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Restoration of the optimal redox state for the photosynthetic electron transfer system by auxiliary oxidants in an aerobic photosynthetic bacterium, *Erythrobacter* sp. OCh 114

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In an aerobic photosynthetic bacterium, *Erythrobacter* sp. OCh 114, photosynthetic electron transfer (photooxidation of cytochromes) and light-driven proton release did not occur under anaerobic conditions. 'Auxiliary oxidants', such as nitrate, nitrite, trimethylamine *N*-oxide (TMAO) and chlorate, restored the optimal redox state for the photosynthetic electron transfer system, resulting in the photooxidation of *c*-type cytochrome and the release of protons from intact cells on illumination. These auxiliary oxidants oxidized *c*-type cytochrome(s) under anaerobic conditions in the dark, suggesting the presence of reductases for these auxiliary oxidants. The redox level of the electron transfer chain shifted towards oxidation in the presence of the auxiliary oxidants and was poised to allow photosynthetic electron transfer. KCN inhibited the photooxidation of cytochromes and the light-driven proton release in the presence of these auxiliary oxidants, except for TMAO. KCN also inhibited the auxiliary oxidant-induced oxidation of cytochromes. This suggests that KCN inhibits the reductases for these oxidants. Antimycin and myxothiazol enhanced the auxiliary oxidant-induced oxidation of cytochrome(s), suggesting the involvement of the cytochrome b-c_1 complex in nitrate, chlorate and nitrite reduction.

Introduction

Erythrobacter sp. OCh 114 is a member of a species of 'aerobic photosynthetic bacteria' which contain bacteriochlorophyll and which can grow aerobically in the dark but cannot grow anaerobically, even in the light [1,2]. The cells have a structure morphologically similar to the intracytoplasmic membranes (chromatophore membranes) of the purple photosynthetic bacteria [3] and a functionally competent photochemical system [3,4]. In fact, the reaction center bacteriochlorophyll and a soluble cytochrome, cytochrome *c*-551, have been shown to be reversibly oxidized on illumination under aerobic conditions [3,4]. Furthermore, the reaction center complex has been purified from this bacterium and its biochemical properties are very similar to those of the reaction center from purple photosynthetic bacteria [5,6]. However, the photochemical system does not operate under anaerobic conditions [4,7], probably because the electron acceptor quinones in the reaction center are easily reduced under

Abbreviations: TMAO, trimethylamine *N*-oxide; Mops, 4-morpholinepropanesulfonic acid.

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anaerobic conditions [4]. The absence of the photosynthetic electron transfer seemed to be partly due to the relatively high midpoint potential of the primary electron acceptor quinone [4] and partly to the incapability of maintaining the optimal redox state under anaerobic conditions, which caused the over-reduction of the electron transfer system.

Common facultative anaerobic photosynthetic bacteria can maintain the optimal redox state under anaerobic conditions. It has been found that in *Rhodobacter capsulatus,* phenazine methosulfate inhibited the generation of the membrane potential probably because it perturbed the redox state, and the acceptor quinones became reduced [8]. 'Auxiliary oxidants' such as nitrate, nitrite, nitrous oxide, trimethylamine *N*-oxide (TMAO) and dimethylsulfoxide restored the optimal redox level by removing the extra reducing equivalents from the photosynthetic electron transfer chain [8,9]. These auxiliary oxidants have been thought to be the electron acceptors in the anaerobic respiration of *Rhodospirillaceae* and to maintain the redox balance during photosynthetic metabolism [10]. We recently discovered that *Erythrobacter* OCh 114 can grow under anaerobic conditions in the presence of TMAO [11]. In this communication, nitrate, nitrite, TMAO and chlorate were shown to restore the cells from over-reduction, resulting in the photooxidation of *c*-type cytochromes and light-induced proton release from intact cells under anaerobic conditions.

