ISSN 1061-9348, Journal of Analytical Chemistry, 2006, Vol. 61, No. 7, pp. 669–672. © Pleiades Publishing, Inc., 2006.

ARTICLES

Electrochemistry and Electrocatalytic Properties of Heme Proteins Incorporated in Lipopolysaccharide Films[1]

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Received May 3, 2005

Abstract—The electrochemical behavior of heme proteins such as hemoglobin (Hb), myoglobin (Mb), and horseradish peroxidase (HRP) was studied in stable thin films composed of the proteins and a natural bacterial endotoxin (lipopolysaccharide, LPS). All three protein-LPS films exhibited a pair of well-defined, quasi-reversible cyclic voltammetric peaks in pH 6.0 phosphate buffers. Moreover, hydrogen peroxide and trichloroacetic acid could be reduced catalytically by the proteins entrapped in the films. Electrochemical parameters, such as the apparent Michaelis–Menten constant (K_m^{app}) and formal potentials (E^0), were obtained. The positions of the Soret absorption band of the proteins suggested that the three proteins could keep their secondary structure in LPS films.

DOI: 10.1134/S1061934806070112

1. INTRODUCTION

Bioelectrochemistry is a well-established discipline both for investigating protein redox properties and for fabricating new kinds of electrochemical biosensors [1, 2]. A key factor for successful bioelectrochemical investigations of redox proteins is to obtain an efficient electrochemical response at some electrode surface. Incorporation of proteins into films is a relatively new approach to realize this response, which may also help to avoid contributions to the experimental data deriving from protein diffusion in the solution. So far, many kinds of films containing heme proteins have been reported to produce quite stable electroactive arrays and well-defined redox peaks [3–15]. Proteins retain the native conformations in these films, and the electron transfer rates are greatly enhanced.

Lipopolysaccharide is a primary constituent of the outer cellular membrane of gram-negative bacteria [16]. The structure of LPS varies among bacterial species but is overall composed of three parts: lipid A, a variable polysaccharide chain, and a core sugar. It has been reported that lipid and polysaccharide films can effectively enhance the direct electron transfer between heme proteins and electrodes [5, 6]. We thus expect that an LPS which contains both lipid and polysaccharide chain may provide a new matrix for immobilization of proteins and may be used as a suitable microenvironment for heme proteins to exchange electrons directly with underlying electrodes.

In this study, three heme proteins including hemoglobin (Hb), myoglobin (Mb), and horseradish peroxidase (HRP) were incorporated in LPS films, and the films were modified on pyrolytic graphite (PG) electrodes. Enhanced reversible electron transfer between the heme proteins and underlying PG electrodes was realized. The protein–LPS films have been characterized by UV–Vis absorption spectra, which show that the proteins are not denatured. Furthermore, electrocatalytic reductions of hydrogen peroxide (H_2O_2) and trichloroacetic acid (TCA) have been observed, showing the potential applicability of the film-modified electrodes as biosensors.

EXPERIMENTAL

Chemicals. Human hemoglobin (MW 66.000), horse heart myoglobin (MW 17.800), horseradish peroxidase (MW 42.000), and lipopolysaccharide were obtained from Sigma. They were all used without further purification. Other chemicals were of analytical grade. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, United States) to a specific resistance above 16 MΩ/cm. The solutions were stored in the refrigerator at a temperature of 4°C when not in use.

Preparation of protein–LPS films. The PG electrode was first polished using rough and fine sand papers. Then its surface was polished to mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Finally, the electrode

[1] The text was submitted by the authors in English.

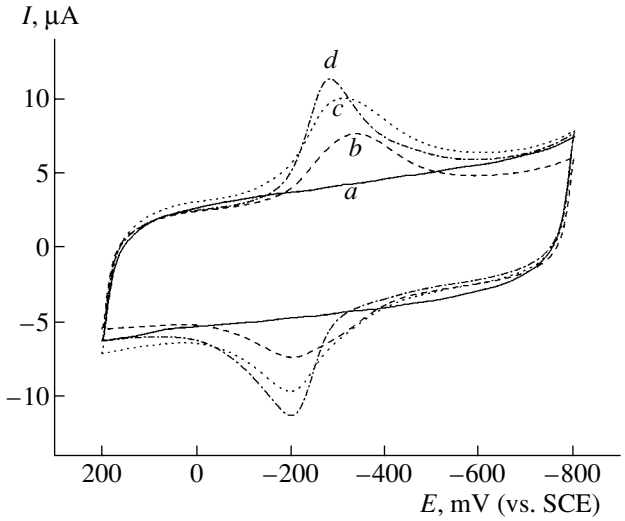

Fig. 1. Cyclic voltammograms at 200 mV/s in 0.1 M phosphate buffer (pH 6.0) for (*a*) LPS film, (*b*) Hb–LPS film, (*c*) Mb–LPS film, and (*d*) HRP–LPS film.

was thoroughly washed by ultrasonicating in both double-distilled water and ethanol for about 5 min.

