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

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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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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