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Bioelectrochemistry 69 (2006) 74 – 81
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Escherichia coli-catalyzed bioelectrochemical oxidation of acetate in the presence of mediators

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Received 30 August 2005; received in revised form 1 November 2005; accepted 10 November 2005
Available online 20 January 2006

Abstract

Bioelectrocatalytic oxidation of acetate was investigated under anaerobic conditions by using *Escherichia coli* K-12 (IFO 3301) cells cultured on aerobic media containing poly-peptone, glucose or acetate as the sole carbon source. It was found that all *E. coli* cells cultured on the three media work as good catalysts of the electrochemical oxidation of acetate as well as glucose with $Fe(CN)_6^{3-}$, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), 2,6-dichloro-indophenol, or 2-methyl-1,4-naphthoquinone as artificial electron acceptors (mediators). Acetate-grown *E. coli* cells exhibited the highest relative activity of the acetate oxidation against the glucose oxidation. On the other hand, all the artificial electron acceptors used work as inhibitors for the catalytic oxidation of acetate at increased concentrations. The inhibition phenomenon can be interpreted in terms of competitive substrate inhibition as a whole. Apparent values of Michaelis constant, catalytic constant, and inhibition constant were evaluated by amperometric methods. Q_0 is an effective artificial mediator as evidenced by a large reaction rate constant between the cell and Q_0 at least at low concentrations (<50 μM). However, $Fe(CN)_6^{3-}$ is a promising mediator in biosensor applications because the inhibition constant is very large and it works as an electron acceptor even under aerobic conditions.

Keywords: *Escherichia coli*; Acetate oxidation; Mediated bioelectrocatalysis; Inhibition

1. Introduction

The substrate-level phosphorylation during the degradation of carbohydrates (in the glycolysis pathway), some amino acids, and several organic acids is the major process to gain ATP in bacteria under anaerobic conditions [1], and acetate is one of the typical products in these reactions. Thus, acetate exists widely in environment.

The standard Gibbs free energy change ($\Delta G^{\circ\prime}$) of the acetate oxidation to CO_2 is -893.73 kJ mol^{-1} [1]. As compared with that of glucose ($\Delta G^{\circ\prime} = -2872.2$ kJ mol^{-1}) [1], waste of acetate leads to waste of chemical energy and environmental pollution. With these issues in mind, microbial fuel cells to convert the chemical energy of acetate to the electric energy were proposed using anaerobic microbes such as *Geobacter sulfurreducens* [2] or the one from activated sludge [3], where direct electron transfer is proposed to occur from the microbes to anode without artificial mediators. Such non-mediated systems are simple and convenient for constructing biofuel cells especially when microbes are used as catalysts. The maximum current density in non-mediated systems may be calculated as in the order of 1 mA cm^{-1} at planner electrodes based on the theory of non-mediated bioelectrocatalysis with multilayer catalysis [4]. In contrast, the current density of mediated systems increase with the concentration of mediators (linear dependence at low concentrations and square root dependence at high concentrations [5]), and may be calculated as in the order of 10 mA cm^{-1} at planner electrodes by considering practical immobilization methods. In addition, the mass transfer of mediators through the outer membranes of microbes is sufficiently fast [6]. Therefore, it seems to be important to characterize mediated bioelectrocatalysis of acetate with microbes as catalysts as well as non-mediated systems. Such microbe-based mediated bioelectrocatalysis of acetate may also open routes to construct acetate biosensors, since oxidoreductase of acetate has not been found.

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doi:10.1016/j.bioelechem.2005.11.003

However, to our best knowledge, there is no report on mediated bioelectrocatalysis of acetate using microorganisms.

Escherichia coli can use acetate as well as glucose as the carbon and energy source under aerobic conditions [7–12]. As shown in Scheme 1, acetate is converted to acetyl-CoA by consumption of ATP and CoA with the aid of phosphotransacetylase, acetate kinase, and acetyl-CoA synthase [9]. Acetyl-CoA is metabolized in the TCA cycle and is oxidized to CO_2 to generate 3 moles of NADH and 1 mole of $FADH_2$ in succinate dehydrogenase (total 8 electrons per molecule) as well as GTP [9–11].

$$\text{acetyl} - \text{CoA} + 3\text{NAD}^+ + \text{FAD} + \text{GDP} + \text{Pi} \rightarrow 2\text{CO}_2 + 3\text{NADH} + \text{FADH}_2 + \text{GTP} + 2\text{H}^+ + \text{CoA}. \quad (1)$$

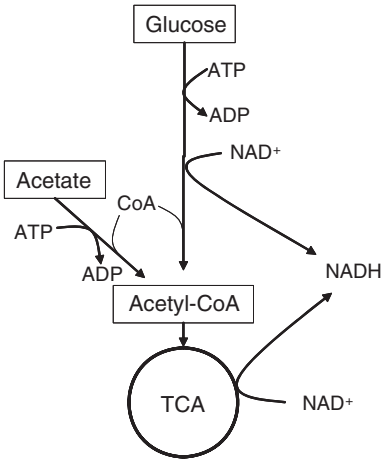
In contrast, glucose is metabolized in the glycolysis pathway to acetyl-CoA to generate NADH and ATP.

$$\text{glucose} + 4\text{NAD}^+ + 2\text{ADP} + 2\text{Pi} + \text{CoA} \rightarrow 2\text{acetyl} - \text{CoA} + 4\text{NADH} + 2\text{ATP} + 2\text{CO}_2. \quad (2)$$

Since acetyl-CoA generated from glucose is metabolized in the TCA cycle as in the case of acetate-derived acetyl-CoA, 10 moles of NADH and 2 moles of $FADH_2$ (total 24 electrons per molecule) are produced in glucose oxidation to CO_2.

