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A cost-effective protocol for the over-expression and purification of fully-functional and more stable *Erwinia chrysanthemi* ligand-gated ion channel

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Abstract

The *Erwinia chrysanthemi* ligand-gated ion channel, ELIC, is considered an excellent structural and functional surrogate for the whole pentameric ligand-gated ion channel family. Despite its simplicity, ELIC is structurally capable of undergoing ligand-dependent activation and a concomitant desensitization process. To determine at the molecular level the structural changes underlying ELIC's function, it is desirable to produce large quantities of protein. This protein should be properly folded, fully-functional and amenable to structural determinations. In the current paper, we report a completely new protocol for the expression and purification of milligram quantities of fully-functional, more stable and crystallizable ELIC. The use of an autoinduction media and inexpensive detergents during ELIC extraction, in addition to the high-quality and large quantity of the purified channel, are the highlights of this improved biochemical protocol.

1. Introduction

The electrical communication between neuron-to-neuron and neuron-to-non-excitable cell is mediated by very fast chemical signaling. At the synaptic cleft, a neuron releases neurotransmitter molecules that bind to a receptor-channel located at the cell membrane of a nearby neuron (i.e., pentameric ligand-gated ion channels, pLGICs). This ligand binding event triggers structural changes at the extracellular domain of the receptor-channel, which are allosterically coupled to the opening of the membrane-embedded channel's pore domain (Fig. 1)[1].

Pentameric Ligand-Gated Ion Channels (pLGICs) are omnipresent membrane proteins expressed at the cell membrane of prokaryotic and eukaryotic cells. They are particularly important for the normal functioning of the human body in which they are responsible for essential cognitive processes, e.g. learning, memory and perception. pLGICs are arranged

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within the cell membrane as a repeat of the same subunit (homo-pentamers) or as an assembly of different subunits (hetero-pentamers)[2–5].

50 years of systematic electrophysiological studies have yielded a clear understanding of the kinetic states that better describe the functioning of this type of molecule[6]. However, a structural description of the pLGIC kinetic cycle lagged, except for the resting state of the nAChR receptor channel from Torpedo electric ray for which a medium-resolution structure was obtained by electron microscopy studies[7, 8]. This experimental approach was limited due to the low resolution of the structural models and the impossibility of performing mutagenesis perturbation analysis to determine the channel's functional-structural correlations.

Of particular importance to the pLGIC research field was the identification of two prokaryotic homologs (ELIC from *Erwinia chrysanthemi* and GLIC from *Gloeobacter violaceus*) that could be expressed in the pervasive, economic, and versatile *E. coli* expression system, albeit in very low quantities[9, 10]. Though a low yield was obtained for these two channels, their high-resolution structures were solved by X-ray crystallography[11–13]. These two structural snapshots represented the first atomic resolution depiction of the “putative” closed and open states for pLGICs.

More recently, the pLGIC research field has made significant strides in the quest to provide a structural framework for this family of ion channels. High-resolution structural information has been obtained for several homo and heteropentameric pLGICs by X-ray crystallography[14–16]. Single-particle electron microscopy has provided us with a first glimpse of the Glycine receptor in the agonist and antagonist bound conformations, which has enriched our understanding of how these ion channels might work[17].

However, in spite of all the pioneering structural work done with pLGICs, many uncertainties preclude drawing direct correlations between the available set of structures and distinct functional states, since no significant conformational changes are detected among them[18, 19]. Furthermore, membrane protein structures obtained in the absence of a lipid bilayer always have the risk of representing a non-physiological conformation that could lead to wrong conclusions[20]. Finally, the comparison of structures from different channels trapped in different kinetic states, although very informative, is a more qualitative than a quantitative description of the structural dynamics underlying the gating process of a pLGIC.

Generating a more precise and quantitative description of the pLGICs gating process is imperative to obtain high-resolution structures of the same channel while “trapped” in different and well-defined kinetic states[21]. Next, a spectroscopic validation by fluorescence or continuous wave electron paramagnetic resonance spectroscopy (CW-EPRs) for these channel's kinetic states must be done while embedded in a lipid bilayer, to ensure their physiological relevance[22]. Finally, a thorough electrophysiological evaluation of these “trapped” kinetic states must be done to establish their structural-functional correspondence.

To undertake this type of structural-functional study, mainstream laboratories must produce milligram quantities of properly folded and functional protein in a cost-effective manner. In

this work, we have systematically tested different *E. coli* strains, growth media, growth temperatures and different detergents for membrane protein solubilization to generate a protocol for the over-expression and economic purification of fully-functional, better-folded and more stable ELIC channel (as evidenced by differential scanning calorimetry). Our novel production and purification scheme allows us to produce ~ 20 times more protein than well-known available protocols.

2. Materials and Methods

All experiments were performed at 4°C, unless otherwise noted. All the reagents were purchased from Fisher, Sigma, BDH or J.T. Baker, unless otherwise stated.

