Recognition of Substrates by Membrane Potential of Immobilized Glucose Oxidase Membranes

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SYNOPSIS

The shifts in membrane potential, caused by the injection of glucose into a permeation cell, were measured using immobilized (entrapped) glucose oxidase membranes. No pH change in the permeation cell was observed upon injection of glucose, but the shift in membrane potential was definitely detected. The shift in membrane potential was observed under nitrogen bubbling (in the absence of oxygen) using initially used enzyme membranes. It was, therefore, suggested that the shifts in membrane potential were not caused by an enzyme-substrate reaction, but by binding of the substrate to the enzyme, which induces a conformational change in the enzyme and leads to a change in charge density in the enzyme membrane. This mechanism is also supported by the fact that the shifts in membrane potential were observed upon injection of not only D-glucose but also L-glucose as reported in our previous study [*J. Chem. Soc. Faraday Trans.*, **87**, 695 (1991)]. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Electrical pulses in living organisms (shifts of membrane potential in living membrane or nerve fibers) are directly generated at internal receptors for perceiving chemical substrates such as hormones or neurotransmitters.[1] Artificial bilayer membranes that do not contain the receptors have also been studied as excitable models of living membranes.[2,3] Aizawa et al.,[4] on the contrary, developed an immunoresponsive membrane for serological tests for syphilis. Membrane potential was monitored for the determination of antibody concentration.

The shifts in membrane potential, caused by the injection of substrates into a permeation cell, were measured using immobilized glucose oxidase membrane from our previous studies.[5,6] No shift in membrane potential was observed with the injection of caffeine and galactose, but there was a shift for D- and L-glucose. Since the enzyme did not react with L-glucose but did react with D-glucose, it was suggested that L-glucose can bind with the enzyme but cannot react with the enzyme and, therefore, gives no product.[5]

The shifts in membrane potential were considered to be generated by a change in the charge density in the enzyme membrane due to the binding of glucose to the enzyme, which induces a conformational change in the enzyme (induced fit of enzyme[7]) and leads to a change in charge density. Since the membrane potential is a function of the charge density in a charged membrane,[8-10] the shifts in membrane potential can be generated by the binding of glucose to the enzyme in the enzyme membrane. One contradiction for this mechanism was, however, that no potential shift was observed under no-oxygen tension.[5] The shifts in membrane potential should be also observed under N_2 bubbling, if the shifts in membrane potential were generated by the binding between host (glucose oxidase) and guest (glucose).

In this study, the pH change in the permeation cells was monitored with the injection of glucose, and the shifts in membrane potential were measured under no-oxygen tension using initially used enzyme membranes. Time hysteresis of shifts in membrane

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potential was also investigated for a enzyme membrane and a cross-linked enzyme membrane. The goal of this study was to confirm that the shifts in the membrane potential are generated by the change in charge density due to the conformational change in the enzyme to bind with the substrate and not by ionic products of the enzyme/substrate reaction.

EXPERIMENTAL

Materials

Poly(γ-methyl-L-glutamate) (PMLG) was kindly supplied by Ajinomoto Co. and purified by precipitation from 5 wt % dichloroethane in methanol. Glucose oxidase (from *Aspergillus niger,* Grade II, 100 units mg^{-1}) was purchased from Nakarai Chemicals (Kyoto, Japan). Other chemicals were of reagent grade and were used without further purification.

Immobilized Enzyme Membrane

Glucose oxidase (GO) was dissolved in a 1 wt % dichloroethane solution of PMLG. The casting solution, for which the GO concentration was 2.5 mg cm^{-3} PMLG solution, was used in this study. Immobilized (entrapped) enzyme membranes (EM) were prepared by casting the enzyme–PMLG solution on flat Petri dishes and then dried at room temperature for 6 days. Cross-linked enzyme membranes were also prepared from the procedure where the enzyme membrane was immersed in an aqueous solution of 5% glutaraldehyde for 24 h at 25°C.[11] The EM and cross-linked enzyme membrane (CEM) were finally dried under vacuum at room temperature for 24 h and then stored at 10°C.

