Biosensors and Bioelectronics 27 (2011) 58–63

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A membrane protein based biosensor: Use of a phosphate – H^+ symporter membrane protein (Pho84) in the sensing of phosphate ions

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ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form 29 May 2011
Accepted 10 June 2011
Available online 25 June 2011

Keywords:
Capacitive biosensor
Membrane protein
Pho84
Phosphate
Symporter

ABSTRACT

A label free biosensor for direct detection of inorganic phosphate based on potential-step capacitance measurements has been developed. The high-affinity Pho84 plasma membrane phosphate/proton symporter of *Saccharomyces cerevisiae* was used as a sensing element. Heterologously expressed and purified Pho84 protein was immobilized on a self-assembled monolayer (SAM) on a capacitance electrode. Changes in capacitance were recorded upon exposure to phosphate compared to the control substance, phosphate analogue methylphosphonate. Hence, even without the explicit use of lipid membranes, the Pho84 membrane protein could retain its capacity of selective substrate binding, with a phosphate detection limit in the range of the apparent *in vivo* K_m. A linear increase in capacitance was monitored in the phosphate concentration range of 5–25 μM. The analytical response of the capacitive biosensor is in agreement with that the transporter undergoes significant conformational changes upon exposure to inorganic phosphate, while exposure to the analogue only causes minor responses.

1. Introduction

Affinity biosensors based on protein recognition have almost exclusively been constructed by use of soluble proteins (Bontidean et al., 2001; Mattiasson et al., 2010; Teeparuksapun et al., 2010) immobilized onto the transducer surface of the sensor. In spite of the fact that cell membranes harbours 20–30% of the cellular proteins (Krogh et al., 2001), many of them responsible for cellular sensing, signalling and transport, this source has so far not been applied extensively in biosensor constructions. This class of trans-membrane proteins represents an interesting group of affinity binders that certainly could offer new opportunities and new analytical challenges when incorporated into biosensors. A drawback however, is the unique properties of this class of hydrophobic integral proteins, which are generally difficult to handle by use of conventional techniques used for soluble proteins. Isolation and purification of these proteins is often accompanied by loss of the functional structure (Carpenter et al., 2008; Junge et al., 2008).

When using a capacitive biosensor where the basis for the measurement is displacement of the ion layer from an insulated electrode surface, it is important to either bind large units that can induce such a displacement, operate with proteins that undergo drastic conformational changes upon binding of small molecules or by using small molecules as displacers of larger structures (Bontidean et al., 1998; Labib et al., 2009). Many membrane proteins bind small signalling substances and convey action by conformational changes. If the intention is to exploit these types of structural dynamics in constructing biosensors, then intact membrane proteins must be used.

In unicellular eukaryotes such as yeast, regulation of inorganic phosphate (P_i) transport is maintained by transduction of nutrient signals across the plasma membrane into the cell and the nutrient controlled transcriptional regulation of the phosphate transport systems. The mechanism involved in the cellular phosphate response of *S. cerevisiae* forms part of a complex cascade pathway, the *PHO* regulon, a genetic regulatory circuit involving five phosphate transporters; two high-affinity transporters and three low-affinity transporters expressed during phosphate-limiting and surplus conditions, respectively (Persson et al., 2003; Mouillon and Persson, 2006; Wykoff et al., 2007). Of these, the high-affinity Pho84 phosphate permease encoded by the *PHO84* gene (Bun-ya et al., 1991) is an integral plasma membrane transporter

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0956-5663/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.bios.2011.06.024

