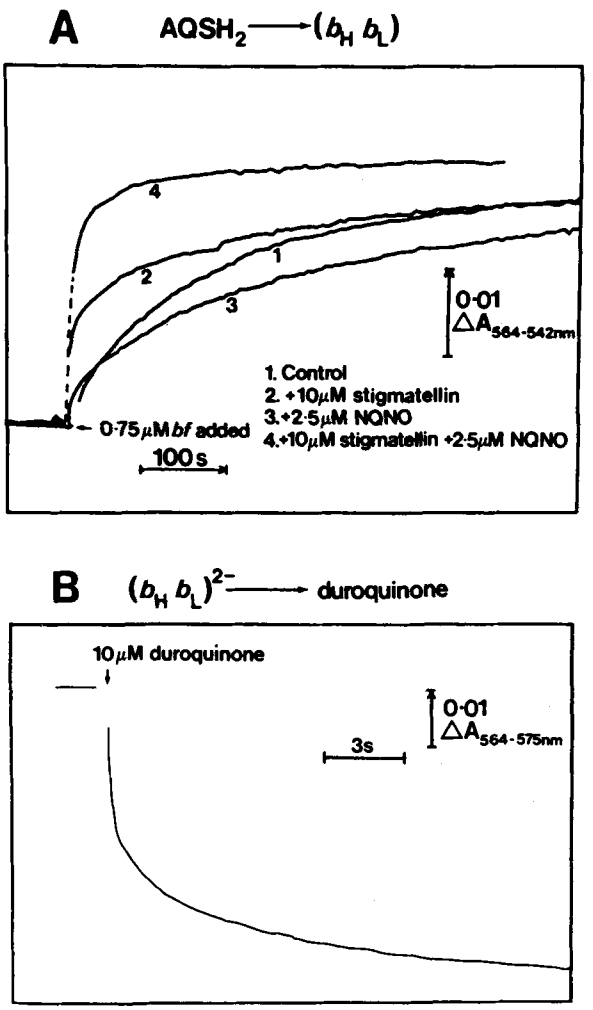

Fig. 12. NQNO sensitivity of quinol-ferrihaem *b* oxidoreductase and ferrohaem *b*-quinone oxidoreductase activities of the cytochrome *bf* complex. In A, a solution of 330 mM glucose, 50 mM Tricine, 400 units/ml catalase, 2500 units/ml glucose oxidase and 100 μM anthraquinone-2-sulphonate at pH 8 was kept anaerobic with nitrogen and photoreduced to around 50% reduction of the quinone system (ambient E_h around −250 mV) [41]. 0.75 μM oxidised cytochrome *bf* complex (1.5 μM haems *b*) which had been pretreated as indicated was anaerobically injected into the solution and the kinetics of haem *b* reduction were monitored at 564–542 nm. The maximum extent of trace 4 indicates full reduction of both haems *b*. In B, 2 μM cytochrome *bf* complex was added to an anaerobic, photoreduced anthraquinone-2-sulphonate solution as above (but which contained in addition 50 μM methyl viologen to further accelerate haem *b* reduction). The haems *b* were around 70% reduced at the point of addition of anaerobic 10 μM duroquinone and their oxidation was monitored at 564–575 nm.

either endogenous plastoquinol or added duroquinol as the donor (Fig. 13). It is clear that the maximal extent of transient haem *b* reduction was significantly decreased, and therefore the rate of haem *b* reoxidation was apparently increased, by the added duroquinol. The effect appeared to not be caused by contaminating duroquinone since 100 μM duroquinone added with duroquinol did not increase the effect.

