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Redox Reactions of Proteins in Polymer Electrolytes

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REDOX REACTIONS OF PROTEINS IN POLYMER ELECTROLYTES

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ABSTRACT

Hemoglobin (Hb), a typical heme-protein, was used to carry out electrochemical redox reactions in solvent-free ion conductive polymers. Hb was chemically modified with poly(ethylene oxide) (PEO) to prepare PEO-Hb. PEO-Hb is soluble in dry PEO (MW = 200) containing 0.5 M KCl, which is a model system for an ion conductive polymer solid. When the system was electrochemically reduced and reoxidized, the rate was small because the diffusion of proteins was the rate-determining step. The PEO-Hb was also reduced and reoxidized by the potential control when it was cast on an ITO electrode and further covered with PEOs with average molecular weights higher than 200 or by other ion conductive polymers containing KCl. The rate did not improve, probably because the electron transfer between adjacent proteins in the cast layer was the rate-determining step.

INTRODUCTION

Polymer electrolytes, so-called ion conductive polymers, are different from polyelectrolytes, so-called charged polymers. Ordinary polyelectrolytes have charges on their chains, i.e., the charges are covalently bound. They provide a large amount of small (counter) ions in an aqueous solution, but most of them act as an insulator in completely dry conditions. Polymer electrolytes, on the other hand, are defined as polymers which enable ion conduction in the solid state without any low molecu-

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lar weight additives. A simple difference between a polyelectrolyte and a polymer electrolyte can be seen in Fig. 1. The former (A in Fig. 1) has charges on the chain, but the latter (B) can dissociate salts and keep charges through the ion dipole interaction force. They are used as supporting electrolytes in an aqueous (or organic) solution and a solid state, respectively.

Solid-state electrochemistry requires electron transfer from an electrode the migration of counterion(s) to compensate for the charge change after electron transfer. Because solid polymer electrolytes can transport small ions in the solid state, the electrochemistry of several functional molecules (even proteins) should be carried out in the solid state. These ion conductive polymers are therefore considered to be polymer solvents [1]. Typical polymer electrolytes are easily prepared by mixing inorganic salts with polyethylene oxide (PEO) [2]. PEO is considered to be a polymerized water from the viewpoint of structural similarity. This is not the same idea (polywater) which was discussed in 1960–1970. PEO certainly solubilizes inorganic salts, dissociates them into ions, and transports the ions without any additives [2].

We are trying to solubilize not only inorganic salts [3, 4] but also organic molecules [5], vinyl monomers [6], polyviologens [7], metal complexes [8], and even proteins [1, 9, 10] in such polymer electrolytes. The electron transfer reactions of these substrates in polymer electrolytes are interesting subjects for developing a "solid state bioelectrochemistry" [1]. The present paper reports the characteristics of redox-active proteins in polymer electrolytes.

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EXPERIMENTAL

Materials

Poly(ethylene oxide) (PEO). PEOs with average molecular weights of 200, 400, and 600 were purchased from NOF Co. PEOs containing no additives, such as anti-oxidants, were dried in vacuo for a week before use.

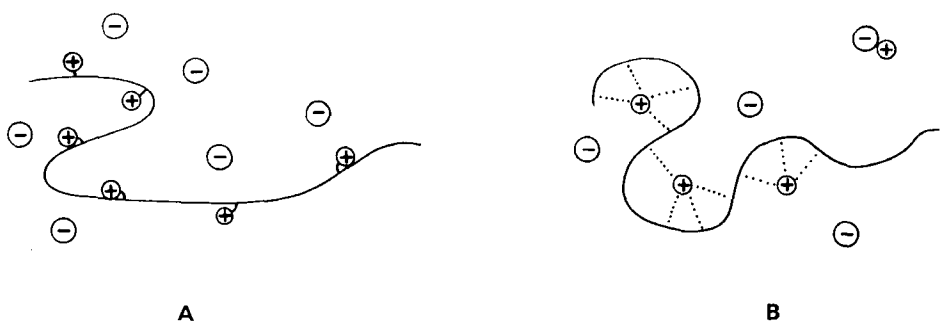

FIG. 1. A schematic comparison of the polyelectrolyte and polymer electrolyte. The polycation (A) contains positive charges on the chain. The polymer electrolyte (B) stabilizes positive charges through the ion-dipole interaction force.

