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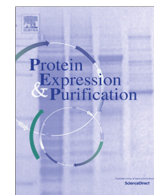

Cell-free expression of a functional pore-only sodium channel

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ABSTRACT

Voltage-gated sodium channels participate in the propagation of action potentials in excitable cells. Eukaryotic Na_vs are pseudo homotetrameric polypeptides, comprising four repeats of six transmembrane segments (S1–S6). The first four segments form the voltage-sensing domain and S5 and S6 create the pore domain with the selectivity filter. Prokaryotic Na_vs resemble these characteristics, but are truly tetrameric. They can typically be efficiently synthesized in bacteria, but production *in vitro* with cell-free synthesis has not been demonstrated. Here we report the cell-free expression and purification of a prokaryotic tetrameric pore-only sodium channel. We produced milligram quantities of the functional channel protein as characterized by size-exclusion chromatography, infrared spectroscopy and electrophysiological recordings. Cell-free expression enables advanced site-directed labelling, post-translational modifications, and special solubilization schemes. This enables next-generation biophysical experiments to study the principle of sodium ion selectivity and transport in sodium channels.
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Introduction

Voltage-gated sodium channels (Na_vs)[2] are membrane proteins from the superfamily of voltage-gated ion channels, closely related to voltage-gated potassium channels and voltage-gated calcium channels [1]. Na_vs are present in all excitable cells, where they participate in the propagation of action potentials by changing the Na^+ permeability of the cell membrane. Most voltage-gated ion channels comprise of similar building blocks and are mainly alpha-helical. Na_vs comprise six transmembrane segments (S1–S6), where S1–S4 form the voltage-sensing domain and S5 and S6 create the pore domain. In bacteria, four of these subunits arrange around a central pore to form a functional channel. In higher organisms, all four subunits are assembled from a single polypeptide chain, which may associate with auxiliary subunits [2].

Eukaryotic sodium channels were discovered first and have been the subject to extensive research for many decades [3]. However, the discovery of a prokaryotic bacterial sodium channel in 2001 [4] was a prerequisite for solving the proteins' three dimensional crystal structure [5], mainly because it enabled production of larger amounts of channel protein. Bacterial Na_vs have 20–25% identity with human Na_vs and are expected to have a similar fold as they have nearly identical hydrophobicity profiles and predicted topologies in each of the pseudo-repeated eukaryotic domains [6]. Despite the available structural information, the mechanisms of molecular ion transport and ion selectivity are still not completely understood. X-ray crystallography catches high-resolution structures of stationary states, but lacks dynamic information. In principle, molecular dynamics simulations can be used to produce dynamical models. However, for potassium channels [7], such simulations have led to radically different proposals for mechanisms for ion transport [8,9]. This emphasizes the need for experimental validation. As we point out below, suitable experiments are today becoming possible. They may require sophisticated and site-selective modifications of the protein, which is asking for efficient production of Na_vs with cell-free (*in vitro*) synthesis.

In cell-free expression, proteins are expressed from exogenous template DNA added to the transcription and translation enzymes extracted from a cell lysate. This *in vitro* synthesis is becoming increasingly popular, particularly because it is possible to produce proteins which aggregate, or proteins which are toxic to host cells [10–14]. Today, typical yields of 0.3 mg to several milligrams of protein per mL reaction mixture can be achieved in batch or in

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[2] *Abbreviations used:* Na_vs, voltage-gated sodium channels; Na_vSp1p, sodium channel from *Silicibacter pomeroyi*; CMC, critical micelle concentration; GUVs, giant unilamellar vesicles.