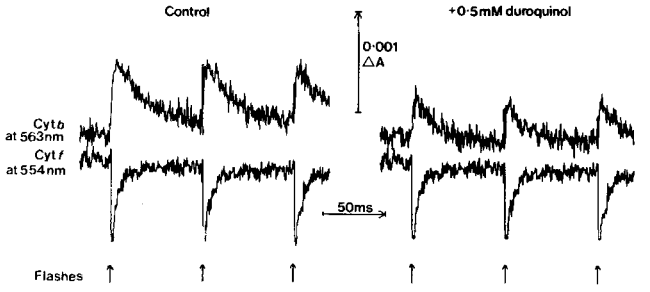

Fig. 13. Stimulation of *b*-563 oxidation by duroquinol in the presence of plastoquinol. Broken chloroplasts were resuspended to 75 μg/ml in 160 mM sucrose, 40 mM KCl, 0.8 mM EDTA and 10 mM potassium phosphate at pH 7.27 and containing 0.1 μM nonactin and 1 μM gramicidin. Duroquinol where added was 0.5 mM. The sample was given 1 s of continuous red illumination in order to cause plastoquinone pool reduction, followed by 2 s of dark adaptation before the measuring flashes were given. Data are the average of ten recordings at each wavelength with a 5 ms time constant.

Discussion

Duroquinol donation to the bf complex and the plastoquinone pool

The data presented in Figs. 2 and 3 strongly indicate that duroquinol is a very poor direct donor to the plastoquinone pool. This is consistent with the known required chemical mechanism for quinol reduction of quinones [48]. In mitochondria, duroquinol reduction of the ubiquinone pool is rapid [49], but is catalysed by a quinol-quinone transhydrogenase activity of the bc_1 complex [39]. The activity presumably arises from a transient donation of two electrons from a quinol to the haem *b* pair via the Q_i site, followed by donation of these two electrons onto another quinone. The midpoint potentials of the haems *b* of the cytochrome *bf* complex are much lower than their bc_1 counterparts, however, and so the unstable intermediate state will be very improbable. Hence, a quinol-quinone transhydrogenase activity is absent (Table II).

The data of Fig. 3 also indicate that duroquinol itself must be a rapid donor to the cytochrome *bf* complex at sufficiently high concentrations. At pH 8, the observed rate constant of cytochrome *f* rereduction was around 100 s^{-1} with 0.5 mM duroquinol. Since two to three plastocyanin molecules are reduced synchronously with the cytochrome *f*, this is consistent with a duroquinol-supported turnover number of the cytochrome *bf* complex of 300–400 electrons s^{-1}, as observed experimentally (see, for example, legend to Fig. 4).

Duroquinol interaction with the chloroplast electron transport chain is often depicted as a donation of electrons to the plastoquinone pool. The present data show clearly that this process is extremely slow. Instead, rapid donation occurs directly into the cytochrome *bf* complex.

The mechanism of action of DBMIB

A number of features of inhibition by DBMIB of the chloroplast cytochrome *bf* complex can be deduced from the present and previously published data.

(i) Under optimal conditions it is an extremely tight binding inhibitor of the quinol oxidation site of the cytochrome *bf* complex with a K_D close to 10^{-9} M (Fig. 2). Its effect is weakened by *decyl*-plastoquinol or duroquinol, in agreement with Trebst [8] and Nanba and Katoh [40], presumably partly because these substrates compete for the quinol oxidation site with $DBMIBH_2$, but possibly also because they accelerate the reduction of the tightly bound oxidised form(s) of DBMIB.

(ii) With pea chloroplasts or isolated *bf* complex from lettuce, all of our data indicate a requirement for 1DBMIB/*bf* monomer for inhibition of electron transfer activity. Hurt and Hauska [27] reported a similar result with isolated spinach enzyme, but other data obtained with spinach chloroplasts have indicated a requirement of less than one DBMIB/*bf* monomer for maximal inhibition, and a dimeric catalytic complex has been proposed [13]. Some of the discrepancy in results may result from a cumulative effect of small possible errors of quantitation which are inevitably inherent in both our data and those of Graan and Ort. However, another possible reason for findings of a substoichiometric titre may lie in the mechanism by which inhibition by DBMIB is actuated. As we have shown, tight inhibition at low DBMIB concentrations requires a number of turnovers of the cytochrome *bf* complex. Hence, if part of the cytochrome *bf* complex population does not turn over a number of times relatively rapidly, perhaps because of the known heterogeneity of component distribution which can occur in the chloroplast membranes [50], then a substoichiometric DBMIB titre could be found. Although we were unable to observe any such effect in our pea chloroplast preparations whether grown at low or high light intensities, other specific growth conditions might promote such an effect.