2.1 Plasmids and Bacterial Strains

MBP-ELIC cloned into the pET28 vector was generously provided by Claudio Grosman (University of Illinois at Urbana-Champaign). This construct was generated using the following scheme: 6×His tag – MBP – 6×His tag – Tobacco Etch Virus cut site – ELIC. Mutations were made in-house by site directed mutagenesis. Recombinant Tobacco Etch Virus Protease (rTEV) was purified in-house using previously published protocols[23]. *E. coli* strains: BL21 DE3 Gold, BL21 DE3 Codon (+), BL21 DE3 Rosetta (Novagen), C41, and C43 (Lucigen) were tested for their ability to express the MBP-ELIC fusion protein in sufficient quantity and quality.

2.2 Cell Type, Media, and Detergent Selection

MBP-ELIC expression levels were tested in four different growth media types: Luria Broth (LB), Terrific Broth (TB), and ZYM-5052 and ZYM-512 auto-induction media, as well as the 5 *E. coli* strains listed above. LB was purchased from Difco. TB was made in-house and consisted of 1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH₂PO₄, and 72 mM K₂HPO₄. The ZYM-5052 auto-induction medium was made as previously described [24]. ZYM-512 media was made using the same protocol, with the exception of an increase to 0.1% glucose from 0.05% glucose. The components used to make the media are as follows: tryptone, yeast extract, MgSO₄, 0.2× trace metals, Na₂HPO₄, Na₂SO₄, KH₂PO₄, NH₄Cl, glucose, lactose, and glycerol.

Extraction tests of MBP-ELIC fusion were performed with the following detergents, which were purchased from Anatrace unless otherwise noted: 1% SDS, 1% Sarcosine, 20 mM Dodecyl Maltoside-DDM, 40 mM Decyl Maltoside-DM, 1.5% Anzergent 3–10, 1.5% Anzergent 3–12, 1.5% Thesit (Sigma), 1.5% Triton X-100, 1.5% Anapoe C₁₂E₁₀, 1.5% Anapoe C₁₀E₉. From this pool, Sarcosine, DDM, Anzergent 3–12, and Triton X-100 were chosen due to the large quantities of protein extracted during the solubilization test. The hydrodynamic properties of the purified ELIC were assessed by Size Exclusion Chromatography (SEC) on a BioRad EnRich SEC 650 10×300 column.

2.3 Large scale expression of the MBP-ELIC Fusion protein using new and optimized conditions

An LB pre-culture was inoculated using transformed BL21 DE3 Rosetta cells and grown overnight. It is important to note that competent cells were freshly made from glycerol stocks for every purification. We noted a decrease in expression of MBP-ELIC if competent cells were frozen and used at a later date. 1 L of desired growth media was then inoculated with 1% pre-culture, grown at 37°C to an $OD_{600} = 1.5$, and was then cooled down to room temperature. The incubator was set to 20°C, and MBP-ELIC fusion was expressed overnight at 250 RPM.

We utilized a protocol published by Spurny et. al. [25] as a reference to compare our new expression and purification strategy.

2.4 Purification of ELIC

Cultures were harvested, pelleted at 6,000 RCF for 20 minutes, and the pellet was resuspended in 20 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4 (Buffer A). If purification on cobalt or nickel is desired, Buffer A must be EDTA free. To this buffer, a full homemade protease inhibitor cocktail (1mM Pepstatin, 1mg/mL Aprotinin, 1 mg/mL Leupeptin, 1mM Pefabloc, 1 mM E64, and 1 mM Bestatin [ThermoFisher]) was added. Typically, 1 L culture would produce 22 g of wet cell weight. The cells were lysed on a French-press microfluidizer (Microfluidics Corporation M-110EH), and the membrane fractions were pelleted by centrifugation at 100,000 RCF for 1 hour. The membrane pellets were resuspended in Buffer A and frozen at -80°C overnight. The next day, thawed membranes were then extracted using Buffer A + 1.5% (vol/vol) Triton X-100 for 2 hours with rotation. The insoluble components were pelleted by centrifugation at 100,000 RCF for 1 hour. The supernatant was mixed with amylose resin, pre-equilibrated in Buffer A + 1 mM DDM, pH 7.4 (8 mL of resin was used per 1 L of culture). The extract was then batched with the amylose resin for 2 hours with constant rotation. The batch was put over a gravity-flow column, then washed with 7.5 CV of Buffer A + 0.17 mg/mL PMSF + 1 mM DDM. The resin was then washed with 2.5 CV of 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (Buffer B), and 1 mM DDM. ELIC was eluted using Buffer B + 1 mM DDM + 20 mM maltose with 2.5 CV. The amount of eluted protein was measured by its absorbance_{280nm}. MBP-ELIC fusion protein eluted at 11.10 mL as a monodispersed peak. rTEV was added to the solution at 1:10 rTEV:MBP-ELIC w/w, and proteolysis occurred overnight with constant rotation at 4 °C. To the proteolysis mixture, 3 mL of cobalt resin per 20 mg of MBP-ELIC fusion was added. Batch binding occurred for 3 hours with constant rotation. The batch was then added to a gravity-flow column, and ELIC as flowthrough was collected. The stability and purity was then assessed by SEC, ELIC eluted as a monodispersed peak at 12.56 mL. As a final note, while the yield was higher when utilizing cobalt or nickel affinity chromatography as compared to amylose, the absence of imidazole in the eluate when using amylose simplifies the cleaning of cut MBP and rTEV.

