Biochimica et Biophysica Acta 933 (1988) 35–41
Elsevier

BBA 42728

Electron conduction between *b* cytochromes of the mitochondrial respiratory chain in the presence of antimycin plus myxothiazol

Ian C. West, Peter Mitchell and Peter R. Rich

Glynn Research Institute, Bodmin (U.K.)

(Received 20 October 1987)

Key words: Electron transfer; Cytochrome bc_1 complex; Antimycin; Myxothiazol; Membrane potential

The *b* haems of the bc_1 complex of bovine heart mitochondria were poised with succinate and fumarate so that only the high-potential haem (*b*-562) was reduced, and then isolated from further redox exchange with the ubiquinone pool by adding antimycin and myxothiazol. A transmembrane electric potential difference was then developed, either by electron flow from $[Ru(NH_3)_6]Cl_2$ to oxygen or by ATP hydrolysis. The small difference spectrum, caused by the electric field, indicated 32–55% oxidation of *b*-562 with concomitant reduction of *b*-566. No lag greater than 0.1 s was detectable between the initiation of respiration and the development of the difference spectrum, thus providing a direct demonstration of (fairly) rapid electron transfer between the *b* haems.

Introduction

The two *b* haems of the bc_1 complex of mammalian mitochondria are currently pictured as lying approx. 2 nm apart (centre to centre) on a line normal to the plane of the membrane [1]. This is based on a proposed folding pattern of the *b*-peptide amino-acid sequences from a number of organisms and the identification of four conserved histidine residues likely to form the axial ligands for the two *b* haem groups. On this basis a more or less symmetrical arrangement would be expected with *b*-566 (b_L, the low potential haem) 1 nm from the outer surface and *b*-562 (b_H, the high potential haem) 1 nm from the inner surface. However, according to the evidence from paramagnetic probes, *b*-566 must lie near the outer surface of the membrane, while *b*-562 lies near the middle [1]. The effect of membrane potential on the redox poise of *b*-562 in antimycin-inhibited particles led to the conclusion that 40% of the membrane potential is sensed between the two haems, in agreement with the above picture [2,3]. From the size of a myxothiazol-sensitive but antimycin-insensitive electrogenic event observed during the flash-induced reduction of cytochrome *b* in *Rhodobacter sphaeroides,* Glaser and Crofts [4] concluded that 35–50% of the full electrogenicity of one turnover of the bc_1 complex lies between the *b* haems. The remaining electrogenicity, being antimycin-sensitive, was ascribed to the re-oxidation of *b* by quinone. Similar observations have been made on the b_6f complex of chloroplasts by Jones and Whitmarsh, who further concluded that

Abbreviations: $\Delta\psi$, electrical potential of inner (matrix) phase relative to that of the outer aqueous phase; ΔpH, pH of inner aqueous phase minus pH of outer aqueous phase; o- (i-) site, site on the bc_1 complex of quinone oxidation (reduction); b_H (or *b*-562) and b_L (or *b*-566), high potential and low potential *b* haems, respectively, of the bc_1 complex; *b*-562*, *b*-562 in the presence of antimycin and myxothiazol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(aminoethylether)tetraacetate.

Correspondence: I.C. West, Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, U.K.

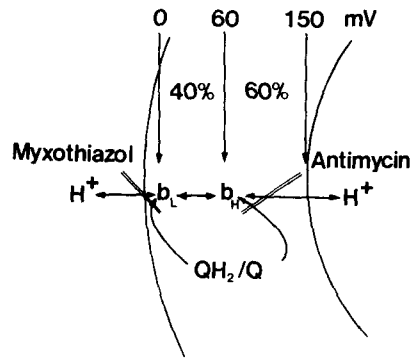

Fig. 1. A summary of the current consensus view regarding the disposition of *b*-haems in bc_1-type complexes.

the o-site is not detectably buried in the membrane dielectric [5]. Against this, Konstantinov has recently argued that *b*-562 is more accessible to the inner surface than *b*-566 is to the outer surface [6]. Nevertheless, the disposition of the *b* haems, summarized by Fig. 1 rationalizes the majority of the available data.

Though it is widely assumed that the two *b* haems form a conducting pathway for the passage of electrons from the o-site (the site of ubiquinone oxidation) to the i-site (the site of ubiquinone reduction), this is not universally accepted [7], and direct electron transfer between the haems has never actually been demonstrated. Some years ago it was shown that, when the i-site is blocked by antimycin, a membrane potential will drive electrons from *b*-562 into the ubiquinone pool with no detectable effect on the redox poise of *b*-566 [2,3].

