Recognition of Hormones by Membrane Potential and Circular Dichroism of Immobilized Protein Membranes

MARIKO HARA,[1] MASAHIRO HIGUCHI,[2] NORIHIKO MINOURA,[2] AKON HIGUCHI[1]

[1] Department of Industrial Chemistry, Faculty of Engineering, Seikei University, 3 Kichijoji Kita-machi, Musashino, Tokyo 180, Japan

[2] Department of Organic Materials, National Institute of Materials and Chemical Research 1-1, Higashi Tsukuba, Ibaraki 305, Japan

Received 5 September 1996; accepted 18 October 1996

ABSTRACT: The shifts in membrane potential, caused by the injection of hormones into a permeation cell, were measured using immobilized (entrapped) serum albumin and γ-globulin membranes. The effective fixed charge density was estimated to increase after the injection of estradiol and testosterone in both albumin and γ-globulin membranes, while the charge density was estimated to decrease after the injection of progesterone in the γ-globulin membranes. Because the change in the charge density originates from the conformational change of proteins in the membranes, the change in the circular dichroism induced by the hormones was measured in the membranes. The α-helix content in both albumin and γ-globulin membranes was found from the circular dichroism measurements to increase when estradiol and testosterone was bound to the proteins, while the α-helix content in the albumin membrane decreased on the binding of progesterone.

Some discrepancy was found between the conformational change of the proteins in the membranes detected by the membrane potential measurements and the circular dichroism measurements. This is explained by the fact that the circular dichroism measurements do not directly contribute to the change in the charge density induced by the binding of hormones to proteins in the membranes. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **65:** 251–259, 1997

Key words: membrane potential; circular dichroism; serum albumin; γ-globulin; hormone

INTRODUCTION

Recognition of organic substrates from their binding to biological macromolecules (i.e., proteins, enzymes, and DNA) is of vital importance in biochemistry, biophysical, and analytical chemistry. Proteins including enzymes and DNA are generally used as host molecules in the recognition in both living and artificial membranes. There are extensive studies[1–9] of their application focussed on the development of biosensors, which detect reaction products generated by the binding between the enzymes and substrates.

Gregg and Heller[5] investigated amperometric biosensors based on a crosslinkable poly(vinyl pyridine) complex of $[Os\text{-}(bpy)_2Cl]^{+/2+}$ that communicated electrically with flavin adenine dinucleotide redox centers of enzymes. The crosslinked, enzyme-containing films were shown to be not only stable but selective and highly active for the catalytic oxidation of glucose.

Novel types of biosensors have also been developed in which membrane proteins (e.g., nicotinic

Correspondence to: A. Higuchi.

acetylcholine receptor,[10–13] auxin-receptor ATPase,[14] antibody,[15] maltose binding protein,[16] and Na^+/D-glucose[17] or H^+/lactose co-transporter[18]) or DNA[19] were utilized as receptor sites.

Zhou and Cass[16] isolated the maltose binding protein (MBP) from Gram negative bacteria. MBP was labelled with a fluorescent probe and was immobilized onto pore-controlled glass using three different chemical methods. The binding of maltose to the labelled MBP led to an energy transfer efficiency increase from 29 to 42%.

In our previous study, the shifts in membrane potential, caused by the injection of D- and L-glucose into a permeation cell, were measured using immobilized glucose oxidase membranes.[20–22] Although the enzyme does not react with L-glucose but does react with D-glucose, both L- and D-glucose generated the shifts in membrane potential. This study prompted us to investigate the potential response of immobilized protein (except enzyme) membranes induced by specific substrates.[23–25] The shifts in the membrane potential were generated by a change in the charge density in the membrane due to the binding of substrates to the protein in the system.

The shifts in membrane potential caused by the injection of amino acids into the permeation cell were therefore measured using immobilized albumin and γ-globulin membranes.[23–26] The conformational change of the immobilized albumin membranes induced by the binding between the protein and amino acids in the membranes was also measured using circular dichroism for the explanation of the membrane potential response induced by the amino acids.[25,26]

In this study, bovine serum albumin and γ-globulin were immobilized (entrapped) in a poly(α-amino acid) network. The shifts in the membrane potential caused by the injection of hormones were investigated at pH 7.0 and were compared with the shifts caused by the injection of amino acids reported previously.[24–26] The conformational change of the membranes induced by the binding of hormones to the serum albumin and γ-globulin was also investigated based on circular dichroism measurements and was compared with not only the results obtained from the shifts in the membrane potential but the results induced by the binding of amino acids to serum albumin reported previously.[26]

EXPERIMENTAL

Materials

Poly(γ-methyl-L-glutamate) (PMLG) was kindly supplied by Ajinomoto Co., Inc., and purified by precipitation from 10 wt % dichloroethane in methanol. Bovine serum albumin (BSA; essentially fatty acid free, 9048-46-8) and γ-globulin (fraction 2, 3, 9007-83-4) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were used without further purification. Ultrapure water was used throughout the experiments.

Immobilized Protein Membranes

BSA and γ-globulin were dissolved in a 1 wt % dichloroethane solution of PMLG, respectively. The concentration of BSA and γ-globulin in the casting solution was 4.0 mg cm^{-3} in this study. Immobilized (entrapped) BSA and γ-globulin membranes were prepared by casting the BSA–PMLG solution and the γ-globulin–PMLG solution, respectively, onto flat Petri dishes and then drying at room temperature for six days. The BSA–PMLG membranes and the γ-globulin–PMLG membranes were finally dried under vacuum at room temperature for 24 h and then stored at 10°C.