Poly(oligo(oxyethylene) methacrylate) (PMEO). Monomer MEO was also obtained from NOF Inc. MEO was polymerized in an isopropanol solution with AIBN at 60°C for 3 hours. PMEO was purified by the reprecipitation method. A supporting electrolyte (KCl) was added to a chloroform solution of the purified PMEO, evaporated, and dried in vacuo for 2 days.

PEO-modified hemoglobin (PEO-Hb). PEO-Hb was a gift from Ajinomoto Co. The activated PEO (PEO succinimidyl succinate; average molecular weight of 3500) was reacted with stroma-free human hemoglobin [11]. The average molecular weight of PEO-Hb containing 5 PEO chains was calculated to be 1.0×10^5 (including a small amount of crosslinked hemoglobin dimers) by laser light-scattering measurement [11].

ITO glass electrode. The ITO glass electrode was prepared by Oji Tobi Co. The ITO layer thickness was 1500 Å, and the surface resistance was about 15 Ω/cm^2. The ITO glass electrode was washed with dehydrated chloroform and dried before use.

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Methods

Thin Layer Cell

In order to detect reactions on the working electrode, electrochemical studies were carried out with a thin layer cell composed of a glass plate and an ITO glass working electrode (0.9 × 0.1 × 3.5 cm). The light pass length was 150 μm [9, 10].

Potentioabsorptometry

PEO-Hb was dissolved in PEO_{200} containing 0.50 M KCl at 40°C with stirring. This PEO-Hb solution (0.50 mL) was slowly introduced into the plastic cell. Then the thin layer cell was set in the plastic cell with Pt and Ag electrodes, and the plastic cell was sealed with a rubber cap. The PEO solution rose in the thin layer cell by capillary action, and the electrode reaction under a potential was analyzed spectroscopically (Shimadzu, UV-2200). The reduction of PEO-Hb was carried out by applying as negative potential, −0.5 to −1.2 V (vs Ag). A potentiogalvanostat (Nikko Keisoku Co., NPGFZ-2501-A) was used for potentioabsorptometry. A 100% reduction of PEO-Hb was carried out by giving a negative potential (−1.0 V vs Ag) to the working electrode for over 3 hours. All experiments were carried out under a nitrogen gas atmosphere to avoid the formation of oxygen adducts with heme-proteins in a reduced state.

Since PEO-Hb was insoluble in PEOs with average molecular weights larger than 400, electrochemical analysis of the PEO-Hb on the electrode was carried out in PEO solution only when PEOs with molecular weights higher than 400 were used. PEO-Hb was dissolved in distilled water and cast onto the ITO electrode with microsyringe. The dried PEO-Hb cast ITO electrode was then attached to a glass plate to prepare a thin layer cell. This was then soaked in PEO oligomers containing KCl as the supporting electrolyte.

PEO-Hb was also cast onto the ITO electrode. The dried PEO-Hb cast electrode was further covered with PMEO containing KCl. Another ITO glass electrode

(as a counterelectrode) was attached instead of a glass plate. A polished silver wire (0.5 mm in diameter), soaked in the PMEO layer, was used as the reference electrode. This setup was used for potentioabsorptometry.

Scanning Tunneling Microscopy

STM analysis was carried out with a high resonance STM made by Dr. Umeda's group in our university.

RESULTS AND DISCUSSION

PEO/Salt Complex Behaves as a Polycation

Polyethylene oxide can dissolve salts and dissociate them into ions through ion-dipole interaction [3]. This was frequently used to introduce ions into nonpolar solvents. For example, PEO helps salts to solubilize in chloroform. It has been experimentally shown that a PEO chain hold small cations in chloroform solution to act as a polycation [10]. For example, PEO with an average molecular weight of 50,000 showed typical viscosity behavior in a chloroform/DMF mixed solvent (4:1 by volume) as shown in Fig. 2. However $PEO_{50,000}$ showed a polyelectrolyte-like behavior when it was mixed with an inorganic salt such as lithium perchlorate. $PEO_{50,000}$ containing 5.0 mol% $LiClO_4$ relative to the ether oxygen unit showed a larger specific viscosity for diluting the solution with the mixed solvent. In this solution, PEO chains keep cationic changes in their domain through ion-dipole interaction. The increase in the specific viscosity of the PEO salt mixed system cannot be observed in polar solvents such as water because both PEO and the ions are fully solvated by only water molecules. The polyelectrolyte-like characteristics can be found only in less polar environments. This interesting characteristic of polycations should also be effective in solid (or bulk) PEO-containing salts.

