Expression, purification, crystallization and preliminary X-ray diffraction analysis of Chloride intracellular channel 2 (CLIC2)

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Running Title: CLIC2 crystallization
Synopsis

Chloride intracellular channel 2 (CLIC2) belongs to a family of intracellular chloride channel proteins that can exist in a soluble form. We report here the expression, purification and crystallisation of human CLIC2 in two different crystal forms.

Abstract

The chloride intracellular channel (CLIC) family of proteins are unusual in that they can exist in either an integral membrane channel form or a soluble form. Here we report the expression, purification, crystallization and preliminary diffraction analysis for CLIC2, one of the least studied members of this family. Human CLIC2 was crystallized in two different forms, both in the presence of reduced glutathione and both diffracted to better than 1.9 Å resolution. Crystal form A displayed P2₁2₁2₁ symmetry, with unit cell parameters of $a = 44.0$ Å, $b = 74.7$ Å and $c = 79.8$ Å. Crystal form B displayed P2₁ symmetry, with unit cell parameters of $a = 36.0$ Å, $b = 66.9$ Å and $c = 44.1$ Å. Structure determination will shed more light on the structure and function of this enigmatic family of proteins.
1. Introduction

The first member of the chloride intracellular channel (CLIC) family was identified based on intracellular chloride channel activity and purified by affinity to a chloride channel inhibitor (Landry et al., 1993). This led to the identification of a number of homologs that all contain a conserved region of approximately 240 residues. Consistent with their original identification as chloride channels, a number of family members are able to insert into artificial membranes in vitro and form ion channels with varying degrees of anion selectivity. Surprisingly, CLIC family proteins can also exist in a soluble form and do not possess any obvious hydrophobic trans-membrane segments, features reminiscent of many bacterial pore-forming toxins (Cromer et al., 2002). Based on very weak sequence similarity between omega glutathione S-transferase (GST) and the conserved region of CLIC proteins, it was hypothesized that, in their soluble form, CLIC proteins adopt the canonical GST fold (Dulhunty et al., 2001). Structure determination for both CLIC1 (Harrop et al., 2001) and CLIC4 (Littler et al., 2005, Li et al., 2006) has confirmed this hypothesis and also revealed that CLIC1 can covalently bind glutathione via a conserved cysteine (Cys24) (Harrop et al., 2001), in a similar manner to omega class GSTO1-1 (Board et al., 2000). The structure adopted by cytosolic GSTs and soluble CLICs comprises two domains, an N-terminal mixed α/β thioredoxin-like domain and an all α-helical C-terminal domain. There is good evidence that that the N-terminus can translocate across membranes (Tonini et al., 2000), a step in channel formation that would require some unfolding of the N-terminal domain. Consistent with this concept, oxidation of CLIC1 favours channel formation and leads to an alternative form of soluble CLIC1 that is dimeric, with considerable unfolding of the N-terminal domain and formation of an intramolecular disulphide between Cys59 and the conserved Cys 24 (Littler et al., 2004).

CLIC2 is a relatively poorly studied member of the CLIC family. The N-terminal domain of CLIC2 lacks the cysteine equivalent to Cys59 of CLIC1 but contains another cysteine Cys33 that, together with the conserved Cys30 (equivalent to Cys24 in CLIC1), forms a CXXC motif similar to glutaredoxin. Based on these cysteine residue differences, we hypothesized that CLIC2 may undergo differential redox regulation and conformational change, relative to CLIC1. To investigate this
hypothesis, we have undertaken structural studies of human CLIC2 and report here the production of well-diffracting crystals that have enabled the determination of the structure of CLIC2, which will be reported elsewhere.

2. Experimental procedures and results

2.1. Cloning, expression and purification

CLIC2 was expressed with a His-tagged ubiquitin fused at the N-terminus. Human CLIC2 was amplified from the EST clone AI129485 and ligated between the BamHI and PstI sites of the pQE-30 vector (Qiagen, Clifton Hills, Australia) to produce pQECLIC2 as described previously (Board et al., 2004). The CLIC2 insert was subcloned as a SacII/HindIII fragment into pHUE (Catanzariti et al., 2004) to create a 6His-ubiquitin-CLIC2 fusion protein. This protein was expressed in BL21(DE3) cells grown over night in the presence of 0.1mM isopropyl thio-D-galactoside and processed by the methods described by Whittington et al. (1999). The recombinant protein was purified by immobilised metal affinity chromatography with Ni-agarose as previously described for His-tagged GSTs (Whittington et al., 1999). Following dialysis to remove imidazole, the 6His-ubiquitin tag was cleaved by digestion with a ubiquitin specific protease (Baker et al., 1994; Cantanzariti et al., 2004) and both the protease and 6His-ubiquitin tag were removed by immobilised metal affinity chromatography with Ni-agarose (Catanzariti et al., 2004). The protein was further purified by gel filtration on a Pharmacia fast protein liquid chromatography Superose 12 column equilibrated with 50mM Hepes, 10% glycerol, pH 7.0.