AFM Images

The membranes of 1–3 μm thickness were prepared by casting the BSA–PMLG solution, γ-globulin–PMLG solution, and PMLG solution on mica plates (10.0 × 10.0 mm).

The images of the surface of the membrane loaded on the mica plate were observed with a NanoScope IIIa MultiMode Scanning Probe Microscope manufactured by Digital Instruments, Inc. (CA). Atomic force microscopy (AFM) images were acquired using the tapping mode. Tapping mode AFM images were obtained using silicon cantilevers with integral tips (length = 125 μm, radius of curvature = 5–10 nm, and spring constant = 20–10 N m^{-1}). The cantilever was oscillated at its resonance frequency from 200 to 250 kHz.

Measurements of Membrane Potential and Shifts in Membrane Potential

The membrane potential $\Delta\phi$ was measured in cells that consisted of two chambers separated by the protein membranes as described in previous articles.[20–25] Each chamber contained 150 cm^3 of 1.0×10^{-3} mol dm^{-3} (M) phosphate buffer solution and NaCl. The concentration of NaCl in the solution was kept constant in one side of the

Table I Recognition of Substrates from CD Spectra and MP Shifts in Immobilized BSA and γ-globulin Membranes ($n = 4$)

	BSA			γ-globulin		
Substrate	$\Delta\theta 208$ (%)	$\Delta\theta 222$ (%)	$\Delta\Delta\phi$ (mV)	$\Delta\theta 208$ (%)	$\Delta\theta 222$ (%)	$\Delta\Delta\phi$ (mV)
Estradiol	2.2	3.2	5.6	3.6	5.6	1.1
Testosterone	2.7	5.8	2.1	0.4	4.8	0.4
Progesterone	−3.6	−3.4	2.1	4.4	0.8	−0.6
Cortisol	1.6	3.8	0.0	−3.5	−0.7	0.0

chamber (side 1), C_1, at $1.0 \times 10^{-3} M$ and also in the other side of the chamber (side 0), C_0, at $0.1 M$. The potential was measured using a digital multimeter (range −99.9999 mV to +99.9999 mV; Model 7561, Yokogawa Electronic Co.) with Ag/AgCl electrodes (TOA HS-205C; TOA Electronics, Ltd.) at 37 ± 0.02°C.

The pH in the cell was monitored with a pH meter (TOA HM-30S; TOA Electronics Ltd.) and was adjusted to pH 7.0 ± 0.02 with the phosphate buffer solution.

After the pH in the cell registered a constant value (pH 7.0 ± 0.02), hormones (β-estradiol, progesterone, testosterone, and cortisol) dissolved in 1 mL ethanol were carefully and quickly injected into the chamber of side 1.

The shift in the membrane potential, caused by the injection of the hormone into the cell, was monitored on a recorder; and the data were transferred to a 32-bit personal computer (PC-9801BX; NEC Corp, Tokyo). The shift in the pH was also monitored during this study.

The solution in the cell was replaced with ultrapure water several times after each set of the measurements to remove the residual hormones. Each of the membranes was capable of withstanding more than 30 measurements over a one-month period.[22,23,25] The membrane potential shifts were reproducible to within approximately ±0.4 mV on repeated runs with the same membranes and ±2.0 mV on repeated runs with different membranes.[23–25] Each point in Table I and Figure 3 is an average of 4 measurements ($n = 4$).

Circular Dichroism of Membranes

Thin membranes (i.e., 1–3 μm) were prepared by casting the BSA–PMLG solution, γ-globulin–PMLG solution, and PMLG solution on quartz plates (6.0 × 0.99 × 0.125 cm), respectively. The membranes loaded on the quartz plate were immersed in the $1.0 \times 10^{-3} M$ phosphate buffer solution with and without hormones at pH 7.0. The final concentration of hormones was $1.0 \times 10^{-3} M$. The circular dichroism (CD) of the membrane loaded on the quartz plate was measured with a JASCO J-600 instrument (JASCO Co., Tokyo) after the membrane was immersed in the solution for 30 min prior to the measurements. Spectra shown in Figures 4–6 were averages of four measurements using different membranes for each hormones.

RESULTS AND DISCUSSION

AFM Images of Membranes

AFM measurements of PMLG, BSA–PMLG, and γ-globulin–PMLG membranes were performed to characterize the surfaces of the membranes. Figure 1 shows AFM images of PMLG and BSA–PMLG membranes. The surface of the PMLG membranes is found to be flat and smooth, while the surface of the BSA–PMLG membranes shows a rather rough and uneven surface. The rough surface of the BSA–PMLG membranes is caused by a nanoscale aggregation of proteins. The topography of the γ-globulin–PMLG membranes was observed to be the same as that of the BSA–PMLG membranes in the AFM measurements (this is not shown in the figure). This probably originates from the fact that both the protein membranes were prepared by the same method (i.e., casting method) and the nanoscale aggregation of proteins is the same in both protein membranes.

