Short Communication

Whole-cell Enzyme Electrodes Based on Mediated Bioelectrocatalysis

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Considerable attention has been given to enzyme electrodes which use electron transfer mediators to couple an enzyme reaction with the electrode reaction. The substrate of the enzyme can be oxidized or reduced at the electrode by an enzymatic reaction that uses the electrode as a final electron acceptor or donor, i.e., by bioelectrocatalysis, resulting in a current whose magnitude depends on the concentration of the substrate. Electrodes relying upon this principle of current response are referred to as mediated amperometric enzyme electrodes and have received much attention as biosensors that can work even under anaerobic conditions.1 We have constructed such biosensors from a variety of dehydrogenases as well as oxidases.2 In this communication, we extend this type of biosensor to that using microorganisms in place of enzymes, in which the oxidoreductases in bacterial membranes are used as catalysts. Although there have been a few reports dealing with the current response for microorganisms in the presence of mediators3,4 these have mainly been directed on phenomena originating from the respiratory activity of microorganisms. We investigated the current originating from the catalytic action of membrane-bound enzymes in bacterial cells.

We used Gluconobacter suboxidans (G.s., IFO 12528), because the properties of the membrane-bound oxidoreductases of this bacterium have been extensively studied.5) G.s. was transferred from a potato extract-agar slant to 25 ml of a culture medium, and cultivation was carried out at 30°C for 24 hr on an orbital shaker.6) Cells were harvested by centrifugation at 10,000 × g for 10 min and washed twice with distilled water. The cell paste was suspended in a 0.1 M acetate buffer (pH 5.0), the suspension having an OD 430nm value of 1.5, and the number of cells was calculated to be 1.2 × 10¹¹ per ml. The bacteria were loaded by pipetting 5 μl volumes on to a gold disk electrode (BAS Co., 2 mm in diameter), and were immobilized by covering with a dialysis membrane (20 μm thick in the dry state) after the solvent had been allowed to evaporate. The dialysis membrane was fixed with a nylon net as described previously.6) Electrochemical measurements were carried out with a three-electrode system in buffer solutions at 25°C. The potential was measured against an Ag/AgCl (saturated KCl) reference electrode.

The gold electrode with immobilized G.s. was immersed in a pH 5.0 acetate buffer solution, and the current at 0.5 V was measured. No current response was obtained with the addition of ethanol. However, when the solution contained 1 mm Fe(CN)₆³⁻, the electrode produced an anodic current with the addition of ethanol as shown in Fig. 1A. The current attained a steady-state after 30 sec, and increased with increasing concentration of ethanol. The dependence of the steady-state current on the concentration of ethanol tended toward saturation, which is typical of enzyme kinetics. The results indicate that alcohol dehydrogenase (ADH) in the cytoplasmic membranes of G.s. had catalytic activity to oxidize ethanol when using Fe(CN)₆³⁻ as an electron acceptor. The Fe(CN)₆³⁻ was reduced by the ADH reaction to produce Fe(CN)₄⁴⁻, which, in turn, was oxidized electrochemically at the electrode to regenerate Fe(CN)₆³⁻, thus serving as a mediator and producing an anodic current. There are a number of factors that can hinder the approach of the substrate and mediator to the active site of the enzyme in a bacterial membrane. The substrate must diffuse into the dialysis membrane and the immobilized bacterial layer to reach the surface of the bacterial cells, and the mediator must shuttle between the electrode and the cells in the layer. Further, both the substrate and mediator must permeate into the multilayer cell walls of the bacteria and, probably, into the cytoplasmic membranes within which ADH is more or less buried.5) Nevertheless, the response time of the electrode (Fig. 1A) was as fast as that of an electrode constructed by using a dialysis membrane and ADH itself (data not shown) and that of substrates of the electrodes prepared in a similar manner with other membrane-bound enzymes.7,8) The result indicates that the response time was mainly controlled by the permeability to the substrate of the dialysis membrane, and that the cell walls and membranes of the bacteria were not dominant barriers. On the other hand, the response of the same G.s. electrode to glucose was rather slow as shown in Fig. 1B; a steady-state current was attained 15 min after the addition of glucose.

Fig. 1. Current (I)-Time (t) Curves for the Oxidation of Substrates at the Electrode with G.s.

The current was measured at 0.5 V in pH 5.0 buffer solutions containing 1 mm Fe(CN)₆³⁻, the solutions being stirred with a magnetic stirrer at 500 rpm. At the times indicated by arrows (A) 0.1 mM ethanol or (B) 0.1 mM glucose was added successively to the buffer solutions.

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to the solution. Glucose dehydrogenase (GDH) of G.s. is located more deeply in the cytoplasmic membranes. This may have been the reason for the sluggish response to glucose; both the mediator and substrate would have to diffuse into the membrane to approach the active site of GDH. This idea is supported by the observation that when the solution contained 0.1% Triton-X100, the current attained a steady state three times faster than that in the absence of the surfactant.

Various factors influencing the magnitude of the current for the oxidation of ethanol were examined. The dependence of the current magnitude on pH was very similar to that obtained with the electrode with immobilized ADH (data not shown) and to the pH dependence of the activity of ADH, with an optimum pH at around 6.0. The current magnitude increased with increasing temperature from 5°C to 45°C; the value at 45°C was almost double that at 5°C. An increase in both the amount of immobilized bacteria and the concentration of the Fe(CN)₆³⁻ mediator caused an increase in the current magnitude. Organic dyes like benzoquinone (BQ) and dichlorophenolindophenol functioned as efficient electron acceptors as well. Ordinary electrodes such as glassy carbon and carbon paste electrodes could be used in place of a gold electrode. When a carbon paste electrode with immobilized G.s. was used in a pH 5.0 buffer solution at 25°C, the electrode showed a linear current response to ethanol from 0.1 mm to 1.0 mm, and the electrode was stable for at least 5 days. Further studies on the stability and the dependence of activity on the immobilized state of the bacteria are being currently pursued.

Gluconobacter suboxydans (G.i., IFO 3260) and Pseudomonas fluorescens FM-1 (P.f.) are bacteria whose membrane-bound oxidoreductases of which have also been studied extensively. D-Fructose dehydrogenase (FDH) and glyceraldehyde dehydrogenase (GLDH) are contained in the membranes of G.i., and D-glucurate dehydrogenase (GADH) in the membranes of P.f. These bacteria were immobilized on electrodes in a similar manner to G.s. The electrode with G.i. produced anodic currents for the oxidation of D-fructose and glyceral, and the electrode with P.f. produced an anodic current for the oxidation of D-glucurate. Pseudomonas aeruginosa (P.s., IFO 3445) and Pseudomonas denitrificans (P.d., IFO 13302), were employed to study the reduction of substrates. These bacteria had nitrite reductase activity when cultured anaerobically in a medium containing nitrate. Both the electrode with P.s. and that with P.d. produced a cathodic current when sodium nitrite was added to the solution, although the current magnitude was small, where hydroquinone (BQH) was used as a mediator. BQH has been reported to be effective as an electron donor for the catalytic reaction of nitrate reductase from P.a. The results show that electrodes employing whole cells of bacteria can be used not only for detecting a variety of substrates, but also for monitoring the activity of membrane-bound enzymes in the intact cells of bacteria.

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References