The purified protein was dialysed into 50 mM HEPES pH 7.5 and 100 mM NaCl and concentrated to 7.25 mg/ml for crystal form A and 15 mg/ml in 20 mM Tris-HCl pH 7.5, 50 mM NaCl for crystal form B. The purified protein was essentially completely monomeric in solution, as indicated by gel-filtration chromatography on a Superdex 75 10/300 chromatography column (GE Biosciences) in 50 mM sodium phosphate, pH 7.4 and 100 mM sodium chloride (data not shown), and was greater than 95% pure, as determined by SDS-PAGE.
2.2. Crystallisation

All crystallization experiments were carried out using the hanging-drop vapour-diffusion technique using 24-well Limbro tissue culture plates (ICN Inc.) at 19 °C. Drops were formed by mixing equal volumes (1 µl) of protein solution and reservoir solution. Two different crystal forms were found (Fig. 1) using different batches of purified protein, at different concentrations and with slightly different reservoir solutions. For crystal form A, the protein concentration was 7.25 mg/ml and the reservoir solution contained 35% - 50% (v/v) PEG 400, 100 mM Tris-HCl pH 8.0 – 9.2 and 5 mM reduced glutathione (GSH). Crystals appeared after 2 to 3 days. For crystal form B, the protein concentration was 15 mg/ml and the reservoir solution contained 30% - 32% (v/v) PEG 400, 100 mM Tris-HCl pH 7.5 and 5 mM GSH. Crystals appeared after 3 days and were used immediately for X-ray data collection. GSH was found to be a necessary ingredient for both crystal forms.

2.3. Data collection

Both crystal forms were frozen in the buffer from the crystallisation drop as the concentration of PEG 400 was sufficient to prevent ice crystal formation. Crystals were mounted in cryo-loops (Hampton Research, CA) and transferred directly into a stream of nitrogen gas maintained at 100K. For crystal form A, X-ray diffraction data were collected on BioCARS beamline 14-ID-B at the Advanced Photon Source in Chicago, USA (Table 1). For crystal form B, X-ray diffraction data were collected in-house using a Rigaku RU200H generator equipped with mirror optics (Xenocs) and a MARResearch 345mm imaging plate detector. Diffraction data were integrated and scaled using HKL (Otwinowski. & Minor, 1997) for crystal form A and MOSFLM (CCP4, 1994) and SCALA (CCP4, 1994) for crystal form B. Data statistics are shown in Table 1. Both crystal forms diffracted to better than 1.9 Å resolution. Crystal form A displayed P2₁₂₁₂₁ symmetry, with unit cell parameters of \( a = 44.0 \text{ Å}, b = 74.7 \text{ Å} \) and \( c = 79.8 \text{ Å} \). Crystal form B displayed P2₁ symmetry, with unit cell parameters of \( a = 36.0 \text{ Å}, b = 66.9 \text{ Å}, c = 44.1 \text{ Å} \) and \( \beta = 99.9^\circ \). The structures of CLIC2 in both crystal forms have now been determined by molecular replacement, using the published CLIC1 structure (Harrop et al., 2001) as a probe, and have been reported elsewhere.
(Cromer et al., 2007). The atomic coordinates and structure factors (accession codes 2R4V and 2R5G for crystal forms A and B respectively) have been deposited in the Protein Data Bank at the Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org). These structures will provide further insights into the structure and function of this intriguing family of proteins.

Acknowledgements

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References


Table 1

Crystal data and X-ray diffraction data collection statistics.

The values in parentheses are for the highest resolution bin.

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Form A</th>
<th>Form B</th>
</tr>
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<tbody>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell dimensions (Å)</td>
<td>44.0, 74.7, 79.8</td>
<td>36.0, 66.9, 44.1</td>
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<td>β (°)</td>
<td>90.0</td>
<td>99.9</td>
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<tr>
<td>Resolution (Å)</td>
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<td>1.86 (1.95 – 1.86)</td>
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<td>No. of crystals</td>
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<td>1</td>
</tr>
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<td>300° (1° x 300 images)</td>
</tr>
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<td>100</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>No. of unique reflections</td>
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<td>16303</td>
</tr>
<tr>
<td>Multiplicity</td>
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<td>6.0 (5.3)</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>93.9 (62.0)</td>
<td>99.5 (84.3)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>31.3 (2.8)</td>
<td>24.2 (5.1)</td>
</tr>
<tr>
<td>R_{merge} (%)²</td>
<td>6.8 (30.6)</td>
<td>4.7 (31.5)</td>
</tr>
</tbody>
</table>

²R_{merge} = Σhk|Σ|I_i| - <I>||<I>|, where I_i is the intensity for the ith measurement of an equivalent reflection with indices h,k,l.
Figure 1. Crystals of CLIC2 in (a) crystal form A and (b) crystal form B.