Membrane-bound respiratory system of *Enterobacter cloacae* strain HO1 grown anaerobically with chromate

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1. SUMMARY

Chromate has been demonstrated to be an electron acceptor in the respiratory chain of anaerobically grown *Enterobacter cloacae* strain HO1. By using potentiometric titration and low temperature spectroscopy, the spectral absorption of the anaerobic membrane vesicles of this organism in the presence of chromate was resolved into six peaks at 548, 549, 550, 555, 556, and 558 nm in the α band of the reduced minus oxidized spectra. The spectra elicited by chromate are attributed to cytochromes c₅₄₈, c₅₄₉, c₅₅₀, b₅₅₅, b₅₅₆, and b₅₅₈. Among these cytochromes, c₅₄₈ was found to be specifically involved in electron transfer to chromate.

2. INTRODUCTION

Chromate is one of the toxic heavy metal ions that are common pollutants of the environment. Several reports have been made of the isolation of *Pseudomonas* strains that, under anaerobic conditions, reduced chromate to the less toxic trivalent form [1–4]. Little is known about the biochemical mechanisms of anaerobic reduction of chromate. We have recently isolated from activated sludge a chromate-reducing bacterium *Enterobacter cloacae* strain HO1 which can reduce chromate under anaerobic conditions [5]. Chromate reductase activity of *E. cloacae* HO1 was observed to be membrane-bound and could be enhanced by adding an electron donor [6]. In this paper, we report that chromate acts as an electron acceptor in the anaerobic respiratory system and that cytochrome c₅₄₈ is specifically involved in electron transfer to chromate.

3. MATERIALS AND METHODS

3.1. Organism and growth conditions

*Enterobacter cloacae* HO1 was isolated and grown anaerobically in KSC medium as described previously [5]. For anaerobic conditions slow stirring, sufficient to prevent sedimentation of cells, was used and anoxic conditions were obtained by flushing the culture with N₂ gas. Cells were
harvested in the late-exponential phase of growth when $A_{660}$ was about 0.2.

3.2. Preparation of membrane vesicles and protein determinations

Membrane vesicles were prepared from freshly grown cells as described by Bilous et al. [7]. Protein was estimated spectrophotometrically using Coomassie brilliant blue R-250 solution as described by Bradford et al. [8].

3.3. Room temperature split beam difference spectroscopy

Membrane samples from anaerobically grown cells were divided into two cuvettes. Both reference and sample cuvettes were reduced with NADH (0.2 mM), and the reference cuvette was then oxidized by adding potassium chromate (0.5 mM) or O$_2$ gas. Aerobiosis and anaerobiosis were maintained by slowly flushing the cuvettes with O$_2$ and N$_2$ gas, respectively. The NADH-reduced minus NADH-reduced spectrum was scanned after slowly bubbling N$_2$ gas for 10 min, and was stored as baseline. NADH-reduced minus chromate-oxidized (or oxygen-oxidized) difference spectra were then recorded at time intervals on a Shimazu UV3000 spectrophotometer.

3.4. Low temperature difference spectroscopy

Membrane vesicles samples (1.0 ml) from anaerobically grown cells were added to cuvettes with a gas-tight syringe fitted with a rubber stopper to maintain the anaerobic state. The cuvettes were then frozen in liquid nitrogen (77 K) and spectra were recorded in the dual wavelength mode of the Shimazu UV3000 spectrophotometer.

3.5. Potentiometric titration

Potentiometric titration was performed anaerobically by the method described by Dutton [9]. Redox mediators used at final concentration of 40 $\mu$M were quinhydrone (midpoint potential $E_m =$ 280 mV), tetramethyl-p-phenylenediamine dihydrochloride (TMPD; $E_m =$ 260 mV), dichloroindophenol ($E_m =$ 217 mV), 1,2-naphthoquinone ($E_m =$ 143 mV), phenazine methosulfate (PMS; $E_m =$ $-145$ mV), 60 $\mu$M of potassium ferricyanide ($E_m =$ 430 mV) was required to reach a potential above 300 mV. Low temperature spectra were scanned from $-230$ mV to $+380$ mV for anaerobically grown cells.

3.6. Data analysis

Difference spectra through all selected potential ranges were obtained by subtracting the selected spectrum from a stored determined oxidized spectrum.

4. RESULTS AND DISCUSSION

4.1. Changes of spectral absorption of cytochromes elicited by chromate or oxygen in the membrane vesicles of anaerobically grown cells

Enterobacter cloacae was found to be chromate-resistant in aerobic and anaerobic conditions [5]. Oxygen was reported to inhibit chromate reduction soon after being added to the anaerobic cell culture in the presence of chromate, and chromate reduction ability could be recovered after the oxygen was completely exhausted in the culture.

