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Preparation of Phosphate-Binding-Protein-Modified Electrode and Its Application to Reagentless Phosphate Sensor

Izumi Kubo and Daisuke Satoh

Graduate School of Engineering, Soka University, Hachioji, Tokyo 192-8577, Japan

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To develop a simple and reagentless phosphate sensing system, phosphate binding protein (PBP) was utilized as a recognition element for the sensor. The preparation of a PBP-modified electrode was investigated. PBP was immobilized on a gold electrode surface through cysteamine and glutaraldehyde. The amount of immobilized PBP was 13.7 ng/mm^2, which corresponded to three layers of PBP. The response of the PBP-modified electrode to phosphate was measured as the potential change of the electrode and the response time was about 2 min. The response to phosphate was –0.505 mV/mM in the concentration range of 10–50 mM of phosphate. To evaluate the nonspecific response to ions other than phosphate, a malic-dehydrogenase-immobilized electrode was compared with the PBP-modified electrode. The response to phosphate of the PBP-modified electrode was specific but those to other ions were almost same as those of the malic-dehydrogenase-modified electrode.

1. Introduction

Phosphate is a major component of living organisms, as a part of nucleic acids, membrane lipids, ATP and phosphorylated proteins. Phosphate is essential for all living things, not only animals but also plants and microorganisms.

Animals acquire phosphate from food, but plants and microorganisms absorb it from their environment. In nature, biological abundance depends on the concentration of phosphate, and sometimes an overabundance of phosphate causes an explosive growth of phytoplankton.

*Corresponding author, e-mail address: kubo@t.soka.ac.jp

Determination of phosphate concentration is necessary for environmental monitoring, food quality control and *in vivo* monitoring. Conventionally, phosphate concentration has been determined by a colorimetric method which is based on the molybdenum-blue method.[1] This method is sensitive to phosphate concentration; however, it requires too much time to be applied to phosphate monitoring.

In place of a colorimetric method, biosensors have been proposed and investigated for the determination of phosphate. Watanabe *et al.* proposed a phosphate sensor based on the inhibition of nucleoside phosphorylase and xantine oxidase by phosphate and using an oxygen electrode as a transducer.[2] d'Urso and Coulet modified this method, coupling it with a hydrogen peroxide electrode.[3] These phosphate sensors were composed of two or three enzymes and a transducer. Usually the optimum conditions for each enzyme are different and the system becomes very complicated. In addition, substrates for enzyme reactions other than phosphate are necessary in these systems.

To establish a simple and reagentless determination system, a novel sensing system is proposed in this study. It is based on phosphate-binding protein (PBP). PBP is a component of a phosphate transport system in *Escherichia coli*, and binds phosphate in the periplasmic space of the cell[4] among various inorganic and organic ions, and releases phosphate to an intrinsic transporter protein. Therefore, a phosphate-sensing system based on PBP will be a reagentless sensing system. PBP is a member of a binding-protein family and is a single polypeptide protein. The characteristics of PBP were investigated by Kubena *et al.*[5] The tertiary structure of PBP and its binding site have been elucidated by X-ray crystallography.[6]

In a previous study, it was demonstrated that phosphate concentration could be determined using PBP in a potentiometric method, in which PBP was immobilized on a membrane.[7] A potential change in the PBP-immobilized membrane was caused by phosphate binding. The system consisted of the PBP-immobilized membrane and two Ag/AgCl electrodes. The transducer was separated from the PBP-immobilized membrane. To make the system easier to use, immobilization of PBP on the electrode surface was investigated.

Recently, modifications of the surface of gold electrodes with a self-assembled monolayer have been investigated and applied to biosensors.[8] As a result of these modifications, a single molecular recognition layer was fabricated and the biological recognition elements were effectively used. In an electrochemical system, a high density of recognition elements on the surface of an electrode enables us to carry out more sensitive determinations of analyte concentration. PBP immobilized on the electrode binds phosphate through hydrogen bonds, and the charge density of the PBP-immobilized layer changes when phosphate binds. Phosphate molecules gather in the PBP layer on the surface of the electrode by binding to PBP (Fig. 1). Phosphate binding causes an increase in charge density and a potential change. Kubo and Nagai have already reported that sulfate binding protein, which is also produced by *E.coli*, was modified on the surface of a gold electrode and used to determine sulfate concentration.[9] However, the preparation of an electrode modified with binding protein has not yet been investigated in detail. Therefore, the conditions for modifying the electrode with PBP are important and were investigated in this study. The PBP-modified electrode was applied to the determination of phosphate concentration.