belonging to the family of phosphate/H^+ symporters (PHS) of the Major Facilitator Superfamily (MFS) (Saier et al., 1999, 2008, 2009). The Pho84 allows a co-ordinated cellular response and adaptation to changes in availability of external free phosphate (Mouillon and Persson, 2006; Wykoff et al., 2007; Lundh et al., 2009). The general mechanisms of substrate signalling and transport of the MFS proteins are still to a large extent unknown, mainly due to the lack of detailed crystal structures. Only a small number of MFS members have been crystallized (Huang et al., 2003; Abramson et al., 2003; Dang et al., 2010). So far, a kinetic model describing the mechanistic features of the Pho84 protein is still lacking. MFS transporters are believed to function via a single binding site in an alternating mechanism accompanied by a rocker-switch movement (Law et al., 2007) resulting in at least three major conformational states during the transport cycle: an inward facing conformation (C_i), and an outward facing conformation (C_o), and a more compact, occluded conformation in the transition state (Lemieux et al., 2004). Recently, it has been shown that the Pho84 possesses the functionality of both a nutrient transporter and a nutrient receptor, hence the name 'transceptor' (Holsbeek et al., 2004; Thevelein and Voordeckers, 2009; Popova et al., 2010). This functionality implies that Pho84 undergoes the three conformational steps, typical for transporter members of MFS. Since these conformational states are expected to have significant impact on the total dimensions of the transporter we propose that these changes should be detectable by use of a capacitive biosensor.

In the present study we have successfully used the Pho84 protein as a biosensing element in a capacitive biosensor integrated in a flow injection analysis (FIA) system using phosphate ions as the target analyte. For a cartoon model of the sensor setup see Fig. 1A. Capacitive biosensors can be constructed by immobilizing recognition elements in thin layers on the electrode and measuring changes in the dielectric properties when an analyte binds to the biosensing element. Capacitive biosensors are based on the principle that for an electrolytic capacitor, the capacitance depends on the thickness and dielectric layer on the surface of a metal (Gebbert et al., 1992).

Setting up a protein-based capacitative transducer (a Dropsens® gold electrode) during this study was challenging as the immobilization and stability of the Pho84 membrane transporter was of vital importance. Capacitive biosensors are built upon an electrode that is well insulated from the surrounding liquid. Often, a self-assembled monolayer (SAM) of alkylthiols on a gold surface is used. For immobilization of conventional soluble proteins there are many examples on how this has been done successfully (Mattiasson et al., 2010). However, when dealing with a membrane protein the immobilization procedure becomes an interesting challenge. In order to maintain its 3-dimensional structure, the protein is complexed with surface-active compounds to block the hydrophobic membrane-transversing units. Upon binding to the surface compounds, it is of pivotal importance that the orientation of the protein is such that it is able to interact with the phosphate, a protein–substrate interaction, which will give rise to a conformational change that results in a measurable signal. The capacitance of the electrode surface from the SAM layer formation to the protein immobilization was followed by cyclic voltammetry (CV) to study the quality of insulation achieved during the progressive level of immobilization of the protein on the gold transducer with the thioctic acid linker molecule.

2. Materials and methods

2.1. Materials

α-Lipoic acid, dodecanethiol, 1-[{3-(dimethylamino)propyl}]-3-ethylcarbodiimide hydrochloride (EDC) lysozyme, protease inhibitor cocktail for use during His-tag-mediated purification, resin coupled with anti-FLAG and anti-FLAG M2 monoclonal antibodies were purchased from Sigma (St. Louis, Mo, USA). Dropsens® electrodes (DRP-220AT) were purchased from Oviedo (Asturias, Spain). Hi-Trap column was purchased from GE Healthcare (Piscataway, NJ, USA). PVDF membranes were purchased from Millipore (Boston, MA, USA). All other reagents were obtained from commercial sources.

2.2. Pho84 isolation and purification

2.2.1. Plasmid construction

The *PHO84* gene was PCR amplified from genomic *Saccharomyces cerevisiae* DNA and cloned into a pTrcHisB expression vector (Invitrogen, UK) as previously described (Lagerstedt et al., 2004). The resulting pTrcHisB-His6/Xpress-Pho84-FLAG construct was transformed into a TOP10F (Invitrogen, UK) cell line.

2.2.2. Growth and expression of Pho84

Cells harbouring the *PHO84* wild type plasmid construct were pre-cultivated over night in 100 mL LB medium supplemented with 50 μg/mL ampicillin. The pre-cultivated cells were harvested by centrifugation and resuspended in Terrific Broth (50 μg/mL ampicillin) and transferred to a 5 L Erlenmeyer flask containing 1.5 L Terrific Broth (50 μg/mL ampicillin) medium giving a spectral absorbance of about 0.1 at 600 nm (A_{600}). The culture was grown aerobically at 200 rpm and 37 °C. At an A_{600} of 0.6, 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added. After 6 h of induction at 200 rpm and 30 °C, cells were harvested by centrifugation at 8000 × *g* for 10 min. Cell pellets were stored at −80 °C.