The PEO/salt mixture is now widely used as a supporting electrolyte medium because of its high ionic conductivity. The ionic conductivity of a salt containing PEO oligomers is around 3×10^{-4} S/cm in a solution state. It decreases to about 10^{-6} S/cm or less in a solid state. PEO oligomers containing a certain amount of inorganic salt are used as model systems for solid polymer electrolytes.

Reversible Redox Reaction of PEO-Hb in PEO Solution

As briefly mentioned in the Introduction, electrochemical reduction requires electron transfer from the electrode to the substrate and the migration of countercations from the bulk to the reduced substrate to compensate for the charge change after electron transfer. Since polymer electrolytes can transport small ions even in the solid state, the electrochemistry of several functional molecules should be carried out in a solid as schematically illustrated in Fig. 3.

PEO-Hb was solubilized in PEO_{200}. The alpha-helix content of Hb was revealed by CD spectroscopy to be slightly reduced by PEO modification [12]. It was confirmed that there was little conformational change in PEO-Hb compared with native Hb. Then the PEO_{200} solution of PEO-Hb (final concentration of 1.0×10^{-4} M) was spectroscopically analyzed. PEO-Hb was moderately soluble in PEO_{200}.

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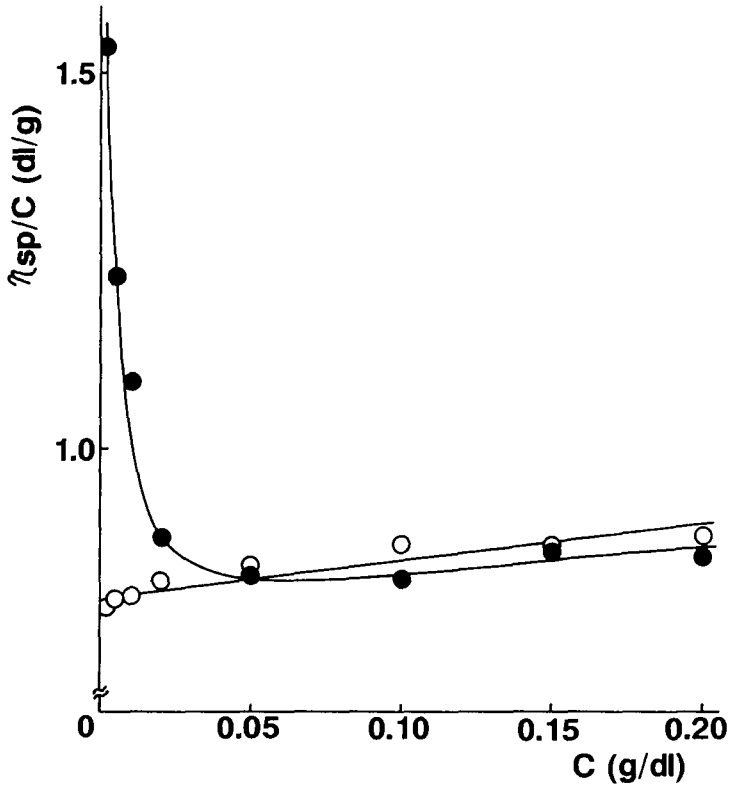

FIG. 2. Concentration dependence of specific viscosity for PEO of PEO/$LiClO_4$ mixture in $CHCl_3$/DMF mixed solvent (4/1 by volume) at 30°C. [—O—]/[Li^+] = 20.0 (by mol), molecular weight of PEO: 50,000. ○: PEO, ●: PEO/$LiClO_4$ mixture.

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The PEO-Hb solution was set in a thin layer cell in order to analyze the visible absorption spectral change of the PEO-Hb in PEO_{200} solution during electrochemical reduction (−0.5 to −1.2 V vs Ag). The spectral change implies a gradual reduction of the PEO-Hb in PEO_{200}, similar to that of the reduction of Hb in an aqueous medium. The reduction of heme proteins is generally confirmed by the shift of the Soret band around 400 nm. The visible spectra for the initial oxidized state and the final reduced state are shown in Fig. 4. In PEO_{200}, PEO-Hb showed absorption maxima (Soret band) at 402.8 and 423.2 nm for the oxidized and reduced form, respectively. The shift of the absorption maximum to a longer wavelength by reduction is similar to the tendency in an aqueous system. The selection of the supporting electrolyte for the reduction of PEO-Hb in PEO oligomers is important. Empirically, potassium chloride (KCl) was selected by us and used to carry out the reversible redox reaction by the potential in the PEO oligomers.