2.5 Differential Scanning Calorimetry

The thermal stability of ELIC was tested using differential scanning calorimetry (DSC, TA Instruments). For DSC experiments, a 1°C/min heating rate was used with a temperature

range of 20°C to 100°C. The storage buffer was adjusted such that experiments were performed using 50 mM Tris-HCl, 150 mM NaCl, pH 8 (Buffer C), and the detergent of interest (5mM DM, 0.15% UDM, 1 mM DDM, 0.15mM TriDM, 0.1mM TetraDM). ELIC was concentrated to 1 mg/mL using an Amicon Ultra 100kD concentrator for all the different detergent thermal stability tests performed in this study. Additionally, ELIC produced using the method described by Spurny et. al. [25] was tested using Buffer C + 0.15% UDM. For all other stability tests, ELIC was purified using our method before detergent exchange on a SEC column for stability characterization. Buffer blanks and ELIC samples were degassed prior to DSC. DSC experiments were repeated at least thrice (n = 3).

2.6 Mass Spectrometry

The in-gel digestion was performed as following: the gel piece was washed with a 1:1 mixture of acetonitrile (ACN)/100 mM NH₄HCO₃ twice for 10 min to destain the gel. Reduction was performed by adding 50 µL of dithiothreitol (DTT) solution (10 mM) for 1 h at 56 °C. After reduction, the protein band was alkylated by adding 50 µL of iodoacetamide solution (55 mM in 40 mM NH₄HCO₃) and incubating in the dark for 30 min. The band was washed one more time with ACN/100 mM NH₄HCO₃ and then dehydrated by adding 100% ACN and air-dried. The digestion was started with 30 µL of trypsin solution (12.5 ng/µL in 25 mM NH₄HCO₃) and left overnight at 37 °C. Peptide extraction was performed twice using 1:1 mix of ACN/water, 0.1% formic acid solution. The extracted peptide solutions were dried in the speed vacuum centrifuge, and the peptides were resuspended in 20 µL of 0.1% formic acid for the nanoflow liquid tandem mass spectrometry (nano-LC–MS/MS) analysis.

The peptides obtained from in-gel digestion were analyzed by nano-LC–MS/MS using an LTQ Orbitrap Velos Mass Spectrometer (Thermo, CA). Chromatographic separation of the peptides was performed using a Dionex nano-HPLC (Ultimate 3000) with a trapping column (C18, 3 µm, 100 Å, 75 µm × 2 cm), followed by a reverse phase column (C18, 2 µm, 100 Å, 75 µm × 15 cm, nanoViper). Peptides were first injected onto the trapping column, which was equilibrated with 1% ACN and 0.1% formic acid in water and washed for 10 min with the same solvent at a flow rate of 300 nL/min. After washing, the trapping column was switched to the reverse-phase analytical column and bound peptides were eluted using solvents A (2% ACN, 0.1% formic acid in water) and B (98% ACN, 2% water, 0.1% formic acid). The gradient was kept constant for the first 10 min at 4% solvent B, followed by a linear increase to 11% solvent B in 20 min. Solvent B was further increased to 60% in 55min, followed by a fast increase in solvent B to 95% over 5 min. The eluted peptides were directed into the nanospray ionization source of the instrument with a capillary voltage of ~1.5 kV. The collected spectra were scanned over the mass range of 400–2000 atomic mass units. Data-dependent scan settings were defined to choose the ten most intense ions for fragmentation and MS/MS spectra were generated by Collision-Induced Dissociation (CID) of the peptide ions at a normalized collision energy of 35%.

The RAW data obtained from LC-MS/MS run was processed using Proteome Discoverer, version 1.4.0.288, and searched with SEQUEST HT search engine against the *Dickeya dadantii* database. Only proteins with FDR less than 1% were accepted for the analysis. The

following parameters were used for the search: enzyme: trypsin; allowed missed cleavage: 2; variable modification: methionine oxidation; fixed modification: carbamidomethylation of cysteine; MS and MS/MS mass tolerance: ± 15 ppm and ± 0.8 Da, respectively.

2.7 Crystallization of ELIC extracted with Triton X-100

ELIC was concentrated to ~ 10 mg/mL in a crystallization buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7.9) supplemented with 3 mM UDM and 0.5 mg/ml *E. coli* polar lipids. The protein was crystallized by sitting drop method at 4 °C. Protein sample was mixed in a 1:1 (vol/vol) ratio with the mother liquor solution containing 50 mM N-(2-acetamido)iminodiacetic (ADA), pH 6.5–6.9, 200 mM $(\text{NH}_4)\text{SO}_4$ and 10–12 % (wt/vol) of PEG 4000. ELIC crystals grew within 2–3 days and after a week they were cryoprotected in 30 % (vol/vol) ethylene-glycol before data collection.

3. Results

3.1 Optimizing conditions for the overexpression of ELIC in the *E. coli* expression system

We have developed a pipeline approach for the economic production of large quantities of better-folded and more stable membrane proteins[26]. Our current approach consists of the optimization of well-known bottlenecks for protein production in bacteria: 1) identification of a suitable *E. coli* strain for ELIC overexpression and 2) screening of growth media and additives that can maximize the quantity of ELIC expressed per cell.