Although Staehelin et al. [50,51] have provided convincing evidence that the *bf* complex is structurally dimeric in the membrane, the notion of a single Q_o site per dimer to make this structural feature of functional significance seems particularly unlikely since four redox centres (two iron-sulphur centres and two haems *b*) would have to have electronic access to the site. Similar titrations of the *bc* complexes with tight binding inhibitors have indicated a requirement for inhibition of one inhibitor *per bc* monomer [14,39,52] and suggestions for a dimeric reaction cycle which have arisen from other types of experiment with this system have involved a single Q_i site, rather than a single Q_o site, per dimer (e.g., Ref. 53).

(iii) In the dark and in the presence of duroquinol,

inhibition by low amounts of DBMIB is slowly, but reversibly, lost. The reversal is not kinetically correlated with cytochrome *f* rereduction and is accelerated by increased duroquinol concentrations. Several turnovers of the enzyme are necessary to reattain maximal inhibition. The extent, but not the rate, of recovery from inhibition slows as the DBMIB concentration is increased, becoming negligible at a concentration of several micromolar.

(iv) $DBMIBH_2$ can be oxidised by the isolated cytochrome *bf* complex. At least some of this oxidation appears to occur through the quinol oxidation site, although in the solubilised system direct reduction of cytochrome *f* via the plastocyanin site may predominate. Even if high concentrations (2 μM) of $DBMIBH_2$ are added to chloroplasts in the dark, inhibition on the first flash is weak.

In our view, these data are most reasonably accommodated in a model in which an oxidised form of DBMIB (i.e., semiquinone or quinone) is the tight stoichiometric inhibitor of the enzyme. In the presence of duroquinol or low ambient potential (< 200 mV), the oxidised DBMIB which is bound will slowly be reduced in the dark to the quinol, $DBMIBH_2$, causing unbinding. Flash activation will lead to a competition between plastoquinol, duroquinol and $DBMIBH_2$ for the quinol oxidation site. Those sites which receive a $DBMIBH_2$ will oxidise it and so form a tight inhibitory complex. Dark adaptation is then necessary to rereduce the oxidised inhibitor to the quinol which then falls off the site. Either the semiquinone or quinone form of DBMIB could be the dominant species in the inhibitory complex. The former may occur because of an insufficiently low potential for haem *b* reduction of the $Q\cdot^-/Q$ couple; the latter may be inhibitory simply because of a very low off dissociation constant. We have previously estimated that the aqueous solution midpoint potential of the $Q\cdot^-/Q$ couple of DBMIB is around +70 mV [48], a value which would be consistent with a thermodynamic barrier to semiquinone oxidation by haem *b*. Malkin [54] was able to estimate that the redox potentials of bound DBMIB at pH 8 are around +20 mV ($Q\cdot^-/Q_{ox}$) and −220 mV ($Q\cdot^-/Q_{red}$), a result which also indicates such a barrier, although electron transfer at a sufficient rate cannot be ruled out by this argument and electron transfer from semiquinone to iron sulphur centre might even occur instead. As pointed out by Malkin [54], these midpoint potentials also demonstrate that both DBMIB and $DBMIB\cdot^-$ bind much more strongly than $DBMIBH_2$, a deduction consistent with our model for the mechanism of inhibition and also consistent with the observed slow rate of reduction of the inhibitory species. Degli Esposti et al. [55] have argued from observations of the effects of DBMIB on mitochondrial cytochrome bc_1 complex that the semiquinone form of DBMIB is likely to be the inhibitory species. As yet, however, none of our experiments can conclusively corroborate this notion.