The protein–LPS films were prepared as follows. Typically, 10 μL of dispersion containing 1.3×10^{-5} M Hb and 0.5 mg/mL LPS was spread evenly onto a freshly abraded PG electrode with a microsyringe for preparing Hb–LPS films. Protein concentrations in similar dispersions for the other films were 4.8×10^{-5} M Mb and 2.1×10^{-5} M HRP. A small bottle was fit tightly over the electrode so that water evaporated slowly and more uniform films were formed. Films were then dried overnight in air.

Analytical procedures. Cyclic voltammetry was performed on a Potentiostat/Galvanostat 283 (Princetin Applied Research, United States) using a three-electrode system. A one-compartment glass cell with a modified PG working electrode, a saturated calomel reference electrode (SCE), and a platinum wire auxiliary electrode were used for the measurements, with a working volume of 5 mL. All the following potentials reported in this work are versus SCE. Buffer solutions were purged with purified nitrogen, and a nitrogen blanket was maintained during scans.

UV–Vis absorption spectra were obtained with a Model UV-2201 spectrophotometer (Shimadzu, Japan). The UV–Vis absorption spectra measurements were performed in a 0.2 mg/mL heme protein solution or mixed solution of 0.2 mg/mL heme protein and 0.1 mg/mL LPS solution (the protein maintained 0.2 mg/mL in the test samples).

RESULTS AND DISCUSSION

Hb–, Mb– or HRP–LPS films modified PG electrodes can give pairs of well-defined reversible cyclic voltammetric peaks in protein-free pH 6.0 phosphate buffers, as is shown in Fig. 1. The anodic and cathodic peaks are located at –0.26 and –0.35 V for Hb, –0.24 and –0.31 V for Mb, and –0.21 and –0.27 V for HRP, respectively. The peak potentials are characteristic for the heme Fe^{III}/Fe^{II} redox couples of the proteins [3, 17, and 18]. Comparably, the PG electrode coated with LPS films alone shows no CV peak in the same potential range (Fig. 1a). Since the bare PG electrode cannot exhibit a voltammetric response effect, the redox peaks in Fig. 1 can be attributed to the redox reactions of the proteins entrapped in the films.

The pairs of the redox peaks of protein–LPS films have an approximately symmetric peak shape and nearly equal heights of reduction and oxidation peaks. The peak currents increase linearly with increasing scan rates from 0.02 to 1 V/s. Integrations of reduction peaks at different scan rates give nearly constant charge (Q) values. All these results are characteristic of quasi-reversible diffusionless thin-layer electrochemistry [19].

The stability of the protein–LPS films modified electrodes was examined. PG electrodes coated with the films are stored in pH 6.0 phosphate buffer, and cyclic voltammograms (CVs) are recorded periodically. All the Hb–, Mb–, and HRP–LPS films demonstrate an excellent stability. The CV peak potentials are at the same positions and the peak currents are nearly stable for at least 2 months.

CVs of the protein–LPS films show a strong dependence on pH. Both the cathodic and anodic peaks potentials of the Fe^{III}/Fe^{II} redox couple shift negatively with an increase in pH. The formal potentials (E^0), estimated as the midpoint of cathodic and anodic peaks potentials, have a linear relationship with pH in the range 3.0–10.0. The linear regression equations are $y = -6.8 - 49.7x$ ($R = 0.996$) for Hb–LPS films, $y = 31 - 51x$ ($R = 0.997$) for Mb–LPS films, and $y = 85 - 54.2x$ ($R = 0.997$) for HRP–LPS films. All these slope values are close to the theoretical value of –57.6 mV/pH at 18°C for a single-proton coupled reversible one-electron transfer [20, 21].

