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On the mechanism of respiratory and photosynthetic electron transfer in *Rhodospirillum rubrum*

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The electron transport in the heterotrophically dark-grown *Rhodospirillum rubrum* strain S1 has been investigated. Membranes isolated from these cells have been shown to contain at least three *c*-type cytochromes with $E_{m,7.0}$ of +330 mV, +260 mV and +125 mV plus at least four *b*-type cytochromes with $E_{m,7.0}$ of +380 mV, +250 mV, +35 mV and −40 mV. The two high-potential *b*-type cytochromes (*b*-380 and *b*-250) are suggested to be components of the oxidase system, since conclusive evidence has also been obtained that aerobically grown *R. rubrum* S1 contains a branched respiratory chain leading to two oxidases. These thermodynamically and spectrally distinct oxidases might also be distinguished by their sensitivity to KCN (I_{50} of 5 μM and 0.5 mM), the high sensitive one being responsible for cytochrome *c* oxidase activity and the other for the alternative (KCN-resistant) CO-sensitive pathway of oxygen consumption. Myxothiazol, UHDBT and antimycin A inhibited both light- and respiratory-dependent electron transport at the ubiquinol/cytochrome *c* oxidoreductase level, while a residual oxidative activity due to the presence of the alternative pathway was sensitive to the quinoline-4-methanol antimalarial drug, mefloquine (I_{50} = 18 μM). Two *c*-type cytochromes (*c*-330 and *c*-260) plus two cytochromes *b* (*b*-35 and *b*-−40) have been shown to be kinetically competent in light-induced cyclic electron flow. These results have been interpreted as evidence for the presence of the previously undetected redox elements of a putative *b*/*c* oxidoreductase complex in *R. rubrum*.

Introduction

Recent studies on membrane-bound electron-transfer chains have definitively established that remarkable similarities exist between electron transport patterns in the *b*/*c* regions of mitochondria, chloroplasts, cyanobacteria and several representative species of bacteriochlorophyll *a*, *b* and *c* containing phototrophs [1–4]. Electron transfer in the *b*/*c* region involves

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; P-870, reaction center primary donor; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; PMS, *N*-methylphenazonium methosulphate; PES, *N*-ethylphenazonium ethosulphate; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; mefloquine, DL-erythro-2-piperidyl-2,8-bis-(trifluoromethyl)-4-quinolinemethanolhydrochloride; E_h, oxidoreduction potential relative to the normal hydrogen electrode; $E_{m,x}$, midpoint oxidoreduction potential at pH = *x*; RC, reaction centre.

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a multiprotein complex (b/c_1 complex) in which two *b*-type haems, one *c*-type haem, and two non-haem irons (Rieske iron-sulphur centre, $g_y = 1.89$) catalyses oxidation of ubiquinol by exogenous cytochrome *c* (ubiquinol-cytochrome *c* oxidoreductase) (see Ref. 1).

In facultative phototrophs, such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, the b/c_1 complex has been shown to interact with membrane-bound *b*- and/or *a*-type oxidases (see Ref. 5). In studies with vesicles derived from aerobically grown cells of the related species *Rhodospirillum rubrum*, one soluble *c*-type cytochrome (c_2) plus three *b*-type components were thermodynamically resolved by dark-equilibrium redox titrations; their oxidation reduction mid-point potentials were +300, +195, +50 and −110 mV at pH 7.0 [6]. A cytochrome *b* with $E_{m,7.8}$ of +50 mV and a *c*-type component with $E_{m,7.8}$ of +275 mV, have recently been shown to operate in photosynthetic electron flow of *R. rubrum* [7]. With regard to the possible role in electron transfer of the other cytochromes, a few suggestions have been made, namely: (i) cytochrome *b*-195 (cytochrome *b*-170 as in Ref. 8) may constitute the CO-sensitive cytochrome '*o*' that forms the oxidase in the respiratory chain of aerobically grown *R. rubrum* [9]; (ii) cytochrome *b*-−110 may be involved in reversed electron flow [10]. Suggestion in (i) is strongly supported by recent evidence [11], that a cytochrome *c* oxidase-deficient mutant of *R. rubrum* (strain CAF10) can aerobically grow in the dark by means of a CO-sensitive oxidase. It has also been shown [11] that most of the cytochrome *c* of *R. rubrum* intact cells can aerobically be oxidized, but only when a functional oxidase is present. This latter finding is clearly in contrast with potentiometric titrations by Niederman et al. [6], who failed to detect any cytochrome with an oxidation-reduction midpoint potential sufficiently high to oxidize ferrocytochrome c_2. On the other hand, the currently accepted schemes for *R. rubrum* respiratory electron transport do not include cytochrome c_2 as a constituent of the aerobic respiratory system so to restrict this soluble cytochrome to photosynthetic electron transport [12–14]. From the literature, it is therefore unclear whether cytochrome c_2 in membranes from *R. rubrum* participate in photosynthetic and/or respiratory electron transfer as previously shown in other species of the Rhodospirillaceae family [13].

Another controversial aspect of the currently accepted schemes of *R. rubrum* electron-transfer chain concerns the lack of thermodynamic and kinetic evidences for the presence in this bacterium of a membrane-bound cytochrome *c* (cytochrome c_1) [7]. The early described cytochrome *c*-552 [14] (α band at 552 nm), which is clearly distinct from cytochrome c_2 (α band at 550 nm), had spectral features similar to those later described by Wood [15] for photosynthetic membrane bound cytochrome c_1. However, the spectral characteristics of cytochrome *c*-552 were not considered by Kakuno et al. [14] sufficiently reliable to suggest the presence in chromatophores from *R. rubrum* of a second *c*-type species in addition to cytochrome c_2. For this reason, cytochrome *c*-552 was reported as cytochrome c_2 in the 'membrane-bound' form. Conversely, recent structural data by Wynn et al. [16] show that chromatophores from photosynthetically grown cells of *R. rubrum* do really contain a *c*-type haem binding peptide of 30 kDa. It has therefore been suggested that, similarly to the b/c_1 complex polypeptide composition of *Rb. sphaeroides*, this membrane-bound *c* might be related to cytochrome c_1. However, thermodynamic and kinetic evidences are required before to assess conclusively its role in photosynthesis and/or respiration.

In the present work we have examined the mechanism of photosynthetic and respiratory electron transport by membranes from *R. rubrum*. We demonstrate that in heterotrophically dark-grown *R. rubrum* operates a putative ubiquinol-cytochrome *c* oxidoreductase which is the common part of a redox chain leading to two oxidases. These oxidases have tentatively been functionally associated with the presence of two cytochromes *b* with $E_{m,7.0}$ of +250 mV and +380 mV (cytochrome *b*-250 and cytochrome *b*-380, respectively). Since cytochrome *b*-250 binds to CO, we speculated that it might function as a *b*-type component of the alternative oxidase, whereas cytochrome *b*-380 would be involved in the oxidation of cytochrome c_2, as previously shown for an analogous high-potential *b* in the closely related species *Rb. capsulatus* [17].

