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Functional *in vitro* assembly of the integral membrane bacterial thermosensor DesK

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ABSTRACT

The *Bacillus subtilis* DesK histidine kinase (HK) is an integral membrane thermosensor that forms part of a regulatory circuit which controls the physical state of membrane lipids. In the pursuit of biochemical and structural approaches to study lipid fluidity-dependent DesK thermosensing, we found that standard expression methods failed to produce enough amounts of a fully functional protein. Here, we describe a high-yield purification method based in an *Escherichia coli in vitro* transcription–translation system. The enzymatic activities of the full-length protein, either solubilized with detergents or co-translationally inserted into liposomes, have been characterized and compared with those measured for the constitutively active cytoplasmic domain of DesK, lacking the transmembrane sensor domain. As expected, the autokinase activity of liposome-inserted DesK was greatly increased when the incubation temperature was decreased from 37 to 25 °C. This is the first report of the spontaneous *in vitro* membrane insertion of a fully functional bacterial HK thermosensor. Moreover, this single step procedure should greatly aid the isolation of a wide range of membrane-associated HKs for biochemical and biophysical studies.

Introduction

The predominant mechanism by which bacteria respond to changing environmental conditions is the classical two-component signal transduction system consisting of a signal sensing protein with histidine kinase (HK)[1] activity and a response regulator (RR), usually a DNA-binding protein. Signal recognition leads ultimately to alterations of the phosphorylation state of the RR, which differentially modulates the RR's propensity to activate or repress transcription of target genes. These signaling systems are found in nearly all bacteria, with most species containing 20–30 HK–RR pairs [1].

HKs harbor an N-terminal input domain that senses a specific external stimulus, either binding to or reacting with a signaling molecule or interacting with the stimulus sensed, and a C-terminal kinase domain. Depending on their membrane topology, HKs can be grouped in three large classes [1]. The first (and largest) group includes the periplasmic (or extracellular) HKs, which possess an extracellular sensory domain framed by at least two transmembrane (TM) helices and a cytoplasmic catalytic domain. Since the sensory and catalytic domains are being located in two different cellular compartments, these proteins require TM signal transduction. This type of membrane topology is typical for sensing solutes and nutrients. A second group includes HKs for which the sensing mechanisms are associated with the membrane-spanning helices, thus lacking an obvious extracellular input domain. The unifying feature of this highly diverse group of sensor kinases is the presence of 2–20 TM segments directly involved in signal perception. Membrane-associated stimuli may include the mechanical properties of the cell envelope or features from membrane-bound enzymes or other integral membrane components. A third group of sensor kinases are membrane-anchored or soluble HKs with cytoplasmic signal-sensing domains. This class of sensor proteins detects the presence of cytoplasmic solutes or other proteins signaling the metabolic or developmental state of the cell. In all cases, signal detection normally results in the induction of an ATP-dependent autophosphorylation of a conserved histidine residue located in the catalytic core of the enzyme.

The DesK thermosensor from *Bacillus subtilis* belongs to the group of HK with sensing mechanisms linked to the TM segments. DesK controls the phosphorylation state of its cognate RR, DesR, which in turn regulates the expression of the cold shock-inducible fatty acid desaturase encoded by the *des* gene [2]. Experimental evidence suggests that DesK detects environmental temperature changes through its hydrophobic N-terminal domain, which consist of four or five TM regions capable of sensing membrane fluidity as the stimulus of DesK-mediated *des* expression [2–4]. Therefore, the regulatory function of DesK is defined by interaction with the

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[1] *Abbreviations used:* HK, histidine kinase; RR, response regulator; TM, transmembrane; TEV, Tobacco Etch Virus; AU, arbitrary units; CMC, critical micellar concentration.

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lipid bilayer and as such represents an ideal model system for studying the biophysical basis of thermodetection and signal transduction.

Our attempts to study the activities of DesK have been hampered by the experimental bottleneck, common to most integral membrane proteins, of obtaining the functional sensor protein in *Escherichia coli* using a variety of expression systems. Furthermore, it is well documented that many transmembrane proteins of Gram-positive organisms, such as *B. subtilis*, are toxic to an *E. coli* overexpressing host, and are difficult to produce in large enough quantities to allow biochemical studies [5]. Here, we report a new approach for *in vitro* high-level expression and purification of DesK as well as a new strategy for liposome-assisted cell-free synthesis of the functional sensor protein. This simple and general methodology will play a key role in attempts to isolate a wide range of HKs, whose molecular study has been vexed by the problem of being removed from the membrane as full-length functional proteins.

Materials and methods

Construction of plasmids

desK and *desK$_{TEV}$* were PCR amplified from plasmid pPA47 [2] with oligonucleotides KINV_UP (5-ATGAGGTAAGCATATGATTA AAAATCATTTTAC) and KINV_DW (5′-CTTTACCCGGGTTTTGAATT ATTAGGAATTGCC) or KINVTEV-DW (5′-CTTTACCCGGGGCCCTG AAAATACAGGTTTTCTTTTGAATTATTAGGAATTGCC), respectively. KINVTEV-DW includes the coding sequence for the cleavage site for the Tobacco Etch Virus (TEV) protease. The resulting amplicons, *desK* and *desK$_{TEV}$*, were cleaved with NdeI and SmaI, and cloned into pIVEX2.3d (Roche Applied Science, Penzberg, Germany). The resulting plasmids were named pAD231 and pMM1, respectively. pIVEX2.3d contains the T7 promoter and a His_6 tag coding sequence so that the expressed proteins, DesK and $DesK_{TEV}$, contain a His_6 tag at the C-terminus. $DesK_{TEV}$ also has a C terminal TEV protease cleavage site located between DesK and the His_6 tag. *E. coli* DH5α strain was used for all gene construction experiments. Transformants were selected in LB-agar plates supplemented with ampicillin (100 μg/ml) at 37 °C.