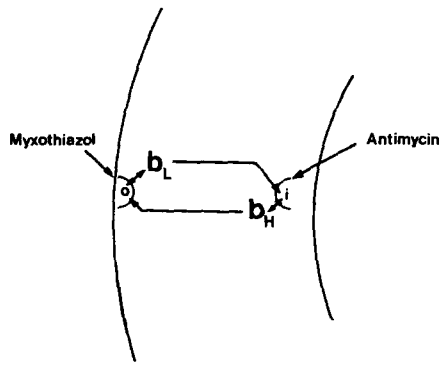

Fig. 2. An alternative picture of the bc_1 complex, compatible with current data, in which the *b*-haems are not connected directly, but via the o- and i-sites.

In the complementary experiment, in which an electric field is imposed in the presence of myxothiazol, one observes reduction of *b*-566 with very little reduction or oxidation of *b*-562 [8], presumably because *b*-562 is still able to exchange electrons with the ubiquinone pool via the i-site. Though these experiments are consistent with electron conduction between the *b*-haems connected in series, they are equally consistent with the *b* haems being connected in parallel to both the o- and i-sites (see Fig. 2).

The experiments described here confirm that there is electronic contact between the haems when the o-site and the i-site are simultaneously blocked. (Part of the present paper was presented previously in preliminary form [8].)

Materials and Methods

Mitochondria were prepared from fresh bovine heart in a KCl/EDTA medium containing bovine serum albumin using a polytron-type tissue disintegrator; the method was based on that of Kuo et al. [9]. The mitochondria prepared in this way were moderately well coupled (respiratory control greater than 2 with succinate) but were considerably depleted of cytochrome *c*. Keilin-Hartree particles were prepared from bovine heart according to King [10].

Myxothiazol and carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP) were obtained from Boehringer Corporation, Hexaminerutheni-um(III) chloride and the quinone mediators were from Aldrich, and other chemicals were from Sigma, except for pyocyanine, which was prepared from phenazine methosulphate by exposure to light [21].

Spectroscopic data were collected using a Sigma (Berlin) ZWS-11 dual-wavelength scanning spectrophotometer with the monochromator exit slits set at 0.5 mm (1.5 nm bandwidth) and a reference wavelength of 575 nm. The 10 mm path-length cuvette could be continuously stirred with a glass rod and an overhead motor. The concentration of bc_1 complex and the contributions of the various cytochrome components to the observed spectra were determined using a matrix deconvolution method [11] using the absorption coefficients shown in Table I.

TABLE I

ABSORPTION COEFFICIENTS USED FOR DECONVOLUTION DUAL-WAVELENGTH SPECTRA

Absorption coefficients for cytochromes *c* [26], c_1 [27] and aa_3 [28] are from published spectra. Spectra for *b*-566, *b*-562 and *b*-562 in the presence of antimycin plus myxothiazol (designated *b*-562*) were obtained as described in the Materials and Methods section, and standardized assuming that the absorption coefficient at the wavelength of maximum absorbance was 20 $mM^{-1} \cdot cm^{-1}$. The reference wavelength was 575 nm. The spectral effects of antimycin plus myxothiazol are treated as though they affect only *b*-562, as discussed in the text.

Wavelength (nm)	Absorption coefficient ($mM^{-1} \cdot cm^{-1}$)					
	c	c_1	*b*-562	*b*-562*	*b*-566	aa_3
545–575	8.3	3.5	1.56	2.42	1.50	−5.77
550–575	21.9	12.5	2.80	2.95	3.71	−4.94
555–575	7.1	16.17	6.00	5.35	5.11	−3.52
561–575	−0.6	4.0	19.48	17.15	10.93	−1.57
566–575	−0.9	0.7	11.27	14.40	20.00	−0.51

The redox-titrations of Keilin-Hartree particles were carried out anaerobically under a gentle stream of oxygen-free nitrogen using the following redox mediators and redox buffers, each at 20 μM: phenazine methosulphate, phenazine ethosulphate, pyocyanine, hexammineruthenium chloride, ferric oxalate, ferric pyrophosphate, substituted 1,4-benzoquinones (trimethyl-, 2,6-dimethyl-, 2,5-di-*tert*-butyl-, tetramethyl-), and substituted 1,4-naphthoquinones (2-methyl-, 2,3-dimethyl-, 2-methyl-3-decyl-, 2-hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone tetraacetyl glucoside). The redox electrode was standardized to ±1 mV against quinhydrone ($E_m^7 = -285.7$ mV at 25 °C [12]).

