Protein Expression and Purification 103 (2014) 38–47

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Successful development and use of a thermodynamic stability screen for optimizing the yield of nucleotide binding domains

Elvin D. de Araujo [a,b], Voula Kanelis [a,b,c,*]

[a] Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada
[b] Department of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Rd., Mississauga, Ontario L5L 1C6, Canada
[c] Department of Cell and Systems Biology, University of Toronto, 25 Harbord St., Toronto, Ontario M5S 3G5, Canada

ARTICLE INFO

Article history:
Received 17 June 2014
and in revised form 8 August 2014
Available online 18 August 2014

Keywords:
ATP-sensitive (K_{ATP}) potassium channel
Sulfonylurea receptor (SUR)
Nucleotide binding domain (NBD)
Nuclear magnetic resonance (NMR) spectroscopy
Thermodynamic buffer screen
Osmolyte

ABSTRACT

ATP sensitive potassium (K_{ATP}) channels consist of four copies of a pore-forming inward rectifying potassium channel (Kir6.1 or Kir6.2) and four copies of a sulfonylurea receptor (SUR1, SUR2A, or SUR2B). SUR proteins are members of the ATP-binding cassette superfamily of proteins. Binding of ATP to the Kir6.x subunit mediates channel inhibition, whereas MgATP binding and hydrolysis at the SUR NBDs results in channel opening. Mutations in SUR1 and SUR2A NBDs cause diseases of insulin secretion and cardiac disorders, respectively, underlying the importance of studying the NBDs. Although purification of SUR2A NBD1 in a soluble form is possible, the lack of long-term sample stability of the protein in a concentrated form has precluded detailed studies of the protein aimed at gaining a molecular-level understanding of how SUR mutations cause disease. Here we use a convenient and cost-effective thermodynamic screening method to probe stabilizing conditions for SUR2A NBD1. Results from the screen are used to alter the purification protocol to allow for significantly increased yields of the purified protein. In addition, the screen provides strategies for long-term storage of NBD1 and generating NBD1 samples at high concentrations suitable for NMR studies. NMR spectra of NBD1 with MgAMP-PNP are of higher quality compared to using MgATP, indicating that MgAMP-PNP be used as the ligand in future NMR studies. The screen presented here can be expanded to using different additives and can be employed to enhance purification yields, sample life times, and storage of other low stability nucleotide binding domains, such as GTPases.

Introduction

ATP-sensitive potassium channels (K_{ATP}) channels are potassium selective channels present in many tissues [1]. By sensing the cellular concentrations of ATP and ADP, K_{ATP} channels couple the metabolic state of the cell to membrane potential [2] and thus play crucial roles in many biological processes. For example, K_{ATP} channels regulate electrical activity and transmitter release in the brain [1], action potential in the heart [1], and insulin secretion in the pancreas [1,3].

K_{ATP} channels are formed from four copies of a pore-forming inwardly rectifying potassium channel (Kir6.1 or Kir6.2) and four copies of a regulatory sulfonylurea receptor (SUR1, SUR2A, or SUR2B) that surround the pore [4,5]. The SUR subunits are members of the C subfamily of the ATP-binding cassette (ABC)[1] transporter superfamily of proteins. ABC proteins consist, at minimum, of two membrane spanning domains (MSD1 and MSD2) and two cytoplasmic nucleotide binding domains (NBD1 and NBD2) [6]. In the SUR proteins and related ABC transporters, these domains are arranged in the sequence MSD1–NBD1–MSD2–NBD2. The transmembrane helices in the MSDs extend into the cytoplasm and are connected by short loops [7–11]. Short segments in these connecting loops, known as coupling helices [12], contact the NBDs. In addition to the minimum ABC protein structure, the SUR proteins contain another MSD (MSD0) that is linked to the N terminus of MSD1 by the cytoplasmic L0 linker [13–16].

Unlike most ABC proteins, the SUR proteins do not possess any transport activity but instead regulate gating of the Kir6.x pore in K_{ATP} channels. Structure–function studies have determined that binding of ATP to the Kir6.x subunit, in the absence of

* Corresponding author at: DV 4042, Department of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Rd., Mississauga, Ontario L5L 1C6, Canada. Tel.: +1 905 569 4542; fax: +1 905 828 5425.
E-mail address: voula.kanelis@utoronto.ca (V. Kanelis).

