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Purification, crystallization and preliminary X-ray diffraction studies of recombinant calcium-binding domain of the small subunit of porcine calpain

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Abstract

The calcium-binding domain of the small subunit of porcine calpain (domain VI) has been expressed in *Escherichia coli*, purified, and crystallized in the presence of Ca^{2+}. Two crystal forms have been obtained by the vapor-diffusion method using PEG 6000 as the precipitant. Crystal form I, belonging to trigonal space group $P3_121$ (or $P3_221$) with cell dimensions $a = b = 79.8$, $c = 57.08$ Å, $\alpha = \beta = 90.0$ and $\gamma = 120.0°$ diffracted to 2.8 Å. The second crystal form diffracts to 1.8 Å and belongs to monoclinic space group $P2_1$ with cell dimensions $a = 50.1$, $b = 79.7$, $c = 57.1$ Å and $\beta = 91.2°$.

1. Introduction

Calpains (E.C. 3.4.22.17) are calcium-dependent cytosolic cysteine proteases, widely distributed in all types of animal cells together with their endogenous inhibitor, calpastatin (Croall & DeMartino, 1991; Sorimachi, Saido & Suzuki, 1994; Suzuki, Sorimachi, Yoshizawa, Kinbara & Ishiura, 1995). These ubiquitous calpains are divided in to two major isoforms μ and m calpains, requiring μ*M* and m*M* calcium concentrations for their activation, respectively. Calpains are heterodimers consisting of a catalytic 80 kDa subunit, and a regulatory 30 kDa subunit. The small 30 kDa subunit is identical for both μ and m calpains and consists of an N-terminal hydrophobic domain (V) and a calmodulin-like domain (VI). Domain VI was predicted to have four calcium-binding *EF*-hand motifs (Mellgren & Murachi, 1990). The large subunit (80 kDa) is made of four structurally distinct domains (I–IV), including a papain-like cysteine protease domain (II) and another calmodulin-like domain (IV). It has been proposed (Tsuji & Imahori, 1981; Yoshizawa, Sorimach, Tomioka, Ishiura & Suzuki, 1995) that in the presence of appropriate levels of Ca^{2+} concentrations, calcium-bound domains IV and VI undergo conformational changes leading to the autolysis of N-termini of the 80 and 30 kDa subunits (resulting in 76 and 28 kDa). Considering that, the autocatalytic modification occurs before the proteolytic function, and the observed increase in sensitivity towards Ca^{2+} is after the N-terminal modifications, Saido, Sorimachi & Suzuki (1994) suggested that the autolysis was an activation step. Since the Ca^{2+} concentration controls the dissociation of 30 kDa leading to the activation of calpain, and the 80 kDa and autolyzed 80 kDa subunits show similar protease activity and Ca^{2+} sensitivity (Yoshizawa *et al.*, 1995), one can argue that the activation is not a consequence of the N-terminal modification, the 80 kDa subunit is the active component of calpain (Suzuki *et al.*, 1995) and the 30 kDa subunit acts as a regulator for the calpain activity.

An increasing number of various endogenous proteins are shown to be susceptible to proteolysis by calpain in a Ca^{2+}-dependent manner. The list of susceptible proteins include myofibrillar proteins, cytoskeletal proteins, hormone receptors, protein kinases and several others (Mellgren & Murachi, 1990). Proteolysis by calpain has been suggested to be an important step in general protein turnover, signal transduction, membrane recognition and fusion events, and regulation of cell differentiation. However, excessive activation of calpain is implicated in a variety of pathological disorders such as stroke (Yokota, Saido, Tani, Kawashima & Suzuki, 1995; Bartus *et al.*, 1995), myocardial ischaemia (Iizuka, Kawaguchi & Kitabatake, 1993), muscular dystrophy (Turner, Westwood, Regen & Steinhardt, 1988) and cataract (David, Shearer & Shih, 1993). A common theme for most of these disorders is cellular Ca^{2+} overload.

In order to understand the mechanisms of calcium-induced calpain activation at the atomic level we have initiated crystal structure determination of various domains of μ calpain. Calpain inhibitors present a potentially novel and powerful means of treating many of the pathological disorders mentioned above. Inhibitor design will be facilitated by these structural studies and specific inhibitors of this enzyme will be useful in exploring the role of calpain in cellular process. Here we report the expression, purification and crystallization of domain VI of porcine calpain which is highly homologous to its human counterpart (Maki, 1996).