Results

Antimycin is well known to cause a significant red shift of the spectrum of reduced cytochrome *b*-562 [13], but there is disagreement in the literature about the effect of antimycin on the midpoint potential of *b*-562 (see Table II, and Discussion). Myxothiazol has been reported to cause a very small spectral change to one of the components of purified bc_1 complex [13,16], but there is disagree-

TABLE II

REDOX MIDPOINT POTENTIALS OF *b*-CYTOCHROMES OF MITOCHONDRIAL bc_1 COMPLEX IN THE ABSENCE AND PRESENCE OF ANTIMYCIN AND MYXOTHIAZOL

Literature values measured at pH other that 7.0 have been recalculated using published [15] pH-dependencies.

Source [Reference]	E_m^7 (mV)							
	control		+ anti		+ myxo		+ anti + myxo	
	b-562	*b*-566	*b*-562	*b*-566	*b*-562	*b*-566	*b*-562	*b*-566
[1]	50	−50						
[2]	61	−49	81	+43				
[15]	77	−55	61	−75				
[33]	35	−35	63	−40				
[33]	28	−77			53	−62		
[23]	45	−30	45	−30				
[24]	90	−30						
[25]	100	−27	100	−27				
Present work	92	−10					92	−10

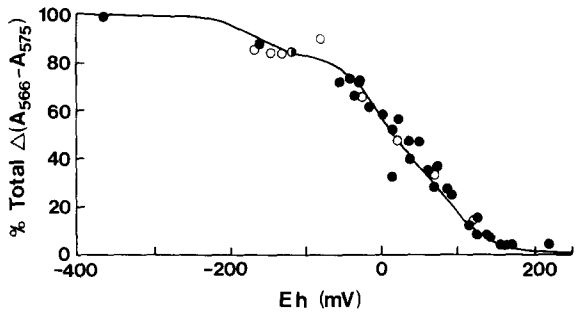

Fig. 3. Redox titration of *b* cytochromes in Keilin-Hartree particles in the presence (●) and absence (○) of antimycin plus myxothiazol. The data from four titrations, containing both reductive and oxidative points, have been superimposed by assuming that *b*-560 has $E_m^7 = -185$ mV, and contributes maximally 16.6% to the $A_{566} - A_{575}$ signal [22]. The solid line describes the titration of three components: $E_m = +92$ mV, 38% of signal; $E_m = -10$ mV, 45% of signal; $E_m = -185$ mV, 17% of signal.

ment as to whether the effect is due to *b*-566 [16] or to *b*-562 [13]. The small difference spectrum looks like a shift spectrum centred at 566 nm [16], but apparently titrates out predominantly at the midpoint potential of *b*-562 [13]. In any case, the myxothiazol difference spectrum is almost entirely missing if cholate is used in place of Triton [16]. In whole mitochondria we did not detect any spectral effect of myxothiazol. For the present investigation it was important to establish both the spectra and the redox midpoints of *b*-562 and *b*-566 when both antimycin and myxothiazol were present together. This was done with a conventional anaerobic redox titration using Keilin-Hartree particles. The midpoint potentials were not significantly affected by the simultaneous presence of antimycin and myxothiazol (see Fig. 3 and Table II). The spectrum of *b*-562(+antimycin + myxothiazol) was obtained by subtracting the absolute spectrum obtained at +130 mV from that obtained at +50 mV. Similarly, that of *b*-566(+antimycin + myxothiazol) was obtained by subtracting the absolute spectrum obtained at 0 mV from that obtained at −100 mV (Fig. 4a), thus avoiding a contribution from the low potential *b*-560 ascribed to complex II [22]. There was no sign of a *b*-562 with a midpoint at +150 mV (c.f. de Vries [14]).