[1] *Abbreviations used:* ABC, ATP-binding cassette; NBDs, nucleotide binding domains; MSDs, membrane spanning domains; TMAO, trimethylamine N-oxide; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; NMR, nuclear magnetic resonance.

http://dx.doi.org/10.1016/j.pep.2014.08.006
1046-5928/

Mg^{2+}mediates channel inhibition, whereas MgATP binding and hydrolysis at the SUR NBDs results in channel opening [2,17–20]. SUR proteins are of vast medical importance. SUR-mediated regulation of K_{ATP} channels is critical for cardiovascular and pancreatic function [1]. Over 150 different mutations that cause either type II diabetes or hyperinsulinism have been identified in multiple regions of SUR1, including in the NBDs [21]. Mutations in the NBDs of SUR2A cause dilated cardiomyopathy [19], atrial fibrillation [22], and increased risk of myocardial infarction [23]. The NBDs also play important roles in drug binding as SUR2A and SUR2B isoforms, which differ only in the final 42 residues of NBD2, have differential responses to potassium channel openers [24,25]. Furthermore, NBD/coupling helix interactions are likely critical for K_{ATP} channel gating, as many diabetes- and hyperinsulinism-causing mutations are found in the SUR1 coupling helices [26,27] and likely disrupt NBD/coupling helix interactions.

The lack of high-resolution structural information for K_{ATP} channels precludes understanding the molecular basis by which disease-causing mutations in SUR1 or SUR2A disrupt normal K_{ATP} channel gating. Structural studies of the NBDs and their interactions would benefit from samples of the isolated SUR NBDs. Although the NBDs are part of a much larger protein complex, the modularity of the SUR proteins, as described above, provides a foundation for studying isolated NBDs. Variability of the domain arrangement and connectivity between different ABC transporters [6,28] provides further evidence of the modularity of this protein superfamily. For example, in several mammalian ABC proteins, including the SURs, the minimum structure is encoded within a single polypeptide chain with the arrangement of MSD1–NBD1–MSD2–NBD2 [6]. By contrast, other ABC proteins, such as human TAP transporters, are complexes of two polypeptide chains, with each chain comprised of one MSD and one NBD [6]. As a further variation, many prokaryotic ABC proteins frequently consist of MSD and NBD domains that are encoded and expressed as independent proteins and co-assemble into the full transporter complex [28]. This variation of how the MSDs and NBDs are arranged on different polypeptide chains in different proteins suggests that the MSDs and NBDs function as independent interacting modules and can be studied in isolation.

We previously demonstrated the heterologous *Escherichia coli* expression and purification of SUR2A NBD1 in a soluble form for studies by nuclear magnetic resonance (NMR) spectroscopy and other biophysical techniques [29]. Our original purification protocol yielded 8 mg of pure NBD1 for each 1 L of culture. We also demonstrated that 2D ^{15}N–^{1}H TROSY-HSQC spectra of SUR2A NBD1 are of high quality [29,30] and that the protein is monomeric at a concentration of 250 μM [30]. 2D ^{15}N–^{1}H correlation spectra of proteins at concentrations of 250 μM or less can be used to monitor global structural changes with mutations or changes in conditions, or to assess binding of the protein to specific ligands [30,31]. However, in order to obtain information at the level of individual residues, each resonance in the 2D ^{15}N–^{1}H correlation spectrum must be assigned to a specific residue. Although ^{15}N–^{1}H TROSY-HSQC spectra of NBD1 at 250 μM can be recorded in ~40 min, NMR experiments required for resonance assignment of the protein require more concentrated samples of NBD1 (⩾0.5 mM or >18 mg/ml) to be stable for many days (>14 days) at 30 °C. Although lower temperature prevents precipitation of NBD1, spectra of NBD1 at lower temperatures suffer from resonance broadening, and hence are not of sufficient quality for detailed NMR studies. Thus, we sought to determine additional conditions that would allow greater yields of soluble NBD1 to be obtained, which would decrease the amount of cell culture used and thus the cost of isotopic enrichment for NMR studies, and would also allow concentrated samples of NBD1 to be stable at 30 °C for many days. Here we describe a cost-effective and convenient method for screening buffer conditions and additives that enhance protein stability. Application of the identified positive screening conditions to the purification protocol of NBD1 resulted in a 10-fold increase in the yield of the purified protein (75 mg NBD1 per liter *E. coli* culture versus 8 mg/L culture reported previously [29]). The thermodynamic screen also provided insights into strategies for long-term storage of NBD1. In addition, we present binding of different nucleotides to NBD1 using fluorescence and NMR spectroscopy. Our NMR data suggests that MgATP, MgADP, and MgAMP-PNP binding to NBD1 cause similar chemical shift changes, which result from the interaction of specific NBD1 residues with nucleotides and protein conformational changes associated with nucleotide binding. Further, NMR spectra of NBD1 with MgAMP-PNP are of higher quality compared to using MgATP, indicating that AMP-PNP should be used as the ligand in future NMR studies. The methods and strategies presented here can be employed to enhance purification yields, sample life times, and storage of other low stability nucleotide binding domains, such as GTPases.