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continuous mode, respectively, and popular reaction mixtures are extracted from *Escherichia coli* or wheat germs [14–16]. As cell-free expression gives direct access to the nascent polypeptide, it facilitates co-translational solubilization of membrane proteins in a wide range of detergents, lipids and nanodiscs [10,17]. Indeed, functional membrane proteins [10–13] including ion channels such as connexins [18], nicotinic acetylcholine receptors [19], drosophila olfactory receptors [20] and potassium channels can be produced by *in vitro* synthesis [17,21,22]. Also, eukaryotic sodium channels protein were synthesized *in vitro* [23]. Most of these examples were produced in cell extracts that yield microgram quantities of proteins, which is sufficient for electrophysiological studies, but not for spectroscopic and crystallographic investigations [18,19,21–23]. Isotope-labelled proteins are easily available as the amino acids are added to the reaction mixture as required [24].

In-vitro synthesis of ion channels is useful, because it may pave the way for the investigation of the molecular details of ion conductance in channel proteins. It has been demonstrated that *in vitro* synthesis enables time-saving direct reconstitution into oocytes [17]. Room temperature spectroscopy methods, such as NMR and vibrational spectroscopy, can in principle be used to study ion channels in native environments. Two-dimensional infrared spectroscopy is particularly interesting, because it can be used to characterize biological processes involving protein conformational change, e.g. transport or charge transfer with picosecond time resolution [25,26]. A specific infrared experiment to study the occupancy in selectivity filters of ion channels has been suggested [27]. Nonetheless, it is usually problematic to assign vibrational spectral signatures to specific sites in proteins, except for when these sites are labelled with isotopes or specific chemical groups [27–29]. *In-vitro* synthesis of protein makes possible site-directed labelling with specific amino acids using amber-stop codon technology [30,31]. To realize this new generation of spectroscopic experiments, reliable *in vitro* production of milligram quantities of ion channels is a prerequisite.

Here we report the production of a bacterial pore-only sodium channel from *Silicibacter pomeroyi* (Na_vSp1p) [32]. Designed and explored by the Minor group, the pore domain folds independently of the voltage-sensing domain into a functional channel protein, which displays selectivity for sodium over potassium ions. When produced in *E. coli*, it is more stable and expresses at higher levels than the complete channel [32]. We show here that cell-free production of a few milligrams of Na_vSp1p is possible, that the protein is folded correctly, and that a functional sodium channel is produced.

Material and methods

Extract cultivation, preparation

S12 extract was prepared from BL21 (DE3):RFI-CBD_3 [31] as described in [33]. Briefly, cells were grown in a 20 L fermentor (Braun Biostat C) at 37 °C in 2xYPTG medium supplemented with choline chloride (Fluka 28.6 mg/L), nicotinic acid (Acros, 25.1 mg/L), p-aminobenzoic acid (Aldrich, 20.0 mg/L), pantothenic acid calcium salt (Fluka, 9.4 mg/L), pyridoxal-5-phosphate (1.8 mg/L), (−)-riboflavin (3.9 mg/L), thiamine hydrochloride (USB Corporation, 17.7 mg/L), betaine hydrochloride (Calbiochem, 33.1 mg/L), D-biotin (MP Biomedicals, 0.1 mg/L), cyanocobalamin (Fluka, 0.01 mg/L), folinic acid calcium salt hydrate (Sigma, 0.075 mg/L), iron (III) chloride hexahydrate (Scharlau Chemie S.A., 20.0 mg/L), sodium molybdate dehydrate (Acros, 3.5 mg/L), boric acid (1.2 mg/L), cobalt sulphate heptahydrate (4 mg/L), copper sulphate pentahydrate (Merck KGaA, 3.4 mg/L), manganese sulphate hydrate (Merck KGaA, 1.9 mg/L), zinc sulphate heptahydrate (Scharlau Chemie S.A., 3.4 mg/L), and amino acids (Asp (28.5 mg/L), Gly (49.1 mg/L), His (9.35 mg/L), Ile (26.2 mg/L), Leu (29.9 mg/L), Lys (31.4 mg/L), Met (14.9 mg/L), Phe (15.3 mg/L), Pro (31.8 mg/L), Thr (37.7 mg/L), Trp (102.1 mg/L), Tyr (37.7 mg/L), Val (117.1 mg/L)). At OD_{600} ~4.5 temperature was decreased to 10 °C by passing the cell suspension through a metal coil immersed in ice water, cells were harvested, washed with extraction buffer (10 mM Tris–acetate (pH 8.2), 14 mM $Mg(OAc)_2$, 60 mM K(OAc), cOmplete EDTA-free (Roche)), and finally resuspended in 10 mL extraction buffer/8 g of wet cells. The cells were lysed by a French press (two passages, 24,000 psi, ThermoFisher), centrifuged at 12,100*g* (10 min, 4 °C), the supernatant was decanted into fresh tubes and incubated for 2 h at a shaking incubator (30 °C, 150 rpm). We removed the release factor 1 protein from the cell extract. This is important for potential subsequent site-directed labelling steps with amber stop codons [30,34]. The S12 extract was passed over chitin resin (New England Biolabs) directly after the incubation and removal was confirmed by Western blotting. After addition of 1 mM DTT the S12 extract was dialyzed twice against extraction buffer supplemented with β-mercaptoethanol (1 mL/L), flash frozen, and stored at −80 °C.