For this purpose, we studied the effect of 5 different *E. coli* strains (C41, C43, BL21 Gold, BL21 Codon (+) and BL21-Rosetta) and 3 different types of growth medium on ELIC expression levels (Fig. 1b). This set of experiments showed that terrific broth, irrespective of the strain used, performed better than the LB medium. Conversely, the pair BL21 Gold/AI medium outperformed the rest of the conditions tested in this experiment (Fig. 1b).

We also know, based on many years of experience expressing membrane proteins in *E. coli*, that scaling up the cell culture volume can deleteriously affect protein expression levels. Thus, we decided to compare ELIC expression level of the three more promising *E. coli* strains (BL21 Gold, BL21 Codon (+) and BL21-Rosetta) using the AI medium (given the large biomass produced by this growth medium) in two different culture volumes, 30 mL (125 mL baffled-flask) versus 1 Liter cell cultures (2.8 L baffled-flask). The pattern of ELIC expression levels for the 3 tested bacterial strains was distinctively different when using a 30 mL versus 1 Liter cell culture (Fig. 1c). In the small flask, ELIC protein expression levels were reduced in a strain-specific fashion, displaying the following sequence: BL21 Gold $\gg$ BL21 Codon (+) $\approx$ BL21 Rosetta. The final biomass yield was very similar, irrespective of the *E. coli* strain that was used (Fig. 1c). On the other hand, when ELIC was expressed in 1 Liter cell culture, the protein expression level was comparable among the 3 tested strains and as high as the best condition identified using the small flask (Fig. 1c), with the advantage that BL21 Rosetta cells produced 30% more biomass. The gold standard to maximize protein production is to express a large quantity of the target protein per cell as well as increasing the culture biomass. The pair BL21-Rosetta/AI medium fulfills this requirement and was selected as the best choice to overexpress the ELIC channel.

A final fine tuning was made by testing the effect of divalent ions (known ELIC channel gating modifiers[27]) or the competitive antagonist acetylcholine (ACh) on ELIC protein expression. Toward this end, expression levels of the ELIC channel in the strain BL21 Rosetta grown in AI medium were assessed in the presence of 10 mM ACh, 10 mM Mg^{2+} and 100 μM Zn^{2+} . The addition of either ACh or Mg^{2+} boosted the expression levels of ELIC ~ 60% (Fig. 1d).

3.2 Extracting ELIC from the cell membrane with more affordable detergents

Pilot solubilization tests were made with a series of more affordable detergents aiming to produce a high-quality and cost-effective preparation of the MBP-ELIC channel. This is an important aspect about the purification of membrane proteins, since the detergents used for their extraction are more than often extremely expensive. Toward this end, we extracted the channel from *E. coli* membrane with the following detergents: 1.5% Sarcosine, 20 mM Dodecyl Maltoside (DDM), 30 mM Decyl Maltoside (DM), 1.5 % Anzergent 3–10, 1.5 % Anzergent 3–12, 1.5% Thesit, 1.5% Triton X-100, 1.5% Anapoe C12E10 or 1.5% Anapoe C10E9 in Buffer A. To determine the quantity of MBP-ELIC extracted by each detergent, we performed western blot analysis (Penta-histidine antibody) of the solubilized product (Fig. 2a) and the relative intensity of the MBP-ELIC band was used as the readout of the solubilization efficiency. 5 out of the 9 tested detergents were capable of extracting a significantly larger amount of the MBP-ELIC channel, which displayed the following efficiency sequence: Sarcosine» Anzergent 3–12≈Anzergent 3–10»DDM≈Triton X-100 (Fig. 2a).

3.3 Large scale purification of ELIC using optimized conditions for protein expression and membrane solubilization

A large-scale production of MBP-ELIC was made in the cells BL21-Rosetta and with the AI medium, and a fresh membrane preparation was made as indicated in the Material and Methods section. Next, the channel was extracted from the membrane suspension using the most promising and affordable detergents identified in *section 3.2*: Sarcosine, DDM, Anzergent 3–12 and Triton X-100. Finally, the channel was purified to homogeneity by metal chelated chromatography (the detergent used during MBP-ELIC's extraction was exchanged for 1mM DDM while it was immobilized in the cobalt column and eluted in 1mM DDM as well).

The hydrodynamic properties and monodispersity of the resulting peak was used as a readout of the quality and purity of the ELIC preparation (Fig. 2b). Sarcosine or Anzergent 3–12 extracted a large amount of MBP-ELIC, except it eluted from the SEC column as an aggregate or unfolded product, respectively. DDM, a mild detergent commonly used for the extraction and purification of membrane proteins, was highly efficient at extracting MBP-ELIC from the *E. coli* membrane. The SEC elution profile of DDM-extracted MBP-ELIC was perfectly monodispersed. Interestingly, Triton X-100-extracted MBP-ELIC (exchanged for 1 mM DDM on the cobalt column) displayed an elution profile remarkably similar to the DDM-extracted MBP-channel (Fig. 2b), with the advantage of being significantly less expensive than DDM. Additionally, we have shown before that Triton X-100 is a very mild detergent for the extraction and purification of membrane proteins with the benefit that its

extracting efficiency can be adjusted by fine-tuning some physical parameters, i.e., temperature, salt concentration, or pH[26].