Regardless of whether the inhibitory state entails the quinone or semiquinone form of DBMIB, the inhibitory complex will have a very low rate constant of dissociation. If the rate limiting step of recovery from inhibition is dissociation of the inhibitory species, then dissociation rate constants are in the range of 0.1 to 0.01 s^{-1} under any of the conditions which we have used. If, however, the rate limiting step is direct reduction of the bound inhibitory species (as seems likely since rate of recovery is accelerated by increased duroquinol concentrations), then dissociation rate constants may be very much less than this. An opposite conclusion was recently reached by Jones and Whitmarsh [10], who deduced that DBMIB was exchanging rapidly between *bf* complexes on the basis that the kinetics of cytochrome *f* rereduction were always monophasic even when inhibition was partial. We fully confirm this experimental observation, but instead attribute it to the very rapid inter-*bf* equilibrating property of plastocyanin. Such an interpretation has already been discussed in detail by Haehnel [56].

The maximum extent of recovery from inhibition decreased at high molar ratios (> 5) of DBMIB/*bf* complex. Presumably, this was caused by an increased fraction of flash-oxidised *bf* complexes being hit by a reduced $DBMIBH_2$, rather than by a duroquinol or a plastoquinol. At a molar ratio of around 10 $DBMIBH_2$/*bf* complex and in the presence of 0.5 mM duroquinol, this probability appeared to be roughly 50%. Electron transfer from a bound $DBMIBH_2$ into the complex via the quinol oxidation site must be significantly slower than from a bound plastoquinol or duroquinol, but must still eventually occur to produce the tightly bound inhibitory species. Hence, at high molar ratios of added DBMIB, relief from inhibition appears to become progressively less, even although the tight inhibitory complex will have defused as normal in the dark. Rich and Bendall [57] previously provided evidence that $DBMIBH_2$ might be an extremely fast donor into the Q_o site of the isolated cytochrome *bf* complex. From the experiments of Fig. 8, however, it now seems likely that cytochrome *f* reduction by $DBMIBH_2$ seen in Ref. 57 was caused mainly by direct donation via the plastocyanin site. This chemical by-pass clearly occurs much less in the intact chloroplast system since maximal inhibition can be so large, and is probably because of partitioning of the inhibitor into the membranous phase.

The mechanism of action of HQNO and NQNO

HQNO and NQNO are well known as inhibitors of the quinone reduction site, and at higher concentra-

tions are inhibitors of the quinol oxidation site, of the *bc*-type complexes [1,15,58]. Our data on inhibition of quinol-quinone transhydrogenase and ferrohaem *b*-duroquinone oxidoreductase in mitochondrial bc_1 complex show clearly that inhibition of the Q_i site is caused by a direct block of the reaction, rather than by a secondary result of elevation of the midpoint potential of haem b_H.

Their action on the chloroplast *bf* complex was first described by Whitmarsh and colleagues [9,10,46]. Observations on the promotion of oxidant-induced reduction of the cytochrome *b* [46], inhibition of the quinol oxidation reaction at high concentrations [10] and raising of midpoint potentials of the haems *b* [18] have prompted the very reasonable interpretation that their actions on the *bc* and *bf* complexes are analogous. However, concentrations which are sufficient to cause a maximal effect on the quinone reduction site completely inhibit steady state electronic turnover of the *bc* complexes but have no effect on steady state electronic turnover [10] or proton translocating stoichiometry [59] of the *bf* complex. Furthermore, at these inhibitor concentrations oxidant-induced reduction of both haems *b* is possible in the *bc* complexes, but oxidant-induced reduction of more than one haem *b* has never been achieved with the *bf* complex [19,60]. These findings have led to views that the cytochrome *bf* complex may have a basic reaction cycle which can be different to that of the *bc* complexes, possibly one in which the quinone reduction site is not absolutely required for steady state turnover, and possibly in a cycle which does not lead to an extra proton translocation [19–21].

The present data extend the information on the action of H(N)QNO on the cytochrome *bf* complex by demonstrating that NQNO does not prevent electrogenic turnover of the enzyme during multiple flashes (Fig. 11), nor prevent access of plastoquinone analogues to haem *b* (presumably via the Q_i site) when the Q_o site has been blocked (Fig. 12). Some results compatible with this latter observation can also be found in [41]. The observations lead us to suggest overall that a normal pathway of haem *b* oxidation by quinone can occur in the presence of NQNO, and that this pathway is electrogenic in the same way as in the unliganded enzyme. This leak through the H(N)QNO-inhibited Q_i site must be capable of supporting maximal turnover rates of the *bf* complex which are in excess of 300 electrons s^{-1} in the presence of duroquinol. It appears that duroquinol itself may be able to increase the leak rate through the site (Fig. 13) although the chemical basis of this effect is unknown.