Materials and Methods

Erythrobacter OCh 114 was cultured at 28°C for 1–2 days in the dark under vigorous aeration in enriched PEPS-II medium [12]. The culture medium contained NaCl, 23.5 g; $MgCl_2 \cdot 6H_2O$, 10.6 g; Na_2SO_4, 3.9 g; $CaCl_2 \cdot 2H_2O$, 1.46 g; KCl, 0.66 g; $NaHCO_3$, 0.19 g; KBr, 0.096 g; $SrCl_2 \cdot 6H_2O$, 0.04 g; H_3BO_3, 0.026 g; NaF, 0.003 g; ferric citrate, 0.1 g; polypeptone (Daigo Eiyokagaku Co.), 2 g; proteose peptone No. 3 (Difco), 1 g; Bacto-soytone (Difco), 1 g and Bacto-yeast extract (Difco), 1 g in 1 liter of distilled water. The pH was adjusted to 7.6–7.8 with NaOH before autoclaving. For measurement of the difference spectra of cytochrome(s), cells were cultured in the light to reduce the content of pink carotenoid pigments and bacteriochlorophyll. If necessary, potassium nitrate (0.2%, w/v) was added in the medium and cells were cultured under anaerobic conditions. Cell growth was monitored by measuring the absorbance of the culture medium at 660 nm with a Hitachi 124 spectrophotometer.

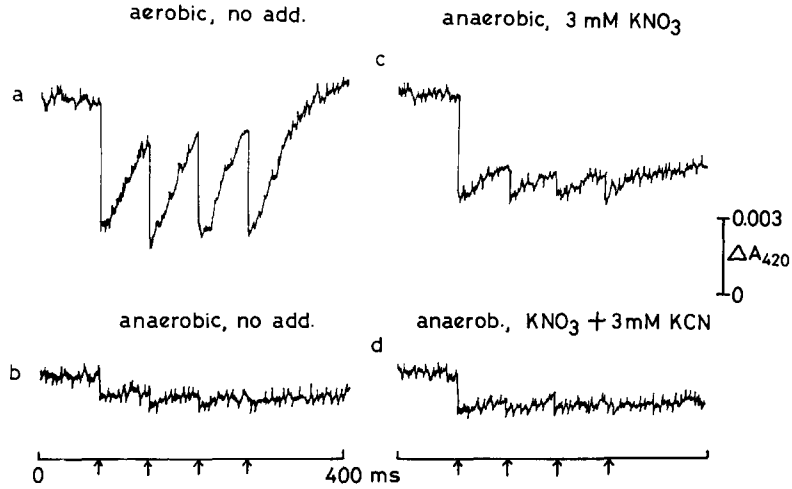

Fig. 1. Effects of nitrate and cyanide on flash-induced cytochrome *c*-551 oxidation in intact cells under aerobic and anaerobic conditions. (a) Under aerobic conditions; (b) under anaerobic conditions; (c) anaerobic, 3 mM KNO_3; (d) same as (c) +3 mM KCN. Oxidation-reduction reactions of cytochrome *c*-551 were measured at 420 nm as described in Ref. 4. Concentration of bacteriochlorophyll, 5 μM (about 1.5 mg cell protein/ml suspension). Arrows on the bottom indicate when the flashes were fired.

For spectroscopic measurements, cells were harvested by centrifugation and suspended in 50 mM Mops (pH 7.0)/3% NaCl (w/v). Flash-induced oxidation of cytochrome *c*-551 was measured as described previously [4]. Difference spectra of cytochromes in intact cells were measured with a Shimadzu UV240 spectrophotometer. Anaerobic measurements were carried out in an anaerobic cuvette into which O_2-free argon gas was continuously streamed. For measurement of pH changes of the intact cell suspension, the harvested cells were suspended in 3% NaCl solution and pH changes were monitored with a Hitachi-Horiba pH meter (Model M8s) connected to a strip chart recorder. The initial pH of the suspension was adjusted to 7.5 by addition of a small volume of diluted HCl or NaOH solution.

Antimycin was purchased from Sigma, and myxothiazol from Boehringer-Mannheim. Other chemicals were purchased from Nakarai Chemicals.