We next proceeded to quantify the yield of pure and properly folded ELIC that can be produced following our optimized method for ELIC expression and detergent extraction. We performed a side-by-side comparison between our method and an already published protocol[25]. ELIC was expressed in the C43 cells and Terrific Broth medium and also in our new experimental conditions, BL21 Rosetta cells and AI medium (see Materials and Methods section). ELIC expression levels using the pair BL21 Rosetta/AI medium were at least 20 times larger than those obtained with the pair C43/Terrific Broth medium, as evidenced by the difference in density of the ELIC band on a protein gel and a western blot membrane (Penta-histidine antibody) (Fig. 3a).

Solubilizing ELIC from the pair C43/terrific broth with 2% Undecyl-Maltoside-UDM (as previously reported[25]) showed a substantial reduction of the extracted channel when compared to our new solubilization strategy using 1.5% Triton X-100 from the pair BL21 Rosetta/AI medium (UDM extracted ~20 times less channel when compared to Triton X-100) (Fig. 3b). In addition to the low yield of the UDM-extracted ELIC, this detergent is outrageously expensive when compared to Triton X-100. The solubilized MBP-ELIC, UDM or Triton X-100 extracted, was purified by affinity chromatography using two new amylose columns. The SEC elution profile displayed a large quantity of a perfectly monodispersed peak for the Triton X-100-extracted sample (BL21 Rosetta/AI medium) (Fig. 3b). Conversely, the UDM-extracted Fusion-channel yielded ~20 times less protein and eluted together with a low-molecular weight contaminant or proteolytic product (Fig. 3b). The purified UDM-extracted Fusion-protein showed a distinctive double-band pattern when observed on a protein gel (i.e., proteolytic degradation), which was absent on the Triton X-100 extracted MBP-ELIC (Fig. 3a). The yield of the Triton X-100-extracted fusion protein was ~ 10 times larger than the UDM-extracted one (~ 18mg/L versus ~ 1.5 mg/L).

After digestion of the Triton X-100-extracted MBP-channel fusion with rTEV enzyme, the purified ELIC eluted as a highly-symmetric and monodispersed peak with a final yield of 7–9 mg/L (Fig. 3c). The amount of channel that can be obtained by using our new and optimized protocol for ELIC expression and purification is ~17–20 times larger and significantly less expensive than the one currently used (~0.4 mg/L using UDM as the extracting detergent, personal communication with Dr. Raimund Dutzler, University of Zurich).

ELIC eluted from the SEC column as a pentamer as evidenced by its elution volume ($V_e=11.94$ ml) and from its migration pattern in a protein gel in which 5 discernible bands were observed, the monomer and dimer being the most prominent (Fig. 3c, inset). Next, pure ELIC was heated in the presence of 4% sodium dodecyl sulfate and ran in a protein gel in which a single band of ~35 KDa was observed. The identity of 68.5% of ELIC's amino acid sequence, expressed and purified by our new protocol, was additionally confirmed by mass spectrometry (Fig. 3d).

3.4 Assessing the biochemical quality of ELIC by Differential Scanning Calorimetry

In addition to producing affordable and large quantities of ELIC to conduct structural and functional studies, it is desirable to produce a biochemical preparation of high-quality and stability. Very often the protein of interest is perturbed by mutagenesis (i.e., site directed, alanine or cysteine scanning and/or genetic deletions) to study how the structure underlies its function. However, perturbation analysis can severely affect the biochemical stability of the protein of interest, hence it is advantageous to produce the most stable protein preparation. To this end, we performed a systematic Differential Scanning Calorimetry (DSC) study on the ELIC channel aiming to quantify its oligomeric stability in relationship to different biochemical parameters, such as: detergent type, age of the preparation and protocol of production.

Typical DSC experiments for a well-folded protein displayed a simple endothermic unfolding event. The temperature at which 50% of the molecules of interest are unfolded is known as the melting temperature, (T_m). Generally, T_m corresponds to the mid-point of the protein unfolding reaction peak and is accepted as the empirical measure of the protein's thermal stability. Another parameter derived from a single DSC experiment is the heat needed to unfold the protein, the calorimetric enthalpy, H_c , which can be obtained from the area under the DSC unfolding experiment [28, 29]. Protein perturbation analysis, detergent types, or physical parameters that can compromise protein stability generally lower the T_m and/or decrease the amount of heat needed for the unfolding reaction.

DSC thermograms of ELIC in 1mM DDM seem to have a single unfolding event with an apparent $T_m \sim 56^\circ\text{C}$ (Fig. 3e). Initially, the data was fit with a single two-state model. However, an optimal fit of the data was achieved by incorporating two models or two unfolding events instead of one (Event 1: $T_{m1} \sim 54.14^\circ\text{C}$ and $H \sim 540.6\text{ K/mol}$ and Event 2: $T_{m2} \sim 58.05^\circ\text{C}$ and $H \sim 525.6\text{ K/mol}$), which strongly suggest that the ELIC unfolding reaction is a convoluted process that involves the melting of at least two different parts of the protein. Since ELIC is a homopentameric channel, the most likely interpretation is that the two-unfolding events correspond to the melting of the extracellular and the transmembrane domains (Fig. 1a).