The question then arises as to why additional oxidant-induced reduction (at least of one haem *b*) is observable with these compounds. We originally assumed [23] that this arose from the fact that HQNO, and therefore presumably also NQNO, causes an elevation of the midpoint potentials of the haems *b* [18]. Hence in the one-electron state of the enzyme, $(b_H b_L Q_i)^-$, the electron would reside mostly on the haems rather than on the Q_i. In this way a greater transient reduction of haem *b* could be observed without the necessity of a block of the quinone reduction site. However, our own redox data (Fig. 9) lead us to seriously doubt such an interpretation, since we observe no significant shift in haem *b* potentials caused by HQNO. Instead, we now favour a model where the reaction of haem *b* reoxidation at the Q_i site is indeed slowed down by NQNO and HQNO, at least for the one-electron state. However, throughput of two electrons to complete the catalytic cycle cannot be impaired to an extent sufficient to cause an observable inhibition of steady state electron transfer or proton translocation. A direct two-electron reduction of NQNO itself at the site can be ruled out as a feasible mechanism since the electrochemical data reveal no suitable redox couple. Similarly, a much weaker binding of HQNO to the two-electron reduced state would have shown up in the redox titration data as a decrease in midpoint potential of one or both haems in the presence of HQNO. Instead, we postulate that a lack of inhibition may arise either because of an electron transfer pathway to quinone which can circumvent or pass through the H(N)QNO molecule, or because of very rapid on/off rate constants of quinone and H(N)QNO at the site such that electron transfer to quinone can still occur at a rapid rate even although the site is, on average, occupied by H(N)QNO. It should be noted that a significant inhibition of the rate constant of haem *b* reoxidation can have a less dramatic effect on the measured turnover number of the enzyme. For example, consider the simple idealised kinetic model:

$$\overset{k_1}{\rightarrow} b_H \overset{k_2}{\rightarrow}$$

If k_2 is initially 3 times k_1 and is inhibited by a factor of 9 by NQNO so that it becomes $1/3k_1$, then the maximum haem *b* observed to be reduced will change from 0.25 to 0.75, and maximum turnover number of the enzyme will also change by only a factor of 3. Inhibition of the haem *b* reoxidation step may be masked further if other reactions in the pathway are limiting. This must be the case in the type of assay used in Fig. 5 of Jones and Whitmarsh [10], for example, since they showed that 1 μM NQNO is without effect on duroquinol → methyl viologen oxidoreductase activity even although it inhibits 3-fold the kinetics of cytochrome *f* rereduction after a flash.

Furbacher et al. [19] recently found that ferredoxin/NADPH caused prereduction of haem b_H alone and that this did not affect the extent of reduc-

tion of haem *b* in the presence of NQNO. They concluded that electron transfer between the haems *b* was unlikely to occur normally and that the action of NQNO was most likely to be a block of a route of haem b_L oxidation which was operative only in cyclic electron transfer. In our opinion, such an unconventional scheme is not warranted by the data. In a Q-cycle model, prereduction of one haem would cause a transient oxidant-induced reduction of the other, as observed experimentally in chromatophores [61,62]. The data of Furbacher et al. [19] are entirely consistent with a Q-cycle formulation, provided again that NQNO fails to sufficiently block reoxidation of the two haems *b* by quinone.