Changes in Membrane Potential

The changes in the membrane potential upon the injection of estradiol, testosterone, progesterone,

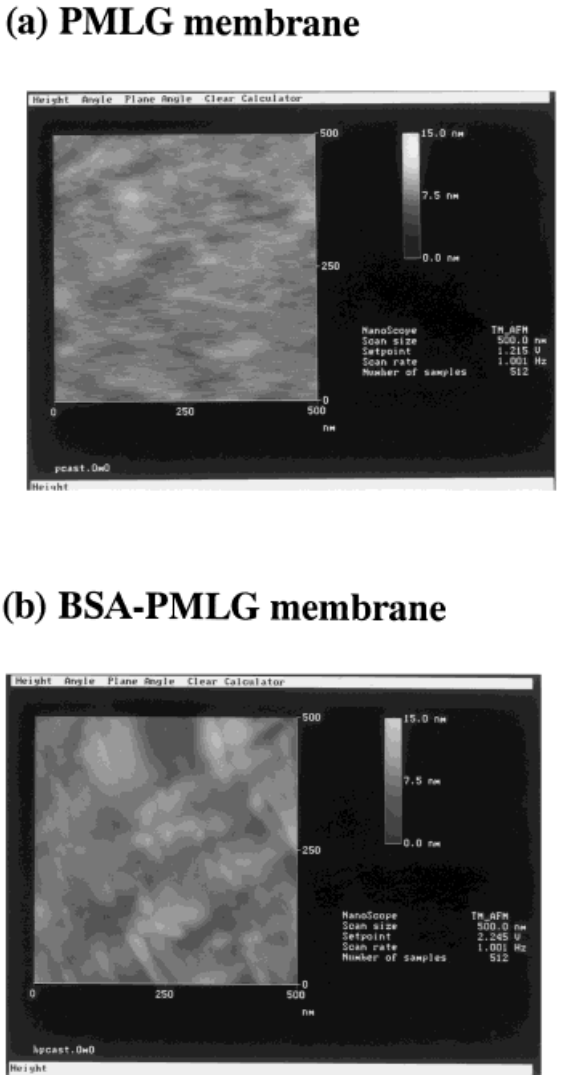

Figure 1 AFM images of PMLG membrane (a) and BSA–PMLG-membrane (b).

and cortisol were measured for the BSA–PMLG membranes and γ-globulin–PMLG membranes at $C_s = 0.001M$ and at pH 7.0, where C_s is the concentration of the injected hormone in the cell of side 1. The time courses of the membrane potential change on the injection of estradiol, testosterone, and progesterone are shown in Figure 2. Changes in the pH after the injection of the hormones were also measured and were not observed within ± 0.01 in this study because the pH in the cell was adjusted to pH 7.0 using buffer solution (see the bottom graph shown in Figure 2).

The membrane potential before the injection of various hormones $\Delta\phi$ (before) was observed to be 12 ± 2 mV at $C_1 = 10^{-3}M$ and $C_0 = 10^{-1}M$. The membrane potential after the injection of substrates $\Delta\phi$ (after) was found to be higher than $\Delta\phi$ (before) for the injection of estradiol, testosterone, and progesterone for the BSA–PMLG membranes. On the other hand, $\Delta\phi$ (after) on the injection of cortisol showed approximately the same value as $\Delta\phi$ (before) for the BSA–PMLG membranes.

The shifts in the membrane potential $\Delta\Delta\phi$ defined by the difference in the potential before and after the injection of the substrates in equilibrium [i.e., eq. (1)] were found to be 0.0 ± 0.5 mV for the PMLG membranes, 0.0–5.6 mV for the BSA–PMLG membranes, and -0.6–1.1 mV for the γ-

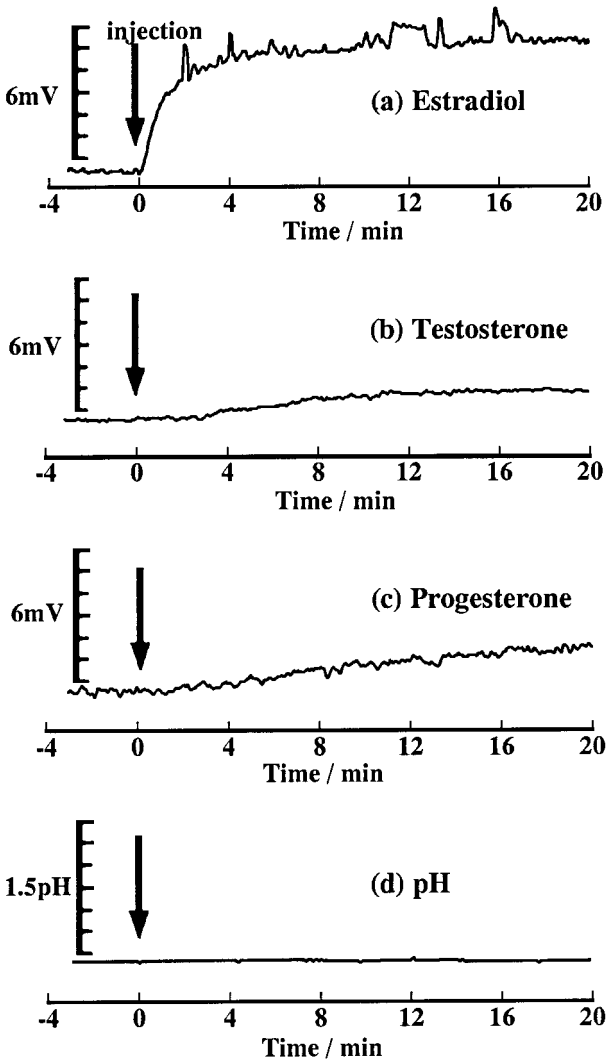

Figure 2 Time course of the membrane potential change [(a)–(c)] and pH change (d) on the injection of estradiol [(a) and (d)], testosterone (b), and progesterone (c) in the BSA–PMLG membrane at $C_0 = 0.1M$, $C_1 = 1$ mM, and 37°C. The arrows indicate the injection time of hormones into the cell.