2.2.3. Protein extraction

Cells thawed on ice were resuspended to a total volume of 30 mL in buffer A containing 50 mM Tris–HCl (pH 7.6), 0.2 M NaCl, 10 mM $MgCl_2$, 20 mM imidazole, 10% glycerol, and 0.1% Triton X-100 (0.45 μm sterile filtered) to which 1 mg/mL lysozyme and protease inhibitor cocktail were added. The suspension was incubated for 30 min on ice. Fractions of 10 mL of the suspension were sonicated for 20 s with a micro-tip sonicator. Triton X-100 and n-Dodecyl-beta-D-Maltoside were added to a final concentration of 0.4% (v/v) and 0.1% (w/v), respectively, and the suspension was incubated for an additional 30 min at 4 °C, with gentle agitation, followed by a repetition of the sonication step. Cell debris and unbroken cells were separated from solubilised proteins by high-speed centrifugation for 30 min at 100,000 × *g*. The filtered (0.22 μm) supernatant was immediately used for dual affinity purification.

2.2.4. Affinity purification

The protein extract was applied at a flow rate of 1 mL/min onto a 1 mL Hi-Trap column, connected online to a FPLC system (Äkta, GE Healthcare), pre-charged with Ni^{2+} according to the manufacturer's protocol. Buffer A with an imidazole concentration of 20 mM was used to prevent binding of protein contaminants having a low affinity for the resin. The enriched Pho84 protein was eluted with 150 mM imidazole in buffer A. The pH of the eluate was verified before batch exposure to 1 mL anti-FLAG resin activated according to the manufacturer's protocol. Contaminants were washed out with 50 mM Tris–HCl (pH 7.5), 150 mM NaCl. Finally, elution of the Pho84 was performed using 50 mM glycine–HCl, pH 3.5. The eluted fractions were brought to a neutral pH by adding 1 M Tris–HCl, pH 8.0.

2.2.5. Analysis of Pho84 expression

Equivalent amounts of solubilised protein (2 μg) were separated on a 12% (v/v) polyacrylamide SDS-gel. Immunoblotting to PVDF