E. coli is frequently utilized as a catalyst for the electrochemical oxidation of glucose to construct biosensors and microbial fuel cells with suitable mediators [13,14]. If parts of the oxidation current of glucose are ascribed to the reducing equivalent generated in the TCA cycle, it can be expected that *E. coli* must be utilized as a catalyst for mediated electrochemical oxidation of acetate.

In this work, we attempted to evaluate the catalytic activity of *E. coli* cells in mediated bioelectrocatalysis of acetate. The effects of cultivation medium on the expression of the acetate oxidation activity were examined, and the overall kinetic parameters were evaluated for several artificial electron acceptors. This work also revealed the inhibition of the mediated bioelectrocatalytic oxidation of acetate by artificial electron acceptors at increased concentrations.



Scheme 1. Schematic picture of the central pathway of glucose and acetate degradation in *E. coli*.

2. Materials and methods

2.1. Cultivation of E. coli cells

E. coli K-12 (IFO 3301) cells were cultured on poly-peptone, acetate, and glucose media. Poly-peptone medium (2%) was prepared by dissolving 20 g poly-peptone in 1 L of distilled water. The buffer system used for acetate and glucose media is composed of 15.15 g of $NaHPO_4 \cdot 12H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.25 g of $MgSO_4 \cdot 7H_2O$, and 0.013 g of $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water, pH being adjusted to 7.0 with HCl. For acetate-defined medium (0.25%), 5.67 g of $CH_3COONa \cdot 3H_2O$ was added to the buffer system, while glucose medium (0.75%) contained 7.5 g of glucose in the buffer system. After the media were autoclaved or filter-sterilized, 1 mL each of filter-sterilized vitamin mixture (2 mg of biotin, 2 mg of folic acid, 10 mg of pyridoxine–HCl, 5 mg of thiamine–$HCl \cdot 2H_2O$, 5 mg of riboflavin, 5 mg of nicotinic acid, 5 mg of D-Ca-pantothenate, 0.1 mg of vitamin B_{12}, 5 mg of *p*-aminobenzoic acid, 5 mg of lipoic acid in 1 L of distilled water) and trace metal solution (0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.18 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.18 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of H_3BO_3, 0.01 g of $Na_2MoO_4 \cdot 2H_2O$, 0.025 g of $NiCl_2 \cdot 6H_2O$ in 1 L of distilled water) was added to 100 mL of the media. *E coli* cells were incubated at 37 °C under shaken and aerobic conditions. When the optical density (OD) reached 3–4 at 600 nm, the *E. coli* cells were harvested by centrifugation and washed twice with distilled water. The cell paste was stocked at −30 °C. Before experiments, the cell paste was suspended in 0.85% NaCl solution. The cell suspension was kept at 4 °C and used within a few days. The cell densities of the suspension were estimated using a hemacytometer and by OD measurements at 600 nm. The suspension with OD = 1.0 contained 7.22×10^7 cells per cm^3. In this work, the cell density will be expressed in the dimension of molar concentration by dividing the cell number by Avogadro number, for convenience in the expression of the catalytic constants of *E. coli* cells [15].

2.2. Chemical reagents

Potassium ferricyanide ($K_3Fe(CN)_6$), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), sodium 2,6-dichloroindophenol (NaDCIP), and 2-methyl-1,4-naphthoquinone (VK_3) were used as mediators (artificial electron acceptors) without further purification. $K_3Fe(CN)_6$ and NaDCIP were dissolved in distilled water, while Q_0 and VK_3 were in dimethyl sulfoxide (DMSO). Dioxygen dissolved in the stock solutions of mediators and substrates was removed by passing argon gas before experiments. All other chemicals used were of reagent grade.

2.3. Electrochemical measurements

Amperometric and voltammetric measurements were carried out in a one-compartment electrolysis cell containing a phosphate buffer (0.1 M, pH 7 and ionic strength = 0.3) at 25 °C on a BAS CV50W voltammetric analyzer (USA).

Glassy carbon (GC) electrodes with 3 mm of a diameter, Ag|AgCl|sat. KCl electrode and Pt wire were used as the working, reference and counter electrodes, respectively. The electrode potential in this work will be referred to the Ag|AgCl|sat. KCl reference electrode. The GC electrodes were covered, if necessary, with a dialysis membrane for constant-potential amperometry and used under stirring, as described in the literature [15].

The catalytic activity of the *E. coli* cells for the oxidation of substrates (acetate or glucose) was evaluated from the initial rate of the decrease in the concentration of an electron acceptor or of the increase in the concentration of its reduced form generated by biocatalytic reactions. The current values were converted to the concentration of the artificial electron acceptor by using calibration curves obtained in separated experiments for the oxidized form of the mediators as described in the literature [15].