Results

Restoration of flash-induced cytochrome oxidation in the presence of nitrate, nitrite, chlorate and TMAO under anaerobic conditions

Figs. 1 and 2 show the effects of nitrate, nitrite, chlorate and TMAO on flash-induced oxidation of cytochromes under aerobic and anaerobic conditions. As shown in the previous study [4], photooxidation of cytochromes (probably a soluble cytochrome, cytochrome *c*-551, see Ref. 13) took place under aerobic conditions and disappeared under anaerobic condition (Fig. 1a and b). Under anaerobic conditions, in the presence of 3 mM KNO_3, the cytochrome was oxidized by flash-activation (Fig. 1c). In the presence of 3 mM KNO_2, the cytochrome was oxidized with a higher recovery rate than in the presence of nitrate (Fig. 2a). In our recent study [23] it is shown that this bacterium constitutively contains nitrate and nitrite reductases. Therefore, added nitrate is probably reduced to nitrite. The effect of nitrate might be due to the nitrite formed by nitrate reduction. Chlorate is thought to be an alternative electron acceptor of nitrate reductase in some bacteria [14–16], but in those cases, nitrite is not produced. As shown in Fig. 2c, cytochromes were

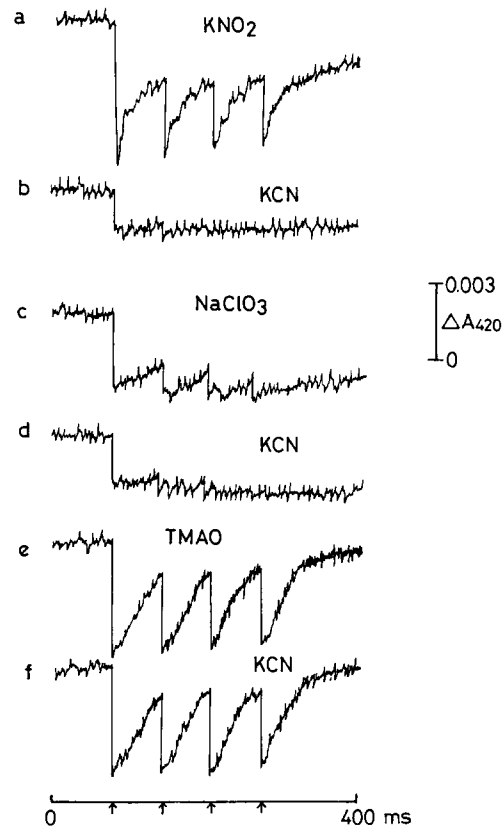

Fig. 2. Flash-induced cytochrome *c*-551 oxidation under anaerobic conditions in the presence of KNO_2, $NaClO_3$ and TMAO. (a) in the presence of 3 mM KNO_2; (b) same as (a) + 3 mM KCN; (c) in the presence of 3 mM $NaClO_3$; (d) same as (c) + 3 mM KCN; e, in the presence of 1 mM TMAO; (f) same as (e) + 3 mM KCN. Other experimental conditions were as in Fig. 1.

also oxidized on illumination in the presence of chlorate, hence the presence of nitrate itself restored the cytochrome photooxidation under anaerobic conditions. Photooxidation of cytochromes took place in the presence of 2 mM TMAO (Fig. 2e) but not in the presence of dimethylsulfoxide [11]. *Rb. capsulatus* used dimethylsulfoxide as an auxiliary oxidant [16].

In all cases of auxiliary oxidants tested, the extent of flash-induced cytochrome oxidation was almost the same as that occurring under aerobic conditions, although the rate of cytochrome re-reduction varied in the presence of different oxidants. As it significantly depended on the redox

level of the electron transfer chain in intact cells [4], the extent of cytochrome oxidation suggested that the redox level of the photosynthetic electron transfer system was restored in the presence of these auxiliary oxidants to nearly the same level as that existing under aerobic conditions.

The flash-induced oxidation of cytochrome disappeared in the presence of KCN (Fig. 2b, d) except for in the case of TMAO. The effect of KCN on photooxidation in the presence of nitrate, nitrite and chlorate suggests that KCN inhibits both nitrate and nitrite reductases, resulting in the inhibition of electron transfer to nitrate and nitrite (see below). KCN did not affect the oxidation of cytochromes in the presence of TMAO, probably because TMAO reductase is a different enzyme from nitrate or nitrite reductase.