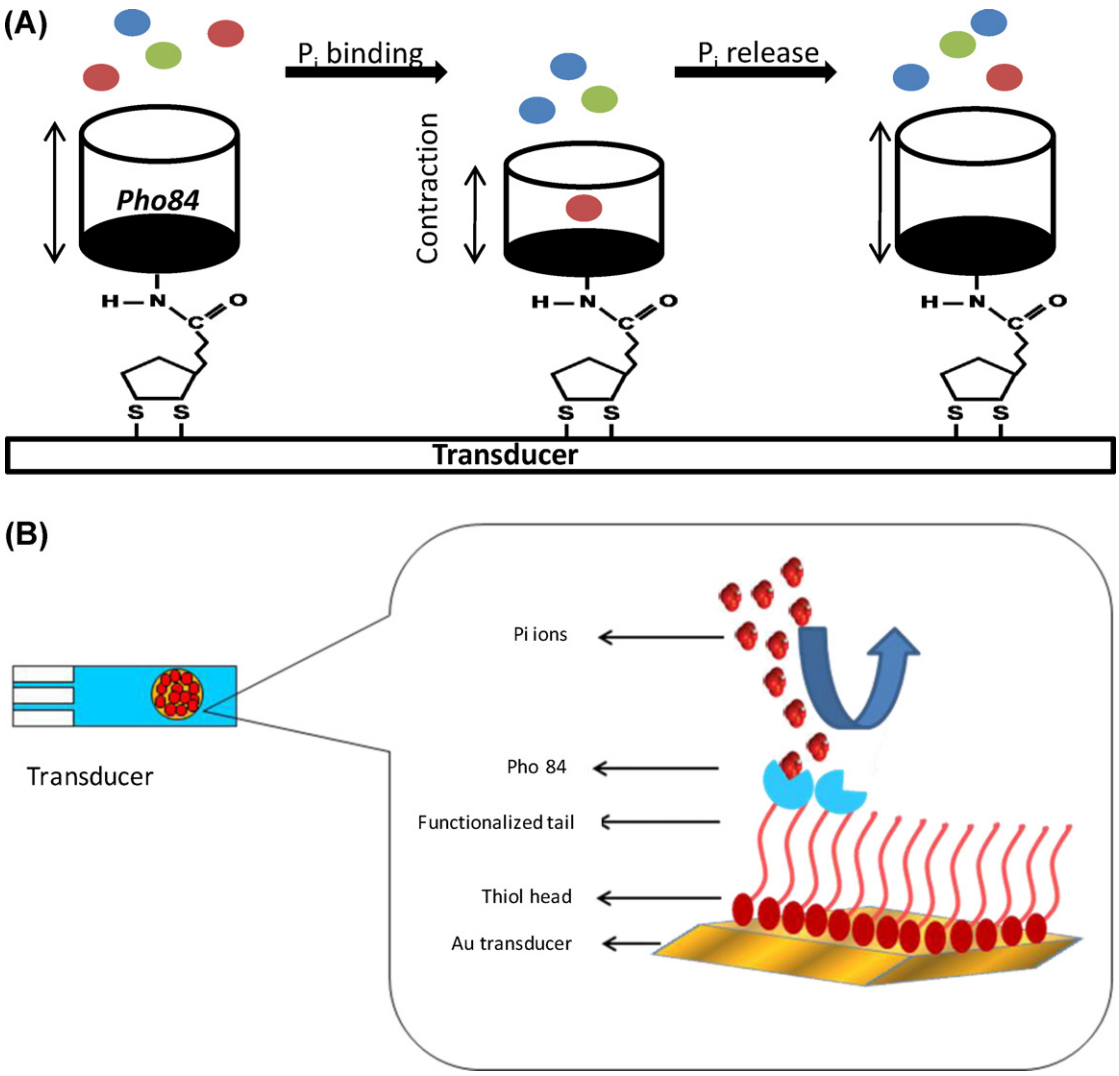

Fig. 1. In (A) a schematic representation of the Pho84 interaction with its analyte (P_i) is shown. (B) Visualize the different stages of the electrode preparation using screen printed gold electrode for P_i sensing using the Pho84 membrane protein immobilized on a thiocticacid SAM layer.

membranes was carried out according to the manufacturer's protocol and probed with primary anti-FLAG M2 monoclonal antibodies, followed by incubation with horseradish peroxidase-conjugated secondary anti-mouse antibodies. Protein was assayed by use of the commercially available Bio-Rad Quick Start Bradford Protein Assay method and bovine serum albumin was used as a standard.

2.3. Preparation of electrodes

The sensing platform was based on a gold transducer using a carboxylic acid terminated alkylthiol. Dropsens® electrodes were initially rinsed thoroughly with ethanol and dried in a nitrogen atmosphere. Thioctic acid (30 μL) was added on the gold surface of each electrode and then placed in a box with an ethanol saturated atmosphere and left overnight at room temperature for the SAM layer formation. Later, the electrode was transferred to a moisture box and incubated in an oven at 55 °C for 1 h. The carboxyl groups of the SAM were then activated using EDC (2% w/v) in water free acetonitrile (dried over molecular sieves) of which 30 μL was added and incubated at room temperature for 5 h. The electrode was then rinsed with running buffer (10 mM Tris–HCl, pH 7.5) and 1.26 μg of the sample protein was added onto the gold surface and left overnight at 4 °C. Complete insulation was achieved by blocking any pin holes (uncovered spots on the transducer surface) by treating the electrode with a long chain alkylthiol, dodecanethiol, for around 30 min and later washing with running buffer to remove any surplus of dodecanethiol (Fig. 1B).