Measurement of Membrane Potential

The membrane potential, $\Delta\phi$, was determined in cells that consisted of two chambers separated by the enzyme membrane.[5] A schematic diagram of the apparatus is shown in Figure 1. The concentration of aqueous NaCl solutions were 1.0×10^{-3} mol dm^{-3} in one side of the chamber (side 1), C_1, and 1.0×10^{-4} mol dm^{-3} in the other side of the chamber (side 0), C_0. The potential was measured using a digital multimeter (range −99.9999–+99.9999 mV, Model 7561, Yokogawa Electronics Ltd.) with Ag/AgCl electrodes (TOA HS-205C, TOA Electronics Ltd.).

Measurement of Shifts in Membrane Potential

NaCl solution, 15 cm^3, was removed from side 0, and, subsequently, 15 cm^3 of D-glucose solution (C_{inj} = 0.1 mol dm^{-3}) were carefully and quickly added to the chamber using a funnel.[5] The shift in membrane potential, caused by the injection of glucose into the cell, was monitored on a recorder and the

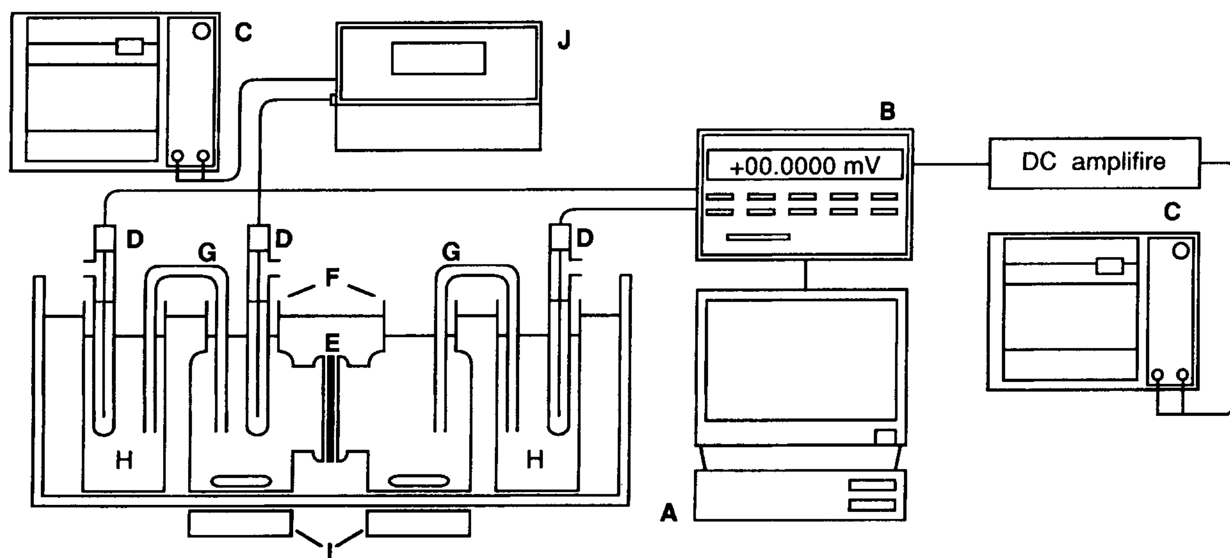


Figure 1 Schematic diagram of apparatus: (A) personal computer; (B) digital multimeter; (C) recorder; (D) electrode; (E) membrane; (F) permeation cell; (G) salt bridge; (H) 3 mol dm^{-3} KCl solution; (I) stirrer; (J) pH meter.

data were transferred to a 16-bit personal computer (PC-9801VX, NEC Corp.).