In order to check whether the proteins have been denatured after being entrapped in LPS films, UV–Vis spectra of the films were recorded. As is well known, the positions of the sensitive Soret absorption band of the heme prosthetic group for heme proteins can provide information about possible denaturation of the proteins [22, 23]. Taking Hb–LPS as an example, the Soret band of Hb is located at 405.20 nm, which is sensitive to variations of the microenvironments around the heme site. Previous studies have shown that the band should diminish or shift if the protein is denatured [23, 24]. Our experimental results reveal that the Soret band for entrapped Hb is also at 405.20 nm (Fig. 2, the dotted curve), suggesting that Hb in LPS films has a secondary structure similar to the natural state. Mb– and HRP–LPS films demonstrate behaviors very similar to Hb–LPS films.

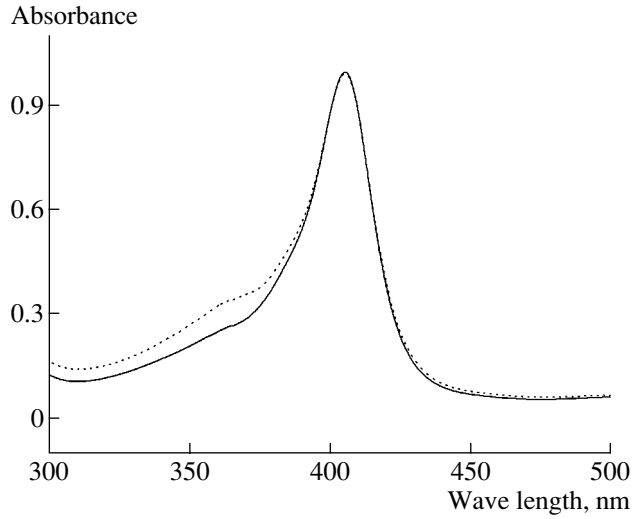

Fig. 2. UV–Vis spectra of Hb solution (the solid curve) and Hb–LPS solution (the dotted curve).

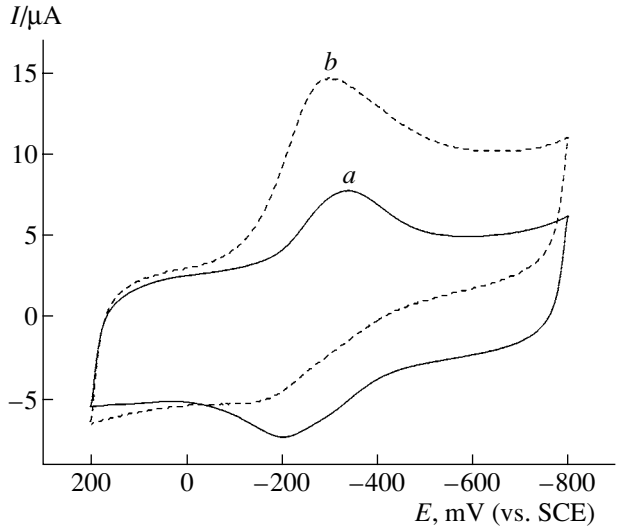

Fig. 3. Cyclic voltammograms obtained at a Hb–LPS modified PG electrode for a 0.1 M phosphate buffer solution pH 6.0 (*a*) before and (*b*) after the addition of 0.15 mM H_2O_2 to the buffer solution.

Further studies reveal that the proteins embedded in LPS films can also exhibit enzymatic activity towards hydrogen peroxide (H_2O_2). The electrocatalytic reduction of H_2O_2 by Hb in LPS films is shown in Fig. 3. When H_2O_2 is added to a pH 6.0 phosphate buffer, an increase in the reduction peak for Hb Fe^{III} at about –0.35 V is observed, accompanied by the disappearance of the oxidation peak for Hb Fe^{II}. However, no reduction peak of H_2O_2 can be obtained at LPS-only modified electrode in this potential range.

The catalytic reduction of H_2O_2 at the protein–LPS film electrodes can be used to determine H_2O_2 quantitatively. Taking Hb–LPS as an example, the linear relationship between the electrocatalytic reduction peak current and H_2O_2 concentration was observed from 1.0×10^{-7} to 8.0×10^{-4} M (Fig. 4). The linear regression equation is $y = 8.152 + 0.04233x$, with a correlation coefficient of 0.999. The detection limit is 4.0×10^{-8} M with a sensitivity of 0.04233 μA/μM H_2O_2. Five independent determinations at a H_2O_2 concentration of 50 μM show a relative standard deviation (R.S.D.) of 3.6%, which displays the nice reproducibility of these measurements. Mb–LPS and HRP–LPS films show similar catalytic behaviors toward H_2O_2. The linear ranges for Mb–LPS and HRP–LPS films are 6.0×10^{-7}–4.0×10^{-4} M and 4.0×10^{-7}–8.0×10^{-4} M, respectively.