A recent DSC study of integral membrane proteins thermal stability as a function of detergent concentration indicated that their extra-membrane domains contributed to the stability of the whole protein. Additionally, high-concentrations of non-denaturing detergents seem to destabilize the extra-membrane domains affecting the protein's thermal stability. Based on these observations, we decided to study the effect of increasing the detergent hydrocarbon chain length (detergent HC-length) on ELIC biochemical stability. DSC experiments were carried out by unfolding ELIC in the presence of the following detergent types: Decyl Maltoside, Undecyl-Maltoside, Dodecyl Maltoside, Tridecyl Maltoside and Tetradecyl Maltoside.

Thermograms of ELIC in all the aforementioned detergents were obtained and best-fitted with a model assuming two unfolding events. ELIC T_{m1} was very sensitive to increments of the detergent HC-length, rising about 14°C when the HC-length was enlarged by 4 carbon atoms (Fig. 3f). On the contrary, ELIC T_{m2} was remarkably more stable changing $\sim 4^\circ\text{C}$

upon the same enlargement of the HC-length (Fig. 3g). Finally, we have demonstrated that ELIC biochemical stability is significantly enhanced when eluted in Maltoside detergents containing a 13 or 14 carbon atoms HC.

Furthermore, we exploited the characteristic ELIC thermal unfolding reaction to assess how the protein thermal stability was affected by the protocol used for expression and purification and its shelf lifetime after purification. Toward this end, DSC experiments were carried out with ELIC samples in UDM (published protocols) versus DDM (our new method) either freshly made or being about a week old. Our experimental results clearly indicated that ELIC made with our new method not only produced more affordable large quantities of protein but also a more stable and long-lasting preparation than the one obtained by previously published protocols (Fig. 3h)

3.5 Functional analysis of recombinant ELIC in artificial lipid bilayers

At the core of any scheme for the expression and purification of a given macromolecular complex is the preservation of its biological function. It is imperative to demonstrate that after purification, a protein remains functional and with high-specific activity. It is well-known, at least in membrane protein biochemistry, that the lack of an inorganic co-factor, a lipid molecule, an accessory subunit, or the occurrence of a local-unfolding event can yield a perfectly pure, monodispersed, but non-functional protein[30].

Accordingly, an ELIC electrophysiological functional evaluation was performed by patch clamp methodology after being purified by our new protocol and reconstituted in giant liposomes. In Figure 4a. a 1 nA ELIC mediated macroscopic current was elicited by rapidly changing the bath solution to 30 mM Propylamine. ELIC reconstituted 100% in the inside-out configuration in pre-formed Asolectin liposomes, which exposes the ELIC extra-cellular domain to the bath solution facilitating its functional characterization. A preliminary quantification of the current activation time constant recorded for the channel in Asolectin liposomes was ~ 400 ms, which is slower than the recorded for ELIC in HEK-293 cells, though they do have different lipid composition (Fig. 4a).

Regarding the ELIC desensitization process, which is a trademark of pLGICs, the current decay was ~ 4000 ms, which is within the range of the current decay measured for ELIC in HEK-293 cells[18, 31, 32]. As far as we know, these are the first macroscopic current recordings of ELIC in an artificial lipid bilayer and this finding opens a unique opportunity to study this channel in structural and functional isolation under fully-controlled experimental conditions, both in steady states and non-stationary conditions.

In addition to the macroscopic current analysis, ELIC permeation properties were studied by measuring its single channel activity in Asolectin liposomes. In Figure 4b. are shown representative ELIC single-channel recordings. ELIC conduction properties after being extracted with Triton X-100 were perfectly preserved, displaying a slope conductance of 154 ± 4 pS (Fig. 4C) in 200 mM $[K^+]$, which is very similar to the one measured in symmetrical 150 mM $[Na^+]$ of ~146 pS[33]. Interestingly, the ELIC channel exhibited at least two clear sub-conductance states, which have been also reported by others[32, 33]. This preliminary functional study of the ELIC channel expressed and purified with our new

protocol validates the high-quality and functional competence of our ELIC preparation. A more detailed characterization of the phospholipid composition's effect on the ELIC channel activity is an ongoing project in our laboratory, which was only possible after improving the method for ELIC expression and purification.

3.5 Crystallization of ELIC solubilized with Triton X-100

To obtain high-resolution structural information of proteins in general, structural biologists require large amount of protein properly folded, fully-functional and suitable for structural studies. Toward this end, ELIC was expressed following our new method, solubilized with Triton X-100, purified and crystallized by sitting-drop method as described earlier[12]. Crystals were grown at 4 °C and appeared in 2–3 days (Fig. 5a). ELIC's crystals of space group $P2_1$ [12] were cryoprotected in 30% (vol/vol) ethylene-glycol and diffracted anisotropically up to ~ 4.5 Å in an in-house X-ray diffraction screening machine (Rigaku Screen Machine) without any optimization. This result strongly suggests the suitability of our ELIC biochemical preparation to be use for structural studies.

3.6 Producing the GLIC channel using a new and improved protocol for the expression and purification of bacterial pLGIC

The published protocol for the expression and purification of the bacterial pLGIC, GLIC, also produced a very low yield of protein (0.2–0.4 mg/L, personal communication with Dr. Pierre-Jean Corringer, Pasteur Institute, France)[9, 11]. For this reason, we decided to test if our new methodological approach could also increase the yield and improve the quality of the produced protein in a cost-effective manner.