Circular Dichroism of Membranes

Thin membranes (i.e., 1–3 μm thickness) were prepared by casting the enzyme–PMLG solution and PMLG solution on quartz plates (6 × 0.99 × 0.125 cm). The membrane loading on the quartz plate was inserted into a quartz cell (1.0 × 1.0 × 4.5 cm) containing pure water or 0.01 *M* D-glucose solution. Circular dichroism (CD) of the membrane loading on the quartz plate was measured with a JASCO J-600 instrument (JEOL), after the membrane was inserted into the solution for 30 min prior to the measurements.

RESULTS AND DISCUSSION

pH Change

The time course of the membrane potential change was measured with the injection of D-glucose into the C_1 side of the chamber using the EM and is shown in Figure 2. The EM has a membrane thickness of 27 μm; 0.025 g of GO was immobilized in the EM; and 0.0015 mol of glucose was injected into the cell having a volume of 150 cm^3. An 8 mV shift in membrane potential was observed in this case. The time course of the pH change was measured and is also shown in Figure 2. No pH change except for a small electrical shock upon injection of the glucose was observed. To measure the change in pH for the reaction of GO and glucose in the bulk solution, 0.0015 mol of glucose was injected into a beaker

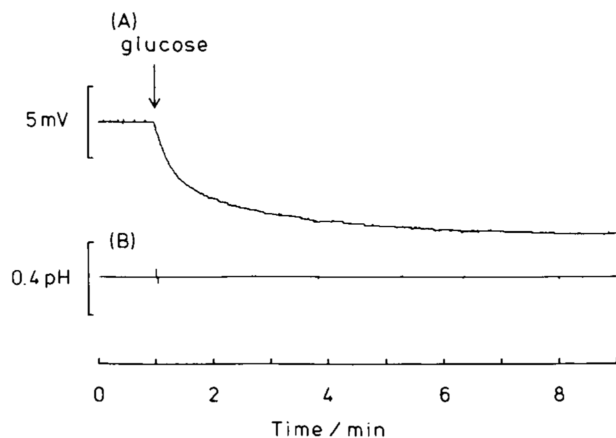


Figure 2 (a) Time course of membrane potential change with the injection of glucose at C_{inj} = 0.1 mol dm^{-3} and 25°C. (b) Time course of pH change with the injection of glucose at C_{inj} = 0.1 mol dm^{-3} and 25°C.

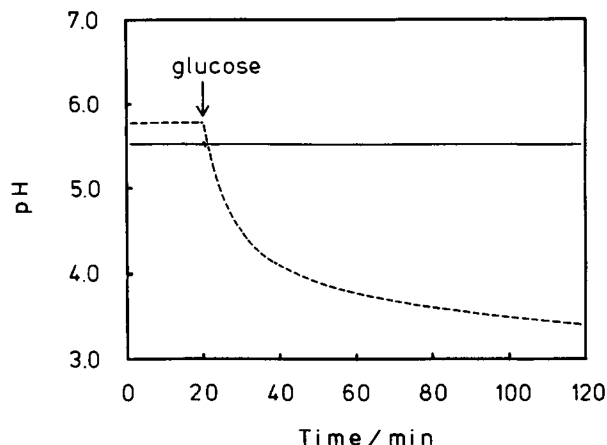
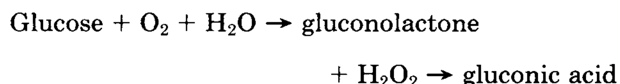


Figure 3 Time course of pH (——) in the permeation cell and (– – –) in bulk in the permeation cell solution at 25°C.

containing 0.025 g GO (i.e., the same amount of enzyme was immobilized in the enzyme membrane) and 150 mL H_2O. The time course of pH for this procedure is shown in Figure 3. pH of the enzyme solution gradually decreased after the injection of glucose. This is due to that the following reaction had occurred and that gluconic acid contributes to the decrease in pH of the solution:



$$\text{Glucose} + O_2 + H_2O \rightarrow \text{gluconolactone} + H_2O_2 \rightarrow \text{gluconic acid}$$

