Crystallization and preliminary X-ray crystallographic studies of transglutaminase 2 in complex with Ca^{2+}

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Transglutaminase 2 (TG2) is a multi-functional protein that has been implicated in a variety of physiological cellular activities, including apoptosis, angiogenesis and cellular differentiation. Two functions of TG2 are protein cross-linking and GTP hydrolysis activities. The protein cross-linking activity of TG2 is positively controlled by calcium; however, the molecular mechanism of its Ca^{2+} -dependent activity is completely unknown. In the present study, full-length human TG2 in complex with Ca^{2+} was overexpressed, purified and crystallized at 20°C as a first step towards elucidating this mechanism. X-ray diffraction data were collected to a resolution of 3.4 Å from a crystal belonging to space group $C222_1$, with unit-cell parameters $a = 133.08$, $b = 216.30$, $c = 166.26$ Å. Based on these data, the asymmetric unit was estimated to contain three molecules.

1. Introduction

Transglutaminase 2 (TG2) is a multi-functional protein that has been implicated in a variety of physiological cellular activities, including apoptosis (Oliverio *et al.*, 1997; Nemes *et al.*, 1997), angiogenesis (Jones *et al.*, 2006; Haroon *et al.*, 1999), wound healing (Upchurch *et al.*, 1991; Haroon *et al.*, 1999), cellular differentiation (Matic *et al.*, 2010; Tee *et al.*, 2010), neuronal regeneration (Eitan *et al.*, 1994) and bone development (Kaartinen *et al.*, 2002). TG2 has received a great deal of attention because it is linked to many human diseases, including neurodegenerative diseases (Lesort *et al.*, 2000; Hoffner & Djian, 2005), diabetes (Porzio *et al.*, 2007) and cancer (Birckbichler *et al.*, 1977; Barnes *et al.*, 1985; Mangala & Mehta, 2005). The involvement of TG2 in cancer has been thoroughly investigated in several reports that have demonstrated its down-regulation in aggressive tumours and metastases (Barnes *et al.*, 1985; Birckbichler *et al.*, 2000). Several mutations in TG2 that impair TG2 activities have also been observed in early stages of type 2 diabetes (Porzio *et al.*, 2007).

Two representative functions of TG2 are protein cross-linking and GTP hydrolysis activities (Lee *et al.*, 1989; Griffin *et al.*, 2002). Protein cross-linking activity by transamidation is positively controlled by calcium and negatively regulated by GTP (Achyuthan & Greenberg, 1987). Human TG2 consists of an N-terminal β -sandwich domain, a catalytic domain and two C-terminal β -barrel domains (Liu *et al.*, 2002). Three crystal structures complexed with guanosine diphosphate (GDP), adenosine triphosphate (ATP) and irreversible inhibitor have been elucidated to date (Han *et al.*, 2010; Liu *et al.*, 2002; Pinkas *et al.*, 2007). These structures showed that TG2 undergoes an extraordinarily large conformational change upon activation by inhibitors. GTP and Ca^{2+} are well known regulators of TG2 transglutaminase activity. GTP inactivates TG2 by promoting transition to the compact conformation (closed form). Although there is evidence that binding of Ca^{2+} induces conformational changes that expose the catalytic triad in TG2 (Casadio *et al.*, 1999), the exact molecular mechanism of Ca^{2+} -mediated activation of the protein cross-linking activity of TG2 is completely unknown.

In the present study, we overexpressed, purified and crystallized full-length human TG2 in complex with Ca^{2+} as a first step towards elucidating this activity. The crystallization conditions of the TG2– Ca^{2+} complex were completely different from previously reported crystallization conditions for the native protein. The details regarding

the atomic structure of TG2 in complex with Ca^{2+} will enable us to understand the regulatory mechanism of TG2 by Ca^{2+} .

