Mediated Electrocatalytic Reduction of Nitrate and Nitrite Based on the Denitrifying Activity of *Paracoccus denitrificans*

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(Received August 14, 1996)

A carbon electrode modified with immobilized cells of *Paracoccus denitrificans* has been found to produce catalytic currents for the reduction of nitrate and nitrite in the presence of durohydroquinone. It has been shown that durohydroquinone functions as an electron transfer mediator between the electrode and the bacterial cells to drive electrochemically the denitrifying reaction of *Paracoccus denitrificans*.

There are a variety of bacterial species that contain particular enzyme systems in the cytoplasmic membranes to oxidize their own substrates using oxygen as a final electron acceptor.[1] It has been shown[2,3] that when the whole cells of such bacteria are immobilized on electrodes the electrodes produce catalytic anodic currents for the oxidation of the substrates in the presence of appropriate redox molecules. The redox molecules function as an electron acceptor in place of oxygen in the enzymatic reactions of the bacteria and serve as mediators allowing the flow of electrons from the bacterial cells to the electrodes. We have observed[4,5] that some bacterial species function as efficient biocatalysts to produce large anodic currents on the time scale of cyclic voltammetry. In view that electrode reactions can be driven in both oxidative and reductive directions by controlling the potential applied to the electrode, we have interested in achieving reductive electrocatalytic reactions using bacterial cells as biocatalysts.[3] As far as we know, only one example of the reductive catalytic current has been reported for the reduction of oxygen using *Thiobacillus ferrooxidans*(ATCC23270) as a biocatalyst.[6] We demonstrate here reductive catalytic currents for the reductions of nitrate and nitrite using whole cells of *Paracoccus denitrificans*. *P. denitrificans* cultivated under anaerobic conditions has an enzyme system for oxidizing lactate by the use of nitrate or nitrite as an electron accepter.[1] Both nitrate and nitrite are reduced in this reaction to nitrogen gas, which is known as denitrifying action of the bacterium.[1] We use durohydroquinone (DQH_2) as an electron donor in place of lactate to drive the denitrifying reaction electrochemically.

P. denitrificans (IFO 12442) was grown to early stationary phase in a 201 medium as recommended by IFO with a slight modification in that 0.2% w/w potassium nitrate was added in the cultivating solution. The cell paste of the bacterium was suspended in a 10 mM phosphate buffer solution (pH7.3). The cell density of the suspension was estimated by means of a calibration curve of dry cell weight from the absorbance at 610nm; the unit optical density was equivalent to 1.25 mg dry cell weight cm^{-3}. To prepare a whole cell-modified electrode, 5 μl of the cell suspension which contained 94 μg in dry cell weight was loaded on a graphite electrode (BAS Inc., No.11-2408, 3 mm i.d.), and the electrode surface was covered with a dialysis membrane (20 μm thick in the dry state) after the solvent had been allowed to evaporate. The *P. denitrificans*-modified graphite electrode with a dialysis membrane is referred to as a *P. denitrificans*-GE in this paper. Electrochemical measurements were performed at 25 °C in a base solution of 10 mM phosphate buffer deaerated with nitrogen gas unless stated otherwise. The solution was stirred at 500 rev. min^{-1}. A platinum disk and an Ag/AgCl/saturated KCl electrode were used as the counter-electrode and the reference electrode, respectively. All potentials in this paper are referred to the Ag/AgCl/saturated KCl electrode.

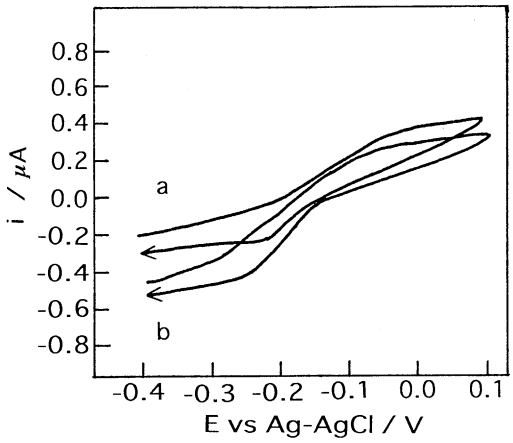

Figure 1. Cyclic voltammograms of 0.1 mM DQH_2 in (a) pH 7.3 phosphate buffer and (b) the buffer containing 80 μM KNO_3. Scan rate: 1mVs^{-1}.

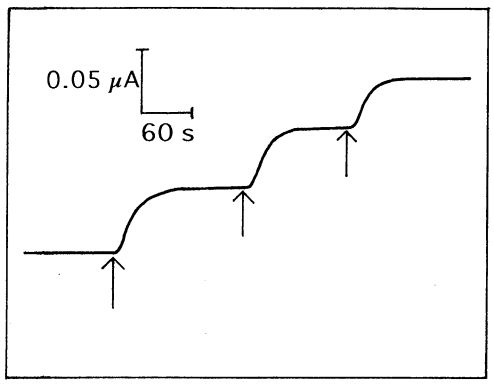

Figure 2. Current-time curve recorded at -0.4 V in the phosphate buffer containing 0.1 mM DQH_2. KNO_3 was added to the solution at 10 μM each at the points denoted by the arrows.