globulin–PMLG membranes, depending upon the hormones injected, as follows:

$$\Delta\Delta\phi = \Delta\phi(\text{after}) - \Delta\phi(\text{before}) \qquad (1)$$

Table I summarizes the $\Delta\Delta\phi$ values for the BSA–PMLG membranes and the γ-globulin–PMLG membranes caused by the injection of various hormones at $C_s = 1$ mM. The data presented in Table I are averages of four measurements, and the standard deviation of $\Delta\Delta\phi$ is calculated to be less than 0.5 mV in this study. The $\Delta\Delta\phi$ for the BSA–PMLG membranes was observed to increase in the following order at $C_s = 0.001M$, 0 mV $\fallingdotseq$ cortisol < progesterone = testosterone < estradiol. On the other hand, the $\Delta\Delta\phi$ for the γ-globulin–PMLG membranes was observed to increase in the following order at $C_s = 0.001M$, progesterone < 0 mV $\fallingdotseq$ cortisol < testosterone < estradiol. The $\Delta\Delta\phi$ after the injection of the hormone for the BSA–PMLG membranes was found to be higher than that for the γ-globulin–PMLG membranes in this study. The $\Delta\Delta\phi$ of testosterone, progesterone, and cortisol obtained in this study was observed to be less than that of amino acids ($=\Delta\Delta\phi_{\text{int}}$ expressed in the previous study[23]) reported previously[23] for the BSA–PMLG membranes.

Shifts in Membrane Potential

The dependence of the shifts in the membrane potential on the concentration (C_s) of estradiol injected in the cell was measured for the BSA–PMLG membrane. This is shown in Figure 3. The specific binding between the host (i.e., BSA or γ-globulin) and the guest molecules (i.e., hormones) in the membrane is important for observation of the shifts in the membrane potential because no intrinsic shifts in the membrane potential were observed for the PMLG membrane. The site saturation mechanism,[27–31] where estradiol binds to BSA in the BSASA-PMLG membrane, is observed from Figure 3 and is expected to explain $\Delta\Delta\phi$ as a function of C_s by eq. (2), as follows:

$$\frac{1}{\Delta\Delta\phi} = \frac{A}{C_s} + B \qquad (2)$$

A linear least squares method was used to fit the data in eq. (2). A and B were selected to be 0.0181 and 0.1578 from the linear least squares method, and the correlation coefficient was obtained to be 0.999 at $C_s \geqq 0.05$ mM in this study. The site saturation mechanism explains the experimental $\Delta\Delta\phi$ well as a function of C_s. It is therefore suggested that $\Delta\Delta\phi$ increases with the increase in bound hormone on the BSA in the BSA–PMLG membrane.

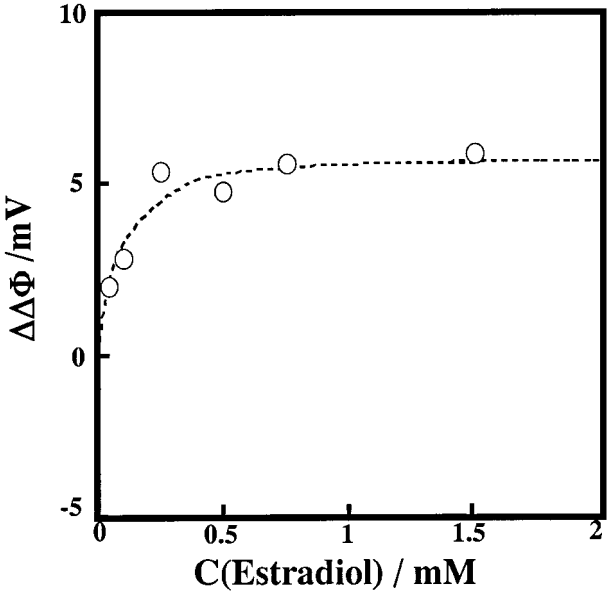

Figure 3 Concentration dependence of membrane potential shifts induced by estradiol injection in the BSA–PMLG membrane at $C_0 = 0.1M$, $C_1 = 1$ mM, and 37°C.

Membrane Potential

The membrane potential is generally expressed by the Teorell–Meyer–Sievers (TMS) theory,[32–38] as follows:

$$\Delta\phi = -\frac{RT}{zF}\left\{\ln\frac{C_1[1 + 4y_0^2]^{1/2} - \alpha}{C_o[1 + 4y_1^2]^{1/2} - \alpha} + U\cdot\ln\frac{[1 + 4y_1^2]^{1/2} - \alpha U}{[1 + 4y_0^2]^{1/2} - \alpha U}\right\} \qquad (3)$$

where U is $[\xi_+ - \xi_-]/[\xi_+ + \xi_-]$, and ξ_+ and ξ_- are the mobilities of the cation (Na^+) and the anion (Cl^-); y_0 is KC_o/C_x; y_1 is KC_1/C_x; C_x is the effective fixed charge concentration;[32–33,36–38] K is the thermodynamic partition coefficient;[32–33,36–38] α has a value of +1 or −1 when the membrane is positively or negatively charged ($\alpha = -1$ in this study); z is the valence of the ion ($z = 1$ in this study); and R, T, and F have their conventional

meanings. The only unknown parameters in eq. (3) are C_x/K and U.