Figure 5 shows the absorption intensity change at 402.8 and 423.2 nm, corresponded to the oxidized and reduced forms, respectively. The figure shows the increase of the reduced fraction and the decrease of the oxidized fraction at the continuous given potential −1.0 V vs Ag. Reduction was slow; it took about 50

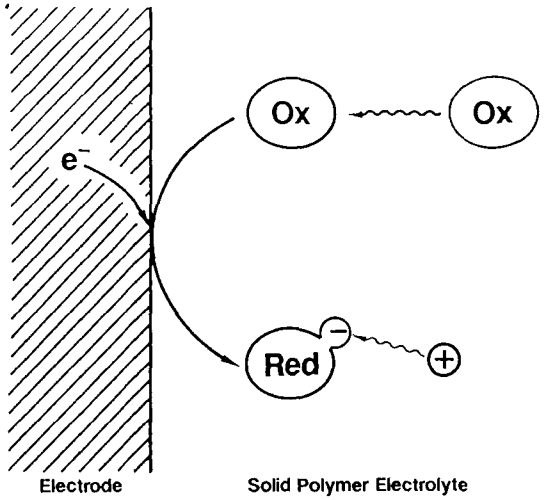

FIG. 3. Schematic illustration of electrochemical reduction of substrate in the polymer electrolyte.

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minutes to reduce to 90% PEO-Hb in PEO_{200} due to the small diffusion coefficient of PEO-Hb in a viscous PEO medium. As shown in Fig. 3, the diffusion of PEO-Hb should be the rate-determining step.

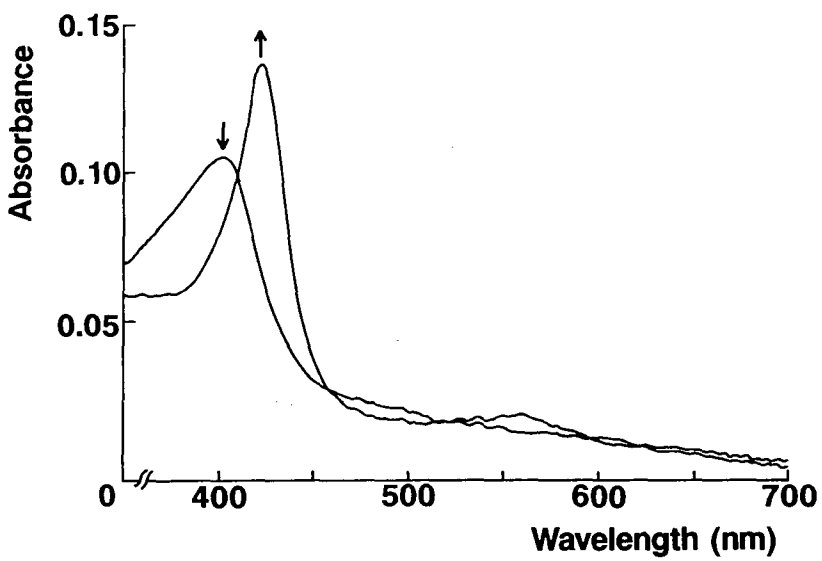

FIG. 4. Visible spectra for the oxidized and reduced PEO-Hb in PEO_{200} containing 0.5 M KCl at 25°C. [PEO-Hb] = 1.0×10^{-4} mol/L. −1.0 V vs Ag was applied for the ITO electrode for 90 minutes.