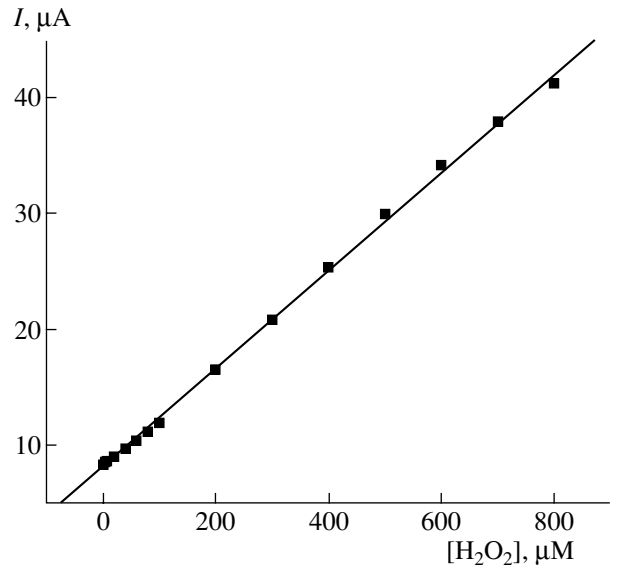

Fig. 4. The linear fitting program of cathodic peak current of Hb for H_2O_2 concentration from 1.0×10^{-7}– 8.0×10^{-4} M.

In order to evaluate the catalytic activity of the properties entrapped in LPS film, we calculated the apparent Michaelis-Menten constant (K_m^{app}) by the Lineweaver-Burk equation [25],

$$I/I_{ss} = I/I_{max} + K_m^{app}/(I_{max}c),$$

where I_{ss} is the steady current after the addition of substrate (with the current derived from heme proteins subtracted), c is the bulk concentration of the substrate, and I_{max} is the maximum current measured under the saturated substrate condition.

K_m^{app}, which gives an indication of the enzyme–substrate kinetics, is thus calculated to be 1309 μM for Hb–LPS films, 1512 μM for Mb–LPS films, and 1201 μM for HRP–LPS films.

The catalytic activity of the proteins in LPS films toward TCA was also investigated. As shown in Fig. 5, after TCA is added to a pH 6.0 phosphate buffer, the Mb

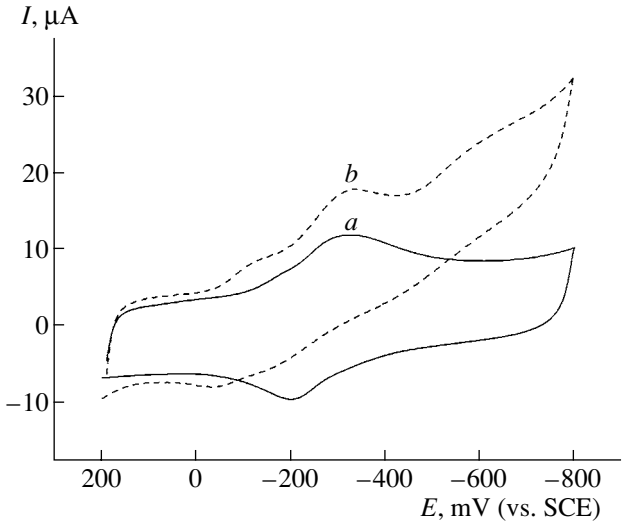

Fig. 5. Cyclic voltammograms obtained at a Mb–LPS modified PG electrode for 0.1 M phosphate buffer solution at pH 6.0 (*a*) before and (*b*) after the addition of 0.02 M TCA to the buffer solution.

Fe^{III} reduction peak of Mb–LPS films at about –0.31 V increases. The reduction peak current for Mb Fe^{III} increases with increasing concentration of TCA in solution. However, no catalytic reduction peak of TCA can be observed at the LPS-only modified electrode in the same potential range, which is characteristic of electrochemical catalysis [26, 27]. For Hb–LPS and HRP–LPS films, similar catalytic behaviors toward TCA can be observed.

ACKNOWLEDGMENTS

We thank the National Natural Science Foundation of China (grant no. 30070214) and the Chinese Ministry of Education (grant no. 01087) for financial support.

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