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Expression, purification, and initial structural characterization of YadQ, a bacterial homolog of mammalian ClC chloride channel proteins

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Abstract YadQ of *Escherichia coli* is a homolog of the mammalian chloride channels of the ClC family. The *yadQ* gene was cloned as a fusion protein with a hexahistidine tag and tobacco etch virus protease site for the removal of the tag. The protein was expressed in the membrane of *E. coli* and extracted with decylmaltoside. Purification was achieved by metal affinity chromatography followed by cation exchange. Circular dichroism revealed a high α-helical content. Size exclusion chromatography suggests that YadQ forms dimers. The similarity in primary, secondary, and quaternary structure and the ability to recombinantly express YadQ in the cell membrane make the protein a good candidate for the structural study of ClC chloride channels.

Key words: ClC chloride channel; YadQ; Ion channel; Skeletal muscle; Kidney; Voltage-gated

1. Introduction

Three classes of chloride channels have been identified: the cystic fibrosis transmembrane conductance regulator, γ-aminobutyric acid and glycine receptors, and the voltage-gated ClC chloride channels [1]. Members of the ClC family perform a wide variety of physiological functions and several are responsible for human diseases. Mutations in ClC-1 result in two forms of myotonia, Thomsen's disease (autosomal dominant myotonia congenita) and Becker's disease (autosomal recessive generalized myotonia) [2–4]. ClC-5 is expressed in kidney, and hereditary hypercalciuric nephrolithiasis (kidney stones) is the result of mutations that reduce the chloride conductance [5,6]. Another ClC expressed in kidney cells, ClC-Kb, is implicated in the renal disease Bartter's syndrome [7]. ClC-2 and ClC-3 are responsible for cell volume regulation [8,9].

It is often difficult to obtain quantities of eukaryotic membrane proteins sufficient for structural studies. To facilitate the study of the structural basis of the function of ClC chloride channels, we turned to bacterial and archaebacterial homologs of this family of voltage-gated channels. The crystal structure of KcsA, a bacterial homolog of eukaryotic voltage-gated potassium channels, provides an example of the utility of this approach [10]. The primary structure of the protein YadQ from *Escherichia coli* is significantly similar to the primary structures of human ClCs, between 14.5% and 19.3% identical as determined by ALIGN [11]. YadQ possesses the same highly conserved sequences found throughout the eukaryotic ClCs, including: GSGIPE, GxEGP, and PxGxFxPxxxxG (Fig. 1). Hydropathy analysis of YadQ suggests that the protein crosses the membrane 10–12 times, in agreement with hydropathy analysis of ClCs and in vitro glycosylation and protease protection assays performed on ClC-1 [12].

2. Materials and methods

2.1. Cloning and expression

E. coli strain MG1655 was purchased from American Type Culture Collection. Genomic DNA was extracted from cells with a Generation Capture Column Kit (Gentra Systems, Inc). and used as the template for PCR amplification of the yadQ gene. Taq polymerase was used with 1 μM of the primers 5′-GATCATTCCATGGGGACTGATACTCCC-3′ and 5′-CGTCTCGAGTCAAGTATTCTCGCTGGC-3′. The reaction was held at 95°C for 5 min before and 72°C for 5 min after 30 cycles of 95°C, 1 min; 58°C, 1 min; 72°C, 3 min. The vector pHIS-Parallel1 encodes a hexahistidine tag and tobacco etch virus (TEV) protease site upstream of the multicloning site [13]. The PCR product and pHIS-Parallel1 were digested with the restriction enzymes *Nco*I and *Xho*I and ligated with T4 DNA ligase. XL-1 Blue cells were chemically transformed with the plasmid using the MBI Fermentas TransformAid Bacterial Transformation System. Cells from a single colony were grown in LB medium and the plasmid DNA was purified from the cells with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced.

Four *E. coli* strains, BL21(DE3), BL21(DE3)pLysS, C43(DE3) and C41(DE3), were electrically transformed with the plasmid. C43(DE3) and C41(DE3) were developed for expression of membrane and/or toxic proteins [14]. Cells from a single colony of each strain were used to inoculate 5 ml of LB. All media contained 50 μg/ml ampicillin. The cells were grown overnight at 37°C and each 5 ml culture was used to inoculate 50 ml of LB. The cells were grown at 37°C until $A_{600} = 0.6$ at which time the temperature was reduced to 26°C and 1 mM IPTG was added to induce expression of rYadQ. After 4 h, 250 μl was removed from each growth and the cells were pelleted by centrifugation. The remainder of the cells were pelleted and frozen at −20°C.