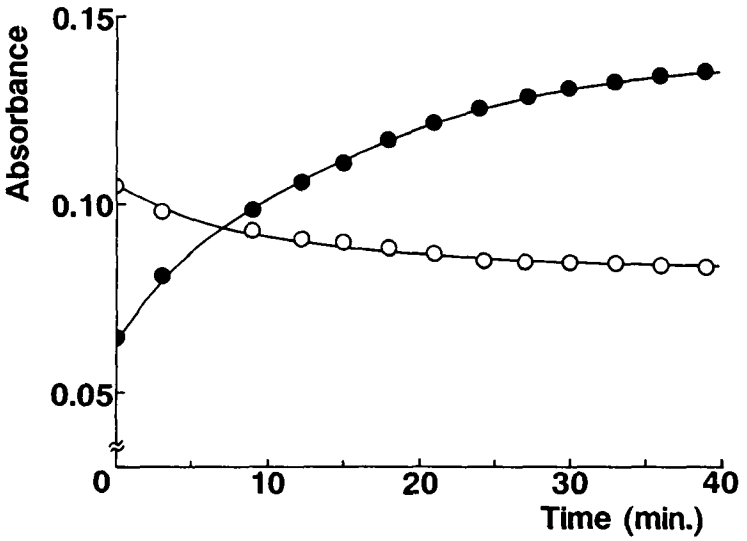

FIG. 5. Visible spectral intensity change for PEO-Hb during reduction in PEO_{200} containing 0.5 M KCl at 25°C. The absorption maxima at 402.8 and 423.2 nm (for oxi- and reduced-PEO-Hb, respectively) were recorded with time. ○: oxidized fraction, ●: reduced fraction.

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The reduced PEO-Hb was reoxidized by using a positive potential such as +1.0 V vs Ag. Figure 6 shows the visible spectral intensity change for PEO-Hb during reversible redox cycling in PEO_{200}. The absorption maxima for oxidized and reduced PEO-Hb changed reversibly but in an opposite direction with each other.

A similar redox reaction was also carried out for PEO-modified myoglobin (PEO-Mb) in PEO_{200} [9]. Since PEO-Hb has a larger molecular weight than PEO-Mb, PEO-Mb showed a larger reduction rate constant. The rate ratio was almost proportional to the ratio of the cubic root of the molecular weight of PEO-Hb to that of PEO-Mb [9].

Redox Reaction of PEO-Hb Cast on the ITO Electrode System

To eliminate the diffusion of PEO-Hb in a very viscous medium, PEO-Hb was cast on an ITO electrode, and the same electrochemical experiments were carried out. Before doing that, the PEO-Hb cast on the ITO electrode was first analyzed by scanning tunneling microscopy (STM). Particles with almost the same size as Hb (60 × 70 Å) were seen (Fig. 7), and they might be the PEO-Hb on the ITO electrode. The surface of the bare ITO electrode also gave particles, but they had a large size distribution. PEO-Hb on the ITO glass electrode was confirmed by the STM technique. The structural change of PEO-Hb on the ITO electrode should be analyzed, but that would be quite difficult.

The PEO-Hb was cast onto the ITO electrode and was faced with a glass plate at a distance of 150 μm to prepare a thin layer cell. The further procedure was the same as that for the PEO_{200} solution system, as mentioned above. A spectral change

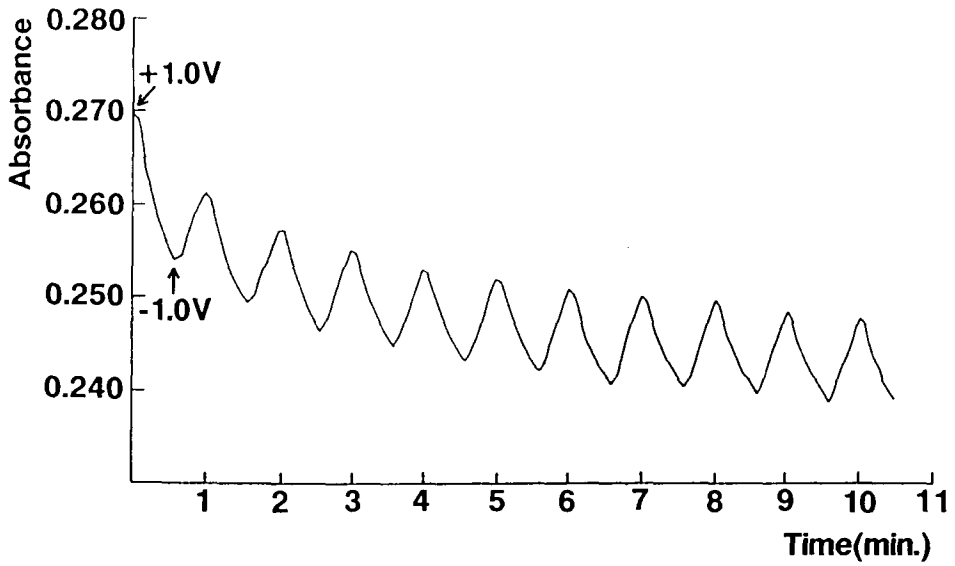

FIG. 6. Changes in the visible spectral intensity for PEO-Hb in PEO_{200} induced by the polarity change of the given potential (−1.0 and +1.0 V vs Ag) at 25°C. The polarity of the given potential was alternated every 30 seconds.