2.4. Bulk electrolysis

Bulk electrolysis was carried out in order to know the recovery of the electron in the acetate oxidation with an artificial mediator as the final electron acceptor. Carbon felt (Toray Co., carbon felt mat, B0050, lot No. 60208B05-016) with surface area of 160 cm^2 cm^{-3} and with a size of 1 cm diameter × 0.1 cm thick was used as a working electrode. The total volume of the electrolysis solution was 0.5 mL. The electrolysis cell arrangement was similar to that one described in the literature [16].

3. Results

3.1. Catalytic activity of E. coli cells for the oxidation of acetate and glucose with an artificial electron acceptor

The first step of the present work was to check the catalytic activity of *E. coli* cells for acetate oxidation with artificial electron acceptors. $Fe(CN)_6^{3-}$ was used as an electron acceptor. Spectrophotometric methods are not convenient to follow such redox reactions in cell suspensions. In contrast, electrochemical methods can be utilized even in turbid samples such as cell suspensions. Therefore, the concentration of $Fe(CN)_6^{4-}$ generated by the catalytic reaction was measured amperometrically at 0.5 V under stirred and anaerobic conditions. *E. coli* cells used in this experiment were grown on the 0.25%-acetate medium. Fig. 1 shows the time course of the amperometric response on the addition of *E. coli* cells, a substrate (acetate or glucose), and $Fe(CN)_6^{3-}$ in this order. When $Fe(CN)_6^{3-}$ was added to the cell suspension at point c, the oxidation current increased linearly with time, as in the case of glucose addition. The linear increase in the oxidation current represents the steady-state reduction of $Fe(CN)_6^{3-}$. The rate of the $Fe(CN)_6^{3-}$ reduction by acetate was 0.433 μM s^{-1} under the conditions, while that by glucose was 0.732 μM s^{-1}. The result clearly indicates that *E. coli* cells work as good catalysts for the oxidation of acetate as well as glucose by $Fe(CN)_6^{3-}$ under anaerobic conditions.

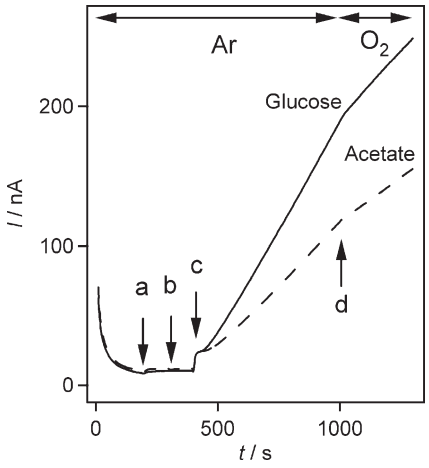


Fig. 1. Amperometric measurements of the acetate and glucose oxidation ability of *E. coli* cells with $Fe(CN)_6^{3-}$ as the artificial electron acceptor in phosphate buffer solution (pH 7.0, 25 °C). The electrochemical cell was in argon atmosphere in the beginning, and (a) suspension of *E. coli* cells cultured on the acetate medium (OD = 1 as the final concentration), (b) glucose or acetate (20 mM), (c) $Fe(CN)_6^{3-}$ (10 mM), and (d) dioxygen were added at the corresponding points indicated by the arrows. The current was measured by the amperometric method under stirring at 0.5 V.

By considering the metabolic pathway in Scheme 1 (and the reaction in Eq. (1)), it is reasonably supposed that the TCA cycle works even under anaerobic conditions in the presence of suitable extracellular electron acceptors, and that $Fe(CN)_6^{3-}$ penetrates in the cell and is reduced by NADH as a whole with the aid of some dehydrogenases such as NADH dehydrogenase and diaphorase [17–19].

$$NADH + 2Fe(CN)_6^{3-} \rightarrow NAD^+ + H^+ + 2Fe(CN)_6^{4-}. \quad (3)$$

$Fe(CN)_6^{3-}$ might also work as an electron acceptor of $FADH_2$ in succinate dehydrogenase.

These reactions would compete with the electron transport system (respiratory chain) under aerobic conditions. In order to verify this point, oxygen gas was introduced into the headspace of the electrolysis cell to make aerobic atmosphere at point d of Fig. 1. Under aerobic conditions, the reduction rate of $Fe(CN)_6^{3-}$ decreased down to 68% and 61% of the anaerobic one, respectively, for acetate and glucose as the substrate. The result supports our hypothesis that the electrons from the substrate are transferred to both dioxygen and $Fe(CN)_6^{3-}$ under aerobic conditions. In the following, the catalytic activity of *E. coli* cells was evaluated under anaerobic conditions.