Materials and Methods

Organism cultivation and membrane isolation. Wild-type *R. rubrum* S1 and the cytochrome *c* oxidase deficient strain CAF10 were grown in the medium of Lascelles [18]. Strain CAF10 is derived from S1 as previously described [11]. Cells were grown in the dark in a fermenter (Microferm; New Brunswick Scient. Co., NJ) at 30 °C for 20 h with 200 rpm stirring and bubbled with humidified air at 1 l/min. The harvested cells were suspended in 50 mM Tricine buffer (pH 7.4) to a concentration of 0.5 g/ml (wet weight) and passed through a pre-cooled (4 °C) French pressure cell at 2 MPa. Whole cells and debris were removed from the crude extract by centrifugation at 20000 $\times g$ for 20 min. The clear supernatant was centrifuged at 106000 $\times g$ for 90 min. The sediment was resuspended in Tricine-buffer (pH 7.4) and used at about 20–30 mg/ml of proteins. Spheroplast-derived membrane fragments were obtained as described in Ref. 19.

Optical spectroscopy. Dark potentiometric titrations of cytochromes were performed under nitrogen in a medium containing 50 mM Mops (pH 7.0), plus 50 mM KCl, using a dual-wavelength spectrophotometer (SIGMA ZW-II) equipped with a rapid-mixing apparatus, according to Dutton and Jackson [8]. E_m (pH 7.0) values were assigned on the basis of a computer-assisted analysis, essentially as in Ref. 8. Cytochrome spectra at a fixed oxidation-reduction potentials were obtained using a computer-linked dual-beam spectrophotometer (Jasco UVIDEC-610 KDB-101).

Respiratory activities. Oxidation of ascorbate-TMPD was measured polarographically at 30 °C in a Yellow Spring (YSI 53) oxygen meter with a jacketed Gilson Oxygraph reaction chamber. Ubiquinol and NADH oxidations were followed either polarographically or spectrophotometrically.

Kinetic spectrophotometry. The kinetics of flash-induced redox changes of cytochromes were measured using a single beam spectrophotometer with a time resolution of 0.5 μs and with a bandwidth of 1.5 nm. Flash excitation was provided by a xenon lamp filtered by a Wratten 88A glass, giving a saturating (more than 90%) flash of 15 μs duration at half maximal intensity. The photomultiplier was protected by a Corning glass filter no. 9782. Rapid digitisation of the photomultiplier linear amplifier output was performed by a Datalab DL905 transient recorder, interfaced to an Olivetti M24 computer. To limit the exposure of samples to light prior to a flash, the monitoring beam was gated shut until approx. 1 s before the flash. Redox poising was done as in Ref. 8.

Other assays. Protein content was estimated by the Lowry method [20] and bacteriochlorophyll was measured at 775 nm in acetone/methanol (7 : 2) extracts using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ [21]. Mefloquine and UHDBT were generous gifts from A.D. Wolfe and B.L. Trumpower, respectively.

Results and Discussion

Respiratory activities

Fenoll and Ramírez [11] have recently shown that NADH and succinate oxidase activities by isolated membranes from *R. rubrum* S1 were partially affected by 0.2 mM KCN. Conversely, the cytochrome *c* oxidase activity was 90% inhibited by 0.2 mM KCN so to suggest that alternative terminal oxidases with different sensitivities to

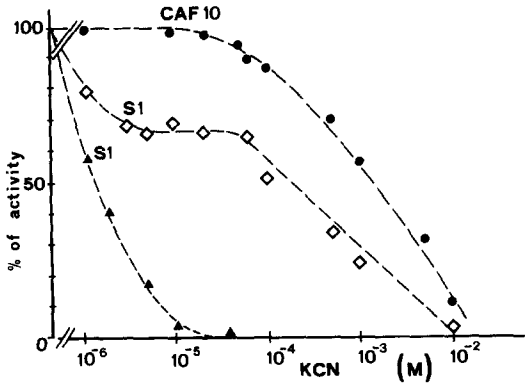

Fig. 1. Cyanide inhibition of the ubiquinol-2 (UQ_2H_2) and ascorbate-TMPD oxidation activities in chromatophores from *R. rubrum*, strains S1 (wild type) and CAF10 (cytochrome *c* oxidase minus mutant). Ubiquinol dependent respiration (●) (◇); ascorbate-TMPD oxidation (▲). Control rates: UQ_2H_2-oxidation, 8 and 3.5 μmol O_2 consumed per h per mg protein in CAF10 and S1 chromatophores, respectively; ascorbate-TMPD oxidation (only present in S1 chromatophores), 2.2 μmol O_2 consumed per h per mg protein. Membranes were suspended at pH 7.2 in 50 mM Mops, 5 mM $MgCl_2$.

cyanide exist. This conclusion was strongly supported by the observation that *R. rubrum* CAF10, a strain deficient in cytochrome *c* oxidase activity, was capable of aerobic dark-growth although at a decreased rate and with a reduced final yield [11]. The results shown in Fig. 1 demonstrate that ubiquinol-2 (UQ_2H_2) and ascorbate-TMPD oxidase activities of membrane fragments from aerobically grown cells of strain S1(wild type) give biphasic and monophasic KCN inhibition curves, respectively. The ubiquinol-2 oxidase in the mutant strain CAF10 shows only one inhibition constant ($I_{50} = 2 \cdot 10^{-3}$ M) for KCN. It is apparent that the ascorbate-TMPD oxidase activity, functional in S1, shows sensitivity to KCN (I_{50} at $2 \cdot 10^{-6}$ M) very similar to that of the most sensitive component of the biphasic UQ_2H_2 oxidation titration curve. Conversely, the ubiquinol-2 oxidase by membranes from CAF10 titrates like the KCN-resistant portion of the parental strain ($I_{50} = 10^{-3}$ M).

The antibiotics antimycin A and myxothiazol typically block electron transport at the ubiquinol-cytochrome *c* oxidoreductase region through binding to the *b*-type haems (see Ref. 22), and thus we anticipated a differential effect of these inhibitors on strains CAF10 and S1. These expectations were confirmed by the observation that 30% of the NADH oxidation in S1 was blocked by 10 μM antimycin A and/or myxothiazol, the same activity being unaffected by equal concentrations of the antibiotics in membranes from CAF10 (not shown). Kinetic information about the role of the Rieske iron-sulphur centre in several photosynthetic and respiratory systems has become available through the use of the inhibitor UHDBT. UHDBT interacts very close to, or with, the Rieske FeS protein, as it blocks the oxidation of the FeS center (see Ref. 22). Additional experiments (not shown) revealed that the kinetics of the NADH-dependent cytochrome *c* reduction (551–542 nm) in membrane fragments from *R. rubrum* S1 and CAF10 were drastically affected by antimycin A (10 μM), myxothiazol (10 μM) and UHDBT (40 μM).