The calculated spectral effect of oxidizing a 1 mM solution of *b*-562(+antimycin + myxothiazol)

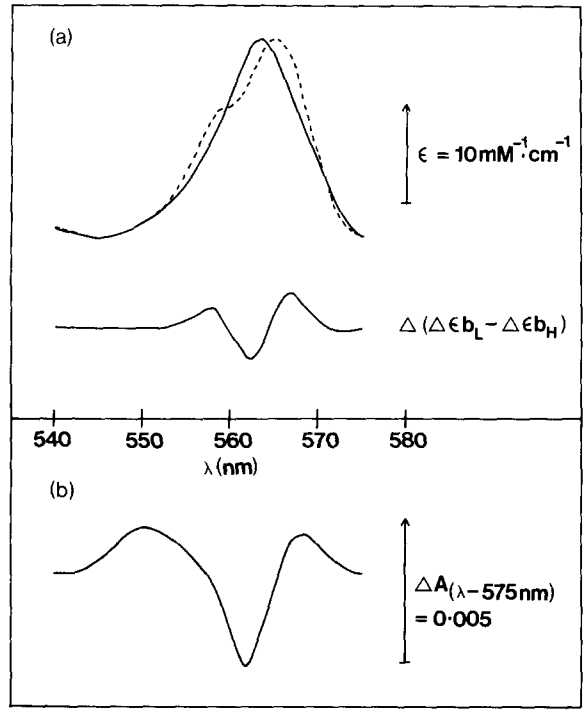

Fig. 4. Dual-wavelength spectra of b_H and b_l in the presence of antimycin plus myxothiazol. (a) Reduced minus oxidized spectra obtained as described in the text, together with the computed difference spectrum for oxidation of b_H with concomitant reduction of b_L. Spectra are the averages of three scans using different batches of particles, and are corrected for small baseline slopes by assuming an isosbestic point at 542 nm. (b) Difference spectrum on imposing a membrane potential across the mitochondrial membrane with b_H initially reduced and b_L initially oxidized. The spectrum is the average of two separate experiments using the same batch of mitochondria, and is corrected for a small baseline slope as above. The cuvette (10 mm light-path) contained the following in a final volume of 1.47 ml: bovine heart mitochondria, 4.5 mg protein; 100 mM KCl; 50 mM Hepes (pH 7.0); 1 mM EGTA; 1.7 mM succinate; 1.7 mM fumarate; 8.5 mM ascorbate; 136 μM $[Ru(NH_3)_6Cl_3]$; 4 μM rotenone; 7.5 μg oligomycin; 2.5 μg antimycin; 11.25 μg myxothiazol. The cuvette was kept aerobic by continuous stirring while a spectrum was taken. FCCP was added (to 1 μM), and the sample rescanned.

and simultaneously reducing a 1 mM solution of *b*-566(+antimycin + myxothiazol) is also shown in Fig. 4a; from the trough at 562 nm to the peak at 567 nm corresponds to an absorption coefficient of 6.6 $mM^{-1} \cdot cm^{-1}$.

In order to detect the movement of an electron from one *b*-haem to the other it is first necessary to adjust the redox potential of the ubiquinone

pool so that one *b*-haem is reduced and the other oxidized. This was done by adding rotenone and then adding equal concentrations of succinate and fumarate, establishing an E_h of +30 mV in the ubiquinone pool.

Two different protocols were used for developing a membrane potential. In the first, electrons were caused to flow from hexammineruthenium(II) to O_2 via cytochrome oxidase. In the second, ATP was hydrolysed. Fig. 4b shows the difference spectrum aerobic minus aerobic with FCCP. The spectrum, when deconvoluted, shows a small amount of cytochrome *c* reduction (0.046 μM), no doubt due to respiratory control in the oxidase. But there is 0.51 μM oxidized *b*-562 and 0.40 μM reduced *b*-566. This represents 55 ± 7% transfer of the electron from *b*-562 to *b*-566. (The concentration of bc_1 complex was 0.827 μM.)

The contents of the cuvette rapidly went anaerobic when not stirred. The membrane potential could be rapidly and reversibly developed by alternately switching the stirring motor on and off. The difference spectrum indicating oxidation of *b*-562 and reduction of *b*-566 developed more rapidly than the 5 s required to obtain a spectrum.