2.4. Cyclic voltammetry

This electrochemical technique is an easy method of obtaining information about the degree of insulation on the gold surface beginning from SAM layer formation to the protein immobilization and correction of the electrode surface with dodecanethiol. Cyclic voltammetry was performed in a potentiostat/galvanostat (Autolab PGSTAT 12, The Netherlands) equipped with a GPES 4.8 software. A standard three electrode system configuration was used in which the gold surface of the Dropsens® electrode served as the working electrode, platinum served as the counter electrode and Ag/AgCl was the reference electrode. A scan rate of 100 mV/s was used during investigating the level of insulation. The degree of insulation was verified using a permeable redox couple 10 mM $K_3[Fe(CN)_6]$ in 100 mM KCl electrolyte solution at a voltage range between −0.3 and +0.6 V.

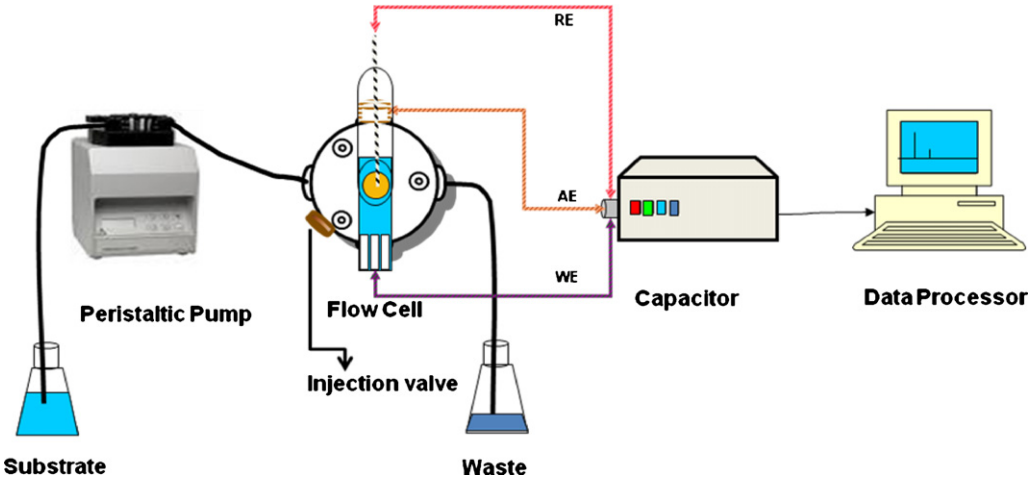

Fig. 2. Schematic view of the experimental set up. RE – reference electrode, AE – auxillary electrode, WE – working electrode.

2.5. Capacitive measurements

In Fig. 2 is shown the basic experimental set up of the flow injection-based capacitive biosensor system. The capacitive sensor was part of a potentiostatic three electrode system which was connected to a potentiostat which was in turn connected to a computer. The three electrode system consisted of the gold electrode (Dropsens® (DS) electrode), reference electrode (Ag/AgCl) and an auxiliary electrode made of platinum. A potential pulse of 50 mV was applied to the working electrode. Running buffer consisting of 10 mM Tris–HCl at pH 7.5 was continuously pumped through the flow-cell at a flow rate of 0.1 mL/min by means of a peristaltic pump. An injector with a 0.25 mL loop was connected to the flow system. The mobile phase (10 mM Tris–HCl, pH 7.5) was filtered through a 0.22 μm filter and degassed prior to use. A 0.1 M stock solution of KH_2PO_4 using the running buffer (pH 7.5) was prepared and serial dilutions were made to obtain solutions with varying concentrations, aliquots of which were injected intermittently and changes in capacitance were measured.