Recently, the quinoline-4-methanol antimalarial drug, mefloquine, was demonstrated to be an effective inhibitor of bacterial respiration [23]. In membrane fragments from the facultative

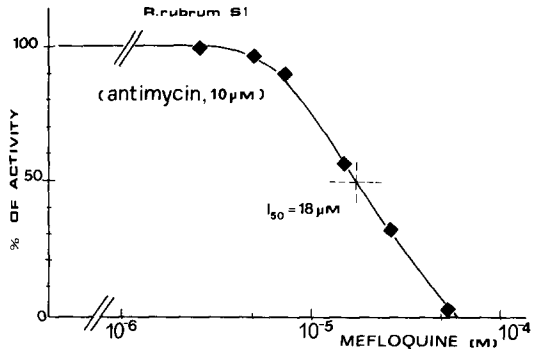

Fig. 2. Ubiquinol-2 oxidation by membrane fragments from *R. rubrum* S1 as a function of mefloquine concentrations in the presence of (10 μM) antimycin A. Activities were 6.5 and 8.5 μmol O_2 consumed per h per mg protein in the presence or the absence of antimycin A, respectively. Experimental conditions as in Fig. 1.

phototroph *Rb. capsulatus*, ubiquinol-oxidation through the KCN-resistant oxidase was shown to be 50% inhibited by 15 μM mefloquine, while reduction of cytochrome *c* was only partially blocked by the same inhibitor concentration. Thus, the primary effect of mefloquine on the alternative-oxidase dependent respiration was clearly distinct from that of antimycin A, previously shown to affect specifically electron transport catalyzed by the b/c_1 complex through binding to cytochrome *b*-562 [24]. In antimycin A-treated membranes from aerobically-grown *R. rubrum* S1, ubiquinol oxidase was 50% inhibited by 18 μM mefloquine (Fig. 2). This latter value is likely to correspond to the inhibition constant for mefloquine of the KCN-resistant alternative oxidase because the NADH-dehydrogenase, measured as either DCIP- or ubiquinone-1 reductase, was unaffected by mefloquine (not shown).

Cytochrome dark-equilibrium redox titrations

Dark-equilibrium potentiometric titrations of membrane fragments from dark-grown cells of *R. rubrum* S1 are shown in Fig. 3. Titrations at 561–575 nm (trace *a*) (pH 7.0) could be resolved into four components, each component behaving closely to theoretical single-electron equivalent ($n = 1$) curve. Computer analysis based on non-linear least-squared minimization gave for these apparent *b*-type cytochromes mid-point potentials equal to +379 mV, 251 mV, 35 mV and −41 mV;

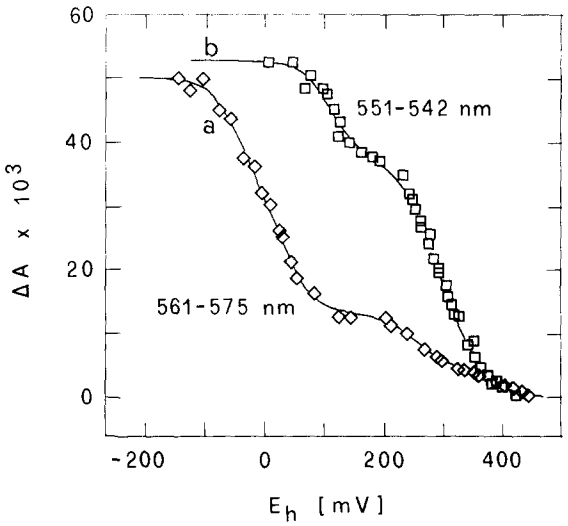

Fig. 3. Dark-equilibrium potentiometric titrations at pH 7.0 of membrane fragments from heterotrophically dark-grown cells of *R. rubrum* S1. Drawn through the points are the curves corresponding to the sum of four Nernstian components ($n = 1$) at 561–575 nm ($E_{m,7.0}$ of +379 mV, +251 mV, +35 mV and −41 mV) and of three components ($n = 1$) at 551–542 nm ($E_{m,7.0}$ of +324 mV, +264 mV and +125 mV). Protein concentrations were of about 5.8 mg/ml and 4.4 mg/ml for (a) and (b), respectively.

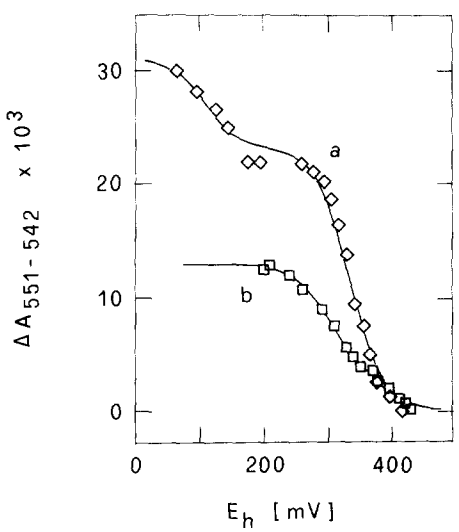

Fig. 4. Dark-equilibrium potentiometric titrations at 551–542 nm of the soluble cell fraction (curve a) and of spheroplast-derived membranes (curve b) from heterotrophycally dark-grown *R. rubrum* S1. Experiments were performed at pH 7.0. Both curves drawn through the points correspond to the sum of two Nernstian components ($n = 1$) with $E_{m,7.0}$ of +335 mV and +115 mV (curve a) and $E_{m7.0}$ of +294 mV and +370 mV (curve b). Protein concentrations were of about 1.5 mg/ml and 3.5 mg/ml for (a) and (b), respectively.

their relative contributions to the total $\Delta A_{561-575}$ change were approx. 10, 20, 35 and 35%, respectively. It is noteworthy that results taken from titrations at 561–575 nm under 50% CO/50% N_2 gas phase indicated that the E_m of the component at +251 mV is affected by CO ($E_{m,7.0}$(+CO) = +290 mV) (not shown).

The results of titrations at 551–542 nm (pH 7.0) (trace *b* in Fig. 3) could be fitted most readily to the sum of three Nernstian components ($n = 1$), to which were assigned E_m values of 324 mV, 264 mV and 125 mV; they comprised 30, 35 and 35% of the signal, respectively. Redox titrations of the soluble cell fraction (106 000 × *g* supernatant, see Materials and Methods) revealed the presence of two cytochromes *c* with mid-point potentials of +335 mV and +115 mV (Fig. 4, trace a). The latter data, when compared to that obtained in spheroplast-derived membranes (Fig. 4, trace b), strongly suggests that in membrane chromatophores the two cytochromes *c* at 324 mV and 125 mV represent loosely-bound *c* type species trapped inside the plasmamembrane vesicles during cell disruption [25], while cytochrome *c*264 (E_m shifted to approx. +290 mV in spheroplasts; see also Ref. 15) is a membrane-bound component.