If the conduction of two electrons between the *b* haems is involved in each complete turnover of the complex, the rate constant for the process must be at least as fast as the maximum turnover number (above 500 electrons/s at 25 °C). In order to improve the time resolution of the experiment, the time-course of the difference signal 568 nm − 562 nm was studied following a pulse of aerobic 150 mM KCl to a stirred anaerobic suspension of

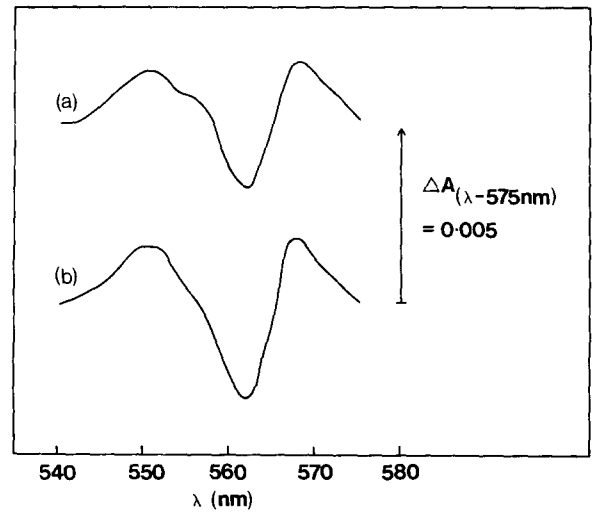

Fig. 6. Difference spectra on imposing a membrane potential across the mitochondrial membrane with b_H initial reduced and b_L initially oxidized. Each spectrum is the average of two experiments. Details as for Fig. 4b, except as follows. (a) Oligomycin was replaced by 1 μg nigericin. The cuvette was scanned anaerobically. ATP (1 μmol) was added and the cuvette rescanned. (b) Oligomycin was omitted but nigericin and ATP added. After scanning, 1.25 μg valinomycin was added and the cuvette rescanned.

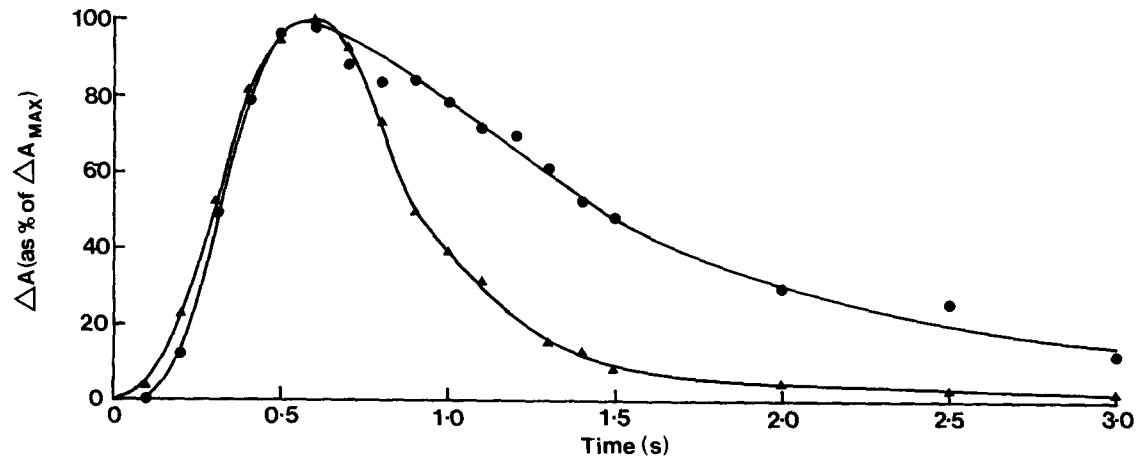

Fig. 5. Time-course of redox changes in the *a* and the *b* cytochromes during a brief burst of respiration. ●, difference signal $A_{568nm} - A_{562nm}$, $\Delta A_{max} = +0.002$. ▲, difference signal $A_{605nm} - A_{575nm}$, $\Delta A_{max} = -0.023$. Details as in Fig. 4b, except that the stirred cuvette was anaerobic. Respiration was initiated by injecting 30 μl air-saturated 150 mM KCl.

mitochondria. This was compared with the time-course of cytochrome *a* oxidation and re-reduction, followed as the difference signal 605 nm – 575 nm in a parallel experiment (Fig. 5). No lag was detectable between the oxidation of cytochrome *a* and the rise of the 568 nm – 562 nm signal at a resolution of 0.1 s, though the latter signal showed a brief lag before following the re-reduction of cytochrome *a*.

In order to determine whether the spectral change was due to $\Delta\psi$ or ΔpH, the experiment of Fig. 4b was repeated in the presence of nigericin. A similar difference spectrum of similar size was obtained on aeration, and was completely abolished with valinomycin.