C_x/K and U before the injection of the amino acid at the isoelectric point of L-alanine were estimated to be 0.24 mM and 0.0638 from the previous study.[23,25] It is known that C_x/K is the predominant influence on the membrane potential;[25] therefore, the positive values of the $\Delta\Delta\phi$ were explained by the fact that the effective fixed charge density increases after the injection of estradiol, progesterone, and testosterone for the BSA–PMLG membranes based on the estimation from eq. (3) and the positive value of $\Delta\phi$ at $C_1 = 10^{-3}M$ and $C_0 = 0.1M$. Whereas, the negative values of $\Delta\Delta\phi$ can be attributed to the fact that the effective fixed charge density decreases after the injection of progesterone for the γ-globulin–PMLG membranes.

We suggest that the increase or decrease in the effective fixed charge density after the injection of hormones originates from the conformational change in the BSA–PMLG membrane and the γ-globulin–PMLG membrane induced by the binding of the hormones to the membranes. Therefore, the CD of the protein membranes was investigated to determine whether the conformational change in the protein membranes was induced by the existence of the guest molecules, hormones.

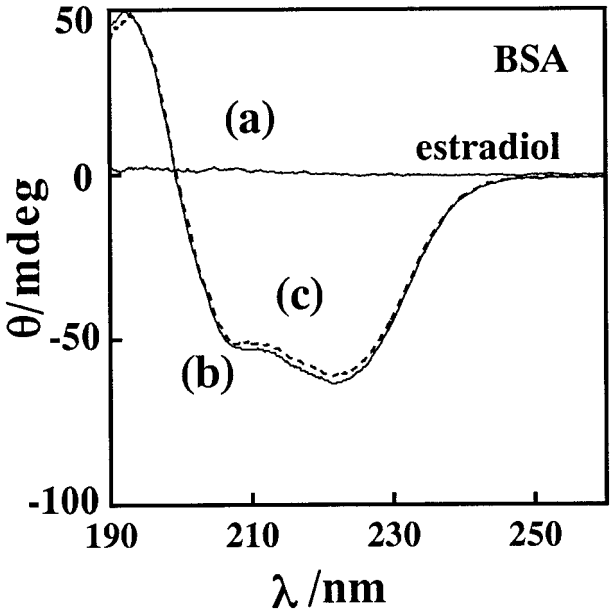

Figure 4-1 CD spectra of 0.001M estradiol solution (a), and BSA–PMLG membranes immersed in phosphate buffer solution (b) and in 0.001M estradiol + phosphate buffer solution (c).

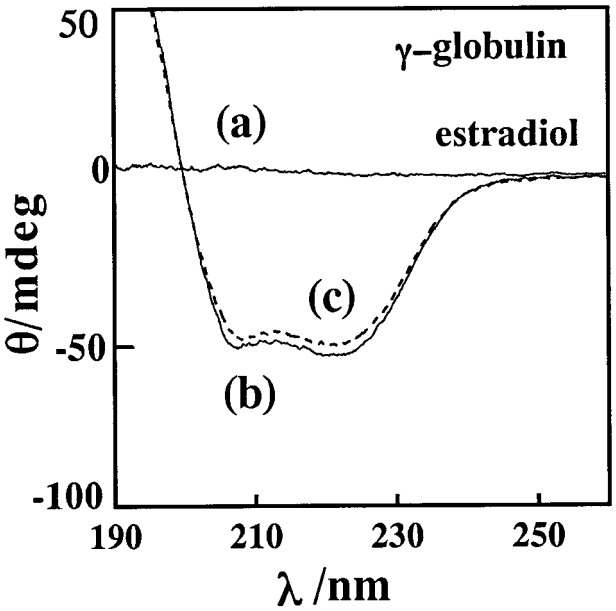

Figure 4-2 CD spectra of 0.001M estradiol solution (a), and γ-globulin–PMLG membranes immersed in phosphate buffer solution (b) and in 0.001M estradiol + phosphate buffer solution (c).

Circular Dichroism of Membranes

Figure 4 shows the CD spectra of the BSA–PMLG membrane and the γ-globulin–PMLG membrane in the phosphate buffer solution at pH 7.0. The CD spectra of these membranes exhibited two negative bands at 208 and 222 nm that are typical of the right-handed α-helix.[39–41] It is known that the native BSA and γ-globulin mainly have an α-helix conformation.[39] These results implied that the immobilized BSA and γ-globulin retain their native structure in the membrane. Figure 4 also shows the CD spectra of the BSA–PMLG membrane and the γ-globulin–PMLG membrane in the phosphate buffer solution with and without estradiol, respectively. Two negative bands at 208 and 222 nm connected with the α-helix conformation were found to decrease in the presence of estradiol. Because the hormone solution did not show any CD as shown in Figure 4 and no CD change in the PMLG membrane was observed in the absence and presence of the hormones (i.e., estradiol, progesterone, testosterone, and cortisol), the spectral changes observed in Figure 4 originated solely from the conformational change of the proteins induced by the binding between the protein and the hormone in the membranes.