Materials and methods

Chemicals

All reagents were purchased from Biobasic or Bioshop unless otherwise specified.

Expression and purification of SUR2A NBD1

As described previously [29], rat SUR2A NBD1 (S615-L933) was expressed in *E. coli* Bl21 (DE3) CodonPlus®(RIL) cells (Stratagene) with an N-terminal His_6-SUMO tag. Cell cultures were grown in minimal M9 media, which contained $^{15}NH_4Cl$ (Sigma Aldrich or Cambridge Isotope Laboratories) for isotopic labeling, with constant agitation at 37 °C. As the cell cultures reached OD_{600} values of 0.4, 0.6, and 0.8, the incubation temperature was progressively decreased to 30 °C, 25 °C, and 18 °C, respectively. Cell cultures were then allowed to incubate at 18 °C for 30 min prior to induction of protein expression by addition of 0.75 mM IPTG. After 20 h the cells were harvested by centrifugation and the pellets were stored at −20 °C.

Purification of SUR2A NBD1 was carried out at 4 °C following the general procedure described previously [29]. Here, we describe the purification procedure and include all modifications to the protocol that allow for larger yields of purified soluble protein. Each 1 g of cell pellet was resuspended in 8 ml of lysis buffer (20 mM Tris–HCl, pH 8.0, 100 mM L-Arg, 2 mM β-mercaptoethanol, 10 mM MgATP, 5 mM imidazole, 0.2% [v/v] Triton-X100, 10% [v/v] glycerol, 2 mg/ml deoxycholic acid, 1 mg/ml lysozyme, 5 mM 6-aminocaproic acid, 5 mM benzamidine, and 1 mM PMSF). The cell lysate was loaded onto a 5 mL Ni^{2+}-NTA affinity column (GE Healthcare) equilibrated with 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 10 mM MgATP, 2% glycerol, and 5 mM imidazole. The Ni^{2+} affinity column was then washed with 10 column volumes of equilibration buffer and the His_6-SUMO-NBD1 fusion protein was eluted using 20 mM Tris–HCl, pH 7.3, 150 mM NaCl, 10 mM MgATP, 2% (v/v) glycerol, and 400 mM imidazole. The elution fractions were immediately diluted 3-fold into a buffer containing 20 mM Tris–HCl, pH 7.3, 10 mM MgATP, 5 mM β-mercaptoethanol, and 2% (v/v) glycerol. The His_6-SUMO tag was cleaved from NBD1 with a His-tagged Ulp1 protease.

The resultant mixture, containing isolated SUR2A NBD1, His_6-SUMO, and His_6-Ulp1, was loaded onto a size exclusion column (Superdex 75, GE Healthcare) in 20 mM Tris–HCl, pH 7.3, 150 mM NaCl, 2 mM MgATP, 5 mM β-mercaptoethanol, and 2% (v/v) glycerol. SUR2A NBD1 was then purified to homogeneity by a reverse Ni^{2+}-NTA affinity column in 20 mM Tris–HCl, pH 7.3,