E. coli cells cultured on glucose or poly-peptone media also exhibited the catalytic activity of the acetate and glucose oxidation. However, the activity depended on the media used for the cultivation (Table 1). The *E. coli* cells grown on the acetate medium exhibited about 8.8 times larger activity of the acetate oxidation than the cells grown on the glucose medium, while the activity of the glucose oxidation was almost equal with each other. The result suggests that acetate induces the acetate-metabolizing enzymes (most probably, the enzymes involved in the conversion of acetate to acetyl-CoA) in *E. coli*. Interestingly, the *E. coli* cells cultivated on the poly-peptone

Table 1
Cultivation medium effects on the rate of *E. coli*-catalyzed reduction of $Fe(CN)_6^{3-}$ (v_M) by acetate and glucose

Medium	Substrate		
	Acetate (20 mM) v_M(A) (μM s^{-1})	Glucose (20 mM) v_M(G) (μM s^{-1})	v_M(A)/v_M(G)
Glucose (0.75%)	0.049	0.746	0.07
Acetate (0.25%)	0.433	0.732	0.59
Poly-peptone (2%)	2.329	5.724	0.41

The rate was determined by the amperometric method at 0.5 V in the presence of 10 mM of $Fe(CN)_6^{3-}$ under anaerobic conditions in the *E. coli* cell suspension of OD 1.0 at pH 7. The relative standard deviations were less than 10% (n=3).

medium exhibited larger catalytic activity toward the acetate and glucose oxidation than those grown on glucose and acetate. However, the acetate-grown *E. coli* cells exhibited the largest relative activity of the acetate oxidation against the glucose oxidation among the cells examined. Therefore, the *E. coli* cells cultured on the acetate medium were used in the following experiments.

3.2. Comparison of artificial electron acceptors in the acetate oxidation

Selection of mediators as well as catalysts is very important to construct mediated bioelectrocatalytic systems. When whole cells are used as catalysts for the substrate oxidations by artificial electron acceptors, the acceptors must penetrate into cytoplasmic membrane as well as outer membrane and are reduced by some redox enzymes in the cells. The reduced form of mediators will diffuse out from the cells and reoxidized at electrodes. Therefore, both of the partition and redox properties will govern the performance of microbe-catalyzing mediated systems [15]. In this work, $Fe(CN)_6^{3-}$, DCIP, VK_3, and Q_0 were used as artificial electron acceptors for the acetate oxidation. The redox potential (formal potential, $E^{\circ\prime}$) of the mediators locate in the potential range of −0.213 V (VK_3) to +0.134 V ($Fe(CN)_6^{3-}$) (see also Table 2). Therefore, the electron transfer from NADH (or $FADH_2$) to the mediators used is down-hill thermodynamically.

The reaction was followed by amperometry at constant potentials for the reduction of the electron acceptors (0 V for $Fe(CN)_6^{3-}$, −0.2 V for DCIP, −0.4 V for Q_0 and VK_3) under anaerobic conditions. Fig. 2 shows the time course of the reductive consumption of the electron acceptors. The initial concentration of the electron acceptors was fixed to 50 μM unless stated otherwise. On the addition of an electron acceptor into the cell suspension containing acetate, the reduction of the electron acceptor started. The relative ratio of the (initial) reduction rate was 82.3 : 21.6 : 11.8 : 1 (1.8 nM s^{-1}) for Q_0 : $Fe(CN)_6^{3-}$: VK_3 : DCIP. Q_0 showed the fastest rate (148.2 nM s^{-1}) among the electron acceptors used, and 50 μM of Q_0 was almost reduced within 500 s. 50 μM of Q_0 added subsequently at point d of Fig. 2 was also reduced almost completely. Details of kinetic parameters will be described later.

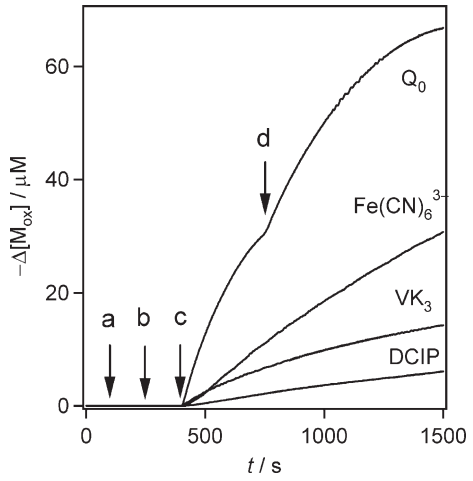


Fig. 2. Time course of the reductive consumption of artificial electron acceptors ($-[M_{ox}]$) by acetate using *E. coli* cells as the catalysts. (a) *E. coli* cell suspension (OD=1), (b) acetate (20 mM), (c) electron acceptor (50 μM), and (d) Q_0 (50 μM) were added at the corresponding points indicated by the arrows. The electron acceptors used are indicated on the corresponding curves. The change in the concentration was detected by the amperometric method under anaerobic conditions.

3.3. Mediated bioelectrocatalytic oxidation of acetate by E. coli cells with Q_0

One of suitable experiments to confirm the mediated bioelectrocatalysis would be cyclic voltammetry. Q_0 gave a quasi-reversible cyclic voltammogram at a bare GC electrode in the absence of acetate (curve a in Panel A of Fig. 3). Curve c is the voltammogram recorded just after the addition of Q_0 to the suspension containing *E. coli* cells and acetate. The increase in the anodic wave represents one of the typical catalytic phenomena. At potentials more positive than the peak potential of Q_0, the reduced Q_0 generated by the *E. coli*–acetate system is re-oxidized at the electrode surface, and then the anodic current increases. This is the first observation of the mediated bioelectrocatalytic oxidation of acetate with *E. coli* cells as the catalysts. Similar catalytic behavior was observed in cyclic voltammetry when glucose was used as the substrate (panel B, curves a and c).