To optimize the amount of GLIC channel expressed per *E. coli* cell, we tested 5 different strains that are known to maximize the expression of membrane proteins (Fig. 5b)[34, 35]. BL21 Gold cells expressed a significantly larger quantity of ELIC in addition to maximizing the final culture biomass, when grown in AI media. Next, we proceeded to maximize the yield of the extracted protein by using the inexpensive detergent Triton X-100 while titrating the [NaCl] during the solubilization step. It has been shown that fine tuning physical parameters like pH, temperature or ionic strength, at this purification step, can significantly extract large quantities of the recombinant protein from the cell membrane[36]. As predicted, a clear increase of the final yield for the MBP-GLIC fusion was observed upon increasing the [NaCl] during the solubilization step (Fig. 5b). About 5–10 times more MBP-GLIC channel was purified from the *E. coli* membrane when extracted in the presence of 600 mM NaCl (Fig. 5c). Additionally, the MBP-GLIC protein eluted from SEC column equilibrated with Buffer A + 1 mM DDM as a single monodispersed peak (Fig. 5d) suggesting that in addition to produce more protein, the biochemical quality of the GLIC preparation was significantly better and highly cost-effective. Generally, the GLIC channel has been extracted from *E. coli* membranes using 2% of the expensive detergent DDM[11, 13].

4. Discussion

A major hurdle for the structural and functional characterization of membrane proteins is the production of large quantities of a properly-folded and fully-functional target in a cost-effective manner. Recently, we have implemented a pipeline approach for the optimization of the expression and purification of membrane protein in *E. coli*[26]. The method involves the systematic screening of different *E. coli* strains (known to increase the expression level of membrane proteins[35]), growth media, additives (i.e., chemical chaperones[37, 38]), blockers[26] or agonists known to stabilize and therefore enlarge the production of a more stable target protein. In this manuscript, we have developed a tailor-made protocol for the overexpression and affordable purification of large quantities of fully-functional and suitable for structural studies, ELIC, which was successfully used to produce milligram quantities of another prokaryotic pLGIC channel (GLIC). For the overexpression of bacterial pLGIC channels, we found that the AI media outperformed the commonly used LB media or Terrific Broth at least by factor of 10. The combined use of the BL21 Rosetta cells and AI media supplemented with a divalent ion (negative modifier) or ACh (competitive antagonist) enhanced the final yield of expressed and purified ELIC about 20 times when compared to previous published protocols.

It follows that a systematic search for an affordable, non-denaturing and highly efficient extracting detergent resulted in the identification of Triton X-100 as a better alternative to the more expensive and commonly used Maltoside detergents, UDM and DDM. The final yield of monodispersed and highly pure ELIC was about 7–9 mg/L of culture using cobalt immobilized metal affinity chromatography, which is between 17 to 20 times more protein than the one obtained using published protocols[12, 39].

The oligomeric state of ELIC was evidenced by SEC chromatography and corroborated by the electrophoretic migration of 5 discernible bands in a protein gel. 68.5% of ELIC's amino acid sequence was confirmed by mass spectrometry.

In this manuscript, we have also implemented Differential Scanning Calorimetry as a tool to quantify the biochemical stability of ELIC and set the precedent as well for other membrane proteins. A decrease in the T_m or a decrease in the amount of heat (H_c) needed for the channel unfolding is a direct manifestation of biochemical instability. DSC experiments using the ELIC protein in solution with detergents differing in their HC length clearly showed that the larger the HC, the more stable becomes the channel. This experimental finding is very important since it implies a better packing and/or folding of the ELIC channel and it can be used to solve the crystal structure at higher-resolution or to extend its storage lifetime. From these DSC experiments, apparent affinities for ligand-binding can be determined and associated to a given kinetic state of the channel. Finally, DSC methodology allowed us to prove that our new method for the expression and purification of ELIC produced a channel with higher thermal stability (better folded) and a long-lasting 4°C storage lifetime.

The functional evaluation of ELIC extracted with Triton X-100 demonstrated that the channel preserved its physiologically relevant conformation and gating properties: activation

and desensitization, as well as its conduction features, during the purification process. It is important to highlight that in this manuscript we are reporting for the first time ELIC-mediated macroscopic currents in an artificial lipid bilayer, which definitely will open the door to a future systematic electrophysiological analysis of this channel in structural and functional isolation. Of particular interest are the sub-conductance states displayed by ELIC during its single channels recordings; an ongoing functional characterization in our laboratory aims to identify the molecular determinant underlying such a peculiar functional behavior.

Additionally, our ELIC biochemical preparation was highly-suitable for structural studies, as evidenced by yielding diffracting crystal up to ~ 4.5 Å in an in-house x-ray diffraction screening machine without any optimization.

Finally, our pipeline approach to develop a protocol for the overexpression and purification of ELIC was satisfactorily used to overexpress and improve the final yield and quality of another bacterial pLGIC. The final yield of highly pure and properly folded GLIC was increased ~ 10 times. As a final note, while the yield was higher when utilizing cobalt or nickel affinity chromatography as compared to amylose, the absence of imidazole in the eluate when using amylose simplifies the cleaning of cut MBP and rTEV.

This optimized protocol for the expression and purification of bacterial pentameric Ligand-Gated Ion Channels (pLGIC) will pave the road for mainstream structural biology laboratories to engage in the structural-functional characterization of this important family of ion channels.