Our previous redox titration data of chloroplasts [43] had been interpreted in terms of an indistinguishable pair of haems of cytochrome *b*-563. Later, it was shown with purified enzyme that the haems had separable midpoint potentials and spectra [18,42]. We have confirmed such a separation of midpoint potentials in purified enzyme. Such a separation might also be accommodated within our original chloroplast data [43], although a single component was assumed at the time. Our present higher quality data with chloroplasts could also be consistent with a separation of midpoint potentials (up to 100 mV at pH 6 and up to 50 mV separation at pH 8, data not shown). These chloroplast data, however, are still complicated by the presence of cytochrome b-559_{LP} [31] which distorts the high potential end of the curve in Fig. 9B, making more quantitative statistical analyses difficult. What is completely clear from our new data, however, is that neither haem *b* potential is significantly affected by HQNO. The demonstration by Clark and Hind [18] of an increase in midpoint potentials of both haems *b* by HQNO is difficult to explain. If this were so, then both haems *b* would become reduced by duroquinol in the dark in the presence of HQNO, but this undoubtedly does not occur. The finding that their titrations had to be fitted with $n = 2$ curves suggests that equilibrium may not have been attained, and this may be the source of their apparent large midpoint potential shifts.

Acknowledgements

This work is supported by grant GR/E53941 of the Science and Engineering Research Council of the U.K. and by the benefactors of the Glynn Research Foundation Ltd. We are grateful to Dr. A.J. Moody for statistical analyses of data and other helpful discussions, to Mr. A.E. Jeal for redox titrations, to Mr. R. Harper for organic syntheses and electrochemical work, to Dr. D.S. Bendall for useful discussions on hydroxquinoline *N*-oxides and to Dr. P. Mitchell for other valuable contributions.

References

1 Von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
2 Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133.
3 Rich, P.R. and Moss, D.A. (1987) in The Light Reactions (Barber, J., ed.), pp. 421–445, Elsevier, Amsterdam.
4 Oettmeier, W., Godde, D., Kunze, B. and Höfle, G. (1985) Biochim. Biophys. Acta 807, 216–219.
5 Malkin, R. (1986) FEBS Lett. 208, 317–320.
6 Rich, P.R. (1987) in Cytochrome Systems. Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L., eds.), pp. 495–502, Plenum Press, New York.
7 Nitschke, W., Hauska, G. and Rutherford, A.W. (1989) Biochim. Biophys. Acta 974, 223–226.
8 Trebst, A. (1980) Methods Enzymol. 69, 675–715.
9 Jones, R.W. and Whitmarsh, J. (1985) Photobiochem. Photobiophys. 9, 119–127.
10 Jones, R.W. and Whitmarsh, J. (1988) Biochim. Biophys. Acta 933, 258–268.
11 Bowes, J.M. and Crofts, A.R. (1981) Arch. Biochem. Biophys. 209, 682–686.
12 Avron, M. (1961) Biochem. J. 78, 735–739.
13 Graan, T. and Ort, D.R. (1986) Arch. Biochem. Biophys. 248, 445–451.
14 Moody, A.J. and Rich, P.R. (1989) Biochim. Biophys. Acta 973, 29–34.
15 Papa, S., Izzo, G. and Guerrieri, F. (1982) FEBS Lett. 145, 93–98.
16 Kunz, W.S. and Konstantinov, A.A. (1983) FEBS Lett. 155, 237–240.
17 Rich, P.R., Jeal, A.E., Madgwick, S.A. and Moody, A.J. (1990) Biochim. Biophys. Acta 1018, 29–40.
18 Clark, R.D. and Hind, G. (1983) Proc. Natl. Acad. Sci. USA 80, 6249–6253.
19 Furbacher, P.N., Girvin, M.E. and Cramer, W.A. (1989) Biochemistry 28, 8990–8998.
20 Hartung, A. and Trebst, A. (1985) Physiologie Vègètale 23, 635–648.
21 Cramer, W.A., Black, M.T., Widger, W.R. and Girvin, M.E. (1987) in The Light Reactions (Barber, J., ed.), pp. 447–493, Elsevier Science Publishers B.V., Amsterdam.
22 Rich, P.R. (1989) in Highlights of Modern Biochemistry (Kotyk, A., Škoda, J., Pačes, V. and Kostka, V., eds.), pp. 903–912, VSP, Zeist.
23 Rich, P.R. (1990) in Proceedings of the VIIIth International Congress on Photosynthesis (Balcheffsky, M., ed.), pp. 239–245, Kluwer Academic Press, Dordrecht.
24 Lee, W.-J. and Whitmarsh, J. (1989) Plant Physiol. 89, 932–940.
25 Moss, D.A. and Bendall, D.S. (1984) Biochim. Biophys. Acta 767, 389–395.
26 Rich, P.R., Heathcote, P. and Moss, D.A. (1987) Biochim. Biophys. Acta 892, 138–151.
27 Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.
28 Witt, H.T. (1979) Biochim. Biophys. Acta 505, 355–427.
29 Hope, A.B. and Matthews, D.B. (1987) Aust. J. Plant Physiol. 14, 29–46.
30 Dutton, P.L. and Wilson, D.F. (1974) Biochim. Biophys. Acta 346, 165–212.
31 Bendall, D.S. (1982) Biochim. Biophys. Acta 683, 119–151.
32 Rich, P.R. (1981) Biochim. Biophys. Acta 637, 28–33.
33 Stidham, M.A. and Siedow, J.N. (1983) Photochem. Photobiol. 38, 537–539.
34 Caspar, T. and Siedow, J.N. (1982) Photochem. Photobiol. 36, 153–158.