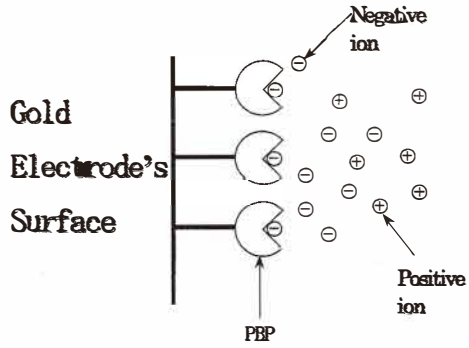

Fig. 1. Scheme of the gold electrode surface modified with PBP.

2. Materials and Methods

2.1 *Preparation of phosphate binding protein*

PBP was derived from *E.coli*. The PBP was extracted from cells collected from the liquid medium of *E.coli* by an osmotic shock method according to a previously reported procedure. It was purified by DEAE column chromatography.[10]

2.2 *Modification of gold electrode with phosphate binding protein*

After the purification, the PBP solution was concentrated and used for the immobilization. PBP was immobilized on the electrode as follows. Gold electrodes (diameter 1.6 mm, AUE) were purchased from BAS. First, the surface of the gold electrode was carefully polished with 0.6 μm aluminum powder. It was sonicated in ultrapure water for 15 min. Immediately after the sonication, the electrode was immersed in the solution of cysteamine (10 mg/ml) for 1 h, and subsequently immersed in glutaraldehyde (2.5%) solution at room temperature. The electrode was rinsed with water to remove excess glutaraldehyde and immersed in a PBP solution of Tris-HCl buffer at 5°C overnight. The concentration of PBP was 0.1–10 mg/ml. The resulting electrode was carefully rinsed with Tris-HCl buffer and used to measure the analyte concentration.

2.3 *Evaluation of mass of immobilized PBP on surface of electrode*

Prior to the evaluation of the mass, modification of the electrode was confirmed using cyclic voltammetry of the hexacyanoferrate (II)/ hexacyanoferrate (III) redox reaction. Cyclic voltammetry was carried out with a computerized electrochemical analyzer CV-50 W and a three-electrode system vs an Ag/AgCl reference electrode.

Evaluation of the mass of PBP on the electrode was carried out using a quartz crystal microbalance. A quartz crystal with a gold electrode was modified with PBP. A frequency analyzer (QCM analyzer QCA917, Seiko EG&G) was used to estimate the mass of PBP on the electrode.

2.4 *Electrochemical setup for potential measurements using PBP-modified electrode*

The potential of the PBP-modified electrode was measured according to the previously reported method. The experimental setup is shown in Fig. 2. As a reference electrode, an Ag/AgCl electrode was used. The PBP-modified electrode and the reference electrode were set in a glass vial containing 10 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM KCl. Solutions of Na_2HPO_4 and K_2HPO_4 were used as the phosphate sample solution. As other ionic solutions, potassium or sodium salts of chloride, and sulfate and nitrate solutions (1 M) were also used. The measurement was carried out while adding these solutions to the vial to the given concentration at room temperature.

3. Results and Discussion

3.1 *Modification of gold electrode*

The modification of the electrode surface with a self-assembled monolayer (SAM) was confirmed by cyclic voltammetry of a hexacyanoferrate (II)/hexacyanoferrate (III) redox couple.[11,12] Modification of PBP was checked. Even at a concentration of 0.1 mg/ml, a decrease in the peak currents of hexacyanoferrate(II)/hexacyanoferrate(III) was observed (Fig. 3). The peak current decreased as the concentration of PBP increased. On the other hand, modification of the gold electrode with cysteamine and glutaraldehyde had no effect on the cyclic voltammetry of the redox couple. Because of the modification, the access of hexacyanoferrate to the electrode surface was hindered by PBP molecules, and the extent of the hindrance depended on the concentration of PBP.

It was suggested that a higher concentration of PBP would result in a higher coverage of the surface. Direct evaluation of PBP immobilized on the electrode was carried out using QCM. The mass of immobilized PBP was estimated from the equation of Sauerbrey.