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Highlights

- A novel protocol for the overexpression of ELIC and GLIC in *E. coli*
- Cost-effective method for the purification of large quantities of a more-stable ELIC.
- A differential scanning calorimetry approach to evaluate the biochemical stability of ELIC.
- A differential scanning calorimetry method to identify simultaneously the unfolding of the extracellular from membrane spanning domains in ELIC.
- The first macroscopic currents recordings of the ELIC channel embedded in an artificial lipid bilayer.

Figure 1. Optimization of conditions for the overexpression of ELIC

1a. A cartoon representation of ELIC highlighting the membrane spanning pore forming domain (red helices), the extracellular domain (yellow B-sheets), and the boundaries of the cell membrane in relation to the channel structure. **1b.** Upper panel, a western blot analysis of the expression levels of the ELIC channel made in the *E. coli* strains: C41, C43, BL21 Gold, BL21 Codon (+) and BL21 Rosetta in the following growth media: Terrific Broth (TB, yellow), Auto-Induction medium (AI, blue) and LB medium (red). Lower panel, normalized ELIC band density from the western blot (Penta-histidine antibody) were plotted against *E. coli* strains grouped by growth media. **1c.** The effect of the cell culture volume on ELIC expression levels and final culture biomass was studied for the three most promising *E. coli* strains: BL21 Gold, BL21 Codon (+) and BL21 Rosetta. **1d.** The effect of Zn^{2+} and Mg^{2+} as channel negative modifiers or the competitive antagonist ACh on ELIC expression levels is represented as a bar plot.

Figure 2. Screening of more affordable detergents for MBP-ELIC membrane solubilization
a. Detergent mediated solubilization of the MBP-ELIC channel was done in the presence of the following detergents: 1% Sarcosine, 20 mM DDM, 40 mM DM, 1.5% Anzergent 3–12, 1.5 % Thesit, 1.5% Triton X-100, 1.5% Anapoe C₁₂E₁₀ and 1.5 % Anapoe C₁₀E₉ and analyzed by western blot. **b.** A size exclusion chromatography analysis of MBP-ELIC extracted with the most efficient detergents identified above. The protein extracted with either Triton X-100, Anzergent 3–10, DDM or Sarcosine was bound to a cobalt column and the detergent was exchanged for 1 mM DDM by extensive washes. Finally, the protein was

eluted with a buffer containing 300 mM imidazole and 1 mM DDM. The SEC column was previously equilibrated with buffer A and 1 mM DDM.

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Figure 3. ELIC purification strategy and differential scanning calorimetry analysis

a. A protein gel and its corresponding western blot contrasting the level of ELIC expression (WC), solubilization efficiency (Sol), flow-through (FT), wash flow-through (wash) and elution between published protocols[13, 25] and our novel protocol for the expression and purification of ELIC. **b.** SEC elution profiles for the MBP-ELIC fusion produced following either of the two protocols. **c.** SEC elution profile of ELIC, $V_e = 11.94$ ml. Inset: a protein gel of ELIC monodispersed peak displaying 5 discernible protein bands **d.** Mass spectrometry confirmed 68.5 % of ELIC amino acid sequence in our biochemical preparation. **e.** A differential scanning calorimetry thermogram for ELIC (1 mg of protein in a buffer containing 1 mM DDM) indicating the melting temperatures for: the total unfolding reaction (T_m), the melting of the membrane spanning domains (T_{m1}) and the melting of the extracellular domains (T_{m2}). **f.** Detergent hydrocarbon chain length (10, 11, 12, 13, or 14 carbon atoms) dependence of ELIC melting temperature for: (Event 1 = T_{m1}) or **g.** (Event 2 = T_{m2}). **h.** An evaluation of ELIC thermal stability according to the protocol used for its expression and purification as well as its time dependent destabilization.

Figure 4. An electrophysiological characterization of recombinant ELIC in an artificial lipid bilayer

a. A representative ELIC-mediated macroscopic current was elicited by fast perfusing 30 mM propylamine into the bath solution. ELIC reconstituted 100% in the inside-out configuration into preformed Asolectin liposomes, which allow its activation by perfusing propylamine into the bath solution. ELIC activated swiftly with a rising time constant ~ 400 ms and desensitized slowly with a time decay of ~ 4000 ms. These parameters are similar to the ones measured in HEK 293 cells. **b.** Representative ELIC single channel recordings measured in symmetrical K^+ concentrations (200 mM $[K^+]$ and 30 mM propylamine) at 50, 100 and 150 mV. The arrows indicate the appearance of at least two distinct subconductance states, which seem to be a trademark of the ELIC channel. **c.** An ELIC current versus voltage relationship – or IV curve – displayed a slope conductance of 154 ± 4 pS in symmetrical 200 mM $[KCl]$

Figure 5. An optimized protocol for the expression and purification of GLIC

a. Crystals of ELIC were grown at 4 ° by sitting-drop method, they appeared within ~ days. **b.** A western blot analysis for the expression of GLIC using different *E. coli* strains in the AI medium. **c.** Effect of increasing the [NaCl] during the solubilization step on the final yield of the MBP-GLIC fusion and on GLIC alone. **d.** An SEC profile for the MBP-GLIC protein showing a pure and single monodispersed peak, $V_e \sim 9.75$ mL.