2. Materials and methods

2.1. Expression and purification

To express C-terminally His-tagged enzyme, the coding region of human TG2 was amplified by PCR using P1 (5'-GGGCA-CATATGATGGCCGAGGAGCTGGTCTT-3') and P2 (5'-GGGCTCGAGGGCGGGGCCAATGAT-3') primers. The PCR product was then digested with restriction enzymes (*NdeI/XhoI*), after which it was inserted into home-made pOKD5 vector that had been cut using the same restriction enzymes. The plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells, after which its expression was induced by treating the bacteria with 0.125 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 25 h at 18°C. Cells expressing full-length human TG2 were then pelleted by centrifugation, resuspended and lysed by sonication in 30 ml lysis buffer [50 mM sodium phosphate buffer pH 7.5, 400 mM NaCl, 5 mM benzimidazole, 1 mM β -mercaptoethanol, 50 μ M ATP, 1 mM PMSF, 0.5% (v/v) Triton X-100, 5 mM imidazole]. The lysate was then centrifuged at 16 000 rev min⁻¹ for 30 min at 4°C, after which the supernatant fractions were applied onto a gravity-flow column (Bio-Rad) packed with Ni-NTA affinity resin (Qiagen). Next, the unbound bacterial proteins were removed from the column using lysis buffer. The C-terminally His-tagged TG2 was subsequently eluted from the column using elution buffer [50 mM HEPES buffer pH 7.0, 100 mM NaCl, 50 μ M ATP, 10% (v/v) glycerol, 300 mM imidazole]. The elution fractions were collected on a 0.5 ml scale to 2 ml and applied onto a Superdex 200 gel-filtration column (GE Healthcare) that had

been pre-equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl. TG2 (molecular mass 78 607 Da) eluted at around 14 ml. A gel-filtration standard (Bio-Rad) containing a mixture of molecular-mass markers (thyroglobulin, 670 000 Da; globulin, 158 000 Da; ovalbumin, 44 000 Da; myoglobin, 17 000 Da; vitamin B₁₂, 1350 Da) was used for size calibration. The purified TG2 was then applied onto a Mono Q ion-exchange column (GE Healthcare) using 20 mM Tris pH 8.0 as a starting buffer and 20 mM Tris pH 8.0, 1 M NaCl as the elution buffer. The eluted TG2 was finally applied onto a Superdex 200 gel-filtration column (GE Healthcare) that had been pre-equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl, 5 mM CaCl₂. Finally, the purified TG2 was collected and concentrated to 6–8 mg ml⁻¹ and the peak was confirmed to contain TG2 by SDS-PAGE.

2.2. Crystallization

The crystallization conditions were initially screened at 20°C by the hanging-drop vapour-diffusion method using screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, Natrix, MembFac, SaltRX, Index HT) and from the deCODE Biostructures Group (Wizard I, II, III and IV). Initial crystals were grown on plates by equilibrating a mixture consisting of 1 μ l protein solution (6–8 mg ml⁻¹ protein in 20 mM Tris pH 8.0, 150 mM NaCl, 5 mM CaCl₂) and 1 μ l reservoir solution No. 26 of the Natrix screen from Hampton Research (10% polyethylene glycol 8000, 0.1 M magnesium acetate tetrahydrate, 0.2 M potassium chloride, 0.05 M sodium cacodylate trihydrate pH 6.5) against 0.4 ml reservoir solution. Crystallization was further optimized using a range of concentrations of protein, salt, pH and additive screening. Crystals appeared within 3 d and grew to maximum dimensions of 0.2 $\times$ 0.05 $\times$ 0.02 mm (Fig. 1) in the presence of 5% polyethylene glycol 8000, 0.1 M magnesium acetate tetra-

Figure 1

Purification of the TG2- Ca^{2+} complex. (a) The first gel-filtration chromatography and SDS-PAGE of the TG2- Ca^{2+} complex. (b) Ion-exchange chromatography and SDS-PAGE. (c) The second gel-filtration chromatography and SDS-PAGE of TG2- Ca^{2+} complex.

Table 1Diffraction data statistics for the TG2–Ca²⁺ complex crystal.

Values in parentheses are for the highest resolution shell.

