Measurement of the redox state of the ubiquinone pool in *Rhodobacter capsulatus* membrane fragments

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The dependence of the respiratory rate on the redox poise of the quinone pool was investigated in wild type and mutant membranes of *Rhodobacter capsulatus*. A linear relationship has been found between these two parameters only when succinate was oxidized by the bc_1 complex. Conversely, a marked nonlinear relationship was observed between the Q-pool reduction level and the respiratory rate when O_2 uptake occurred via the alternative oxidase. In addition, it was found that this latter pathway was not engaged until Q-pool reduction level reached approximately 25%. These results are discussed within the framework of a homogeneous pool regulating both photosynthetic and respiratory fluxes.

Alternative oxidase; Quinone redox level; Respiration; *Rhodobacter capsulatus*

1. INTRODUCTION

Purple non-sulphur bacteria (*Rhodospirillaceae*) are facultative anaerobes which accommodate the pigments and the redox components necessary for both photosynthesis and respiration on the same continuous cytoplasmic membrane [1]. Several observations [2–6] support the concept that photosynthetic and respiratory apparatuses share common redox component(s) such as, for example, soluble cytochrome *c*. The situation is, however, complicated by the fact that the respiratory chain branches at the level of ubiquinone/bc_1 complex into separate pathways leading to distinct oxidases, a Cyt *c* oxidase inhibited by 50 μM KCN and a quinol oxidase inhibited by 3–5 mM KCN and CO [7,8]. This implies that the quinone pool is a key redox element in partitioning electrons through the respiratory branches. In addition, since it has previously been shown that membrane fragments catalyse a light-driven oxygen uptake [9], quinone must necessarily connect the photosynthetic and respiratory apparatuses.

Early data on the redox state of the Q-pool in membranes of *Rb. capsulatus* indicated that the level of quinone reduction induced by respiratory substrates during steady-state respiration is approx. 40% of the total Q present [7]. The level of reduction was shown to increase to only 50% under conditions in which the Cyt *c* oxidase was blocked by CN^- (50 μM), suggesting that the Q-pool was in redox equilibrium with the alternative oxidase-containing pathway. A major difficulty with such studies, obtained through the use of the extraction technique introduced by Kröger and Klingenberg [10], is the lack of a fast and continuous monitoring of the Q redox state. Here, the level of reduction of the Q-pool has been determined voltametrically, a technique recently used to investigate the relationship between the redox state of the Q-pool and respiratory fluxes in plant mitochondria [11–13]. In the present study, we have simultaneously measured the steady-state redox level of the Q-pool and oxygen consumption, in wild type and respiratory mutant strains of aerobically dark-grown *Rb. capsulatus*. The data have been interpreted to show that the partitioning of electron flow between the bc_1 containing pathway and the alternative oxidase branch is determined by the redox state of the ubiquinol pool.

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Abbreviations: AA, antimycin A; CN^-, cyanide; Cyt, cytochrome; Myx, myxothiazol; Q, ubiquinone; Q_r, reduced quinone under steady-state respiration; Q_t, fully reduced ubiquinone; Q-2, ubiquinone-2; RC, reaction center

2. MATERIALS AND METHODS

Semiaerobic growth of *Rb. capsulatus* was carried out in the dark at 30°C in RCV- (MR126 and R126 strains) and MPYE- (MT1131 and MT-GS18 strains) media, as previously described [9,14]. Cells were harvested at late log growth phase. After harvesting and washing cells in 50 mM Mops/5 mM $MgCl_2$ (pH 7.2) chromatophores were prepared by the French press method [9] and resuspended at approx. 30 mg protein/ml. Strains used: *Rb. capsulatus* R126 is a green derivative of strain Y11, a normally pigmented, non-phototrophic (Ps^-) mutant of *Rb. capsulatus* [15]. MR126 is a phototrophic (Ps^+) derivative of R126, constructed using a gene-transfer agent (GTA) [16]. Strain MT-GS18 is a non-

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photosynthetic (Ps^-), Cyt c_1 and Cyt c_2 deficient (c_1^-,c_2^-) mutant constructed via GTA [14]. Strain MT1131 is a 'green derivative', phototrophic competent (Ps^+) of SB1003 [17].

Protein concentration was determined by the Lowry method [18].

The concentration of cytochrome c_1 was determined from oxidized minus reduced difference spectra (552.5–540 nm, ϵ = 19 $mM^{-1} \cdot cm^{-1}$). The concentration of reaction centers (RC) was measured at 542 nm (ϵ = 10.3 $mM^{-1} \cdot cm^{-1}$) as described in [19].