During the mediated bioelectrocatalytic experiments of acetate, it was found that the intensity of the catalytic current depended on the order of the addition of acetate and Q_0. When

Table 2
Kinetic parameters of acetate oxidation of *E. coli* with mediators as the electron acceptors

Mediator	$E^{\circ\prime}$*(mV)	K_M (mM)	$n_S k_{cat}$ (s^{-1})	$n_S k_{cat} K_M^{-1}$ (M^{-1} s^{-1})	K_i (mM)
VK_3	−213	0.91	2.82×10^6	3.10×10^9	0.71
Q_0	−38	0.06	1.39×10^7	2.32×10^{11}	0.02
DCIP	−5	0.02	5.14×10^4	2.57×10^9	0.05
$Fe(CN)_6^{3-}$	134	203.56	1.54×10^8	7.57×10^8	0.60

*$E^{\circ\prime}$ means standard redox potential at pH 7 against Ag/AgCl. The relative standard deviations of the kinetics parameters were less than 15% (n=3).

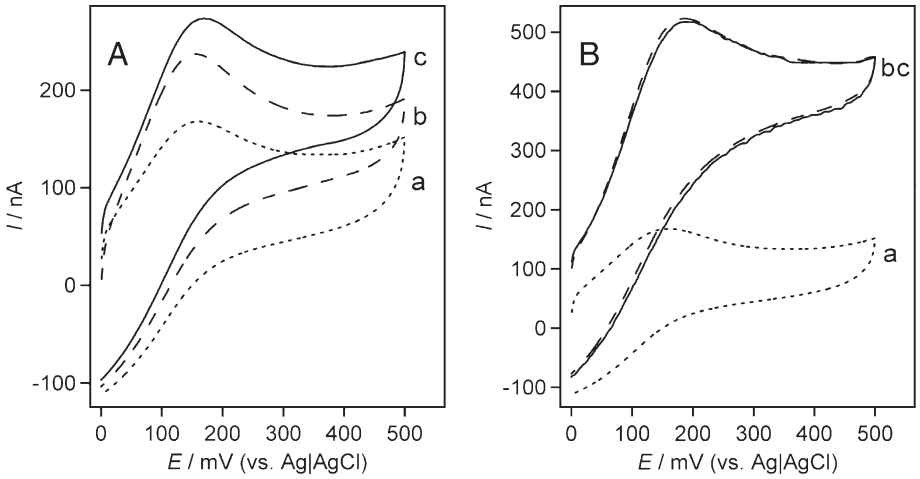


Fig. 3. Cyclic voltammograms of mediated bioelectrochemical oxidation of (A) acetate and (B) glucose. Curves (a) are voltammograms of Q_0 (100 μM) in the *E. coli* cell-containing buffer without substrate. Curves (b) were recorded after addition of the substrate (A: 2 mM acetate, B: 1 mM glucose) in *E. coli* cell suspension (OD = 1) containing Q_0 (100 μM). Curves (c) are voltammograms recorded just after addition of Q_0 (100 μM) in *E. coli* cell suspension (OD = 1) containing the substrate (A: 2 mM acetate, B: 1 mM glucose). The scan rate was 2 mV s^{-1}.

acetate was added last to the cell suspension containing Q_0, the catalytic peak current decreased as shown by curve b in panel A, where the exposure time of the cell with acetate was fixed to be the same as that of curve c. Such phenomena were not observed when glucose was used as the substrate at the concentration of Q_0, as compared with curves b and c in panel B of Fig. 3.

Even in the absence of acetate, Q_0 can oxidize the residual reducing equivalent in the resting cells. During of the oxidation of the reducing equivalent, parts of NADH in the cells would be oxidized to NAD^+. On the other hand, the rate of NADH production is inherently faster in glucose oxidation than in acetate oxidation. Considering these factors, the decrease in the catalytic activity by the oxidative pretreatment of *E.coli* cells with Q_0 seems to be strongly related to the increase in the NAD^+/NADH ratio in the cells. In the following kinetic measurements, the mediator was added last to *E. coli* cell suspension in the presence of acetate.

3.4. Kinetic parameters of the acetate oxidation by E. coli with artificial mediators

For detailed understanding of the catalytic activity of *E. coli* cells for the acetate oxidation, kinetic parameters were examined by amperometric methods. At a given concentration of electron acceptors, the reduction rate increased with the acetate concentration at very low concentrations and reached to the maximum value around 10 mM (data not shown). Therefore, the acetate concentration was fixed to 20 mM in the following experiments. *E. coli* cells (OD = 1), acetate (20 mM), and the electron acceptor were added in this order. The rate of the decrease in the concentration of the artificial electron acceptors was measured amperometrically under stirring.