Demura et al.[12] reported that gluconic acid is predominant as compared to gluconolactone in the reaction of GO and glucose at pH 3.0–6.8 from ^{14}C measurements. Since no pH shift was observed upon injection of glucose during the membrane potential measurements, the shifts in membrane potential were not caused by an enzyme–substrate reaction, but by binding of the substrate to the enzyme. The conformational change in the enzyme (i.e., induced fit of enzyme[7]) to bind the substrate is probably the main reason for the change in the charge density of the enzyme membrane, which contributes to the change in membrane potential.[5,6] This is additional evidence to support the above mechanism in that the shifts in membrane potential were observed upon injection of not only D-glucose but also L-glucose.[5] It was suggested that L-glucose can bind with the enzyme, but cannot react with the enzyme and, therefore, gives no product.

Circular Dichroism of the Enzyme Membrane

The circular dichroism (CD) of PMLG and EMs was measured and is shown in Figure 4. Dual peaks

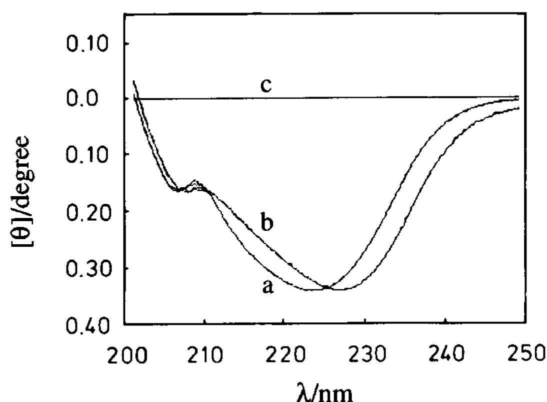


Figure 4 (a) CD spectra of enzyme membrane immersed in pure water and in 0.01M D-glucose solution; (b) CD spectra of PMLG membrane immersed in pure water and in 0.01M D-glucose solution; (c) CD spectrum of 0.01M D-glucose solution.

at 207 and 222.5 nm, which indicate mainly an α-helix conformation, were observed in the CD spectra of the enzyme membrane, whereas the CD spectra of the PMLG membrane show red-shifted dual peaks (i.e., 208 and 226.5 nm) compared to the CD spectra of the enzyme membrane. Exactly the same spectra are obtained when the CD spectra of the enzyme membrane (or PMLG membrane) inserted into pure water are compared with the CD spectra of the enzyme membrane (or PMLG membrane) inserted into 0.01M D-glucose solution.

The conformational change of the GO induced by the binding of D-glucose could not be detected in CD measurements in this study, although a significant difference between CD spectra of immobilized BSA membranes immersed in pure water and in L-tryptophan solution is observed in our measurements.[13] It is suggested that the binding of D-glucose to GO leads to the increase of positive charge per unit volume in the enzyme membrane (i.e., decrease of the effective fixed charge density in the previous study[6]) but does not contribute to the increase or decrease of α-helix, β sheet, and random coil contents in the enzyme membrane.

Oxygen Tension

One contradiction for the mechanism that the binding of glucose to the enzyme induces a shift in the membrane potential is that shifts in membrane potential were not observed in the absence of oxygen (under nitrogen bubbling) in a previous study.[5] The shifts in membrane potential should be observed in the absence of oxygen, because the shifts in membrane potential were not caused by the reaction of the enzyme with the substrate but by the binding of the substrate to the enzyme. It was suggested in the previous study[5] that the binding site of glucose on the enzyme decreased with decreasing oxygen tension and that glucose could not bind to the enzyme in the absence of oxygen.

This explanation is unrealistic, since the reaction of the substrate and enzyme was sometimes reported in the absence of oxygen.[14] Careful experiments concerning shifts in membrane potential were performed under nitrogen bubbling in this study and are shown in Figure 5. The shift in membrane potential was most definitely observed using initially used enzyme membranes in this study.