X-ray source	BL-5A, PAL
Wavelength (Å)	1.0000
Space group	C222 ₁
Unit-cell parameters (Å)	<i>a</i> = 133.08, <i>b</i> = 216.30, <i>c</i> = 166.26
Resolution limits (Å)	50–3.4
No. of observations	318301
No. of unique reflections	29265
Mean <i>I</i> / σ (<i>I</i>)	29.6 (8.9)
Completeness (%)	99.9 (100)
<i>R</i> _{meas} (<i>I</i>)	12.5 (42.3)

hydrate, 0.1 *M* potassium chloride, 1 mM CaCl₂, 0.05 *M* sodium cacodylate trihydrate pH 6.4. The crystals were rectangular and diffracted to a resolution of 3.4 Å.

2.3. Crystallographic data collection

For data collection, the crystals were transiently soaked in a solution corresponding to the reservoir solution supplemented with 8%(v/v) glycerol. The soaked crystals were then cooled in liquid nitrogen. A 3.4 Å resolution diffraction data set was collected on beamline BL-6C at the Pohang Accelerator Laboratory (PAL), Republic of Korea. The data sets were indexed and processed using *HKL*-2000 (Otwinowski & Minor, 1997). The diffraction data statistics are given in Table 1.

3. Results and discussion

As a first step towards elucidating the activity controlling the mechanism of TG2 by Ca²⁺, we overexpressed, purified and crystallized full-length human TG2 in complex with Ca²⁺. His-tag affinity chromatography followed by ion-exchange chromatography and gel-filtration chromatography produced 90% pure TG2 (Figs. 1*a*, 1*b* and 1*c*). The calculated monomeric molecular weight of TG2 including the C-terminal His tag was 78 607 Da and it eluted at approximately 80 kDa, suggesting that it exists as a monomer (Fig. 1).

An initial small crystal from condition No. 26 of the Natrix screen from Hampton Research that diffracted poorly was obtained. Optimization of the crystallization conditions using a range of concentrations of protein, polyethylene glycol 8000, magnesium acetate

Figure 2

Crystal of the human TG2–Ca²⁺ complex. Crystals were grown in 3 d in the presence of 5% polyethylene glycol 8000, 0.1 *M* magnesium acetate tetrahydrate, 0.1 *M* potassium chloride, 1 mM CaCl₂, 0.05 *M* sodium cacodylate trihydrate pH 6.4. The approximate dimensions of the crystals were 0.2 × 0.05 × 0.02 mm.

tetrahydrate, potassium chloride and pH led to diffracting crystals (Fig. 2). The crystallization conditions of the TG2–Ca²⁺ complex introduced in this study were completely different from the previously reported crystallization conditions for TG2 crystals. The optimized crystals grew to dimensions of 0.2 × 0.05 × 0.02 mm in 3 d and diffracted to 3.4 Å resolution. The crystal belonged to space group C222₁, with unit-cell parameters *a* = 133.08, *b* = 216.30, *c* = 166.26 Å.

Assuming the presence of three molecules in the crystallographic asymmetric unit, the Matthews coefficient (*V*_M) was calculated to be 2.55 Å³ Da^{−1}, which corresponds to a solvent content of 51.72% (Begg *et al.*, 2006; Matthews, 1968). The diffraction data statistics are given in Table 1. The data set was indexed and processed using *HKL*-2000 (Otwinowski & Minor, 1997). The molecular-replacement phasing method was conducted with *Phaser* (McCoy *et al.*, 2007) using the structure of the complex of TG2 with ATP (PDB entry 3ly6; Han *et al.*, 2010) as a search model. All three chains were used. A clear solution with *Z*-scores for the rotation function and translation function of 14.7 and 23.1, respectively, was initially obtained. Initial refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the initial *Phaser* model gave rise to an *R*_{work} of 32.8% and an *R*_{free} of 36.7%. Based on the initial structure, two candidate Ca²⁺-binding sites were identified. Further structural refinement is in progress.

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