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similar to that found during reduction of the cast PEO-Hb in PEO oligomers with molecular weight of over 400. Figure 8 shows the visible spectra for PEO-Hb cast on the ITO electrode before and after electrochemical reduction in PEO_{400} containing 0.2 M KCl. The absorption maxima for oxidized and reduced PEO-Hb (cast on the ITO electrode) in PEO_{400} was 407.6 and 422.8 nm, respectively.

The spectral intensity change for PEO-Hb during reduction was also monitored with time, as shown in Fig. 9. In spite of a continuous decrease in the amount of oxidized PEO-Hb fraction caused by the reductive condition, the absorption intensity at 422.8 nm (attributed to the reduced fraction) did not change at the initial stage and then gradually increased. At present we have no idea of why this occurs. It is not due to the decrease of the protein concentration in the thin layer cell. The absorption intensity at the isosbestic points (e.g., 448.7, 525.1, and 577.8 nm) was also monitored to check the baseline drift. The unexpected intensity change at 422.8 nm (10 minutes after reduction) was confirmed not to be due to the decrease of the baseline. It might be due to any remained water molecule coordinated to the heme during casting from aqueous solution. It was empirically found that this initial decrease became small when the cast PEO-Hb layer was soaked in PEO oligomers and there was a wait of about an hour before the potential was given. The details, however, are still not clear.

Poly(oligo(oxyethylene) methacrylate) (PMEO) is known to be an excellent ion conductive solid polymers [13–15]. The electrochemical redox reaction of PEO-Hb was carried out in this typical ion conductive polymer. PEO-Hb was similarly cast on an ITO glass electrode and further covered with PMEO (containing 0.2 M KCl). This was then contacted with a PMEO (also containing 0.2 M KCl) coated

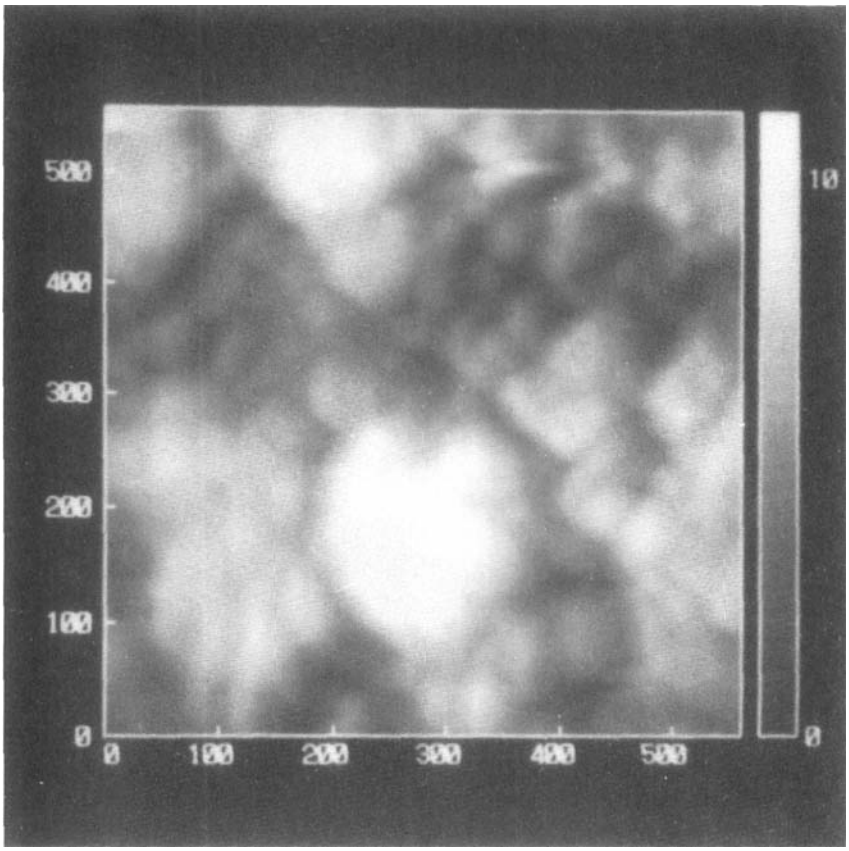

FIG. 7. Scanning tunneling micrograph for cast PEO-Hb on the ITO electrode.