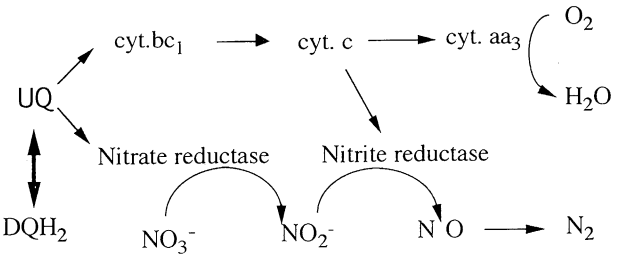

Figure 3. Schematic diagram of respiratory chain related to denitrification in *P. denitrificans*. Adapted after Figure 1 in Ref.9. UQ: ubiquinone, cyt: cytochrome.

Figure 1 shows cyclic voltammograms recorded with the *P. denitrificans*-GE in (a) 0.1 mM DQH_2, and (b) (a) plus 80 μM KNO_3 in the potential range between -0.4 and 0.1V. As seen in figure 1a, DQH_2 produces anodic and cathodic currents starting from -0.2V and -0.15V, respectively, which are attributable to the electrochemical reaction of the redox couple of DQH_2 and duroquinone (DQ). The voltammogram is not peak-shaped but more like sigmoidal, suggesting that the anodic and cathodic limiting currents are mainly controlled by the permeation process of DQH_2 through the immobilized cell-layer and/or the dialysis membrane-layer on the electrode, that is, that the concentration polarization of DQH_2 in the solution is small under the present experimental conditions. The cathodic current is increased in the magnitude by the addition of KNO_3 to the solution and the anodic current is reduced (Figure 1b). In the basal solution not containing DQH_2, the *P. denitrificans*-GE did not produce any observable wave, and the addition of KNO_3 to the solution had no effect on the voltammogram (data not shown). Figure 2 shows the current-time curve recorded at a fixed potential of -0.4V with the *P. denitrificans*-GE in the solution containing 0.1 mM DQH_2. The current starts to increase after successive additions of KNO_3 at 10 μM each, and attains a steady state in about 80 s after each addition of KNO_3. The steady-state current increased with increasing concentrations of nitrate to approach saturation at 80 μM. The magnitude of the steady-state current also depended upon the concentration of DQH_2; the current at 0.1 mM KNO_3 increased with increasing concentrations of DQH_2 from 0.05 to 0.4 mM, at which the current attained a saturation value. These saturation behavior suggests that the current is due to the catalytic action of nitrate reductase in the bacterial membrane as mentioned below, that is, that nitrate is electrocatalytically reduced at the *P. denitrificans*-GE by the mediation of DQH_2.

When KNO_2 was added in place of KNO_3, a current-time curve similar to that in Figure 2 was obtained though the current magnitude at a given concentration of KNO_2 was smaller than the current obtained at the same concentration of KNO_3. When the measurements were carried out under air-saturated conditions, the catalytic currents for the reduction of both nitrate and nitrite were greatly reduced. We have tested hexacyanoferrate(II), $Fe(CN)_6^{4-}$, as a mediator in place of DQH_2 and found that $Fe(CN)_6^{4-}$ works as a mediator for the electrocatalytic reduction of nitrite, but not for the reduction of nitrate.

It has been reported[7,8] that DQH_2 works as a substitute for ubiquinone in the respiratory chain of *P. denitrificans* to donate electrons to the nitrate reductase and cytochrome bc_1. As illustrated in Figure 3, the former catalyzes the reduction of nitrate, and the latter donates electrons to nitrite reductase via cytochrome c to reduce nitrite, yielding nitrogen gas as a final product. On the basis of this scheme, we may explain above results at the *P. denitrificans*-GE. Nitrate and nitrite are reduced by the *P. denitrificans*-catalyzed reactions using DQH_2 as an electron donor, and DQH_2 is continuously generated by the electrochemical reaction at the electrode to produce the catalytic cathodic currents. DQH_2 may be considered to permeate rather rapidly through the bacterial cell membrane to reach the reacting sites in the membrane, which allows us to observe the cataytic currents on the time scale of cyclic voltammetry. The decrease in the current magnitude under air-saturated conditions is understandable by that the pathway using oxygen as an electron acceptor becomes competitive with the denitrifying pathways under aerobic conditions. Considering that the redox potential of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple is much more positive than that of DQ/DQH_2, we may postulate that $Fe(CN)_6^{4-}$ can donate electrons to nitrite reductase, but not to nitrate reductase. Thus, it works as a mediator only for the electrocatalytic reduction of nitrite.

The electrochemical system of using *P. denitrificans* as a biocatalyst is expected to provide a means of monitoring nitrate and/or nitrite at such low concentrations as 10-100 μM.

This work was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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