3. Results and discussion

3.1. Purification of the Pho84 membrane protein

In order to increase the purity and yield of Pho84, dual affinity purification was utilized. For this, the *PHO84* gene was cloned in the pTrcHisB expression plasmid together with a flanking sequence encoding the FLAG antibody epitope (DYKDDDDK) at the C-terminal end of Pho84. We have previously observed that the FLAG-tagged Pho84 expressed under control of its natural promoter in a pRS416 plasmid has a retained capability to transport phosphate in *PHO84Δ* yeast cells under phosphate-limiting conditions (Lagerstedt, Pattison-Granberg and Persson, unpublished results). Similarly, the functionality of the Pho84 protein with the hexa-histidine and Xpress epitope sequences fused to the N-terminus has been demonstrated in a reconstituted system (Fristedt et al., 1999). This dual affinity system has the following advantages: (i) using the affinity of the hexa-histidine for chelated Ni^{2+} as a first, bulk purification step allows for the collection, enrichment and concentration of essentially all extracted Pho84 protein, (ii) the use of a second specific affinity purification step effectively diminishes the presence of contaminants in the final protein preparation, and (iii) the use of affinity tags at the extreme N- and C-terminal ends ensures that only full-length protein with an intact primary structure is purified. The final concentration of the dual purified Pho84 sample was 84 μg/mL, of which 1.26 μg was used for the sensor analysis.

3.2. Electrochemical performance of the immobilization process

Cyclic voltammetry is a technique to monitor the successive insulating layer formations on the gold substrate. Reduction in the redox peaks were clearly visible on the gold surface using thioactic acid when compared to the bare gold surface as the surface was now less accessible to the redox species $K_3[Fe(CN)_6]$. Decrease in the double layer capacitance confirmed SAM formation by thioactic acid and successive immobilization with protein and dodecanethiol on the gold surface.

3.3. Capacitance measurements

The capacitance measurements were performed by using the Dropsens® electrode as the working electrode in the electrochemical flow cell (Fig. 2). Measurements were made using KH_2PO_4 as the analyte. The total capacitance was measured at the working electrode/analyte interface and was continuously monitored. The behaviour of the capacitive measurements depended upon the interaction between phosphate ions and the immobilized Pho84 membrane protein. Capacitance signals were expected as a result of the conformational changes in the protein due to the binding of phosphate ions. The sensitivity of the membrane protein to phosphate ions is represented in Fig. 3. Upon injecting KH_2PO_4, there is a clear concentration dependent difference between the control electrodes as can be seen in Fig. 3B (without the protein) as compared to the response registered when Pho84 is immobilized on the electrode surface (Fig. 3A). To verify whether the registered response is due to P_i interaction, a similar concentration series of $(NH_4)_2SO_4$ was injected. There was no noticeable concentration dependent signal upon $(NH_4)_2SO_4$ injection, which is a clear indication that the sensor favours P_i. The specificity of the membrane protein to phosphate ions was also evaluated by use of a substrate analogue, methylphosphonate (MP), which has the properties of binding to the Pho84 transporter but gets poorly transported across the membrane (Pratt et al., 2004) (Fig. 4).

MP concentrations ranging from 5.5 to 27.5 μM were injected alternating with 100 μM of KH_2PO_4. The amplitudes of the capacitance response on injection of the two compounds clearly show the difference between the specificity of Pho84 for phosphate ions and MP. However, injection of MP did produce some response when compared to the control electrode as MP is a recognizable