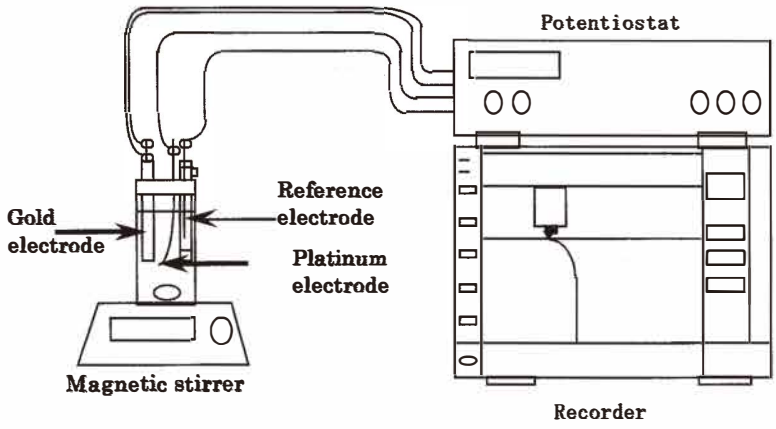

Fig. 2. Electrochemical setup. The potential of the PBP-modified electrode was measured vs a saturated calomel reference electrode in the buffer of 50mM Tris/HCl, pH 8.5.

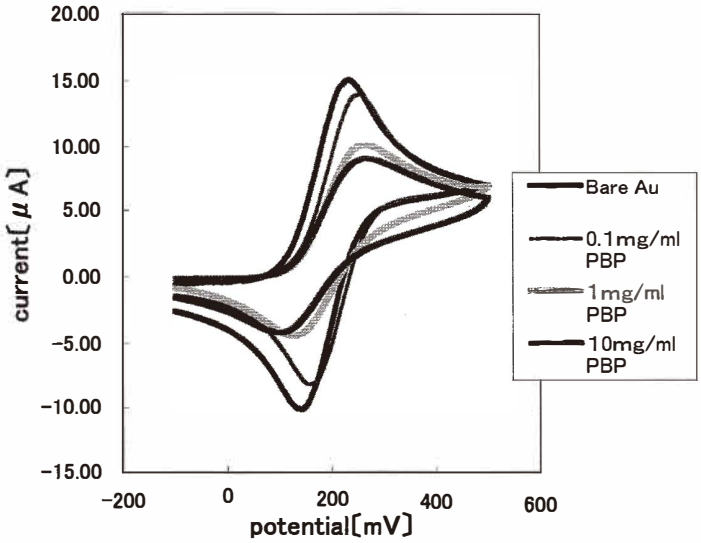

Fig. 3. Cyclic voltammmogram of hexacyanoferrate (II) /hexacyanoferrate (III) redox couple. 10mM hexacyanoferrate (II), scan rate 10mV/sec.

The area of quartz is circular. The frequency of the AT-cut quartz was 9 MHz in this study. The estimated mass is shown in Table 1. The modified mass of PBP increased as the concentration of PBP increased during the immobilization and the density was 9.48 to 13.7 ng/mm^2.

On the other hand, in the case of complete coverage of the surface of the QCM, the expected mass of PBP can be calculated as follows. It is assumed that PBP is immobilized on one side of the gold electrode surface of the QCM. From the area of the electrode (a circle 5 mm in diameter) and dimensions of the PBP molecule (3.5 nm×4.0 nm×7.0 nm), the number of PBP molecules (N_{PBP}) can be estimated as 1.40×10^{12}.

From the molecular weight of PBP, the calculated mass of the single layer of PBP is 79.8 ng (4.07 ng/mm^2). The mass measured with PBP was approximately 2.2 to 3.4 times greater than the calculated one. As PBP is a water-soluble protein, it is hydrated and the apparent mass of PBP including water molecules might be measured by QCM. The gold on the QCM was sputtered onto the quartz crystal, and small grains of gold particles gathered on the surface. Therefore, the area of the QCM might be underestimated in our calculation.