In view of the experimental uncertainty, the E_m values obtained from potentiometric titrations have been the following, rounded to the closest value multiple of 5 mV. The cytochromes detected in dark-equilibrium redox titrations will be therefore indicated as follows: cytochrome *c*-330, cytochrome *c*-260, cytochrome *c*-125; cytochrome *b*-380, cytochrome *b*-250, cytochrome *b*-35 and cytochrome *b*-−40.

Cytochrome absorption spectra

The detection by redox potentiometry of cytochrome *b*-−40 and cytochrome *c*-260 from heterotrophically dark-grown *R. rubrum* S1 (shown in Fig. 3) was of special interest because analogous cytochromes are integral components of the b/c_1 complex in other related Rhodospirillaceae (see Ref. 22). In addition, since two high-potential cytochromes *b* such as cytochrome *b*-380 and cytochrome *b*-250 have not been described previ-

ously in membranes of *R. rubrum* S1 [6], difference spectra were obtained at a fixed oxidation-reduction potentials to characterize these components further. Because it seemed possible that the presence of significant quantities of soluble cytochromes *c* (cytochrome *c*-330 + *c*-125) might partially obscure the membrane bound *b*- and *c*-type components, spectra (Fig. 5) were taken

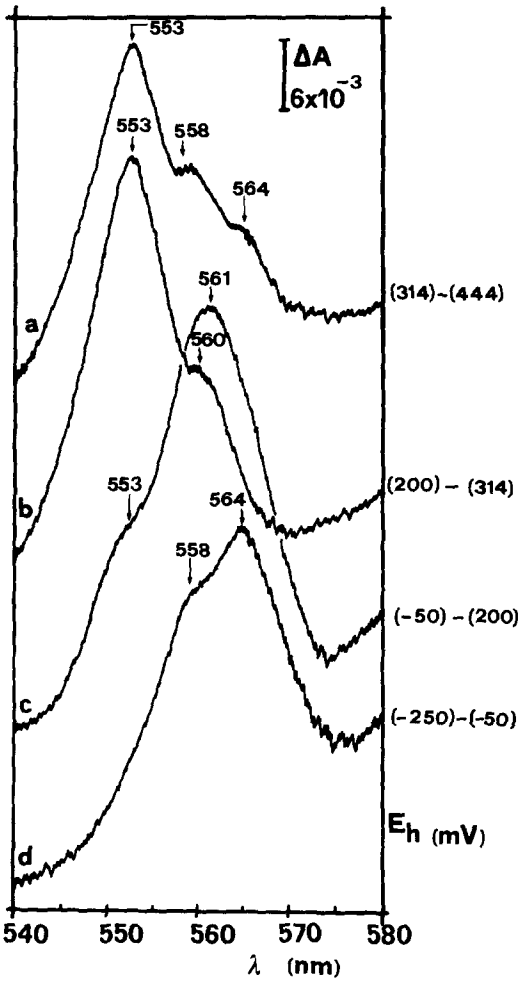

Fig. 5. Absorption spectra of spheroplast-derived membranes from aerobically grown cells of *R. rubrum* S1 at fixed oxidation-reduction potentials. The spectra were obtained with a computer-linked spectrophotometer. Spectra over the range from 540 nm to 580 nm were measured approx. every 30 mV during reductive titration over a range from 444 mV to −250 mV and stored for later analysis. Traces *a* to *d* indicates the differences between spectra obtained at +444 mV and +314 mV, +200 mV and +314 mV, −50 mV and +200 mV, −250 mV and −50 mV. See text for more details.

in spheroplast-derived membrane fragments. After subtraction of the spectrum recorded at +444 mV (fully oxidized sample) from that obtained at +314 mV (cytochrome *c*-290 and cytochrome *b*-380 approx. 30% and 90% reduced, respectively), two significant α-bands with absorption maxima at 553 nm and 558 nm, plus a shoulder at 564 nm, were apparent (spectrum *a*). This spectrum indicates that cytochrome *c*-260 has its α-band at 553 nm (cytochrome c_1), whereas cytochrome *b*-380 presents two α-bands at 558 nm and 564 nm. In Fig. 5, the differences between spectra obtained at +200 mV (cytochrome *b*-250 plus cytochrome *c*-260 reduced) and +314 mV, −50 mV (cytochrome *b*-35 reduced) and +200 mV, and −250 mV (cytochrome *b*-−40 reduced) and −50 mV, are also shown. It may be concluded that cytochrome *b*-250 (trace *b*) has an absorption maximum at 560 nm close to that of cytochrome *b*-35 (trace *c*) which is at 561 nm. Lastly, cytochrome *b*-−40 presents a double α-absorption band with maxima at 564 nm and 558 nm (trace *d*). The sloping baseline seen in the spectrum at the lowest potential cytochrome is caused by the absorption due to the redox mediator 2-hydroxy-1,4-naphthoquinone [26].

The effects of antimycin A and UHDBT on light-induced cytochrome c oxidation

The dark-equilibrium redox titrations reported in Figs. 3 and 4, along with the spectra of Fig. 5, suggest that membrane fragments from heterotrophically dark-grown cells of *R. rubrum* S1 contain both the *b* and *c* type elements which are diagnostic, together with the Rieske iron-sulphur center, of a putative b/c_1 complex [1].

In chromatophores from photosynthetic bacteria, the mid-point potentials of the Rieske centers range from 265 mV to 290 mV [27]. These values raise by 70 mV in the presence of the ubiquinone analog UHDBT [28], suggesting that the binding site of UHDBT is at the iron-sulphur center level. Several authors have also provided strong evidence for the involvement of the Rieske center in light-dependent electron flow. One of the arguments is the increase in the amount of oxidized cytochrome *c* after a flash on the addition of UHDBT [29]. However, in contrast to what observed in *Rb. capsulatus* and *Rb. sphaeroides*, Van