35 Laviron, E. (1986) J. Electroanal. Chem. 208, 357–372.
36 Rich, P.R. (1982) Faraday Discuss. Chem. Soc. 74, 349–364.
37 White, C.C., Chain, R.K. and Malkin, R. (1978) Biochim. Biophys. Acta 502, 127–137.
38 Forbush, B. and Kok, B. (1968) Biochim. Biophys. Acta 162, 243–253.
39 Zweck, A., Bechmann, G. and Weiss, H. (1989) Eur. J. Biochem. 183, 199–203.
40 Nanba, M. and Katoh, S. (1986) Biochim. Biophys. Acta 851, 484–490.
41 Moss, D.A. and Rich, P.R. (1987) Biochim. Biophys. Acta 894, 189–197.
42 Hurt, E. and Hauska, G. (1982) J. Bioenerg. Biomembr. 14, 405–424.
43 Rich, P.R. and Bendall, D.S. (1980) Biochim. Biophys. Acta 591, 153–161.
44 Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284.
45 Joliot, P. and Joliot, A. (1986) Photosynthesis Research 9, 113–124.
46 Selak, M.A. and Whitmarsh, J. (1982) FEBS Lett. 150, 286–292.
47 Cox, R.P. (1979) Biochem. J. 184, 39–44.
48 Rich, P.R. and Bendall, D.S. (1980) Biochim. Biophys. Acta 592, 506–518.
49 Van Hoek, A.N., Van Gaalen, M.C.M., De Vries, S. and Berden, J.A. (1987) Biochim. Biophys. Acta 892, 152–161.
50 Staehelin, L.A. (1986) in Photosynthesis (Encyclopedia of plant physiology; New series, Vol. 5–6, 19) III. Photosynthetic membranes and light harvesting systems (Staehelin, L.A. and Arntzen, C.J., eds.), pp. 1–72, Springer-Verlag, Berlin.
51 Morschel, E. and Staehelin, L.A. (1983) J. Cell Biol. 97, 301–310.
52 Thierbach, G. and Reichenbach, H. (1981) Biochim. Biophys. Acta 638, 282–289.
53 De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 41–53.
54 Malkin, R. (1981) FEBS Lett. 131, 169–172.
55 Degli Esposti, M., Rotilio, G. and Lenaz, G. (1984) Biochim. Biophys. Acta 767, 10–20.
56 Haehnel, W. (1982) Biochim. Biophys. Acta 682, 245–257.
57 Rich, P.R. and Bendall, D.S. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliariello, E., Siliprandi, N. and Slater, E.C., eds.), pp. 187–190, Elsevier/North Holland, Amsterdam.
58 Izzo, G., Guerrieri, F. and Papa, S. (1978) FEBS Lett. 93, 320–322.
59 Hope, A.B. and Rich, P.R. (1989) Biochim. Biophys. Acta 975, 96–103.
60 Rich, P.R. (1988) Biochim. Biophys. Acta 932, 33–42.
61 Glaser, E.G. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 322–333.
62 Meinhardt, S.W. and Crofts, A.R. (1983) Biochim. Biophys. Acta 723, 219–230.