Fig. 4 shows the dependence of the reduction rate of the electron acceptor (the oxidized form of the mediators) (v_M) on the concentration of the electron acceptor ($[M_{ox}]$). The v_M values increased with $[M_{ox}]$ at low concentrations, and reached the maximum values around 700 μM for VK_3, 40 μM for Q_0, 30 μM for DCIP, and 10 mM for $Fe(CN)_6^{3-}$. After reaching the maximum value, the v_M values decreased with an increase in $[M_{ox}]$. The result clearly shows that the electron acceptors work also as inhibitors at increased concentrations. In addition, these characteristics are very similar to that observed in substrate inhibition in isolated enzyme kinetics [20]. As described above, some dehydrogenases must involve in the reduction of the artificial electron acceptors (see for example Eq. (3)), and the acceptors are the second substrate of the dehydrogenase(s). NADH (and succinate) is the most probable first substrate of the dehydrogenase(s). However, acetate should be the primary electron donor in the overall reaction:

$$\text{Acetate} + (n_S/n_M)M_{ox} \rightarrow \text{product} + (n_S/n_M)M_{red} \quad (4)$$

where M_{ox} and M_{red} are the artificial electron acceptor and its reduced form, respectively, and n_S and n_M are the number of electrons of acetate and M_{ox}, respectively. The overall reaction in Eq. (4) closely resembles the enzyme reactions with two substrates. Therefore, the rate equation for the overall reaction of Eq. (4) with an inhibition by the second substrate (M_{ox} in this case) may be written as follows at a constant concentration of the first substrate (acetate in this case), as in the case of usual two-substrate enzyme models with second substrate inhibition [20],

$$v(=(n_M/n_s)v_M) = \frac{v_{max}}{1 + K_M/[M_{ox}] + [M_{ox}]/K_i} \quad (5)$$

where K_M and K_i are the apparent Michaelis constant and inhibition constant of the electron acceptor, respectively. However the parameters are involved with the distribution property of the mediator between bacterial inner medium and outer solution [15]. v_{max} is the maximum reaction rate in cell suspensions, and is written as:

$$v_{max} = k_{cat}[B] \quad (6)$$

where $[B]$ and k_{cat} are, respectively, the concentration of the bacterial cells in the suspension and the catalytic constant of

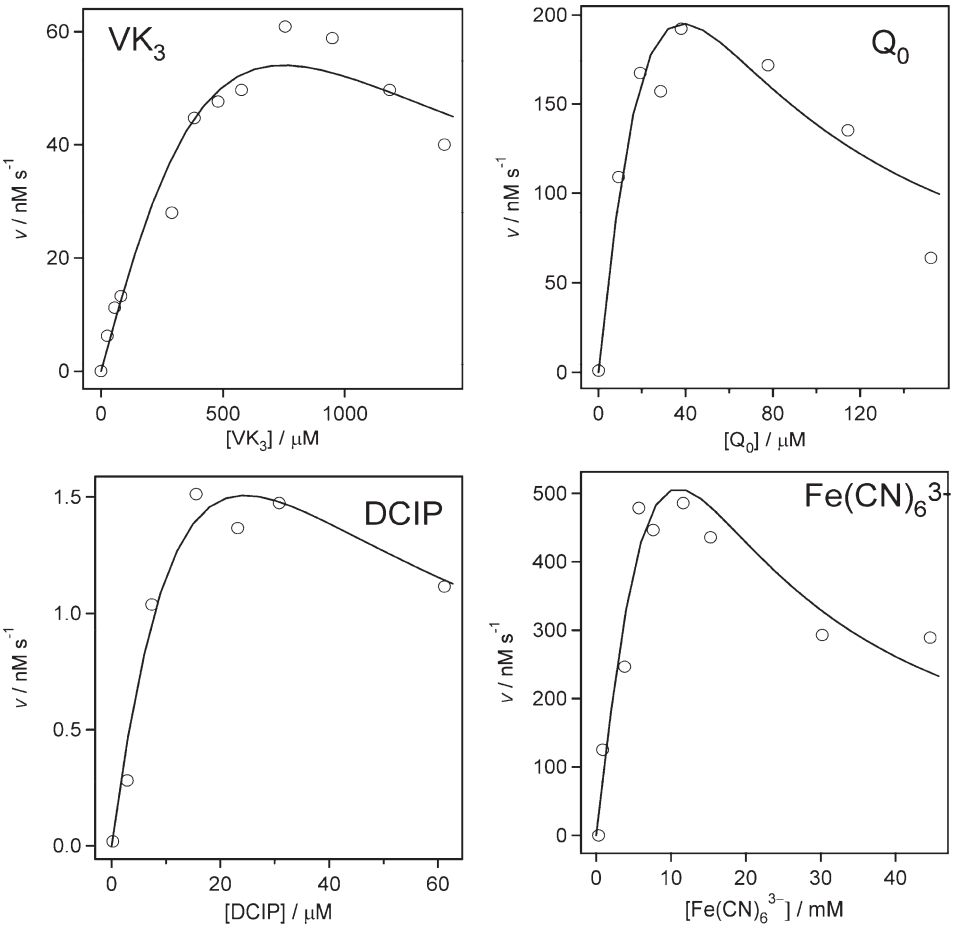


Fig. 4. Electron acceptor concentration dependence of the acceptor reduction rate (v_M) in *E. coli* cell suspension (OD = 1) in the presence of 20 mM acetate. The solid lines are the regression curves analyzed with Eq. (5). The refined parameters are summarized in Table 2.

the bacteria at unit concentration of suspension. In this work, [*B*] was expressed as the molar concentration for convenience [15].