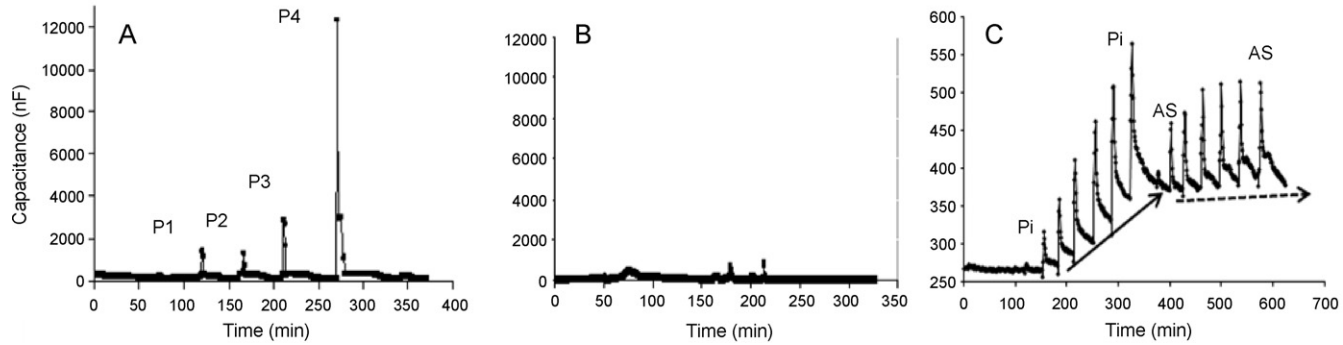

Fig. 3. Real time capacitance measurements as a function of time using the Pho84 membrane protein in order to study sensitivity and specificity. (A and B) Responses of the Pho84 protein to injections of phosphate ions, P_i (P1 – 10 μM, P2 – 25 μM, P3 – 50 μM, P4 – 100 μM) using a gold electrode with and without protein respectively. (C) Time course of capacitance change upon injection of different ascending concentrations of P_i followed by the same concentrations of $(NH_4)_2SO_4$ (AS). The solid arrow indicates the the increase in capacitance in case of P_i and the dashed arrow indicates the near straight baseline upon consecutive injections of AS as the negative control.

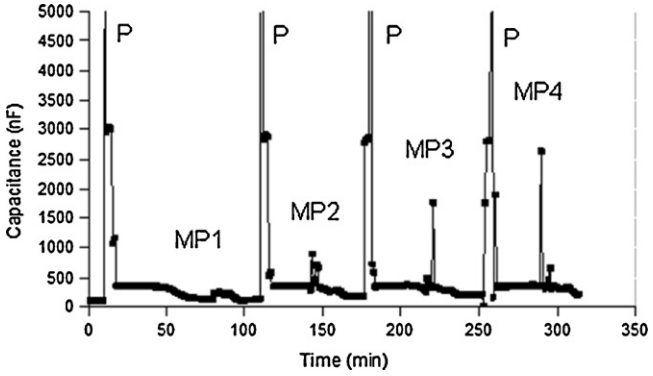

Fig. 4. Alternate injections of phosphate ions (*P*) and methyl phosphonate (MP), MP1 – 5.5 μM, 11 μM, 16.5 μM, 27.5 μM.

non-metabolite substrate. Since it is proposed for proteins belonging to the MFS that a major conformational "switching" mechanism accounts for ligand transport across the membrane, one would expect that MP, being a substrate with poor transport characteristics, initiate only a minor conformational change in comparison with inorganic phosphate. The obtained results from the studies clearly indicate a difference in conformational dynamics and is thus in support of this hypothesis.

The average value of ΔC was calculated by taking the average value of 5 points before the injection of substrate and from the baseline on reaching stabilization after the release of substrate. The requirement of evaluating the capacitance measurement using five average values has been proved by calculation (Ramanviciene and Ramanavicius, 2004).

It was also noted that the concentration of the injected substrate played an important role in the saturation of the working electrode and the subsequent release of it. Discrete pulse injections of the substrate in order of increasing concentrations revealed that the time taken for $H_2PO_4^-$ release after injection increased with increasing concentration (Fig. 5) implying that the retention time for $H_2PO_4^-$ molecules on the working electrode was dependent on the concentration of the substrate injected into the flow cell. The prominent slope observed for every concentration revealed that the electrode tended toward saturation beyond 25 μM, which is in the same order or magnitude as the observation of Berhe et al. (1995), who showed the K_m value for the high-affinity Pho84 to be around 24 μM. The decrease in capacitance thereafter also indicates that there is no requirement for a separate step of regeneration of the working electrode. A linear increase in capacitance change versus concentration was observed in the concentration range 5–25 μM (Fig. 6). This serves as proof of principle for phosphate sensing by the Pho84 membrane protein.

