Expression, Purification, Crystallization and Preliminary Crystallographic Analysis of the Human Intracellular Chloride Channel Protein CLIC4

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Abstract: The human chloride intracellular channel protein CLIC4 has been crystallized by the hanging-drop vapour-diffusion technique using trisodium citrate as the precipitant. The best crystals were obtained by the microseeding method. The crystals diffracted to 2.2 Å resolution and were found to belong to space group P121, with unit-cell parameters a = 73.19, b =86.05, c = 73.38 Å, β = 112.99° and three molecule per asymmetric unit.

Keywords: CLIC4, human chloride intracellular channel protein, purification, crystallization, preliminary crystallographic studies

1. INTRODUCTION

Chloride ion channels are involved in variant functional performance of the physiological processes. They are located both within the plasma membrane and other internal cell membranes [1,2] and participate in the control of secretion and absorption of salt, regulation of membrane potentials, organellar acidification, and cell volume homeostasis [3]. To date, seven members of the CLIC family have been identified: CLIC1 ,CLIC2,CLIC3, CLIC4, CLIC5, p64, and parchorin. Each of these proteins exists both in soluble and integral membrane forms [4-6]. The chloride intracellular channel protein CLIC4 is an important member of the CLIC family and the first human CLIC to be identified recently [7]. It is expressed in a wide variety of tissues [8-10]. CLIC4 gene expression has been linked to different physiology functions such as developmental regulation [11], cell differentiation [12] and cell apoptosis [13, 14]. In this paper we will report the gene cloning, expression, purification, crystallization and preliminary crystallographic analysis of a soluble form of the intracellular chloride channel protein CLIC4 from *homo sapiens*.

2. MATERIALS AND METHODS

2.1. Cloning

The coding sequence for protein CLIC4 (28.7 kDa) was amplified from RT-PCR products from human haemopoietic stem cells using the polymerase chain reaction (PCR) method. The PCR product was purified and restricted with NdeI and XhoI. The fragment was purified and ligated into NdeI- and XhoI-restricted sites of the pET22b(+) vector (Novagen Inc.) with a His6-tag to the C-terminus. The plasmid was amplified in TG1-competent *E. coli* cells. This construction was confirmed by DNA sequencing.

2.2. Expression and Purification

The reconmbinant plasmid was transformed into *E. coli* host strain BL21 (DE3) and grown on the LB plate containing ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) overnight at 310 K. The single clone was picked into 5 ml LB medium and cultured overnight. Then 1.5 ml culture was subcultured into 50 ml of fresh LB. Three hours later, 15 ml culture cells were grown at 310 K in 500 ml LB medium containing 100 mg ml^{-1} ampicillin until the OD600 value reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and culture continued at 310 K for a further 6 h. Cells were collected by centrifugation at 4000 rev min^{-1} for 30 min. The pellet was resuspended in 20 ml lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole) and sonicated. The lysate was clarified by centrifugation at 16 000 rev min^{-1} for 10 min at 277 K to remove the cell debris. The supernatant was applied to a Ni^{2+}- chelating column [Novagen Inc.] and the contaminant protein was washed off with wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole). The target protein was eluted with elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole). The protein was concentrated to 1 ml with an Amicon Ultra 10 kDa molecular weight cutoff filter unit [Millipore] and was purified on a Superdex-75 column (Pharmacia) in buffer A (50 mM Tris–HCl pH 8.0).

2.3. Crystallization

The purified protein was concentrated to about 20 mg ml^{-1} in ultrapure water for crystallization. All crystallization experiments were performed with the hanging-drop vapour-diffusion method using Hampton Research Index and Crystal Screens. 1.5 μl protein solution and 1.5 μl reservoir solution were mixed and equilibrated with 400 μl reservoir solution

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in each well at 293 K. The initial microcrystals were obtained in 1.6 M trisodium citrate pH 6.5. The crystallization condition was optimized at 293 K by varying the concentration of precipitates (trisodium citrate), pH, and the additives, but none of the procedures resulted in suitable crystals for X-ray diffraction. Finally, suitable crystals were obtained by macro-seeding screening with different equilibration times and equilibration concentration of precipitates.

2.4. Data Collection and Processing

Diffraction data were collected at Rigaku R-Axis IV^{++} image plate using with seal-tube X-ray source and Cu *K*_ radiation (λ =1.5418 Å) from a rotating anode operating at 40 kV and 20 mA with 0.1 mm cofocus incident beam diameter in the National Key Laboratory of Biomacromolecules, Institute of Biophysics, CAS. The data were collected at 85 K with a crystal-to-detector distance of 100 mm, Δφ=1˚ and 300 s exposure time. A total of 180 frames were collected. Diffraction data were scaled and integrated with MOSFILM [16]. All data sets were processed by *mosflm6.2.3* program [15] and scaled using CCP4 program *SCALA* [16].