Ubiquinone-10 was extracted from chromatophores according to [7] and its concentration determined by HPLC analysis (high-pressure liquid chromatography) [19]. Standards were the kind gift of Dr J. Baush and Dr H.E. Keller of Hoffmann-La Roche (Basel).

Oxygen consumption was measured polarographically in 1.8 ml of reaction medium containing 50 mM Mops buffer/5 mM $MgCl_2$/5 mM KCl, all adjusted to pH 7.2, in a specially constructed cell (University of Sussex Workshops) housing a Rank oxygen electrode, a glassy carbon, and a platinum electrode.

The redox state of exogenously added Q-2 was measured voltametrically using a glassy carbon working electrode and a platinum electrode (Anachem, London) connected to an $Ag|AgCl_2$ reference electrode. The working electrode was poised at −360 mV with respect to the reference electrode as described previously [20]. Membrane fragments (3–5 mg protein) were incubated with 1 μM Q-2 and 5 mM succinate was added to initiate oxygen consumption. The concentration of Q-2 used had no effect upon the respiration rates or sensitivity to inhibitors. Fully oxidized Q was taken as the base of the trace following addition of Q and membranes. Total reducible quinone (Q_t) was estimated as the amount reduced under anaerobic conditions. Illumination of the reaction chamber was provided with a 150 W spot-lamp with a round flask filled with water as a heat filter and focusing device between the lamp and the electrode.

Antimycin and myxothiazol were purchased from Sigma and Boehringer Mannheim, respectively.

3. RESULTS AND DISCUSSION

Fig. 1A shows the simultaneous measurement of oxygen uptake and steady-state level of reduction of quinone by *Rb. capsulatus* MR126 membrane fragments whilst oxidising succinate. It is apparent that the addition of succinate resulted in a rapid (60 s) reduction of the quinone pool (to 30% of the level achieved upon anaerobiosis, Q_t) and initiated oxygen consumption. Conversely, in the absence of an oxidisable substrate, there is negligible respiratory activity and the quinone pool is substantially oxidised. Notably, the level of reduction of quinone is only slightly increased by light with no appreciable effect on the rate of respiration.

Fig.1B–D shows the traces of a series of experiments in which respiratory electron flow was inhibited by either CN^-, antimycin A (AA) and/or myxothiazol (Myx). The most significant results can be summarized as follows: (a) addition of 50 μM CN^- to respiring membranes induces a further reduction of the quinone pool (Q_r = 70% of Q_t) with a parallel 50% inhibition of the oxygen uptake (Fig. 1B); (b) Myx (5 μM) and CN^- (50 μM) show similar effects, i.e., partial inhibition of respiration with a consistent increase of the steady-state level of Q_r (Fig. 1B and D); and (c) AA (5 μM) blocks 50% of oxygen uptake with only a slight effect on the redox state of the quinone pool (Fig. 1B and C). Previous data on the reduction kinetics of *b*- and *c*-type cytochromes in membranes of *Rb. capsulatus* MR126 indicated that Myx is highly effective in inhibiting reduction of Cyt's *c* ($c_1 + c_2$) whereas AA had a significant, but partial, effect on *c*-type reduction [21]. In contrast, no appreciable change on the reduction kinetics of Cyt *b*-562 was observed upon addition of either AA or Myx although a complete block of Cyt *b* reduction could be induced by addition of both antibiotics [21]. According to the protonmotive 'Q-cycle' scheme [22], Myx acts at a redox center, defined as Q_o, in such a way that it blocks quinol oxidation catalyzed by both Cyt *b*-566 (plus Cyt *b*-562) and Rieske iron-sulphur protein [23–25]. Conversely, AA blocks reduction of quinone by Cyt *b*-562 through binding to a redox center, defined as Q_i, in which *b*-562-heme forms