The shifts in the ellipticity of the CD spectra at 208 and 222 nm induced by the injection of

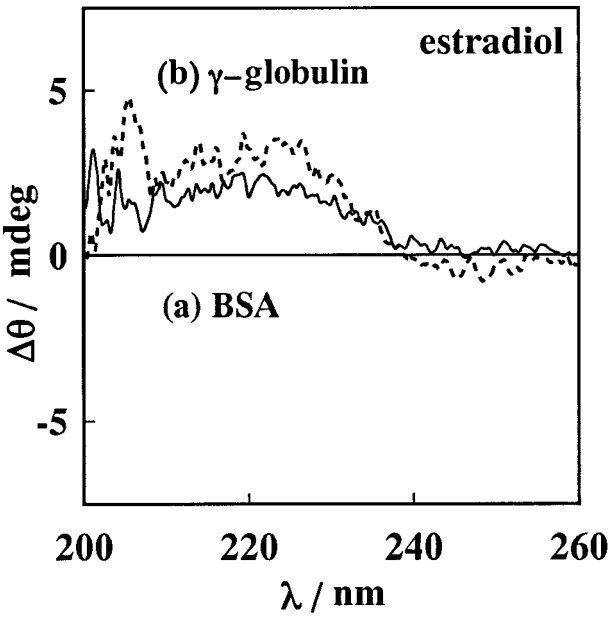

Figure 5-1 CD spectra ($\Delta\theta$) of BSA–PMLG membranes immersed in 0.001M estradiol + phosphate buffer solution (a) and of γ-globulin–PMLG membranes immersed in 0.001M estradiol + phosphate buffer solution (b).

hormones $\Delta\theta_{208}$ and $\Delta\theta_{222}$ are defined in this study as follows:

$$\Delta\theta_{208} = 100 \cdot [\theta_{208}(\text{hormone}) - \theta_{208}(\text{buffer})]/\theta_{208}(\text{buffer}) \quad (4)$$

$$\Delta\theta_{222} = 100 \cdot [\theta_{222}(\text{hormone}) - \theta_{222}(\text{buffer})]/\theta_{222}(\text{buffer}) \quad (5)$$

where θ_{208}(buffer) and θ_{222}(buffer) are the ellipticities of the membranes in the phosphate buffer solution at 208 and 222 nm, and θ_{208}(hormone) and θ_{222}(hormone) are the ellipticities of the membranes in the hormone solution at 208 and 222 nm, respectively. $\Delta\theta_{208}$ and $\Delta\theta_{222}$ for estradiol, progesterone, testosterone, and cortisol are summarized in Table I. The values for $\Delta\theta_{208}$ and $\Delta\theta_{222}$ presented in Table I are the averages of four measurements, and the standard deviation of $\Delta\theta_{208}$ and $\Delta\theta_{222}$ is calculated to be less than 1.2% in this study. It is found that $\Delta\theta$ values at 208 and 222 nm for estradiol and testosterone are positive, which indicates that the content of the α-helix conformation in BSA–PMLG membrane and γ-globulin–PMLG membrane decreases when the hormone binds to the membranes. On the other hand, $\Delta\theta$ at 208 and 222 nm is found to be negative for progesterone in the BSA–PMLG membranes and for cortisol in the γ-globulin–PMLG membranes, which indicates that the content of the α-helix conformation in the membranes increases when the hormones bind to the membranes. In these cases, the binding of hormones to the membrane induces the stabilization of the α-helical conformation of BSA and γ-globulin in the membranes. Figure 5 shows $\Delta\theta$ values for estradiol, progesterone, testosterone, and cortisol in the BSA–PMLG membrane and the γ-globulin–PMLG membrane where $\Delta\theta$ is calculated from θ(buffer) − θ(hormone). θ(buffer) and θ(hormone) are the ellipticities of the membranes in the phosphate buffer solution and in the hormone + phosphate buffer solution, respectively. It is found that $\Delta\theta$ induced by estradiol and testosterone shows the same tendencies in both the BSA–PMLG membrane and the γ-globulin–PMLG membrane, while $\Delta\theta$ induced by progesterone and cortisol in the BSA–PMLG membrane gives the opposite sign of $\Delta\theta$ observed in the γ-globulin–PMLG membrane. The characteristics of proteins are observed in the CD spectra when progesterone and cortisol bind to the host proteins in the membranes.

In the previous study, we investigated the changes in the fixed charge density and conforma-

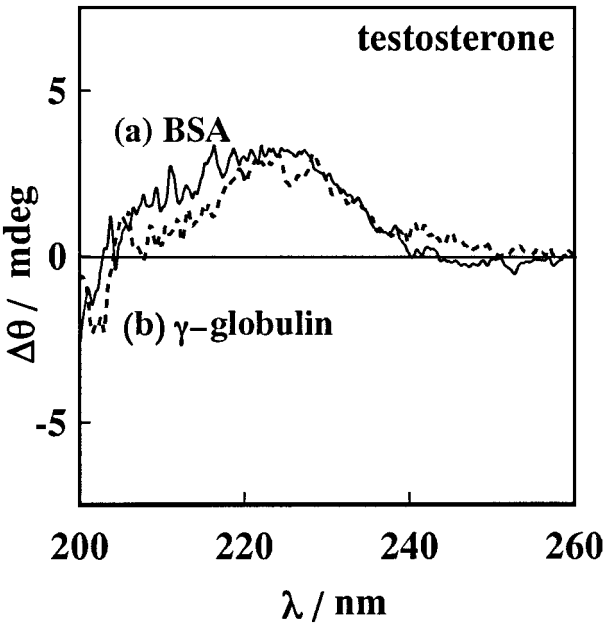

Figure 5-2 CD spectra ($\Delta\theta$) of BSA–PMLG membranes immersed in 0.001M testosterone + phosphate buffer solution (a) and of γ-globulin–PMLG membranes immersed in 0.001M testosterone + phosphate buffer solution (b).

tion of the BSA–PMLG membrane induced by the amino acids binding to the BSA in the membrane. We could not find a direct relationship between the fixed charge density and conformation of the membrane due to the pH change in the cell on the injection of amino acids into the cell;[23] and the pH change induced a change in the fixed charge density in the protein membranes, which caused the membrane potential shifts. In this study, we therefore used neutral molecules, hormones, as substrates to eliminate the change in the fixed charged density originating from the pH change induced by the injection of substrates into the cell.