ITO counterelectrode. A polished silver wire was then put into the PMEO phase as a reference electrode. This was set in a cell holder, and a potential was given to the ITO working electrode. A similar reduction of PEO-Hb was also carried out by a negative potential (< -0.6 V vs Ag). The oxidized PEO-Hb showed maximum absorption at 412.2 nm. This Soret band decreased, and a new Soret band increase at 423.0 nm was attributed to the reduced one. The maximum absorption wavelength was a function of the degree of PEO modification onto the heme proteins [12]. It was also affected by the average molecular weight of the matrix PEO, already mentioned above.

Figure 10 shows the visible spectral change of PEO-Hb during reduction. The spectral change certainly showed that PEO-Hb was reduced electrochemically, even in PMEO. However, diffusion of PEO-Hb is unnecessary for the reduction process,

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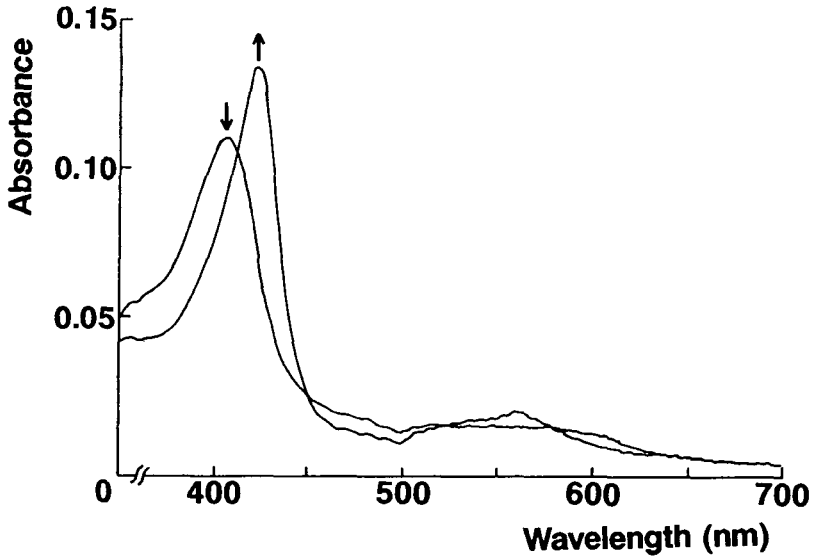

FIG. 8. Visible spectra for oxidized and reduced PEO-Hb cast on the ITO electrode in PEO_{400} containing 0.2 M KCl at 25°C. A potential of −1.0 V vs Ag was continuously applied to the ITO electrode for 90 minutes.

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which was very slow (see Fig. 10). This might be due to the characteristics of electron transfer between layered PEO-Hb. The cast system is schematically drawn in Fig. 11. We speculate the following mechanism to explain this slow reduction.

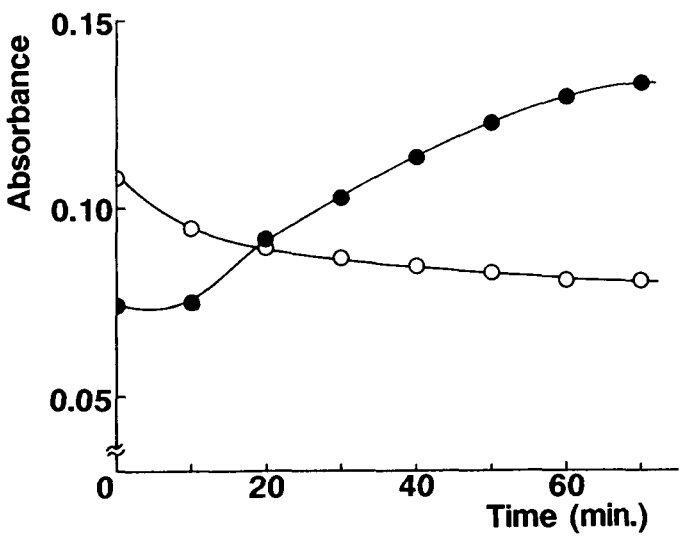

FIG. 9. Visible spectral intensity change for PEO-Hb cast on the ITO electrode during reduction in PEO_{400} containing 0.2 M KCl at 25°C. The absorption maxima at 407.6 and 422.8 nm (for oxi- and reduced-PEO-Hb, respectively) were recorded with time. ○: oxidized fraction, ●: reduced fraction.