2.2. Analysis of expression and localization

Cells from the 250 μl aliquots were resuspended in SDS containing

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Abbreviations: TEV, tobacco etch virus; rYadQ, recombinant YadQ; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; PBS, phosphate buffered saline; DM, decylmaltoside

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sample buffer and the proteins were separated by SDS-PAGE. The proteins were then transferred to PVDF membranes for Western analysis. Western blotting was performed with penta-His antibody (Qiagen) followed by anti-mouse IgG (Promega). The secondary antibody was stained with BCIP/NBT (Sigma). The expression strain that showed the highest level of expression by Western analysis was selected as the expression host for further experiments.

Localization of rYadQ was determined by Western analysis of the membrane and soluble fractions of the expression host. Frozen cells were thawed and resuspended in PBS pH 7.5, 20 ml/l of culture. Fractionation was accomplished by passing the resuspended cells through a French press three times. The lysed cells were centrifuged at $RCF_{av} = 13\,000 \times g$ for 20 min at 4°C to pellet unlysed cells and cells debris. The supernatant was removed and centrifuged at $RCF_{av} = 196\,000 \times g$ for 60 min at 4°C in a Beckman 50.2Ti rotor to pellet the membrane fraction. Aliquots of the supernatant and pellet from each spin were subjected to Western analysis as described above.

2.3. Solubilization and purification

Twelve detergents, including ionic, nonionic neutral, and zwitterionic, were tested for the ability to extract rYadQ from the membrane. 10 μl aliquots of membranes containing rYadQ were mixed with 90 μl of a detergent at approximately 10 times the critical micelle concentration. After a 1 h incubation at room temperature, the mixtures were centrifuged in a Beckman Airfuge at $\sim 100\,000 \times g$ to pellet insoluble material. The amount of rYadQ in the pellet and supernatant was compared on SDS-PAGE gels.

The methods described below were for the purification of rYadQ from 1 l of cell culture. Membranes were prepared as described in Section 2.2. The membrane pellet was resuspended in 15 ml of 20 mM Tris–HCl pH 8.0, 18 mM DM and placed on a rocker platform for 1 h at room temperature to disrupt the membranes. 4 ml of Talon Superflow Metal (Cobalt) Affinity Resin (Clontech) was equilibrated with 40 ml wash buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM decylamltoside (DM)). The concentrations of detergent and NaCl in the membrane containing solution were reduced to that of the wash buffer by the addition of 30 ml of 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The equilibrated resin was added to the solubilized membranes and the mixture was placed on a rocker for 1 h at room temperature. The solution was loaded onto a 15 cm × 1 cm Kontes Chromoflex column and connected to a Bio-Rad Biologic Workstation. The resin was washed with 65 ml wash buffer+10 mM imidazole and rYadQ was eluted with 20 ml wash buffer+250 mM imidazole; the flow rate was 2 ml/min throughout.

The peak fractions were concentrated with Amicon Centriprep filters (30 000 MWCO). The concentrated fractions were exchanged into buffer CE (50 mM Na phosphate pH 7.0, 5 mM DM) with Bio-Rad 10DG columns. A Bio-Rad UNO S-1 column was connected to the Biologic Workstation and equilibrated with buffer CE. Following injection of the protein sample, the column was washed with 24 ml of buffer CE. The proteins were eluted with a 50 ml gradient from 0 mM to 500 mM NaCl at 2 ml/min. The peak fractions were collected and analyzed by SDS-PAGE. Fractions containing pure rYadQ were pooled. Protein concentration was determined by measuring the absorbance at 280 nm using a calculated extinction coefficient of 50780 M^{-1} cm^{-1}.

2.4. Circular dichroism (CD)

CD spectra were collected from purified rYadQ, 0.21 mg/ml in 10 mM malic acid/imidazole pH 7.0, 150 mM NaCl, 3.6 mM DM. A quartz cuvette with a 0.1 mm path length was used in a Jasco 600 spectropolarimeter. Forty scans were collected from 250 to 190 nm with a step size of 0.2 nm, at a rate of 50 nm/min, and a bandwidth of 1.0 nm. The mean residue ellipticity was calculated from the averaged CD spectra using $[\Theta]_{mrw} = \Theta \times M_{mrw}/10 \times c \times l$, where l is the path length in cm, c is the protein concentration in mg/ml, and $M_{mrw} = 107.37$ is the mean residue molecular weight.