The values of $|\Delta\theta_{208}|$ and $|\Delta\theta_{222}|$ for estradiol were found to be less than those for testosterone and progesterone when the BSA–PMLG membranes were immersed in the 0.001M hormone + buffer solution, but the membrane potential shift $|\Delta\Delta\phi|$ of estradiol was higher than the $|\Delta\Delta\phi|$ of other hormones at C_s = 0.001M from Table I. On the other hand, the values of $|\Delta\theta_{208}|$ and $|\Delta\theta_{222}|$ for estradiol were found to be greater than those for testosterone, progesterone, and cortisol when the γ-globulin–PMLG membranes were immersed in the 0.001M hormone + buffer solution; also, the membrane potential shift $|\Delta\Delta\phi|$ of estradiol was higher than the value of $|\Delta\Delta\phi|$ of testosterone, progesterone, and cortisol at C_s = 0.001M from Table I. Therefore, some discrepancy was found between the conformational change of BSA and γ-globulin in the membranes detected by the membrane potential measurements and the CD measurements. This is due to the fact that the CD measurements can detect an increase or decrease in the α-helix, β sheet, and random coil contents but do not contribute to the detection of the change in the charge density due to the presence of hormones in the BSA–PMLG membranes and the γ-globulin–PMLG membranes.

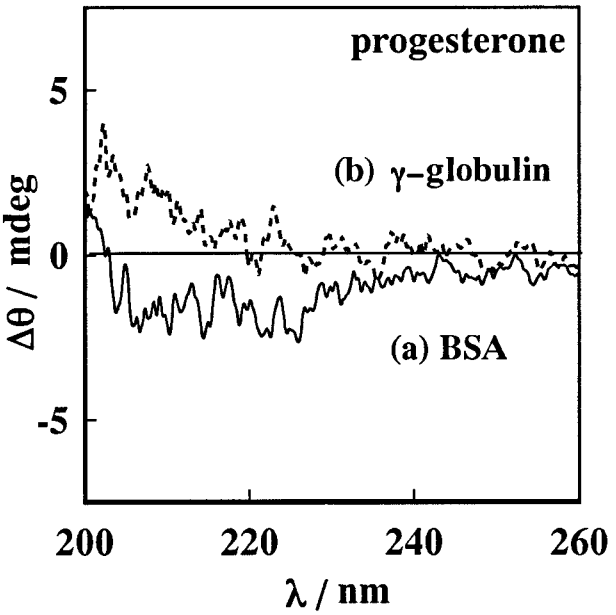

Figure 5-3 CD spectra ($\Delta\theta$) of BSA–PMLG membranes immersed in 0.001M progesterone + phosphate buffer solution (a) and of γ-globulin–PMLG membranes immersed in 0.001M progesterone + phosphate buffer solution (b).

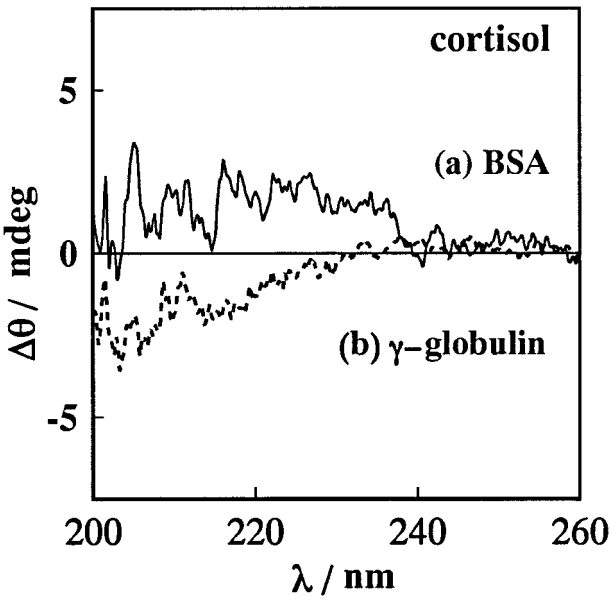

Figure 5-4 CD spectra ($\Delta\theta$) of BSA–PMLG membranes immersed in 0.001M cortisol + phosphate buffer solution (a) and of γ-globulin–PMLG membranes immersed in 0.001M cortisol + phosphate buffer solution (b).

We are grateful to Dr. M. Iwatsuki and Dr. Y. Miyachi (Ajinomoto Co., Inc.) for the gift of PMLG solution. This research was partially supported by the Asahi Glass Foundation.

REFERENCES

1. K. Imai, T. Shiomi, K. Uchida, and M. Miya, *Biotechnol. Bioeng.,* **28,** 1721 (1986).
2. J. Janata and A. Bezegh, *Anal. Chem.,* **60,** 62R (1988).
3. Y. Ikariyama, M. Furuki, and M. Aizawa, *Anal. Chem.,* **57,** 496 (1985).
4. F. F. Orlando, A. S. Ahmad, G. G. George, and J. L. Glenn, *Anal. Chem.,* **60,** 2397 (1988).