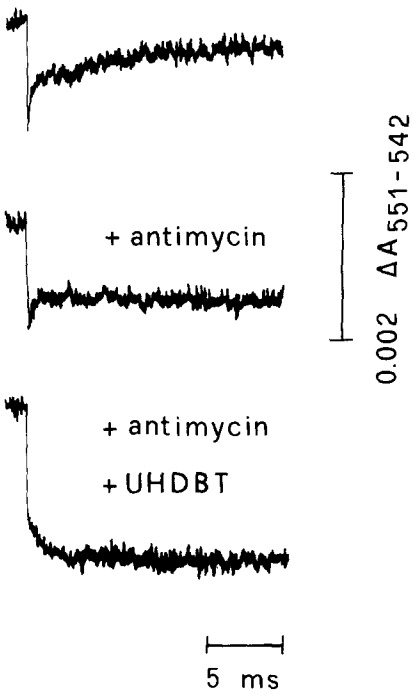

Fig. 6. Single flash-induced cytochromes *c* oxidation measured at 551–542 nm in the presence or in the absence of antimycin A and UHDBT in *R. rubrum* S1 chromatophores. Membranes were suspended to 6.5 μM bacteriochlorophyll in 50 mM Mops/100 mM KCl (pH 7.0) containing 1 μM each of PMS, PES, DAD and 1-hydroxy-*N*-methylphenazonium ethosulphate (pyocianine); 10 μM each of 1,4-naphthoquinone, 1,2-naphthoquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone and *p*-benzoquinone; 20 μM valinomycin and 2 μM nigericin. Antimycin A and/or UHDBT were present where indicated at concentrations of 10 μM and 40 μM, respectively. Experimental conditions were as follows: $E_{h,7.0}$, +110 mV∓10 mV; traces were an average of 32; filter RC, 20 μs; sweep, 20 ms; time between each measurement, 45 s.

der Wal and Van Grondelle [7] failed to detect any UHDBT-induced stimulation of the amount of cytochrome *c* oxidized after a flash in photosynthetically grown *R. rubrum* S1. This latter result is clearly discordant with our observation that the NADH-dependent cytochrome *c* reduction by membranes from *R. rubrum* S1 is strongly affected by UHDBT (not shown). To characterize the UHDBT inhibition-mechanism further, we have therefore studied the oxidation of cytochromes *c* at 551–542 nm, following a single flash of light in the absence or in the presence of UHDBT and/or antimycin A. Traces in Fig. 6 clearly indicate that in the presence of both antimycin A and UHDBT the extent of cytochrome *c* oxidation is approx. 50% stimulated as compared to the extent of the oxidation reached in the presence of antimycin A alone. The re-reduction of cytochrome *c* appears to be completely inhibited over a 20 ms time period. It is also clear that cytochrome *c* oxidation in the presence of UHDBT is distinctly biphasic, being characterized by a fast and a slow phase with $t_{1/2}$ of less than 20 μs and approx. 0.6 ms, respectively. The traces shown here demonstrate therefore that UHDBT inhibits the re-reduction of the cytochromes monitored at 551–542 nm. Whether this inhibition is simply due to the effect of UHDBT in raising the E_m value of the Rieske center [28] or to a separate kinetic effect cannot be determined from these experiments. From the kinetics shown in Fig. 6, it is also impossible to establish the number of *c*-type species involved in light-induced electron transport. Owing to this, the redox titration of the extent of flash-induced cytochrome *c* oxidation at 551–542 nm has been performed. Experimental points were obtained from the extent of the absorbance changes induced by a series of four actinic flashes, in the presence of antimycin A (10 μM) and UHDBT (40 μM), at a series of E_h values at pH 7.0. The results of such a titration (Fig. 7a) indicate that the experimental points nicely fit with a computer-generated two-component ($n = 1$) Nernst curve with $E_{m,7.0}$ of +334 mV and +244 mV. For comparison, a one-component curve with E_m = 304 mV (dashed trace) is drawn through the points. It is apparent that these results are in contrast with an earlier report [7], in which no evidence for two cytochromes *c* involved in electron transport of *R. rubrum* S1 was obtained.

The thermodynamic resolution of two cytochromes *c* competent in cyclic electron flow is strongly supported by their spectral and kinetic resolution. In Fig. 7b, the time-resolved spectra are shown of the flash-induced oxidation of the two *c*-cytochromes. The spectra were obtained in the presence of UHDBT, to inhibit re-reduction by the Rieske-type FeS center [29] and were corrected for the absorption changes due to the reaction center (see below). The spectrum of the change occurring from just before the flash (0 time) to approx. 50 μs after the flash, has a peak at 550