2.5. Size exclusion chromatography

A Pharmacia Superdex 200 FPLC column was connected to the Biologic Workstation and equilibrated with buffer S (50 mM Na phosphate pH 7.0, 150 mM NaCl, 3.6 mM DM). Purified rYadQ in buffer S was loaded onto the column and eluted with 30 ml of buffer S at a flow rate of 0.25 ml/min. Absorbance at 280 nm was monitored and peak fractions were analyzed by SDS-PAGE.

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ClC-0   PQAVGSGIPELKTIIRGAVLHEYLTLRTFVAK
ClC-1   PQAVGSGIPEMKTILRGVVLKEYLTMKAFVAK
ClC-2   PQAVGSGIPEMKTILRGVVLKEYLTLKTFIAK
ClC-3   PYACGSGIPEIKTILSGFIIRGYLGKWTLMIK
ClC-4   PYRCGSGIPEIKTILSGFIIRGYLGKWTLLIK
ClC-5   PYACGSGIPEIKTILSGFIIRGYLGKWTLVIK
ClC-6   PVAAGSGIPEVKCYLNGVKVPGIVRLRTLLCK
ClC-L   PSSGGSGIPEVKTMLAGVVLEDYLDIKNFGAK
ClC-K   PSSGGSGIPELKTMLAGVILEDYLDIKNFGAK
YadQ    PEAGGSGIPEIEGALE--DQRPVRWWRVLPVK
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Fig. 1. Segment of a sequence alignment between YadQ, human ClCs, and ClC-0 from *Torpedo marmorata*. Invariant residues are shaded in dark grey, consensus residues are shaded light grey, and residues similar to a consensus residue are underlined.

3. Results

3.1. Expression and purification

Western analysis of whole cells revealed that rYadQ was expressed equally well in C43(DE3) and C41(DE3) and the level of expression was higher than in BL21(DE3) and BL21(DE3)pLysS. The strain C43(DE3) was chosen as the expression host for subsequent experiments. Analysis of the membrane and soluble fractions of cells in which the protein was expressed revealed that rYadQ is localized in the membrane (Fig. 2). The presence of rYadQ in the pellet from the low speed spin after fractionation may be an indication that some of the protein is in inclusion bodies; however, the pellet also contained unlysed cells which are at least partially responsible for the appearance of rYadQ in the low speed pellet.

Several nonionic and zwitterionic detergents extracted rYadQ from the membrane, including zwittergent 3-12, C_8E_4, and DM. However, rYadQ only bound well to the cobalt resin in the presence of DM and this detergent was used throughout the large-scale solubilization and purification of the protein. Substantial purification was achieved with metal affinity chromatography, but several proteins bound to and eluted from the resin under conditions required for the binding and elution of rYadQ necessitating another step in the purification. It was determined that rYadQ bound to the UNO S-1 column at pH 7.0 and high level purification was achieved by eluting with a linear salt gradient (Fig. 3). The current yield of pure rYadQ is 50 μg/l of culture.

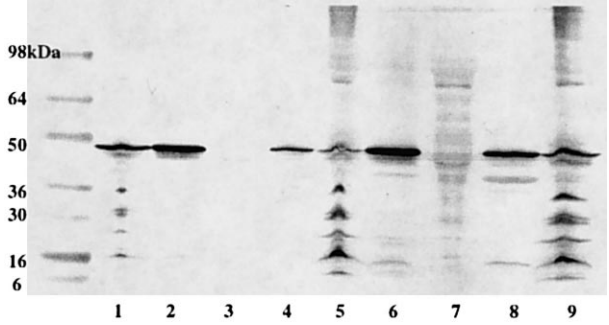

Fig. 2. Western blot showing localization of rYadQ in the membrane. Lane 1, whole cells; 2 and 6, membranes; 3 and 7, supernatant from high speed centrifugation; 4 and 8, pellet from low speed centrifugation; 5 and 9, supernatant from low speed spin.