Restoration of light-induced proton release in intact cells in the presence of the auxiliary oxidants

Under aerobic conditions, intact cells of *Erythrobacter* OCh 114 reversibly ejected protons

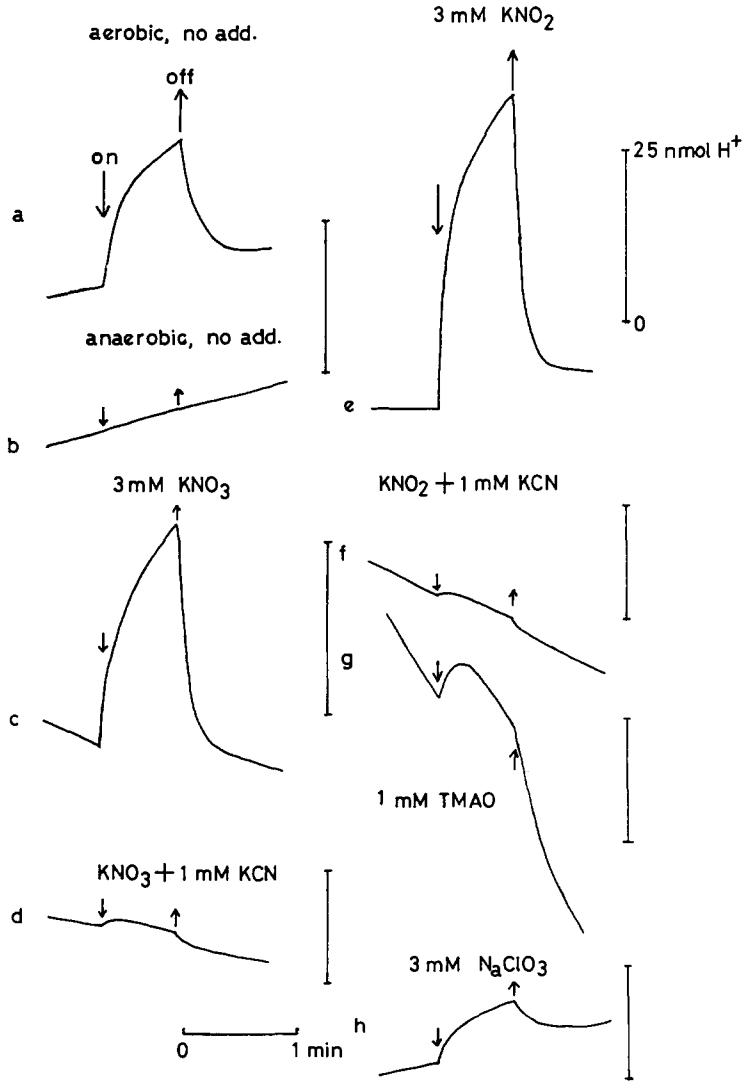

Fig. 3. Proton release from intact cells on illumination under various conditions. For experimental details, see text. Bacteriochlorophyll concentration, 10 μM (about 3 mg cell protein/ml suspension). Downwards and upwards arrows indicate when the actinic light was turned on an off, respectively.

on continuous illumination (Fig. 3a, see also Ref. 4). Under anaerobic conditions, reversible proton release disappeared (Fig. 3b, Ref. 4). The absence of proton release was coincident with the disappearance of cytochrome photooxidation under the anaerobic conditions described above. Under anaerobic conditions, in the presence of the auxiliary oxidants, the reversible proton release was restored (Fig. 3c, e, g, h). The extent of the restored steady-state proton release was similar to that observed under aerobic conditions without auxiliary oxidants. This suggests that the activity of the light-induced proton translocation was restored to the optimal level, as observed under aerobic conditions. The restored proton release was inhibited in the presence of 1 mM KCN (Fig. 3d, f).