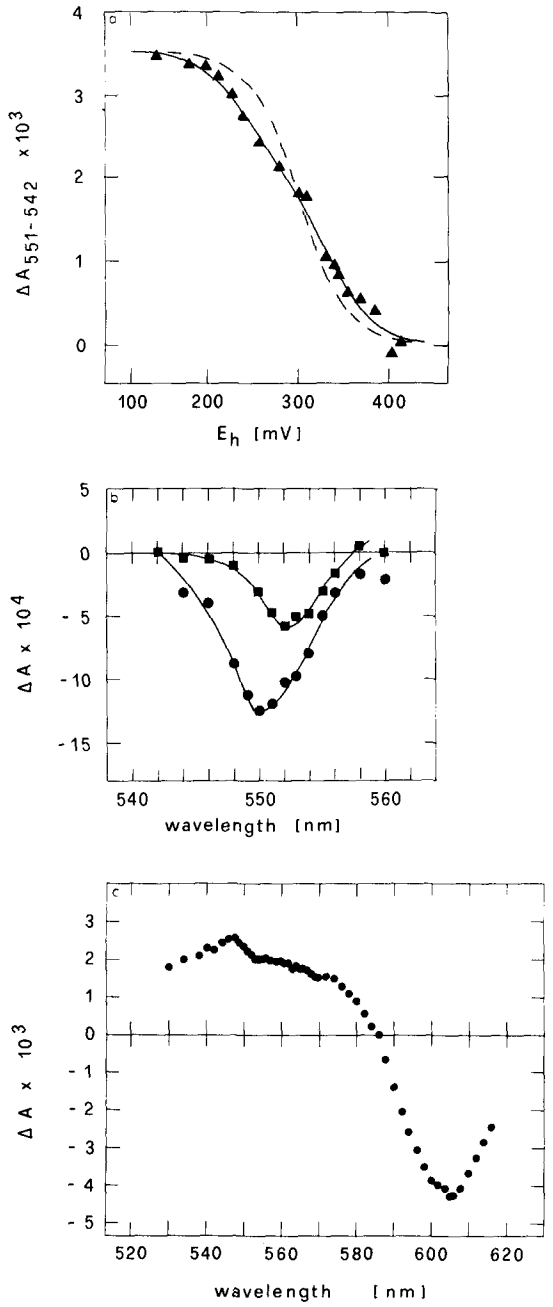

Fig. 7. (a) Redox titrations of the flash-induced cytochromes *c* oxidation measured at 551–542 nm in chromatophores of *R. rubrum* S1. Membrane fragments were suspended to 7 μM bacteriochlorophyll in 50 mM Mops/100 mM KCl (pH 7.0). Kinetic traces (average of eight, 200 ms sweep, 500 μs filter RC) were averaged. Flash trains of four flashes, 30 ms apart, were given every 45 s. Through the points are drawn computer generated, one- (dashed line) and two-component(s) (continuous line) curves ($n = 1$) with $E_{m7.0}$ of +304 mV and 334 mV and 244 mV, respectively. Present as uncouplers were valinomycin (20 μM) and nigericin (2 μM). Also present were antimycin A (10 μM) and UHDBT (40 μM). The mediators present were: 2 μM DAD, 60 μM *p*-benzoquinone, 20 μM 1,2-naphthoquinone and 100 μM each of potassium ferricyanide and ferrocyanide. (b). Time-resolved spectra of cytochrome changes induced by a single-flash excitation in chromatophores from *R. rubrum* S1. Bacteriochlorophyll concentration, E_h value, mediators and uncouplers as for Fig. 6. Antimycin (20 μM) and UHDBT (40 μM) were present. Kinetic traces (average of 32) were accumulated and stored at each point in the spectrum and corrected for the reaction center change (see Fig. 7c). Symbols: ●, changes occurring between 0.0 and 0.050 ms; ■, changes occurring between 0.050 and 2 ms. (c). Difference spectrum of the reaction center in chromatophores from heterotrophycally dark-grown *R. rubrum* S1. Chromatophores were suspended to about 10 μM bacteriochlorophyll in 50 mM Mops/100 mM KCl (pH 7.0) containing 20 μM valinomcin, 1 mM each of potassium ferricyanide and ferrocyanide. Four kinetic traces were averaged at each wavelength. The points show the absorbance change 18 ms after a single flash. The instrument response time was 500 μs. The time between each measurement was 60 s.

nm. The change in the absorption from 50 μs to 2 ms after the flash gives a spectrum with a peak at 552 nm. Comparison of these spectra with those of purified cytochrome c_2 [14,15] and of cytochrome c_1 in spheroplast-derived membranes (this work) indicate that the component with α-band at 552–553 nm is cytochrome c_1 (cytochrome *c*-260) and that with α-band at 550 nm is soluble c_2 (cytochrome *c*-330).

The spectra shown in Fig. 7b were corrected for the contribution of the reaction center by subtraction of the reaction center change calculted at each wavelength from the reaction center difference spectrum and using 542 nm as a reference wavelength. The spectrum of the reaction center change (Fig. 7c) was obtained by single flash excitation of chromatophores posied at high redox potential (+440 mV), so that cytochrome c_2, the rapid electron donor to the photo-oxidized RC, was oxidized before excitation. The spectrum exhibits an isosbestic point at 586 nm and a characteristic bleaching at 605 nm, which correspond almost exactly to those in the light-induced difference spectrum in *Rb. sphaeroides* [30].

The role of soluble cytochrome c_2 (cytochrome c-330) in photosynthesis and respiration

While it is clear (this work) that two cytochromes *c* (cytochrome *c*-330 + *c*-260), and from the

effect of UHDBT, also the FeS center, are intermediate carriers between the ubiquinol/cytochrome *c* oxidoreductase complex and the reaction center, their relative positions are more ambiguous. As previously shown in *Rb. sphaeroides* [31], the most likely arrangement is that FeS is oxidized by cytochrome c_1 (*c*-260) and cytochrome c_2 (*c*-330) by the reaction center. This conclusion is consistent with the kinetic behaviour of cytochromes *c* (Fig. 7b) and with the observation that the flash-induced signal at 551–542 nm in the presence of UHDBT accounts for the absorption change of both *c*-type cytochromes. In Fig. 8, the traces for the light-induced kinetics of oxidation of *c*-type cytochromes and reaction centers in spheroplast derived-membrane fragments are shown. In these membrane fragments most of the cytochrome c_2 is lost during preparation (shown in Fig. 4) and a large fraction of them have an opposite orientation compared to chromatophores [32]. As expected, following a train of eight flashes, the extent of the oxidation of cytochrome *c* was markedly lower than in chromatophores, without any detectable re-reduction of the cytochromes. Addition of cytochrome c_2 at low concentration (1.6 μm) to the suspension of spheroplast-derived membranes increased both the re-reduction rate of the reaction center and the difference in absorption change between the first and the following flashes at 551–542 nm. This clearly demonstrates that the added cytochrome c_2 was able to react with the reaction centers as well as with the b/c_1 complexes exposed to the external medium thus reconstituting a cyclic electron flow.

A possible explanation for the discrepancy between the present data and those of Van der Wal and Van Grondelle [7], who were unable to obtain evidence for the presence of two *c*-type cytochromes in photosynthetically grown *R. rubrum* S1, may be based on the fact that in photosynthetic membranes the amount of cytochrome *c* may even be as low as 0.1–0.2 per reaction center due to a massive loss of soluble *c* [33]. Conversely, in membrane vesicles from heterotrophically dark-grown cells (this work), the cytochrome *c*/RC ratio raises to 0.5. This latter value does not depend on repression of the bacteriochlorophyll synthesis under aerobic dark-growth (with low O_2 tension; see

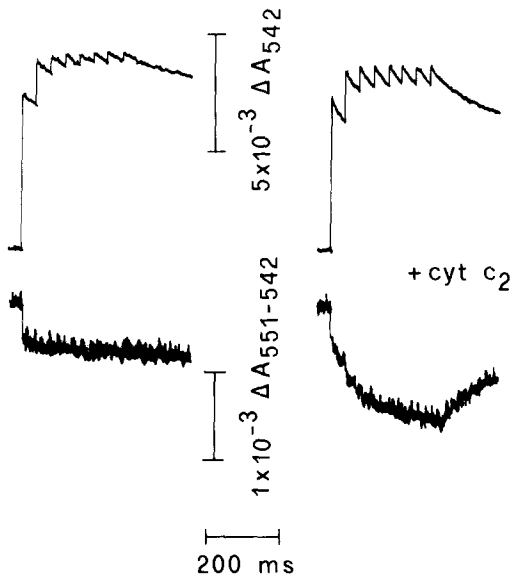

Fig. 8. Changes in the redox state of cytochromes (cyt) *c* and reaction centers after flash activation of spheroplast derived vesicles from *R. rubrum* S1. Experiments were performed at 551–542 nm (cytochrome *c*) and at 542 nm. Membranes (7 μM bacteriochlorophyll) were suspended in 50 mM Mops/100 mM KCl (pH 7.0) at an ambient redox potential of +110∓10 mV. Mediators and uncouplers as in Fig. 7. No inhibitors were present. Kinetic traces (500 μs flter RC, 500 ms sweep, eight flashes, 40 ms apart) were averaged (average of four). Traces on the left: spheroplast-derived vesicles. Traces on the right: spheroplast-derived vesicles plus 1.6 μM cytochrome c_2.