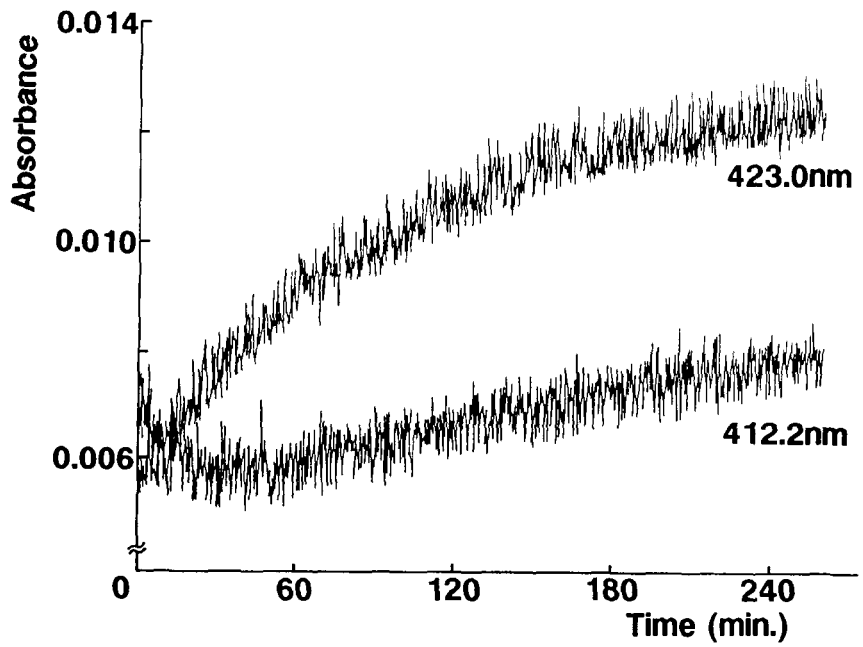

FIG. 10. Visible spectral intensity change for PEO-Hb cast on the ITO electrode during reduction in PMEO containing 0.2 M KCl at 25 °C. The absorption maxima at 412.2 and 423.0 nm (for oxi- and reduced-PEO-Hb, respectively) were recorded with time.

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The electron transfer reaction may be possible only when two PEO-Hbs are in a certain space arrangement suitable for electron transfer. This process should therefore be governed by the rotational diffusion coefficient of the PEO-Hbs in the cast

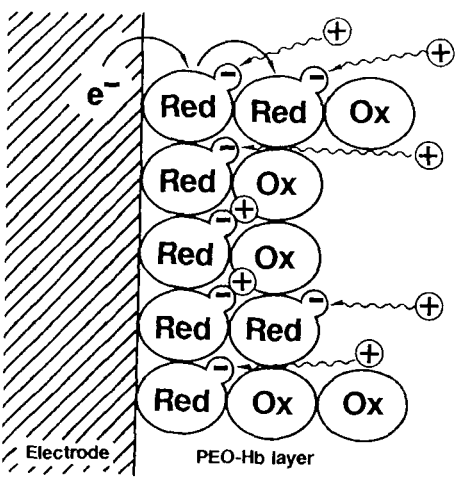

FIG. 11. Schematic illustration of the electrochemical reduction of substrates cast on the electrode in the polymer electrolyte.

layer. The bulk ionic conductivity is also considered to be effective for reduction. The ionic conductivity for PEO_{200} and PEO_{400} containing KCl is about 3×10^{-4} S/cm at 25°C. On the other hand, PMEO containing 0.2 M KCl shows an ionic conductivity of about 2×10^{-5} S/cm at 25°C. This relatively low ionic conductivity may involve slower migration of counterions. Acceleration of electron transfer is one of the remained tasks to be solved. Our study of the reversible reduction of PEO-modified heme proteins, which were coated as a single layer on the ITO electrode, will be reported elsewhere.

CONCLUSION

Reduction of Hb and Mb was carried out in PEO derivatives when they were modified with PEO. Reoxidation and further alternative redox reactions were also successful in these systems. Reduction and further reoxidation were not as fast in polymer electrolytes. The diffusion coefficient of PEO-modified proteins was very small in PEO oligomers. However, layered PEO-proteins gave a small electron transfer rate. Electron transport between adjacent proteins in the layer might be slow.

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