The upper limit in saturation was verified by subjecting the electrode to a continuous analyte infusion of an equivalent con-

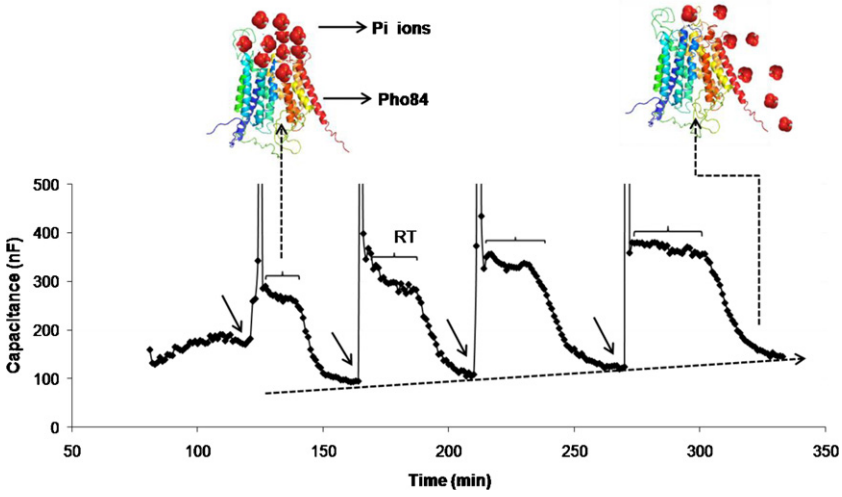

Fig. 5. Capacitance changes after pulse injections of phosphate ions (substrate) to a gold electrode with immobilized Pho84. Arrows indicate the point of substrate injection. RT – retention time (min.) indicated the binding of P_i ions to the Pho84 and relaxation followed by a drop in capacitance, indicative of the release of P_i by the Pho84.

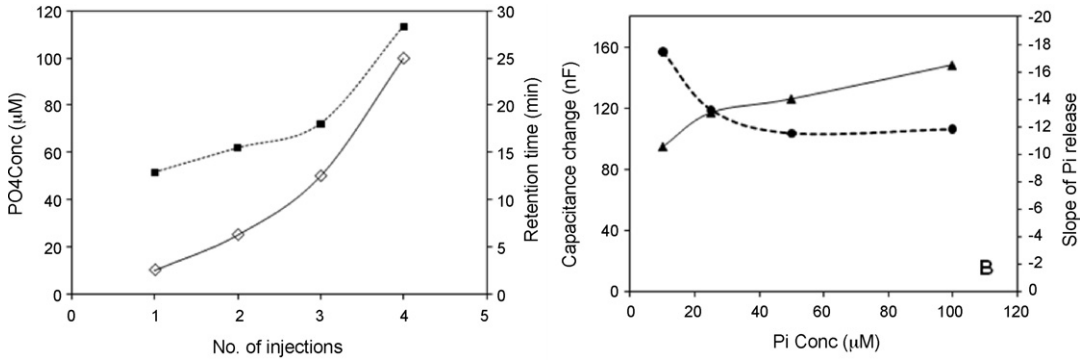

Fig. 6. Stepwise increase in the retention time (-■-) was observed with increasing concentrations of P_i (-□-) C. Capacitance change (-▲-) and slope value (-●-) indicates that the rate of release of excess phosphate ions have a limit of saturation between 20 and 30 μM.

centration to that of the running buffer, i.e. 10 mM KH_2PO_4. An increase and a near stable capacitance were continuously observed and a total saturation of the electrode was assumed to occur though not affecting the sensitivity of Pho84. This indicates that the sensor becomes saturated when the analyte concentration exceeds 5 mM.

4. Conclusion

In this study we show that it is possible to make use of a MFS membrane protein in a biosensor as a sensing element without the use of lipid membranes. From an earlier concept of using whole cells, or at least membrane vesicles, it may now also be possible to operate with cloned and purified membrane proteins. One crucial point is whether the membrane protein will bind and show the conformational changes in the new environment. Such changes are the basis for their function *in situ* within the membrane. The present study clearly shows that even though the protein is in a different environment with surfactants blocking the hydrophobic trans-membrane regions, the Pho84 membrane protein has retained its capacity of selective substrate binding, with a phosphate detection limit in the range of the apparent *in vivo* K_m. Not only is this a strong indication that the protein has retained its conformational dynamics, but it also serves as proof for the sensing capacity of Pho84 *in vivo*.

Acknowledgements

This work was supported by The Swedish International Development Cooperation Agency (SIDA) through the VR Research Link Program.

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