![Fig. 1. Time course of spectral absorption changes in NADH-reduced minus oxygen-oxidized difference spectra for membrane vesicles of anaerobically grown cells. Reference side: membrane vesicles + NADH (0.2 mM) + O$_2$; sample side: membrane vesicles + NADH (0.2 mM) (protein, 2.3 mg/ml).](https://academic.oup.com/femsle/article-abstract/78/1/11/585888)
Fig. 2. Time course of spectral absorption changes in NADH-reduced minus chromate-oxidized difference spectra for membrane vesicles of anaerobically grown cells after the addition of chromate. Reference side: membrane vesicles + NADH (0.2 mM) + chromate (0.5 mM); sample side: membrane vesicles + NADH (0.2 mM) (protein, 2.3 mg/ml).

We therefore used the oxygen-elicited respiratory system as a basal system to compare with the chromate-reduction system. As shown in Fig. 1, oxygen elicited changes of spectral absorption with peaks around 550 nm (α band) and 520 nm (β band) and shoulders around 560 nm (α′ band) and 530 nm (β′ band) which are attributed to cytochrome c₅₅₀ and b₅₆₀, respectively. These two cytochromes were also observed in chromate-elicited spectra (Fig. 2), but a more rapid absorption change and a higher ratio of c- to b-type cytochromes was elicited by chromate as compared to oxygen (Figs. 1 and 2). These data indicate that both chromate and oxygen appeared to behave as electron acceptors in the respiratory system of *E. cloacae* anaerobically grown cells. Similarly, both nitrate and oxygen serve as electron acceptors in the respiratory system of anaerobically grown *E. coli* [11]. The high ratio of c- to b-type cytochromes in Fig. 2 may indicate that c-type cytochrome could play an important role during chromate reduction. It is worthwhile to note that cytochrome d₆₃₀ was also observed in the oxygen-elicited spectrum (data not shown) but could not be detected in the chromate-elicited one. We therefore examined more precisely only cytochromes b and c.

4.2. Potentiometric titration of membrane cytochromes of anaerobically grown cell

To determine which b- and c-type cytochromes participate in chromate reduction, low temperature potentiometric titration and subsequent data analysis were used to assay the cytochromes in various redox potential ranges. Peak characteristic of both b- and c-type cytochromes appeared from electro-positive potential (+317 mV) to electro-negative potential (−230 mV) (Fig. 3). Significant changes of spectral absorption were obtained by subjecting the redox potential ranges of (−230 to −130 mV), (−130 to +166 mV), (+166 to +260 mV) and (+260 to +380 mV) to data analysis against the selected baseline −130, +166, +260 and +380 mV, respectively (Fig. 4) which are
attributed to c_{550}, b_{558}, (Fig. 4, curve A), b_{555} (Fig. 4, curve B), c_{548} (Fig. 4, curve C), and c_{549} and b_{556} (Fig. 4, curve D), respectively. Low temperature potentiometric titration on the membrane vesicles of aerobically grown cells (i.e. in the presence of oxygen) showed that cytochrome c_{550}, b_{558}, b_{555}, c_{549} and b_{556}, but not c_{548}, also appeared from electropositive potential (+190 mV) to electronegative potential (−210 mV), and c_{550}, b_{558} and b_{555}, were within the same redox potential range as those obtained from anaerobically grown cells (data not shown). These results correspond with the result shown in Fig. 2, suggesting that c_{548} might be involved in the chromate reduction but not oxygen reduction, and might play a role in dividing the electron transfer between chromate- and oxygenrespiratory system.

4.3. Low temperature spectra of cytochrome in membrane vesicles of anaerobically grown cells oxidized by chromate or oxygen at various redox potentials

To confirm that c_{548} is responsible for the electron transfer division between chromate and...
oxygen respiratory systems, we recorded spectral changes oxidized by chromate and oxygen at 80 mV and 280 mV of redox potentials which correspond to the upstream and downstream of electron transfer chain through cytochrome c_{548}. The chromate-oxidized minus oxygen-oxidized spectra were subsequently subjected to data analysis. Figs. 5 and 6 show that both cytochromes c_{549} and b_{556} were observed in chromate- and oxygen-oxidized spectra at 80 mV. The peaks at 549 and 556 nm changed markedly when oxidation reached completion (110 min and 70 min for chromate and oxygen-oxidized spectra, respectively). Similar results were also obtained at 280 mV (data not shown). These are consistent with the results in Fig. 4, curve D showing that b_{549} and c_{556} appeared at the oxidized state, i.e. high electropositive potentials. However, the subsequent data analysis revealed a significant peak only at 548 nm at redox potential of 80 mV (Fig. 7a) and a small peak at the same wavelength at 280 mV (Fig. 7b) which means that c_{548} might be a component responsible for the electron transfer division between chromate and oxygen respiratory system though c_{549} and b_{556} might be located toward the more oxidized end of the chromate respiratory system. This is also consistent with Fig. 4, curves C and D.

Based on the above results, we suggest that chromate acts as an electron acceptor in the anaerobic respiratory system of E. cloacae HO1 strain and cytochrome c_{548} is a component specifically involved in chromate reduction.

REFERENCES