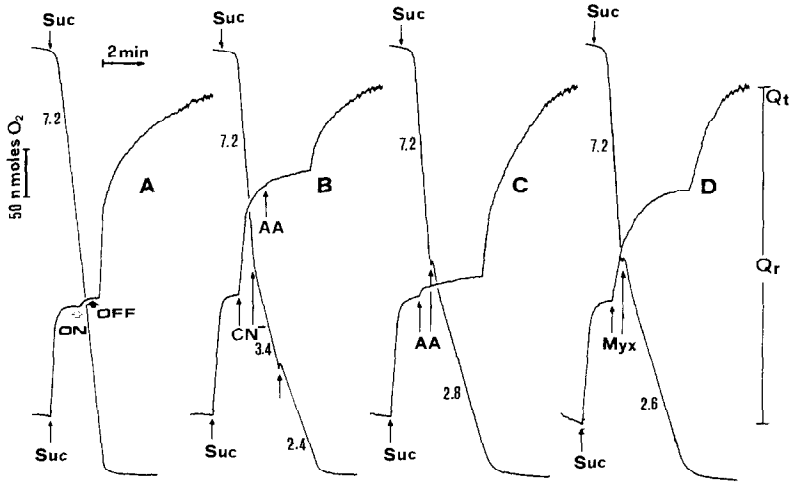

Fig. 1. Simultaneous measurement of O_2 consumption and steady-state reduction level of Q with membranes from *Rb. capsulatus* MR126. Respiration was initiated by the addition of 5 mM succinate (Suc). Membrane concentration was 2.55 mg/ml. Inhibitor additions were as indicated at a concentration of 50 μM and 5 μM for cyanide (CN^-) and antimycin A (AA) or myxothiazol (Myx), respectively. In A, illumination was switched ON and OFF, as indicated. Numbers on the respiratory traces are in μequiv. O_2 per h per mg protein. Further details in section 2.

the catalytic site [26]. On the basis of these considerations results of Fig.1A–D suggest, at least, two partial conclusions, namely: (i) inhibition of the Cyt *c* oxidase branch by CN^- strongly limits the oxidation of quinol; and (ii) inhibition of Q_o and/or Q_i sites by Myx and/or AA results in a differential effect on the redox state of the Q-pool.

In chromatophores from *Rhodobacter sphaeroides* Ga and *Rb. capsulatus* MR126 it has previously been shown that the EPR line shape of the Rieske-center is affected by the redox state of the Q-pool [27]. Parallel experiments in membranes from *Rb. capsulatus* R126 (a non-phototrophic mutant) indicated that the R126 lesion involves quinone function at the Q_o site, i.e. quinone is excluded from the site necessary for Q_o catalysis. In contrast, the Q_i site of R126 is fully functional as compared to its wild-type derivative strain (MR126), and also both primary and secondary quinone acceptors (Q_a and Q_b), *plus* the Q-pool function are intact [28]. Fig.2A shows that in membranes from R126, approx. 34% of the total reducible quinone (Q_t) can be reduced by succinate under steady-state respiration. As expected, this reduction level was not affected by Myx and/or illumination.

To test the role of both Q_o and Q_i sites on the control of the Q redox state, with respect to the oxidation of reduced Q, a strain lacking bc_1 complex, designated as MT-GS18 [14], was analyzed. *Rb. capsulatus* MT-GS18 is a double mutant (c_2^-,c_1^-) unable to grow phototrophically but which maintains the capacity of aerobic growth by means of the alternative oxidase branch. Fig. 2B shows that in MT-GS18, similarly to R126, approx. 37% of Q_t is reduced by respiration. The Q reduction level was insensitive to AA and Myx.

Fig. 3 shows a typical titration of the Q reduction level in wild-type membranes (MT1131 strain) as a function of increasing malonate concentrations (from 0.05 to 11.6 mM) to provide a progressive inhibition of succinate oxidase activity. Clearly, as the malonate concentration was increased, the steady-state level of Q reduction by succinate showed a progressive oxidation until a final reduction level of ca 4–5% was reached. Fig. 4 shows the results of a number of these experiments analysing the relationship between electron flux via the alternative oxidase and/or the bc_1 complex and the reduction level of the Q pool in both MT1131 and MT-GS18 membranes. The data are presented as the ratio of v/V_o (where v is the initial rate of oxygen uptake in the presence of inhibitor of electron input and V_o is the uninhibited rate) plotted vs the proportion of Q in the reduced state (Q_r/Q_t). The results obtained in MT1131 (w.t.) membranes indicate that under uninhibited conditions (open circles), the relationship approximates linearity over the full range of Q reduction level (between 4–5% and 30%). However, the relationship becomes markedly nonlinear when the Cyt *c* oxidase dependent branch is inhibited by CN^- (closed