Plant reported that the single or double layers of alkanethiol on the gold electrode completely suppressed the redox reaction with hexacyanoferrate.[11] The mass of these layers was too small to be detected by QCM. However, the extent of the suppression of the redox reaction was large but the immobilized mass of alkanethiol was much smaller than that of PBP modification. It was considered that PBP was hydrophilic and its isoelectric point was about 7.0. There was not complete suppression of the redox reaction in spite of larger amount of immobilization. At the concentration of 0.1 mg/ml of PBP there remained sufficient space between PBP molecules on the electrode for hexacyanoferrate to access its surface. Since such space was almost entirely filled with PBP molecules at the concentra-

Table 1
Concentration of PBP in the immobilization and the amount of PBP estimated from the frequency shift of QCM.

PBP conc. (mg/ml)	0.1	1	10
PBP(ng)	182	218	268

tions of 1 mg/ml and 10 mg/ml, the redox current decreased markedly. However, the redox current was observed even after the modification at 10 mg/ml of PBP because PBP molecules were hydrolyzed and a little space still remained for hexacyanoferrate to pass through.

The surface of the bare gold electrode was observed by atomic force microscopy (Fig. 4). The surface was not smooth, and grains of 50–100 nm in diameter covered the surface.

As can be seen in Fig. 4(b), on the PBP-modified surface was mostly covered with small particles, which looked like PBP, at the concentration of 10 mg/ml. The scattered PBP molecules did not cover the surface of the gold grains at a concentration of 0.1 mg/ml of PBP (data is not shown). From these observations, a higher concentration of PBP resulted in greater coverage of the surface, but the mass of immobilized PBP did not depend linearly on the concentration of PBP.

3.2 *Response to phosphate of PBP-modified electrode*

The response to phosphate of the PBP-modified electrode was compared at different concentrations of PBP. Figure 5 shows a typical time response to phosphate of the PBP-modified electrode. The response time was within 2 min and there was no difference between the concentrations of PBP in the modifications. The response of the electrode modified in 10mg/ml of PBP to phosphate is shown in Fig. 6. The slopes of each electrode were determined by the method of least squares, and are listed in Table 2. The larger the amount of PBP on the electrodes, the higher the response to phosphate. PBP-modified electrodes at 0.1 mg/ml and 1 mg/ml showed a saturated response at concentrations greater than 40 mM of phosphate.

Among these electrodes, the immobilization at 10 mg/ml of PBP resulted in the greatest sensitivity to phosphate. The PBP-modified electrode was prepared hereafter at a concentration of 10 mg/ml of PBP.

3.3 *Selectivity of PBP-modified electode*

Using the PBP-modified electrode, selectivity to other ions was investigated. Responses to chloride, sulfate and nitrate were compared with that to phosphate. The responses of the bare gold electrode and the malic dehydrogenase (MDH)-modified electrode were compared. As the isoelectric point of MDH is 7.0 and is the same as that of PBP, the MDH-modified electrode was chosen for the comparison with the PBP-modified one.

The slopes of the responses to various ions are listed in Table 3. A bare gold electrode responded weakly to the ions examined. There was not much difference between the response of the MDH-modified electrode and that of the PBP-modified electrode except for phosphate. However, responses to phosphate of the PBP-modified electrode were three

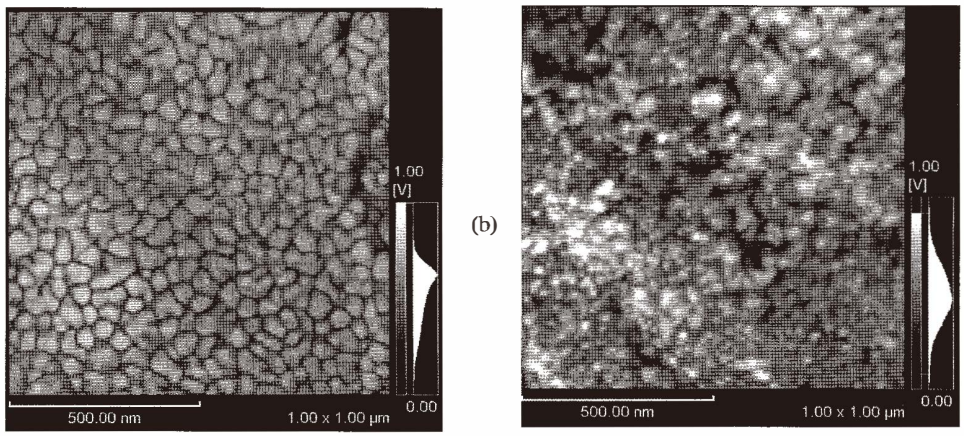

Fig. 4. AFM image of bare Au and PBP-modified electrode. (a)Bare Au, (b)PBP-modified at the concentration of 10 mg/ml PBP.