2. Materials and methods

2.1. *Expression*

The cDNA fragments for domain VI of porcine μ calpain were cloned into the *BamH*I site of pET-3d vector, and expressed as described previously (Takano, Ma, Yang, Maki & Hatanaka, 1995). The resulting plasmid (pET-S-CaMLD) was transformed into *E. coli* strain BL21(DE3)pLysS. For expression of the recombinant protein, a single colony of the *E. coli* BL21(DE3)pLysS cells harboring pET-S-CaMLD was grown at 310 K in LB medium containing 50 μg ml^{-1} ampicilin and 34 μg ml^{-1} chloramphenicol until the absorbance of the culture reached 1.0 at 600 nm. The cultures were diluted 1:100 in fresh LB medium containing ampicilin, chloramphenicol, 0.2% glucose, and grown at 310 K. At the mid-log phase of growth isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 m*M* and the culture was grown for an additional 4 h. The expressed protein was soluble and could be purified under native conditions.

2.2. *Purification*

Approximately 2.7 g of wet cells in 0.5 l culture were harvested by centrifugation and resuspended in 20 ml of lysis buffer (50 m*M* sodium phosphate buffer, pH 8.0, containing 50 m*M* sodium chloride, 0.1 m*M* PMSF and 5 m*M* β-mercaptoethanol). After stirring for 30 min, the lysate was subjected to sonication on ice (3 × 30 s bursts per 1 min cooling, 300 W). The lysate was centrifuged at 125 000*g* for 30 min. The supernatant containing recombinant protein was chromatographed on DEAE cellulose column equilibrated with 50 m*M* sodium phosphate, 50 m*M* sodium chloride, 2 m*M* EDTA, 5 m*M* β-mercaptoethanol, pH 8.0. After washing the column thoroughly with the equilibration buffer, the bound protein was eluted with 50 m*M* cacodylate buffer, pH 5.8, containing 0.5 *M* NaCl, 0.1 m*M* PMSF and 5 m*M* β-mercaptoethanol. The peak fractions (2.5 ml) were collected and dialyzed against 2 l of 10 m*M* cacodylate buffer, pH 7.0, containing 2 m*M* EDTA, 1 m*M* $CaCl_2$ and 5 m*M* β-mercaptoethanol with several changes of buffer. The dialyzed sample was concentrated approximately to 5 ml by Amicon Ultrafiltration. After centrifugation the sample was loaded on to Sephacryl S-200 gel-filtration column (2.6 × 100 cm) equilibrated with the dialysis buffer. The protein was eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. The peak fractions (5 ml) were pooled and concentrated. The purified protein migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis with molecular mass of about 21 kDa. The final yield was approximately 70 mg from 0.5 l starting culture. For crystallization, the solution was further dialyzed against 2 l of 5 m*M* cacodylate buffer, pH 7.0, containing 2 m*M* EDTA, 1 m*M* $CaCl_2$ and 5 m*M* β-mercaptoethanol.

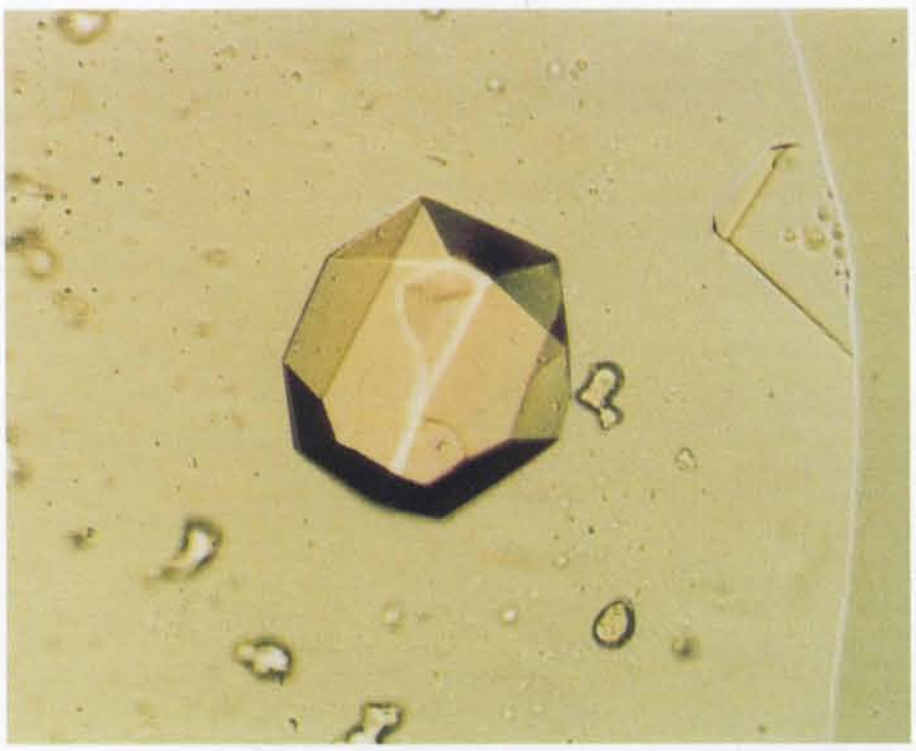

Fig. 1. Crystal form I. Trigonal crystals in space group $P3_121$ or $P3_221$.