In Fig. 6 the membrane potential was developed by the hydrolysis of ATP. Nigericin was present to minimize ΔpH. The upper trace shows the difference spectrum with ATP minus without ATP. The lower trace shows the difference spectrum ATP without valinomycin minus ATP with valinomycin. The very similar spectra again show 5–8% reduction of cytochrome *c*, and show respectively 32% and 44% transfer of an electron from *b*-562 to *b*-566.

Discussion

The experiments reported here are believed to be the first direct demonstration of electron transfer between the *b* haems of the bc_1 complex. The time resolution of the present experiments cannot establish that the rate of electron transfer is adequate to account for the maximum observed turnover rates (at least 500 electrons/s). In experiments on the analogous complex of *Rhodobacter sphaeroides,* no transient reduction of *b*-566 could be observed, even on a 100 μs time-scale, unless *b*-561 was already reduced [19], so the rate constant for $b \rightarrow b$ transfer, if that step is involved, would have to be greater than 10^4 s^{-1}. But Von Jagow and Engel have shown [20] that electron transfer from ubiquinol to the *b*-haem pair is slowed down by the simultaneous presence of antimycin and myxothiazol to an apparent rate constant of $1.8 \cdot 10^{-3}$ s^{-1}. The rate of electron transfer observed here is thus at least $5.5 \cdot 10^3$-times too fast to be accounted for by an electron transfer pathway that includes the o- and the i-sites.

It is not normally possible to observe the *b*-566 haem in the reduced state when the accompanying *b*-562 haem is oxidized. Therefore, there have previously been no good grounds for believing that the spectrum ascribed here to *b*-566 is indeed appropriate; the familiar spectrum, with a peak at 566 nm and a shoulder at 558 nm, might belong only to the species containing two electrons on the haem pair. The present experiment suggests, however, that the 566/558 spectrum shown in Fig. 3, or something rather like it, does apply when only one electron is present on the haem pair, but residing on the low potential haem.

Kunz and Konstantinov [17] reported a small spectral shift in the region 560–565 nm when a protonmotive force was developed in dithionite-reduced, cyanide-inhibited, submitochondrial particles. They concluded that it might be an electrochromic effect of the electric field on the *b*-haem chromophores, or the result of a conformation change. In any case, it is distinct from the effect discussed in this paper; their difference spectrum had a trough at 560 nm and a peak at 565, and the trough-to-peak difference amounted to only 0.4 absorbance units $\cdot$ $mM^{-1} \cdot cm^{-1}$.

From the data of Mitchell and Moyle [18], it may be estimated that the membrane potential ($\Delta\psi$) developed during respiration in the presence of oligomycin and EGTA is in the region of -200 mV [18], while that developed by ATP hydrolysis may be somewhat less. The redox potential of *b*-566(+antimycin + myxothiazol) is -100 mV relative to *b*-562(+antimycin + myxothiazol). If there were an electric field placed across the membrane such that the electric potential at *b*-566 were +100 mV relative to that at *b*-562, one would expect a single electron on the *b*-562-*b*-566 system to reside equally on the two haems, representing 50% transfer from *b*-562 to *b*-566. The observed 32–55% transfer of the electron in the present experiments implies that 30–55% of the membrane potential may be sensed between the two *b*-haems of the bc_1 complex. This calculation ignores the possibility that the pH-dependency of both *b*-562 and *b*-566 might involve the electrogenic taking up of a proton from, or release of a proton into, the bulk aqueous phases. This uncertainty, together with uncertainties concerning the midpoint of the *b*-haems (Table II) and the possi-

bility of a small electrochromic effect [17], makes it impossible to be more precise. However, the results appear to be consistent with Fig. 1 and with the conclusions of other workers [4,5] regarding the topology of the *b*-haems in related bc_1 and b_6f complexes.

Direct electron transfer between the haems of cytochrome *b* is a feature of both the Q-cycle [29] and the semiquinone cycle [30] models of turnover. Hence, the present demonstration does not distinguish these alternatives. However, the recent demonstration that neutral semiquinone, QH•, but not charged semiquinone, Q^-, appears to move between the o- and i-sites in the chloroplast system under certain conditions [31] can explain the observations of Rich and Wikström [32], whilst strongly favouring the Q-cycle as the mechanism that operates during normal turnover.

Acknowledgements

This work was supported by the Medical Research Council and The Glynn Research Foundation Ltd. I.C.W. is on leave-of-absence from the University of Newcastle upon Tyne. We gratefully acknowledge the technical assistance of Alan Jeal and Robert Harper.

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