Materials and Methods), but it is due to a generalized increase of the *b*- and *c*-haem content on a molar/protein basis [34].

Previous reports on respiratory electron transport by membrane fragments from aerobically grown cells of *R. rubrum* did not include cytochrome c_2 as a constituent of the respiratory chain [12]. On the other hand, recent studies have demonstrated that most of the cytochrome *c* in intact cells can aerobically be oxidized, on condition that a functional oxidase is present [11]. These latter results seem therefore to lay some doubt about previous interpretation of the role of cytochrome c_2 in respiration of *R. rubrum*. In Table I, some respiratory activities catalyzed by membrane vesicles and spheroplast-derived membranes from heterotrophically dark-grown cells of *R. rubrum* S1, are summarized. It is apparent that cytochrome c_2-depletion produces a drastic de-

TABLE I

SOME RESPIRATORY ACTIVITIES IN CHROMATOPHORES AND SPHEROPLAST-DERIVED VESICLES FROM *RHODOSPIRILLUM RUBRUM* S1

The rates are expressed as μmol of oxygen consumed per h per mg protein. Substrate concentrations: NADH, 1 mM; sodium ascorbate (Asc), 5 mM; TMPD 0.2 mM.

Activity	Additions	Chromatophores	Spheroplast-derived membranes
NADH oxidase	–	16.8	12.0
NADH oxidase	50 μM cytochrome *c* *	20.5	15.5
NADH oxidase	1.5 μM cytochrome c_2	19.5	14.5
Asc-TMPD oxidase	–	10.5	2.0
Asc-TMPD oxidase	50 μM cytochrome *c* *	10.5	5.0
Asc-TMPD oxidase	1.5 μM cytochrome c_2	10.5	4.5

* Horse-heart cytochrome *c*.

crease of cytochrome *c* oxidase activity which is partially restored by addition of either 1.6 μM cytochrome c_2 or 50 μM horse-heart cytochrome *c*. These and the above reported data unequivocally demonstrate a common role of cytochrome c_2 in photosynthetic and respiratory electron transport by membrane vesicles from heterotrophically dark-grown cells of *R. rubrum*.

Time-resolved spectra of light-induced cytochrome b reduction

It has previously been shown that two components with Soret bands at 433 nm and 428 nm were kinetically competent in photosynthetic electron transfer of *R. rubrum* [7]. However, no clear indication of their spectral features and mid-point potentials were presented, although $E_{m,7.8}$ around 50 mV and less than 0 mV for *b*-428 and *b*-433, respectively, have been suggested.

Fig. 9a shows spectra of the absorption changes in *R. rubrum* chromatophores induced by each of two consecutive flashes, fired 30 ms apart, in the presence of antimycin. Both spectra, after correction for the reaction center change, indicate contributions from cytochromes *c* and cytochromes *b*. The spectrum of the absorbance change induced

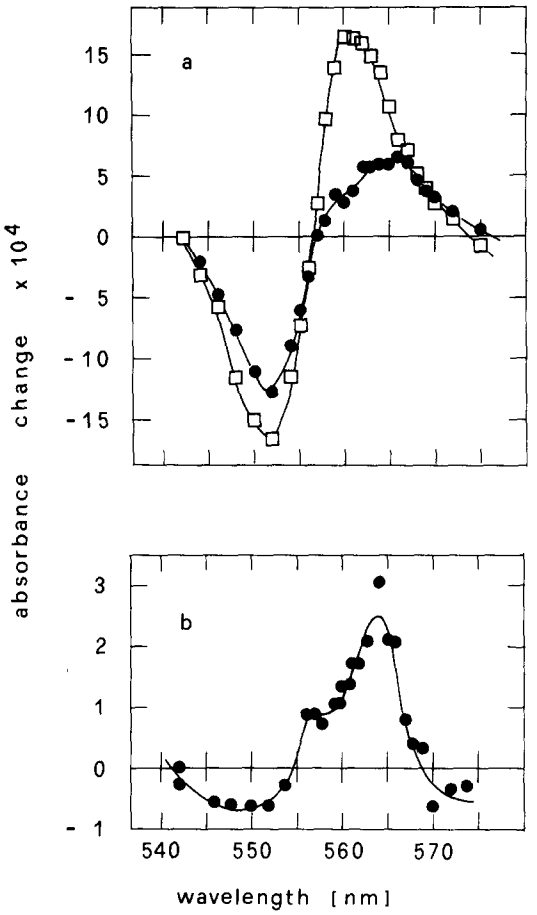

Fig. 9. (a) Light-minus-dark differences spectra of cytochrome changes in chromatophores from heterotrophically dark-grown *R. rubrum* S1. Chromatophores were suspended to 7 μM bacteriochlorophyll in 50 mM Mops/100 mM KCl (pH 7.0) at $E_h = 170 \mp 10$ mV. Mediators and uncouplers as for Fig. 7. Antimycin A, 20 μM, was also present. At each wavelength, chromatophores were subjected to two 15 μs xenon flashes with 30 ms interval between each flash. Each kinetic trace (200 ms sweep, 500 μs filter RC) was the average of eight signals (60 s dark period between measurements). Symbols: (□) The change 27 ms after the first flash; (●) The change 22 ms after the second flash, using the point 27 ms after the first flash as the baseline. The contribution of the reaction center change was removed by subtraction of the appropriately normalised change recorded at 542 nm. (b) Spectrum of the myxothiazol-sensitive changes following a single flash excitation at an ambient redox potential of 0 ∓ 10 mV. S1-chromatophores were suspended to 6.5 μM bacteriochlorophyll in 50 mM Mops/100 mM KCl (pH 7.0), with the following mediators: 1 μM Pes, 1 μM pyocyanine, 10 μM duroquinone. Valinomycin 20 μM, nigericin 2 μM were present as uncouplers. Antimycin A, 20 μM, was also present. Kinetic traces were the average of 16 measurements with an instrument response time of 500 μs. The spectrum show the changes that occurred 20 ms after the flash.

by the first flash resembles in the cytochrome *b* region that attributed to cytochrome *b*-35 (α band max. at 561 nm, this work Fig. 5), although a small contribution from other components is apparent as a broadening of the spectrum at 564–566 nm. The spectrum of the change induced by the second flash closely resembles that of cytochrome *b*-−40 (split of the α band with maxima at 558 nm and 564 nm in the reduced oxidized difference spectrum; see Fig. 5), providing evidence that the low potential cytochrome *b*-−40 is kinetically competent in light-dependent electron flow in a millisecond time scale. In Fig. 9b, we show the spectrum of the myxothiazol-sensitive changes following a single flash illumination under ambient redox conditions (E_h, approx. 0 mV) in which most of the cytochrome *b*-35 is chemically reduced before the flash. The difference spectrum of the absorbance changes occurring 20 ms after the flash shows a component with a double α-band of similar shape and with the same values for λ_{max} as that of the redox-difference spectrum of Fig. 5 (trace *d*) and the time resolved spectrum of Fig. 9a attributed to reduction of the low potential cytochrome *b*-−40.