The previous data[5] for membrane potential change under nitrogen bubbling were obtained using reused membranes (i.e., the enzyme membrane was washed and stored in pure water for 12 h after the measurements of membrane potential). The shifts in membrane potential were also not observed for reused membranes in this study. There should be residual glucose in the membranes, although the enzyme membranes were repeatedly washed in pure water. When the reused membranes were set on the cells and were under nitrogen bubbling before the measurements, the residual glucose diffuses to the enzyme in the membrane and the oxidized enzyme was reduced by the reaction of enzyme and substrate under nitrogen bubbling. Since there is no oxygen in the cell, reduced enzyme cannot be oxidized and, therefore, cannot bind to the substrate. This is the reason that the initially used membrane, which contains no residual glucose, generates the shifts in membrane potential, and the reused membrane cannot generate any shifts in membrane potential.

Reproducibility of Shifts in Membrane Potential

Reproducibility of shifts in membrane potential was tested for an entrapped EM and CEM and is sum-

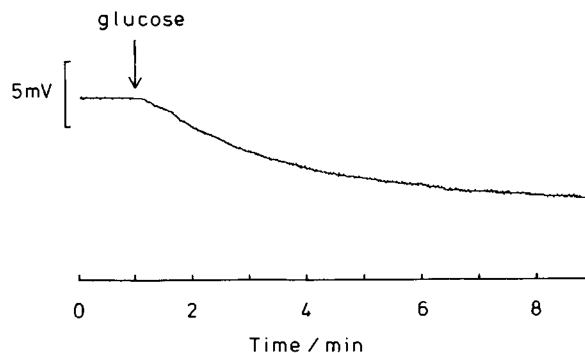


Figure 5 Potential shift using initially used enzyme membrane with nitrogen bubbling at C_{inj} = 0.1 mol dm^{-3} and 25°C.

Table I Reproducibility of Shifts in Membrane Potential Using Enzyme Membrane (EM) and Cross-linked Enzyme Membrane (CEM)

Run	Time (h)	Shift (mV)	
		EM	CEM
1	0	20.0	5.9
2	12	17.3	5.4
3	24	11.2	5.2
4	36	11.8	5.1
5	48	12.1	6.2
6	60	10.3	4.4
7	72	10.9	5.3
8	84	10.2	5.2
Average		13.0	5.3
SD		3.4	0.50

marized in Table I. Each experiment to measure the shifts in the membrane potential was performed every 12 h. A gradual decrease in the shift of membrane potential was observed for the entrapped enzyme membrane from run 1 to run 3, whereas no significant variation in shifts of membrane potential was observed after run 3 within ±1 mV. The EM still shows 10 mV shifts after 84 h of measurement. The CEM shows exactly the same shifts in membrane potential for any experimental run, but gives rather small shifts in membrane potential, 5 ± 1 mV, compared to the entrapped enzyme membrane (i.e., 10–20 mV). This is because the binding sites of substrates in the cross-linked enzyme membrane are partially destroyed by cross-linking between amides in the enzyme and membrane. Most of data presented in the previous[5,6] and present studies were the data where the enzyme membrane was not cross-linked and the shifts in membrane potential were observed to be stable values (i.e., after run 3).

CONCLUDING REMARKS

No pH change in the permeation cell was observed upon injection of glucose, but the shift in membrane potential was observed. The shift in membrane potential was observed in the absence of oxygen using initially used enzyme membranes. Shifts in membrane potential were observed upon injection of not only D-glucose but also L-glucose.[5]

It is suggested that the shifts in membrane potential were not caused by an enzyme–substrate reaction, but by binding of the substrate to the enzyme, which induces a conformational change in the enzyme and leads to a change in the charge density of the EM. Since the membrane potential is a function of the charge density in the charged membrane, shifts in membrane potential can be generated by the binding of glucose to the enzyme.

This method, therefore, leads to the measurement of binding (interaction) between host and guest molecules without any reaction, i.e., not only by enzyme/substrate but also by antibody/antigen and protein/specific ligand interactions.

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