The solid lines in Fig. 4 are the regression curves obtained by a non-linear regression analysis according to Eq. (5) with the parameters of $(n_S/n_M)k_{cat}$, K_M, and K_i. The v_M vs. $[M_{ox}]$ data were fairly reproduced by the model given by Eq. (5). The refined parameters are summarized in Table 2. The n_S value should be 8 when CO_2 is the final product of acetate (see Eq. (1)), but the product $n_S k_{cat}$ values are given in Table 2 because stoichiometric experiments could not be done.

$Fe(CN)_6^{3-}$ gave the largest value of $n_S k_{cat}$ and K_M among the electron acceptors examined. The large $n_S k_{cat}$ value might be due to the large value of the free energy change ($\Delta G^{\circ\prime}$) in the electron transfer from NADH to $Fe(CN)_6^{3-}$ (because of positive value of $E^{\circ\prime}$ of $Fe(CN)_6^{3-}$), while the large K_M value seems to be due to its large negative charge and hydrophilic characteristics, which makes the ion difficult to penetrate into the cell outer membrane composed of lipopolysaccharide and phospholipids. The parameter $n_S k_{cat}/K_M$ in Table 2 represents the electron transfer rate constant from the cell to the electron acceptor. Q_0 gave the largest value of $n_S k_{cat}/K_M$ among the mediators used, and we can conclude that Q_0 is an effective artificial electron acceptor at low concentrations.

On the other hand, most of K_i values evaluated here were in the order of the corresponding K_M values except $Fe(CN)_6^{3-}$, of which the K_i value is much lower than K_M. Therefore, the reaction rate v_M in Fig. 4 did not reach the expected maximum value given by $(n_S/n_M)k_{cat}[B]$. Q_0 showed the strong inhibition effects on the acetate oxidation activity. Judging from the K_M, K_i, and $n_S k_{cat}$ values, the acetate oxidation rate is in the order of $Q_0 > Fe(CN)_6^{3-} > VK_3 > DCIP$ at the electron acceptor concentration of 50 μM. This calculation is in accord with the result given in Fig. 2.

3.5. Bulk electrolysis with the inhibitory and non-inhibitory concentration of VK_3

We performed bulk electrolysis also in order to evaluate the electricity recovery in the mediated acetate oxidation and to reconfirm the hypothesis on the substrate inhibition from the artificial mediators. VK_3 was selected as a mediator first, because it has relatively large K_i and $n_S k_{cat}$ values. The carbon felt was used as the working electrode and the experiments were carried out under argon atmosphere. Curve A in Fig. 5 shows the current–time curve during the mediated bulk electrolysis of acetate at 0.5 mM of VK_3, the concentration being slightly lower than the K_i value. The *E. coli* cells and VK_3 were added at

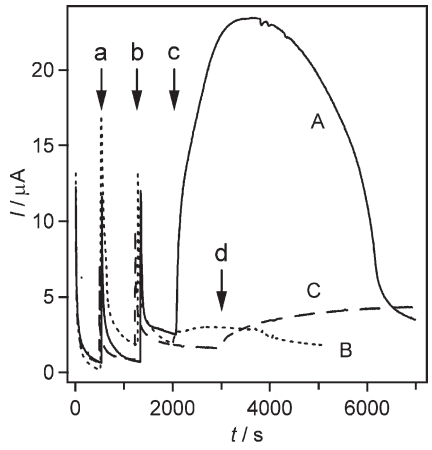


Fig. 5. Current–time curves in bulk electrolysis of mediated oxidation of acetate (A, B) and glucose (C). For curves A and B, (a) *E. coli* cell suspension (OD=8), (b) VK_3 (0.5 mM for A or 1.5 mM for B)), and (c) acetate (1 mM) were added at the corresponding points indicated by the arrows. For curve C, (a) *E. coli* cell suspension (OD=8), (b) VK_3 (0.5 mM), and (d) glucose (1 mM) were added at the points indicated. The bulk electrolysis was performed at 0.5 V under stirring in phosphate buffer solution (pH=7) bubbled continuously with moistured argon gas.

points a and b, respectively. On the addition of acetate (1 mM) at point c, the catalytic oxidation current increased rapidly up to about 23 μA and then gradually decreased with time. The electricity was calculated as 0.0216 C from the area under the current–time curve during the 5000-s electrolysis from point c. The electricity corresponds to 19% of the theoretical electricity for the 8-electron oxidation of the total amount of acetate added.

The electrolysis efficiency increased up to 36% when 10 mM of $Fe(CN)_6^{3-}$ was used as a mediator, while the value decreased down to 6.3% when 40 μM of Q_0 was used. Similar (but somewhat smaller) current–time response was observed by subsequent addition of acetate. Judging from the result and the fact that the K_M value of dioxygen may be in or less than the order of 10^{-5} M [15], these low values of the electrolysis efficiency (or the low recovery of the electricity) seems to be in part due to competitive electron transfer to very low concentrations of dioxygen as contaminant in argon gas introduced continuously in the electrolysis cell. When glucose (0.5 μM) was used as a substrate in place of acetate, the electrolysis efficiency was 15% and 17%, respectively, with VK_3 (0.5 mM) and Q_0 (40 μM) as mediators, by assuming the 24-electron oxidation of glucose.