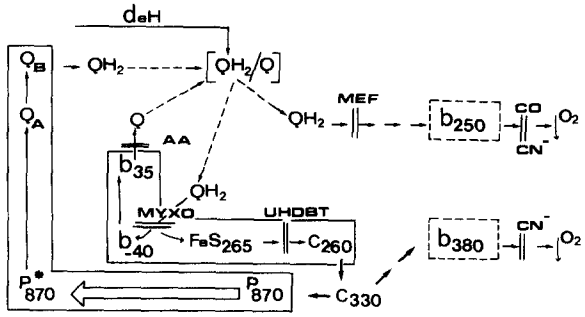

Fig. 10. A model for photosynthetic and respiratory electron transfer by membranes from *R. rubrum* S1. The postulated sites of inhibition by antimycin A (AA), UHDBT, myxothiazol (MYXO), mefloquine (MEF), KCN and CO, are shown. The scheme depicts electron transfer in the forward direction, from either a dehydrogenase (deH) to either branch (under pre-steady-state conditions) or a reaction center bacteriochlorophyll (P-870) to the Q_{bc}-containing pathway. Solid and broken arrows represent electron-transfer reactions and quinone diffusion, respectively. The brackets enclosing the Q/QH_2 couple symbolises the ubiquinone pool. The enclosed boxes (continuous line) represent multiprotein redox complexes. P-870* is the reaction center in the excited state. The two membrane-bound oxidases are indicated as enclosed (dashed) boxes. Rhodoquinone is not included because under aerobic growth with low oxygen tension it does not exceed 9% of the total quinone content [37]. Cytochromes are indicated with their $E_{m7.0}$ (∓5 mV). The E_m of the Rieske FeS center (at pH 7.6) is taken from Ref. 27.

Conclusions

The conclusions concerning the respiratory and photosynthetic electron transfer by membranes isolated from dark-grwon cells of *R. rubrum* S1 are schematically depicted in Fig. 10. According to this model, the main features of *R. rubrum* respiratory chain can briefly be summarized as follows.

(1) The respiratory apparatus is branched, since two oxidative pathways may be distinguished by cyanide sensitivity.

(2) The branch must occur before the antimycin A-, myxothiazol-, UHDBT- and mefloquine-sensitive sites, because oxidative activities mediated by the two branches display different sensitivities to these inhibitors.

(3) A putative ubiquinol cytochrome *c* oxidoreductase complex operates in respiratory electron transfer, because of its sensitivity to myxothiazol and antimycin A along with evidence for the presence of the haem-type elements of isolated b/c_1 complexes.

(4) Cytochrome *b*-380 is suggested to be part of the cytochrome *c* oxidase system, its E_m being thermodynamically consistent with this component functioning at the oxidative side of the soluble cytochrome c_2 (*c*-330).

(5) Cytochrome c_2 is a priori required for respiration through the CN-sensitive pathway, since in cytochrome c_2 depleted membranes, the cytochrome *c* oxidase activity is drastically repressed.

(6) Differently from cytochrome *b*-380, cytochrome *b*-250 is likely to be involved in the alternative CN^--resistant oxidase activity because a shift towards higher values of its E_m can be demonstrated in dark-redox titrations under an atmosphere of N_2/CO.

With regard to the *b*- and *c*-type cytochromes operating in cyclic electron flow, we have given strong evidence that in *R. rubrum* S1 the mechanism of electron transfer exhibits thermodynamic and kinetic properties similar to those described in *Rb. capsulatus* and *Rb. sphaeroides* [35,36]. This

conclusion implicates an arrangement of the redox carriers, which differs considerably from earlier proposed schemes [7,12,14]. New experimental evidences supporting the scheme in Fig. 10 are as follows.

(1) Two different cytochrome *c* components can be identified by time-resolved spectra. One species of cytochrome *c*, which differs from the classical cytochrome c_2, is present as a membrane bound component analogous to cytochrome c_1 of mitochondria [15]. Evidence for the analogy between the bound cytochrome c_1 of chromatophores and the mitochondrial c_1 has been obtained on the basis of the thermodynamic and spectral differences and differential extractability from spheroplasts, which distinguish cytochrome c_2 and cytochrome c_1.

(2) The indirect demonstration, that Rieske iron-sulphur center, cytochrome c_1 and cytochrome c_2 operate in series. Indeed, we have seen that electron transfer by membranes from *R. rubrum* S1 is blocked by UHDBT, whereas the flash-induced oxidation of cytochromes *c* ($c_1 + c_2$) is enhanced by this ubiquinone analogue. Furthermore, we have demonstrated that light-dependent oxidation of cytochrome c_1 is markedly repressed in cytochrome c_2-depleted membranes. Since it has largely been established that UHDBT binds to the Rieske centre of the ubiquinol/cytochrome *c* oxidoreductase (see Ref. 22), the Rieske FeS is likely to be the electron donor to the cytochromes *c*.

(3) In the presence of antimycin A, two cytochromes *b* with $E_{m,7.0}$ of -40 mV and $+35$ mV are rapidly reduced following flash excitation. Cytochrome *b*--40 has its alfa band splitted into two peaks at 558 nm and 564 nm and it is therefore analogous to cytochrome *b*-566 ($E_m = -90$ mV) of isolated b/c_1 complexes. Conversely, cytochrome *b*-35 has an alfa band at 561 nm and corresponds to the second *b*-type haem involved in ubiquinol/cytochrome *c* oxidoreductase. Reduction of both cytochromes is strongly dependent on ambient redox potential (E_h) and when cytochrome *b*-561 is oxidized ($E_h > 60$ mV) before flash excitation in the presence of antimycin A, cytochrome *b*-566 changes following a single flash are much smaller, perhaps indicating a transient reduction followed by oxidation. However, a fuller reduction of cytochrome *b*-566 can readily be observed if a second flash occurs before cytochrome *b*-561 goes reoxidized. Conversely, when cytochrome *b*-561 is chemically reduced before flash activation ($E_h < 0$ mV), rapid myxothiazol-sensitive reduction of cytochrome *b*-566 can be seen following a single flash. As shown in Fig. 10, these results are most easily interpreted in terms of an electron-transfer chain in which cytochrome *b*-566 accepts electrons from a donor, and passes them to cytochrome *b*-561 in a simple linear sequence. Antimycin A would therefore inhibit the oxidation of the two *b*-type cytochromes through binding to cytochrome *b*-561.

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