Cytochrome(s) oxidation by auxiliary oxidants under anaerobic conditions

The results described above suggest that nitrate, nitrite, chlorate and TMAO could serve as electron acceptors under anaerobic conditions, resulting in restoration of the optimal redox state in the photosynthetic electron transport chain of this bacterium. If this is the case, the addition of these auxiliary oxidants will oxidize cytochrome(s) in the electron transfer chain under anaerobic conditions. Fig. 4A shows steady-state cytochrome oxidation by the anaerobic addition of these auxiliary oxidants. The maximum wavelength of the α-band (approx. 551 nm) showed that the *c*-type cytochrome(s) was oxidized in all cases and suggested that cytochrome *c*-551 [13], which was also oxidized on flash-activation under anaerobic conditions (see above and Ref. 4), was the most probable candidate. There was no indication of the involvement of *b*-type cytochromes. This was in contrast to the case of *Rhodobacter sphaeroides* f. sp. *denitrificans*, in which only *b*-type cytochromes were oxidized by the addition of nitrate and both *b*- and *c*-type cytochromes were oxidized by the addition of nitrite [17]. In the case of *Erythrobacter* OCh 114, the presence of KCN (3 mM) inhibited cytochrome oxidation in the presence of nitrate, nitrite and chlorate (Fig. 4B). The

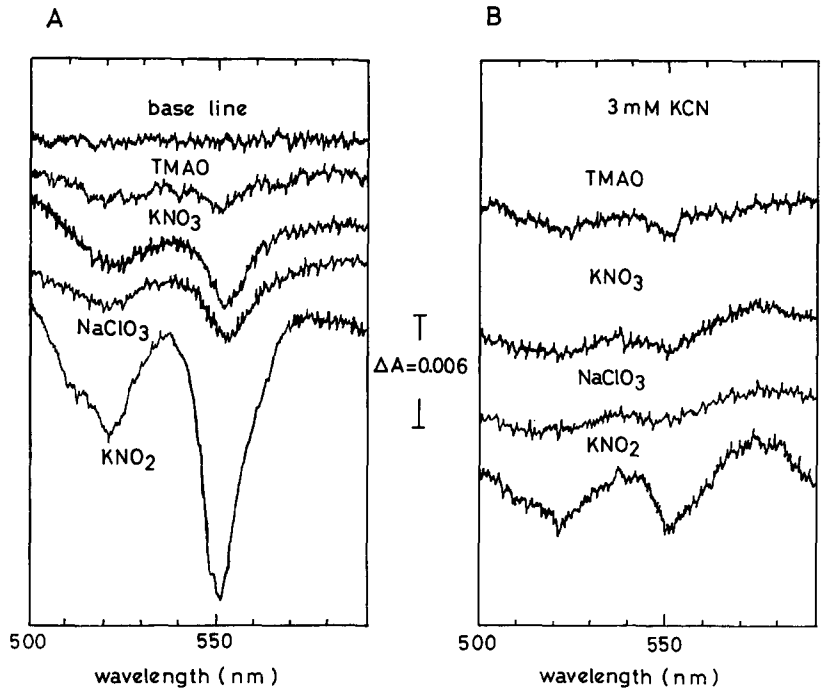

Fig. 4. Oxidized-minus-reduced difference spectra of cytochrome(s) induced by the auxiliary oxidants under anaerobic conditions in the absence (A) and presence (B) of 3 mM KCN. Concentrations of oxidants were 3 mM, except for 1 mM TMAO. Cell concentration was about 6 mg cell protein/ml suspension.

results shown in Fig. 4 indicate that these compounds function as auxiliary oxidants for the electron transfer chain under anaerobic conditions and that cytochrome *c*-551 is involved in the electron transfer chain. KCN is an inhibitor of the electron transfer chain(s), and probably inhibits both the nitrate and nitrite reductases.

Fig. 5 shows the effect of antimycin and myxothiazol on cytochrome oxidation in the presence of these auxiliary oxidants under anaerobic conditions. In this experiment, both reference and sample cuvettes contained cell suspensions supplemented with one of the auxiliary oxidants under anaerobic conditions, and the base lines were measured. Then, antimycin or myxothiazol (each 10 μM) was added to the sample cuvette and the difference spectra were measured. Addition of the same amount of pure ethanol (3 μl) had no effect. The addition of antimycin or myxothiazol shifted cytochrome *c*-551 towards a more oxidized level. It was shown using lower concentrations (2 μM) of the inhibitors that the cytochrome *b*-c_1 complex was involved in TMAO-induced electron transfer [11]. These results suggest that the electrons passed through the cytochrome *b*-c_1 complex to cytochrome *c*-551, and then to these auxiliary oxidants. In fact, membranes from this bacterium had ubiquinol–cytochrome-*c* oxidoreductase activity, which was inhibited by either antimycin or myxothiazol (Fig. 6). Furthermore, anaerobic growth in the presence of 0.2% nitrate was also inhibited in the presence of antimycin or myxothiazol (not shown), suggesting that the cytochrome *b*-c_1 complex was involved in nitrate and/or nitrite reductions.