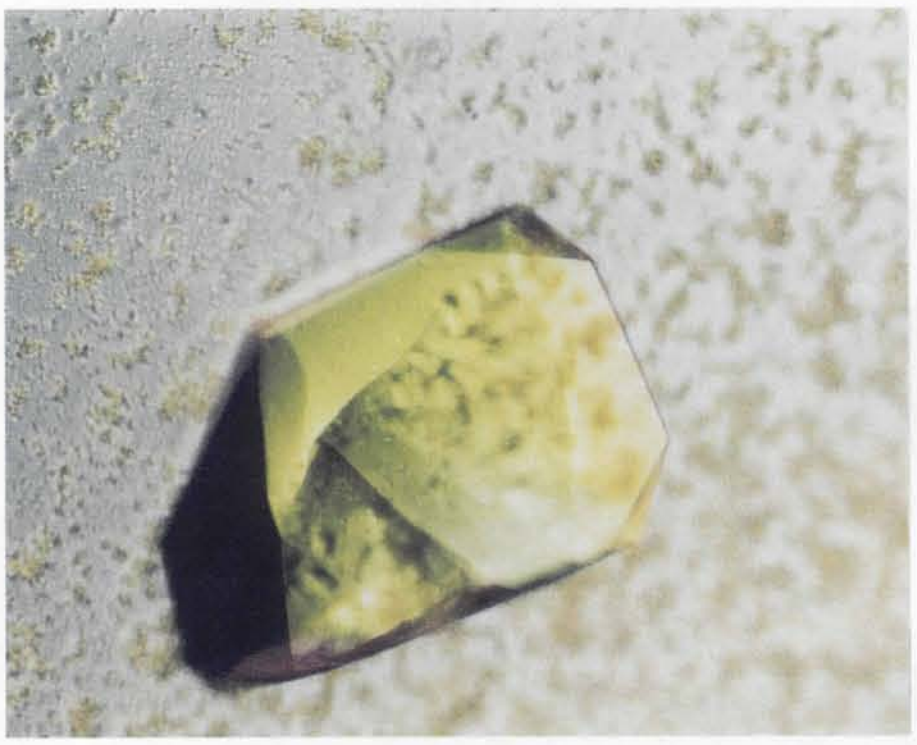

Fig. 2. Crystal form II. Crystals belong to monoclinic space group $P2_1$.

2.3. *Crystallization and data collection*

Crystallization of the recombinant domain VI of porcine calpian was achieved with the hanging-drop vapor-diffusion method at 295 K. The protein concentration used for crystallization was near to 50 mg ml^{-1}. The reservoir solution was 50 m*M* cacodylate buffer, pH 7.4, containing 12.5% PEG 6000 and 20 m*M* $CaCl_2$. Optimal conditions for crystallization were found by mixing 3 µl of protein solution, 3 µl of reservoir solution, and 1 µl of deionized water and equilibrating against 1 ml of reservoir solution. Two crystal forms were observed at similar conditions, but each drop had one type of crystal. Trigonal form (crystal form I) crystals appeared after few hours and reached a maximum size of 0.8 × 0.8 × 0.6 mm within 3 d (Fig. 1). These crystals belong to space group $P3_121$ or $P3_221$ with cell dimensions $a = b = 79.8$, $c = 57.1$ Å, $\alpha = \beta = 90.0$ and $\gamma = 120.0°$. Crystals diffracted to 2.8 Å resolution. The estimated solvent content is 50% and $V_m = 2.48$ Å^3 Da^{-1}, when one molecule per asymmetric unit is assumed.

The monoclinic crystals (crystal form II) grew to a size of 1.0 × 0.8 × 1.0 mm in two weeks (Fig. 2) and belong to space group $P2_1$ with unit-cell dimensions $a = 50.1$, $b = 79.7$, $c = 57.1$ Å, $\beta = 91.2°$. These crystals diffracted to 1.8 Å and both the crystal forms were radiation stable. The crystal form II was used for X-ray diffraction analysis. Diffraction data were collected on a Rigaku RU-300 rotating-anode generator (40 kV and 80 mA) using Cu $K\alpha$ radiation and were processed with *X-gen* software (Howard *et al.*, 1987). The combined data collected from two crystals had 76 677 observations which were merged into 24 832 unique reflections to 2.08 Å resolution. $R_{sym} = 5.49\%$ and 93.2% of the possible data were recorded to 2.08 Å with $I/\sigma > 10$ in the last resolution bin.

Using the known molecular weight, the calculated $V_m = 2.7$ Å^3 Da^{-1} and two molecules are estimated to be in the asymmetric unit with a solvent content of 48% in the unit cell. The self-rotation function calculated using *X-PLOR* (Brünger, 1992) did not give any clear orientation of a twofold non-crystallographic symmetry axis in the $\chi = 180.0°$ plane. We are currently screening for isomorphous heavy-atom derivatives.

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