3. RESULTS

The gene CLIC4 pET22b(+) vector (Novagen Inc.) with a his tag was successfully cloned. By transforming the plasmid into the *E. coli strain* BL21(DE3), the recombinant protein was expressed at a level of about 20 mg in 0.5 L LB liquid medium.

Hampton Research kit Index^{TM} and Crystal Screens^{TM} were used to supply conditions for initial screening. After one week the initial microcrystals were obtained in 1.6 M trisodium citrate pH 6.5. To optimize the growth of the crystals, the gradient of concentration of precipitates and equilibration time of reservoir solution were used to macro-seeding. Finally the best crystals (0.10x2x0.4 mm in size) (Fig. **1**) that qualified for data collection were obtained with the recipe of 1.5 μl protein solution mixed with 1.5 μl reservoir solution which contained 1.1 M trisodium citrate, 0.1 M Tris pH 7.5 with 0.1 M magnesium chloride and 3% ethanol as additive, and it was equilibrated for 8 hours in 400 μl reservoir solution before seeding.

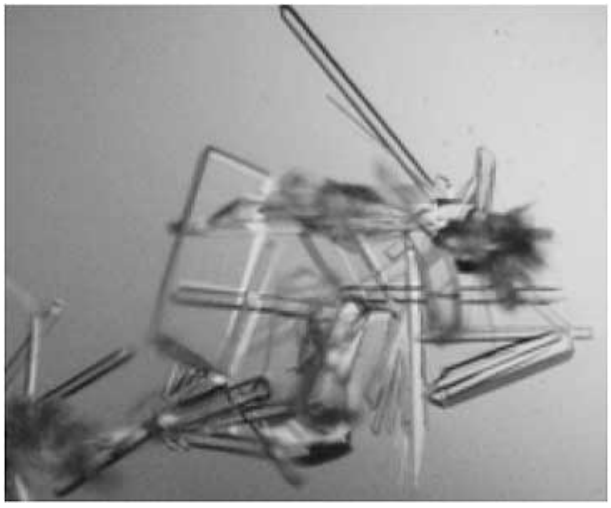

Figure 1. Crystals of CLIC4.

The diffraction data were collected at a resolution 2.2 Å (Fig. **2**) and the data statistics are summarized in Table **1**. The results showed that the crystal belongs to space group P 1 21. The unit cell parameters are a =73.18 Å, b =86.05 Å, c = 73.38 Å, β = 112.99˚. This data are compatible with the presence of three molecules in the asymmetric unit, with a Matthews coefficient of 2.50 Å^{3}Da^{-1} and a solvent content of 51%.

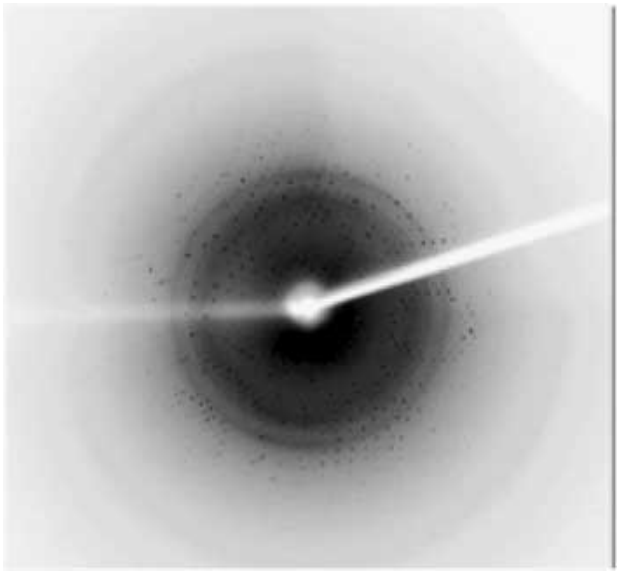

Figure 2. The image of the CLIC4 diffraction pattern of a resolution 2.2 Å.

Table 1. Crystal data and Data-Collection Statistics. Values in Parentheses are for the Highest Resolution shell (2.32-2.20 Å)

Wavelength(Å)	1.5418
Space Group	$P2_1$
Unit-cell parameters(Å)	a=73.18,b=86.05,c=73.38, β(°)=112.99
Molecules per AU	3
Matthews coefficient(Å^{3}Da-1)	2.5
Resolution range(Å)	23.49-2.20
Unique reflections	43497
Completeness (%)	99.7(97.7)
Average I/σ (I)	7.8(17.7)
Rsym(%)	6.6(36.5)

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