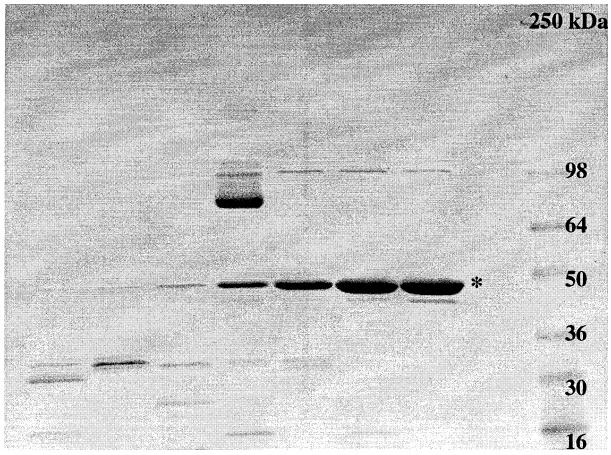

Fig. 3. SDS-PAGE gel of peaks from ion exchange purification of rYadQ. The asterisk marks the location of rYadQ. Lanes 5, 6 and 7 are from the first peak in the elution, 1–4 are each from peaks which eluted later in the salt gradient. Bands other than that labeled by the asterisk are impurities.

3.2. CD spectroscopy

CD spectra were collected from rYadQ after ion exchange purification. Forty spectra were collected from buffer with and without rYadQ. The spectra were averaged and the baseline spectrum was subtracted from the protein spectrum. Minima in the baseline subtracted spectrum at 208 and 220 nm are indicative of a predominantly α-helical content (Fig. 4).

3.3. Size exclusion chromatography

A Superdex 200 FPLC column was calibrated with proteins of known molecular weight. rYadQ eluted between aldolase, 158 kDa, and bovine serum albumin (BSA), 67 kDa, data not shown. The calculated molecular weight of rYadQ with the histidine tag and rTEV protease site is 53 576 Da. The Stokes radii of aldolase and BSA are 4.6 and 3.5, respectively. The Stokes radius of rYadQ determined from the protein's elution time is 4.4, close to that of aldolase. Because integral membrane proteins solubilized with detergents exist as protein–detergent complexes, the mass of the detergent molecules surrounding the protein must be considered when analyzing size exclusion chromatography of these proteins. The elution time of rYadQ suggests that the protein forms dimers.

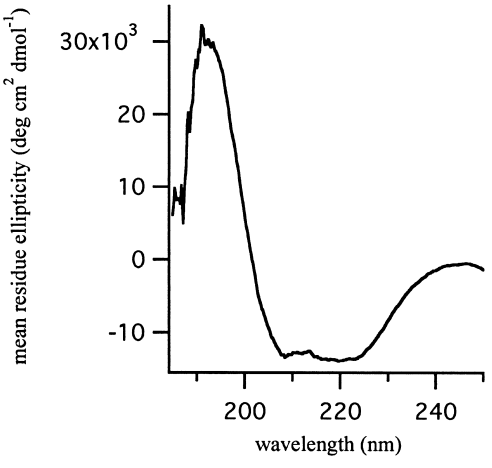

Fig. 4. CD spectrum of purified rYadQ.

4. Discussion

ClC chloride channels perform diverse physiological functions in a wide variety of cell types. The biochemical and electrophysiological characterization of these proteins is ongoing. To fully understand the molecular basis of the function of ClCs, detailed structural information is necessary. An increasingly popular means of overcoming expression and purification obstacles associated with the study of eukaryotic membrane proteins is the identification of structurally and functionally similar bacterial and archaebacterial homologs.

YadQ is related through evolution to the ClCs found in humans, as evidenced by the primary structure conservation. Analyses of the sequences and experimental studies of ClCs have led to the conclusion that the proteins contain α-helices which cross the lipid bilayer 10–12 times. Experimental investigation of several ClCs has revealed a dimeric stoichiometry [15]. This study shows that recombinant expression and high level purification of a bacterial homolog of mammalian ClCs is possible. Additionally, rYadQ has a secondary and quaternary structure similar to that found in ClCs. We conclude that it is justified to use rYadQ to study the structure and function of ClC chloride channels. Crystallization experiments are currently under way in our laboratory. We note that another lab has very recently published a paper that also describes expression, purification and characterization of YadQ [16].

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