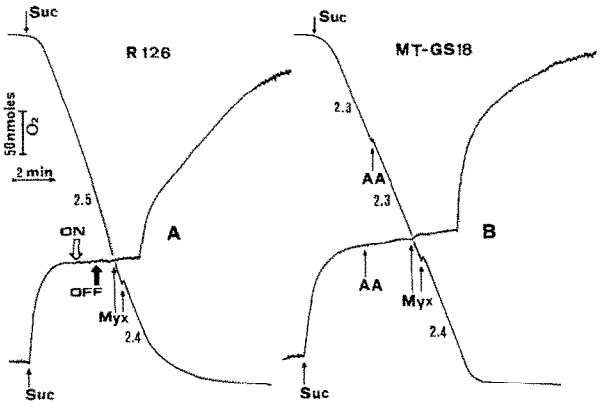

Fig. 2. Simultaneous measurement of respiration and steady-state reduction level of Q in membranes from *Rb. capsulatus* mutant strains R126 and MT-GS18. Membrane concentrations were 2.6 and 2.1 mg/ml for R126 and MT-GS18, respectively. Numbers on the respiratory traces are in μequiv. O_2 per h per mg protein. Additions and abbreviations as in Fig. 1.

circles) and/or CN^- *plus* AA and Myx (closed squares). In this latter case, the Q_r/Q_t level at which a significant rate of electron flux can be seen, is shifted from 4–5% to approx. 25% indicating that in MT1131 membranes the alternative oxidase will not be engaged unless the steady-state Q-pool reduction is maintained above this

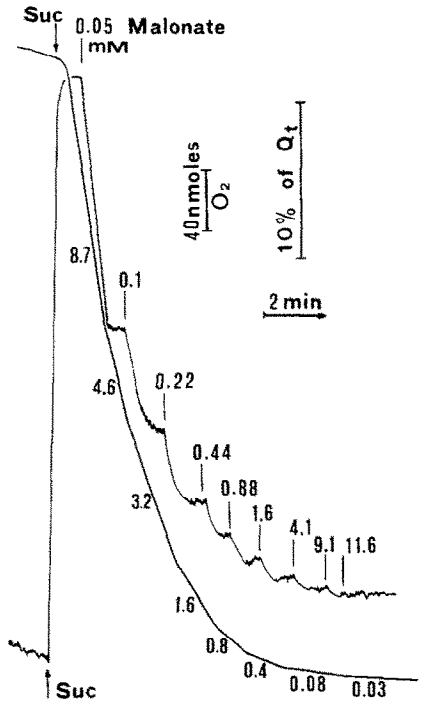

Fig. 3. Steady-state reduction level of Q and oxygen uptake as a function of increasing concentrations of malonate in membranes from *Rb. capsulatus* MT1131. Malonate additions were as indicated up to a final concentration of 11.6 mM. Numbers on the Q-trace are in mM whereas those on the respiratory trace are in μequiv. O_2 per h per mg protein. Membrane protein concentration was 1.4 mg/ml. Other conditions as in Fig. 1.

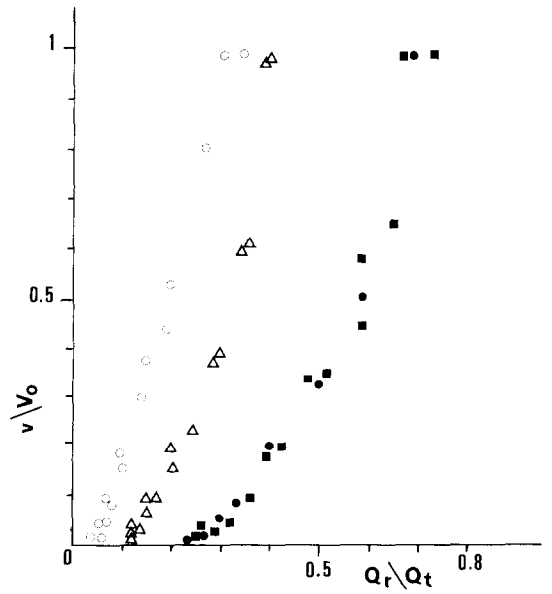

Fig. 4. Dependence of respiratory flux on quinone redox state in membranes from *Rb. capsulatus* MT1131 and MT-GS18. Abbreviations as in Fig. 1. (○) MT1131 in the absence of inhibitors; (●) MT1131 in the presence of 50 μM CN^-; (■) MT1131 in the presence of 50 μM CN^- plus 5 μM AA and 5 μM Myx; (△) MT-GS18. Data represent a summation of two separate experiments. See text for further details.

level. In Fig. 4 the values of v/V_o vs Q_r/Q_t in membranes from MT-GS18, are also reported (open triangles). In this mutant strain, the alternative oxidase is not engaged to any significant extent until the level of the Q pool reaches 12%. Below this level the rate of electron flow is negligible. These data suggest that in MT-GS18, in which the total respiratory electron flow depends on a quinol oxidase activity, the quinol oxidase is engaged at a lower Q reduction level, possibly reflecting a higher oxidase activity of the mutant. This point is currently under investigation (Zannoni and Daldal, in preparation).