In vitro protein synthesis

Plasmid DNA (1 μg, prepared from *E. coli* cells with a midi prep kit (SIGMA)) was incubated in Roche RTS 100 HY Kit reactions (Roche, Penzberg, Germany) for 4 h in accordance with the manufacturer's instructions. Detergents or liposomes were added as supplements where indicated. Once the reactions were finished, the RTS lysate containing the synthesized protein was centrifuged at 20,000*g* for 10 min at 4 °C with an Eppendorf F45-30-11 rotor. The pellet and supernatant fractions were treated with acetone, resuspended in 1× Sample Buffer (5× Sample Buffer: 60 mM Tris–HCl (pH 6.8), 25% v/v glycerol, 2% w/v SDS, 144 mM 2-mercaptoethanol and 0.1% w/v bromophenol blue) and analyzed by SDS–PAGE followed by Western blot. Preparative scale cell-free expression of soluble DesK was performed in a 1 ml cell using the RTS 500 ProteoMaster *E. coli* HY Kit (Roche, Penzberg, Germany) and the expression plasmids pAD231 or pMM1 (30 μg) for 18–20 h at 26 °C, in the presence of either 0.45% w/v Brij58 or 4 mg/ml 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes.

Protein purification protocols

For DesK purification in micelles of Brij58, the 1 ml reaction mix was first half diluted with buffer A (50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 5 mM imidazole and 0.45% w/v Brij58) and then centrifuged at 4 °C at 20,000*g* for 10 min. DesK was purified by affinity chromatography using a 1 ml HisTrap- HP (GE Healthcare) at 4 °C with a stepwise elution at 20, 100, 200 and 500 mM imidazole in buffer A. DesK mainly eluted at 500 mM imidazole. Suitable fractions were pooled, washed out and concentrated to 1.6 mg/ml using a Vivaspin 4 ml concentrator (Vivascience AG, Hannover, Germany) with a 10 kDa cutoff. Purified DesK in detergent micelles (DesKm) migrates in an SDS–PAGE with an apparent molecular mass of 43.5 kDa. The His_6 tag fusion proteins DesKC and DesR were overexpressed in *E. coli* and purified as described [3,4]. The protein profile of all purified proteins was determined by SDS–PAGE.

Liposome preparation

To obtain large multilamellar vesicles, 20 mg of DOPC lipids (Avanti Polar Lipids, USA) were hydrated in 1 ml of hydration buffer (20 mM Tris–HCl, pH 8.0, 250 mM sucrose and 100 mM K_2SO_4), and the suspension was incubated at 37 °C in a vortex mixer for 3 h. The suspension was then extruded using a hand held extrusion device obtained from Avanti. For liposome generation, DOPC lipids dispersions were passed >20 times through a 400 nm filter.

Sucrose gradient ultracentrifugation

To separate DesK-containing proteoliposomes from the reaction mixture, sucrose gradient ultracentrifugation was performed. The reaction mixture (1 ml) was placed on the bottom of a step sucrose gradient (1.6, 1.2 and 0.2 M sucrose) and centrifuged overnight at 30,000 rpm in a SW40 rotor at 4 °C. After centrifugation, proteoliposomes were washed with 30 mM Tris–HCl pH 8.0 and ultracentrifuged again for 1 h at 4 °C. Finally, DOPC proteoliposomes containing DesK (DesKp) were resuspended in hydration buffer and used for protein concentration determinations, SDS–PAGE followed by Western blot and biochemical characterization.

Orientation of DesK in proteoliposomes

Proteoliposomes containing $DesK_{TEV}$ were resuspended to a final protein concentration of 1 μg/μl in hydration buffer. The TEV cleavage site localized between the C-terminus of the protein and the histidine tag was used to determine the orientation of DesK in liposomes. In this sense, only the cleavage sites located on the outside of the liposome's membrane are accessible for the TEV protease (1 OD_{280} of TEV protease per 80 OD_{280} of $DesK_{TEV}$). A sample without TEV treatment was used as control. Reaction mixtures were incubated for 16 h at room temperature. The samples were then subjected to SDS–PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blot using an anti-His antibody.

In vitro phosphorylation, dephosphorylation and TLC analysis

Unless otherwise indicated, all phosphorylation assays were carried out in P buffer (50 mM Tris–HCl, pH 8, 200 mM NaCl, 1 mM dithiothreitol, 20% v/v glycerol, 50 mM KCl, 1 mM $MgCl_2$, 25 μM ATP and 0.25 μCi/μl [γ-^{32}P]ATP (NEN Life Science Products) [3]. For the autokinase assay, DesKm, DesKp or DesKC were incubated in P buffer at room temperature; different time-points aliquots were taken in 5× Sample Buffer and subjected to SDS–PAGE on 12% polyacrylamide gels.

For the phosphotransfer assay, enzymatic preparations were first allowed to autophosphorylate for 8–10 min in P buffer, followed by addition of an equal volume of purified DesR (0.6 μg/μl) in the same buffer without ATP. Aliquots were withdrawn at different time points, mixed with 5× sample buffer to stop the chemical reaction and analyzed by SDS–PAGE as above.

To obtain phosphorylated DesR, DesKp (50 μg) was first allowed to autophosphorylate for 10 min as described. The proteoliposome