5. B. A. Gregg and A. Heller, *Anal. Chem.,* **62,** 258 (1990).
6. D. T. Pierce, P. R. Unwin, and A. J. Bard, *Anal. Chem.,* **64,** 1795 (1992).
7. L. Shufang and S. R. Walt, *Anal. Chem.,* **61,** 1069 (1989).
8. A. Higuchi and T. Nakagawa, *Bull. Chem. Soc. Jpn.,* **63,** 3209 (1990).
9. M. Demura and T. Asakura, *J. Membr. Sci.,* **59,** 39 (1991).
10. M. Gotoh, E. Tamiya, M. Momoi, Y. Kagawa, and I. Karube, *Anal. Lett.,* **20,** 857 (1987).
11. M. E. Eldefrawi, S. M. Sherby, A. G. Andreou, N. A. Mansour, Z. Annau, N. A. Blum, and J. J. Valdes, *Anal. Lett.,* **21,** 1665 (1988).
12. R. F. Taylor, I. G. Marenchic, and E. J. Cook, *Anal. Chim. Acta,* **213,** 131 (1988).
13. K. R. Rogers, J. J. Valdes, and M. E. Eldefrawi, *Biosens. Bioelectron.,* **6,** 1 (1991).
14. M. Thompson, U. J. Krull, and M. A. Venis, *Biochem. Biophys. Res. Commun.,* **110,** 300 (1983).
15. M. Aizawa, S. Kato, S. Suzuki, Y. Nagamura, R. Shinohara, and I. Ishiguro, *Kobunshi Ronbunshu* (in Japanese), **34,** 813 (1977).
16. L. Q. Zhou and A. E. G. Cass, *Biosens. Bioelectron.,* **6,** 445 (1991).
17. N. Sugao, M. Sugawara, H. Minami, M. Uto, and Y. Umezawa, *Anal. Chem.,* **65,** 363 (1993).
18. H. Kiefer, B. Klee, E. John, Y.-D. Stierhof, and F. Jahnig, *Biosens. Bioelectron.,* **6,** 233 (1991).
19. Y. Okahata, Y. Matsunobu, K. Ijiro, M. Mukae, A. Murakami, and K. Makino, *J. Am. Chem. Soc.,* **114,** 8299 (1992).
20. A. Higuchi, S. Ogawa, and T. Nakagawa, *J. Chem. Soc. Faraday Trans.,* **87,** 695 (1991).
21. A. Higuchi, S. Chida, and T. Nakagawa, *J. Chem. Soc. Faraday Trans.,* **87,** 2723 (1991).
22. A. Higuchi, M. Hara, K.-S. Yun, and T.-M. Tak, *J. Appl. Polym. Sci.,* **51,** 1735 (1994).
23. A. Higuchi, Y. Ando, and T. Nakagawa, *Polym. J.,* **25,** 747 (1993).
24. K.-S. Yun, T.-M. Tak, S. Shimada, T. Nakagawa, M. Hara, and A. Higuchi, *J. Appl. Polym. Sci.,* **55,** 343 (1995).
25. M. Hara, M. Higuchi, N. Minoura, and A. Higuchi, *J. Appl. Polym. Sci.,* **58,** 2025 (1995).
26. M. Hara, M. Higuchi, N. Minoura, S. Oh-uchi, C.-S. Cho, T. Akaike, and A. Higuchi, *Nippon Kagaku Kaishi,* 483 (1996).
27. D. R. Paul and W. J. Koros, *J. Polym. Sci., Polym. Phys. Ed.,* **14,** 675 (1976).
28. T. Uragami, H. B. Hopfenberg, W. J. Koros, D. K. Yang, V. T. Stannett, and R. T. Chern, *J. Polym. Sci., Polym. Phys. Ed.,* **24,** 779 (1986).
29. S. A. Stern and V. Saxena, *J. Membr. Sci.,* **7,** 47 (1980).
30. A. Higuchi, F. Fushimi, and T. Iijima, *J. Membr. Sci.,* **25,** 171 (1985).
31. A. Higuchi, M. Hara, T. Horiuchi, and T. Nakagawa, *J. Membr. Sci.,* **93,** 157 (1994).
32. A. Higuchi and T. Iijima, *J. Appl. Polym. Sci.,* **31,** 419 (1986).
33. A. Higuchi and T. Nakagawa, *J. Chem. Soc. Faraday Trans.,* **85,** 3609 (1989).
34. T. Teorell, *Proc. Soc. Exp. Biol. Med.,* **33,** 282 (1935).
35. K. H. Meyer and J. F. Sievers, *Helv. Chim. Acta,* **19,** 649 (1936).
36. H. U. Demisch and W. Pusch, *J. Colloid Interface Sci.,* **69,** 247 (1979).
37. A. Higuchi and T. Nakagawa, *J. Membr. Sci.,* **32,** 267 (1987).
38. T. Kinoshita, T. Yamashita, T. Iwata, A. Takizawa, and Y. Tsujita, *J. Macromol. Sci. Phys., Ed.,* **B22,** 1 (1983).
39. N. Greenfield and G. D. Fasman, *Biochemistry,* **8,** 4108 (1969).
40. N. S. Simmons, C. Chohen, A. G. Szent-Gyorgyi, O. B. Wetlaufer, and E. R. Blout, *J. Am. Chem. Soc.,* **83,** 4766 (1961).
41. E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry,* **8,** 3205 (1969).