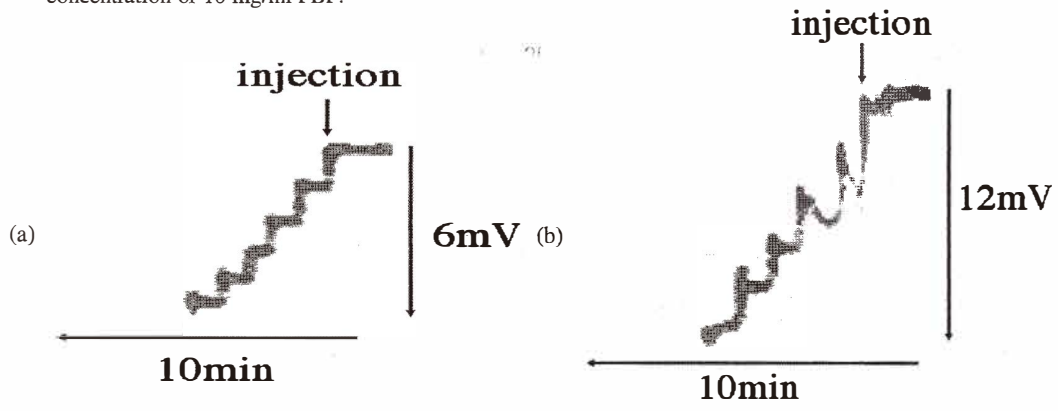

Fig. 5. Typical response curves of PBP-modified electrode: (a)response to KCl, (b)reponse to K_2HPO_4.

times higher than those of the MDH electrode. This result demonstrated that the PBP-modified electrode showed a selective response to phosphate.

Although the response of the PBP-modified electrode was selective, it did respond to other ions. Protein molecules are generally polyionic, and nonspecific ionic interactions occur and result in nonspecific responses. Therefore, a reference electrode such as the MDH-modified electrode is indispensable for practical use when the PBP-modified electrode is used as a phosphate sensor.

We have been working on a sulfate-binding-protein-modified electrode.[9] In this case, nonspecific responses to ions other than sulfate were not observed, and they can be eliminated using an appropriate protein-modified electrode as a reference.

Binding-protein-modified electrodes do not need any reagent to detect the target ion. Therefore, the system is quite simple. The possibility of using binding proteins as recognition elements was demonstrated in this study.

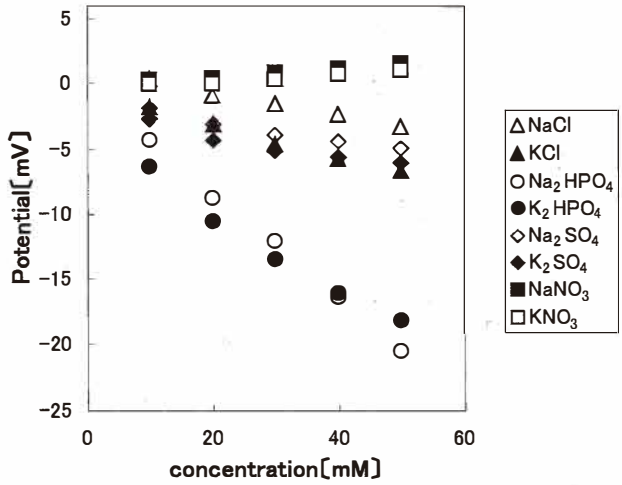

Fig. 6. Response of PBP-modified electrode to various ions. The electrode was modified with PBP at the concentration of 10 mg/ml.

Table 2
Concentration of PBP in the immobilization and the response to phosphate. The slope of the response (mV/mM) is listed.

PBP conc., mg/ml	0.1	1	10
Response, mV/mM	–0.384	–0.451	–0.505

Table 3
Comparison of the responses of PBP-modified electrode, bare Au and MDH-modified electrode. The slope of each response is listed.

Eelectrode	Cl^-	HPO_4^{2-}	SO_4^{2-}	NO_3^-
Bare Au	–0.03	0.098	–0.007	0.042
MDH modified	–0.134	–0.174	–0.061	–0.01
PBP modified	–0.122	–0.505	–0.073	–0.029

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