Discussion

Restoration of the optimal redox state of the photosynthetic electron transfer system by auxiliary oxidants and involvement of the cytochrome b-c_1 complex and cytochrome c-551

As shown in our previous study and brief comments in Introduction, absence of photochemical

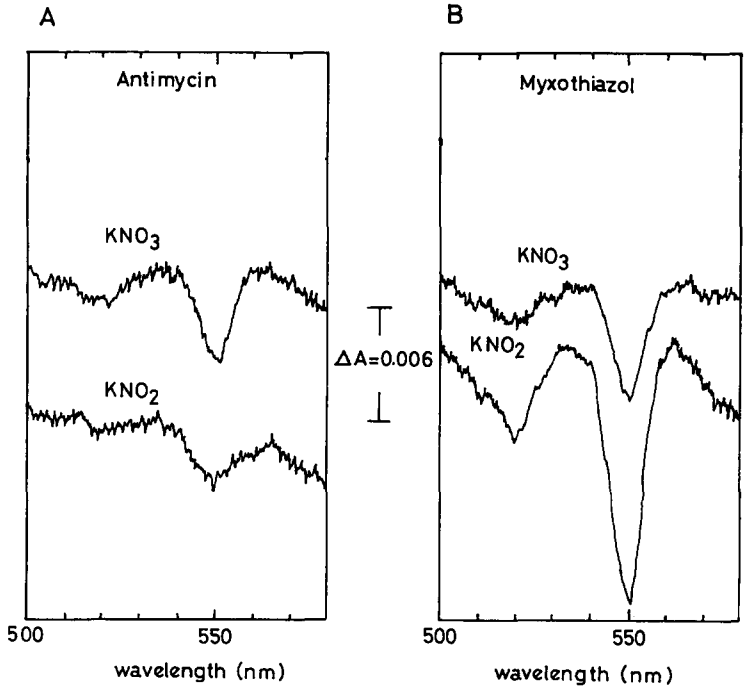

Fig. 5. Difference spectra of cytochromes in the presence and absence of antimycin (A) or myxothiazol (B) under anaerobic conditions. Experimental conditions were as in Fig. 4. To both reference and sample cuvettes, the auxiliary oxidants were added under anaerobic conditions. The base lines were then measured. Ethanol solution of antimycin or myxothiazol (each 10 μM, 3 μl) was added to the sample cuvette and the difference spectra were measured.

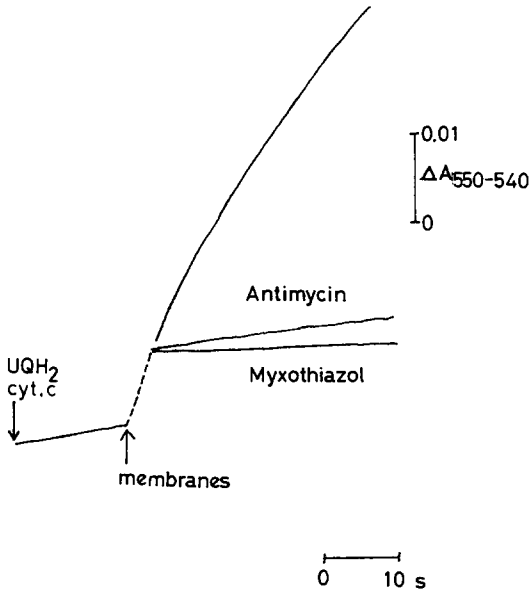

Fig. 6. Ubiquinol–cytochrome-*c* oxidoreductase activity in the membrane preparation of *Erythrobacter* OCh 114. The reaction mixture contained 16 μM horse-heart cytochrome *c*, 100 μM KCN, 15 μg/ml of ubiquinol-1. KCN was used to inhibit a cytochrome oxidase in the membranes which reoxidizes cytochrome *c* enzymatically reduced by ubiquinol-1. The membrane preparation was added at the time indicated by the arrow (138 μg protein/ml). 10 μg of antimycin or myxothiazol were added as indicated.