However, it is supposed that the low recovery of the electricity is also due to the inhibition by the artificial electron acceptors. This hypothesis is based on the dependence of the electricity recovery on the K_i value of the mediator used: the larger the K_i, the larger electricity recovery is. In order to make sure this hypothesis, 1.5 mM of VK_3 was used in the bulk electrolysis of acetate, the concentration being higher than the K_i value. Under the conditions, no obvious catalytic current was observed, as shown by curve B in Fig. 5. The result indicates the almost complete inhibition of mediated oxidation of acetate by VK_3 at increased concentrations.

The inhibition is strongly related to the oxidative treatment of the cells by increased concentrations of mediators or by the pre-electrolysis of the resting cells. Similar phenomena are reported for isolated NADH dehydrogenase, which is inhibited by artificial electron acceptors including $Fe(CN)_6^{3-}$, DCIP, and VK_3 [17]. Therefore, it is likely that the inhibition of the acetate oxidation occurs in the electron transfer reaction from NADH to the mediators catalyzed by NADH dehydrogenase(s). This means that such inhibition is expected even in the mediated bioelectrochemical oxidation of glucose by strong oxidative pretreatment of the cells. Therefore, the mediated electrolysis was carried out for glucose with 0.5 mM VK_3. The result is given by curve C in Fig. 5. Glucose was added at point d after sufficient pre-electrolysis of *E. coli* cells with VK_3. The pre-electrolysis inhibited the mediated glucose oxidation also. The inhibition phenomena are very similar to that observed in mediated acetate oxidation at an increased concentration of VK_3 (curve B in Fig. 5). During the pre-electrolysis, the reducing equivalent (probably NADH) in the cells would be almost completely oxidized to NAD^+. Therefore, it can be reasonably assume that NADH dehydrogenase(s) is susceptible to the inhibition at increased NAD^+/NADH ratios.

4. Discussion

This is the first report on the mediate bioelectrocatalytic oxidation of acetate with *E. coli* cells as catalysts. Acetate will be converted to acetyl-CoA and is oxidized to CO_2 in the TCA cycle to generate 3 moles of NADH and 1 mole of $FADH_2$. Artificial electron acceptors are routinely used to measure enzyme activity of dehydrogenase in cells including those involved in the TCA cycle. Therefore, it is likely that some dehydrogenases such as NADH dehydrogenase and diaphorase catalyze the electron transfer from NADH to mediators penetrated into the cell. Mediators might also work as an electron acceptor of $FADH_2$ in succinate dehydrogenase. However, electrolysis efficiency (=total coulomb/(8 electron×total mol of acetate)) was about 40% at most. It is important to elucidate the detailed pathways of the electron transfer from acetate to mediators.

All *E. coli* cells grown on poly-peptone, glucose, and acetate media expressed the enzyme system for the oxidation of both acetate and glucose. Acetate-grown *E. coli* cells exhibited the highest relative activity of the acetate oxidation against the glucose oxidation. Other researchers also showed the evidence of acetate-induced expression of acetate oxidation-related enzymes in *E. coli* on the basis of the enzyme analysis [12] and global gene expression analysis [11]. Our result on acetate-induced 8.8-fold expression of the acetate oxidation activity is in accord with the report that acetate kinase is up-regulated 9.5-fold higher in acetate medium than that in glucose medium [11]. On the other hand, it is believed that acetate as a by-product in protein degradation can enhance the production of acetate kinase [9,10]. This seems to be in accord with our result that poly-peptone medium is suitable to enrich the enzymes for the acetate oxidation as well as those for the glucose oxidation.

This research also revealed the inhibition phenomena by artificial mediators in mediated electrocatalysis for acetate (and

also glucose) oxidation. The overall kinetics of the *E.coli*-catalyzed acetate oxidation was well interpreted on the model of second substrate inhibition for two-substrate enzyme reactions. In our reaction, acetate is the first substrate and the artificial electron acceptor is the second one. However, the inhibition might occur in NADH dehydrogenase(s) at increased NAD^+/NADH ratios, as mentioned in Section 3.5. There are two possibilities in the inhibition. One is a competitive inhibition by artificial electron acceptor against the first substrate NADH at low NADH concentrations; the other is a product inhibition by NAD^+. The latter case was proposed for isolated NADH dehydrogenase [17], but the details remain to be elucidated in our whole-cell level reactions. Acetate oxidation is more susceptible to the inhibition in mediated bioelectrocatalysis than the glucose oxidation. This would be due to the fact that the rate of NADH production is inherently slower in acetate oxidation than in glucose oxidation. Lowering the concentration of mediators and keeping the acetate concentration at sufficient level can minimize the inhibition effect, although further research is needed to overcome the drawback in utilizing the mediated bioelectrocatalysis of acetate for microbial fuel cells. In sensor application, $Fe(CN)_6^{3-}$ may be utilized as a mediator at low concentrations because the K_i value is rather high. At such low concentrations of $Fe(CN)_6^{3-}$, the signal would be very small because the K_M value is very large. However, the current density is not the important factor in sensor application. The fact that $Fe(CN)_6^{3-}$ works an competitive final electron acceptor against dioxygen under aerobic conditions is also suggestive of further application of the mediated system to acetate sensors.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Science, and Technology of Japan, COE for Microbial-Process Development Pioneering Future Production Systems (COE program of MECSSTJ), and the National Science Council of the Republic of China under Contract No. NSC94-2211-E-006-033.

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