Cell-free protein expression was performed in batch mode as described by [33]. Briefly, plasmid 0.01 μg/μL DNA, 14–20 mM $Mg(OAc)_2$, all 20 amino acids (1 mM each, besides Gln (4 mM) and Ser (2 mM)), 27.4 mM NH_4OH, 212 mM D-Glu, 230 mM KOH, 52.5 mM Hepes-KOH (pH 7.0), 1.1 mM ATP, 800 μM GTP, 800 μM CTP, 800 μM UTP, 640 μM cAMP, 68 μM folinic acid (BioXtra), 1.7 mM DTT, 51.6 mM creatine phosphate (Roche), 4.4 mM L-(−)-malic acid (Fluka), 1.5 mM succinate (SAFC), 1.9 mM α-ketoglutaric acid (Fluka), 175 μg/mL tRNA (Roche), 8 U/mL RiboLock RNase Inhibitor (Thermofischer), 1xcOmplete EDTA-free (Roche), 50 μg/mL T7RNA polymerase (prepared according to [35,36]), 125 μg/mL creatine kinase (Roche), 31% (v/v) S12, and detergents were mixed. Brij®-58 (polyoxyethylene (20) cetyl ether) and Brij®-78 (polyoxyethylene (18) octadecyl ether) (Sigma) were used at a final concentration of 10 times excess of the critical micelle concentration (CMC), 0.8 mM and 0.46 mM, respectively. DDM (*n*-dodecyl-β-D-maltoside) and DM (*n*-decyl-β-D-maltoside) (Anagrade Affymetrix) were used at 3 × CMC, 0.6 mM and 5.4 mM, respectively. Mixture was incubated for two hours at 30 °C and 800 rpm. For every batch of S12 the optimal Mg^{2+} was determined by GFPcyc3 expression. GFPcyc3 fluorescence was measured with a FluoStar plate reader (BMG Labtech, 390 nm (excitation), 520 nm (emission)).

To verify protein expression, Western blotting was performed following the manual for XCell II™ Blot Module and ONE-HOUR Western™ Basic Kit (Mouse) (GenScript) using Anti-His Antibody (GE Healthcare). Chemiluminescence was detected using a Fujifilm Las-1000 Luminescent Image Analyzer Chemi Fuji together with its software.

Overexpression of Na_vSp1p in E. coli cells

Na_vSp1p cloned into pHM3C-LIC, a vector containing a N-terminal hexa-His tag, a maltose-binding protein and a HRV 3C cleavage site was a kind gift from Daniel Minor [32]. The construct was transformed into Escherichia coli BL21 (DE3) and expression was performed as described [32].

Purification of Na_vSp1p from cell-free synthesis and expression in E. coli

We adapted a purification scheme from Shaya et al., 2011. After completed cell-free synthesis, the reaction mixture was centrifuged (16,000*g*, 20 min, 4 °C) and the supernatant was loaded

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