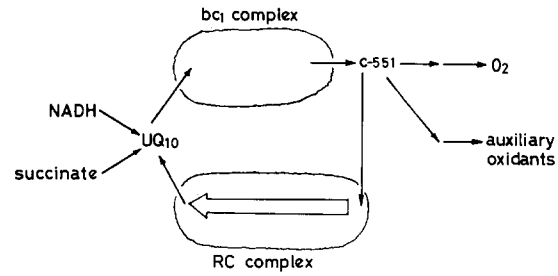

Fig. 7. The scheme of the electron transfer system in *Erythrobacter* OCh 114. Arrows indicate the flow of electrons. UQ_{10}, ubiquinone-10; *c*-551, cytochrome *c*-551.

and related reactions under anaerobic conditions seemed to be due to the over-reduction of the electron transfer chain. This over-reduction caused the reduction of the primary electron acceptor quinone in the reaction center, resulting in the absence of charge separation on illumination. Under anaerobic conditions, the addition of the auxiliary oxidants caused the oxidation of cytochrome *c*-551. This implied that the redox level of the electron transfer chain was shifted towards a more oxidized state by removal of the reducing equivalents from the chain to the auxiliary oxidants. The extent of cytochrome oxidation and proton release restoration in the presence of auxiliary oxidants was similar to that occurring under aerobic conditions (Figs. 1–3). The redox level of cytochrome *c*-551 in the presence of KNO_2 (Fig. 4A) was also similar to that existing under aerobic conditions (not shown, see Ref. 4). These results suggest that the optimal redox level was restored for the photosynthetic electron transfer system by auxiliary oxidants. For proton release, there is another possibility – that $\Delta\mu_H$ across the membrane in the dark varied under different conditions, and hence affected the extent of light-induced proton release.

The fact that antimycin and myxothiazol enhanced the oxidation of cytochrome *c*-551 by oxidants under anaerobic conditions suggests that the cytochrome *b*-c_1 complex as well as cytochrome *c*-551 was involved in the chain. The restoration of flash-induced oxidation of cytochromes and of proton-release in the presence of auxiliary oxidants led us to conclude that the photosynthetic electron transfer chain partly shared the auxiliary oxidant-supported electron transfer chain in this bacterium. A proposed scheme of the electron transfer system of *Erythrobacter* OCh 114 is shown in Fig. 7.

A few recent reports show that neither antimycin nor myxothiazol inhibit nitrate reduction or nitrate-induced cytochrome *b* oxidation in *Rb. sphaeroides or Rb. capsulatus*, which is indicative of absence of involvement of the cytochrome *b*-c_1 complex [17–19]. By contrast, in *Erythrobacter* OCh 114 cells, antimycin and myxothiazol enhanced cytochrome oxidation induced by nitrate and nitrite (Fig. 5). The presence of the cytochrome *b*-c_1 complex was shown in this bacterium (Fig. 6). This suggests that the cytochrome *b*-c_1 complex was involved in the nitrate- and nitrite-induced electron transfer. The absence of the reduction of *b*-type cytochromes by antimycin may be peculiar to this bacterium. The involvement of the cytochrome *b*-c_1 complex was indicated in the case of nitrite reduction in *Rb. sphaeroides* f. sp. *denitrificans* [20]. Thus, involvement of the cyto-

chrome b-c_1 complex in the auxiliary oxidant-induced respiration may depend on both the bacterial and the oxidant species used.

Inhibitory effect of KCN on th restoration of optimal redox state

KCN is known to be an inhibitor of both nitrate and nitrite reductase in *Rb. sphaeroides* f. sp. *denitrificans* [21,22]. In the present study, nitrite restored neither the flash-oxidation of cytochrome *c*-551 nor the light-induced proton release in the presence of KCN, suggesting that KCN was an inhibitor of nitrite reductase. In the presence of KCN, neither nitrate nor chlorate restored these reactions under anaerobic condition (Fig. 1). Furthermore, KCN inhibited nitrate- and chlorate-induced oxidation of cytochrome *c*-551 under anaerobic condition (Fig. 4B). These results indicate that KCN inhibits electron transfer between cytochrome *c*-551 and these auxiliary oxidants.

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