It is noteworthy that the Q_r values observed in MT1131, R126 and MT-GS18 strains were confirmed by experiments in which the redox state of the Q-pool was determined by combining chemical extraction [10] and HPLC analysis (see section 2) (Table I).

Table I

Comparison of the Q-pool reduction levels (Q_r/Q_t) during steady-state succinate oxidation by membranes of *Rb. capsulatus*, as determined by either the voltametric technique (Q-electrode) and/or by extraction/HPLC analysis

Strains	Extraction/HPLC	Q-electrode
MT1131	0.32	0.35
MT-GS18	0.30	0.37
R126	0.28	0.34

Note: results are the means of two experiments; see text for further details.

4. CONCLUSIONS

This study demonstrates, for the first time, how electron flow via the alternative pathway, i.e., quinol oxidase, of *Rb. capsulatus* is regulated by the reduction level of the Q-pool. Unlike the bc_1 complex, through which electron transport appears to be linearly related to Q_r/Q_t (between 4–5% and 30%), net flux through the Q_r-oxidizing step of the alternative pathway is strongly limited until the Q-pool reduction level reaches approx. 25%. Obviously this is dependent on the relative rates of input and output of electrons from the Q-pool which, in turn, will vary with different substrates.

It has previously been shown that in photosynthetic membranes of *Rb. sphaeroides* electron flow is modulated by changes in the pool redox state only through the quinol oxidase site (Q_o site) [19]. The apparent K_m of QH_2 at this site was calculated to be approx. 3.5 Q per RC. Semiaerobically grown *Rb. capsulatus* MT1131 contains ca 60 quinones per RC (this work, not shown). Since we have demonstrated that, under steady-state respiration, the bc_1 complex is reduced when the Q reduction level reaches 4–5% (Fig. 4), this means that the K_m of QH_2 at the Q_o site is approx. 2.4–3 Q per RC, a value close to that found in phototrophically grown *Rb. sphaeroides*. If we define the quinol-oxidizing step of the alternative oxidase as Q_{ox} site, it can also be concluded that in MT1131 membranes (Fig. 4, closed symbols) the K_m of QH_2 at this site is approx. 15 Q per RC, since the alternative oxidase is only engaged when the Q pool has been approx. 25% reduced.

The kinetic model of homogeneous Q-pool behaviour as proposed by Ragan and Cottingham [29] provides a mechanism to explain the observed first-order kinetics of oxido reduction of Q, via the cytochrome chain under conditions of apparent equilibrium saturation. More recently, Reed and Ragan [30] demonstrated how modification of the homogeneous pool model, to incorporate the existence of an unfavorable equilibrium associated with the quinol oxidase step, may cause marked deviations from first-order kinetics. A marked non-linearity of the relationship between alternative pathway activity and reduction level of the Q-pool has been shown to be present in soybean (*Glycine max*) mitochondria where the CN^--insensitive pathway is not engaged until Q_r/Q_t ratio reaches 35–40% [12]. Here we demonstrate that a similar non-linearity between these two parameters is also observed in membranes from *Rb. capsulatus* under conditions in which the bc_1-containing branch is either absent or inhibited.

In plant mitochondria, the non-linearity of the relationship between CN-resistant pathway activity and the reduction level of the Q-pool, has been suggested to limit the extent of 'energetically wasteful' respiration

[12]. This proposal is not applicable to *Rb. capsulatus*, since observations made with the respiratory mutant R126 indicate that the quinol oxidase pathway is linked to energy transduction [31]. Furthermore, it has also been observed that the restriction of respiratory electron flow by light and the uncoupling of electron flux from $\Delta\tilde{\mu}_{H+}$, involves the quinone-reducing step (NADH dehyd) rather than the quinol-oxidizing pathways [32,33]. These findings suggest that, under aerobic conditions in the light, the alternative oxidase of *Rb. capsulatus* will be poorly engaged because of a limited substrate-dependent electron input. Conversely, a net flux through the Q_{ox} site of the alternative pathway will be observed under conditions of high influx rate, e.g., when the reducing power level is large enough to induce uncoupling of complex I [33].

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