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Purification, crystallization and preliminary X-ray analysis of a μ-like calpain

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The X-ray structure of m-calpain in the absence of Ca^{2+} has been described, but it has not been possible to obtain sufficient μ-calpain for structure determination. Comparison of the two structures is of interest in attempting to understand their different Ca^{2+} requirements. Here, the crystallization in the absence of Ca^{2+} of an inactive mutant hybrid calpain (MW $\simeq$ 100 kDa), which contains 85% of the rat μ-calpain sequence and is well expressed in *Escherichia coli*, is described. The properties of this calpain in its active form and particularly its Ca^{2+} requirement are close to those expected for wild-type μ-calpain. Clusters of plate-shaped crystals were obtained by vapour diffusion with polyethylene glycol ($M_r \simeq 6000$) as precipitating agent in the presence of detergent. The crystals diffract to a resolution of 2.7 Å at a synchrotron source. The space group is $P2_1$, with unit-cell parameters a = 72.7, b = 184.6, c = 86.3 Å, β = 100.7°. There are two molecules in the asymmetric unit, corresponding to a solvent content of 57.1%.

1. Introduction

The two calpains, μ-calpain and m-calpain, are heterodimeric Ca^{2+}-dependent cysteine proteases found in most mammalian tissues (Croall & DeMartino, 1991; Suzuki *et al.*, 1995; Sorimachi *et al.*, 1997). Their physiological and pathological functions are only partially understood and have been reviewed elsewhere (Goll *et al.*, 2003). It remains unclear in evolutionary terms why these two apparently very similar enzymes continue to coexist in most tissues. One major difference between them is in the amount of Ca^{2+} required *in vitro* to cause activation (K_d), μ-calpain requiring $\sim$25 μM Ca^{2+} and m-calpain requiring $\sim$325 μM (Goll *et al.*, 1992). This difference is necessarily a property of the large (80 kDa) subunits, since the small subunit (28 kDa) is the same in both enzymes. The amino-acid sequence identity between the μ-calpain and m-calpain 80 kDa large subunits is $\sim$62%, with a further 30% of conserved and semi-conserved replacements. Simple examination of the sequence alignment therefore does not offer any obvious explanation for the difference in K_d.

The structures of rat and human m-calpain in the absence of Ca^{2+} have recently been described (Hosfield, Elce *et al.*, 1999; Strobl *et al.*, 2000). Success in resolving these structures depended on the relatively high expression of rat m-calpain in *Escherichia coli* and of human m-calpain in Sf9 cells. It is obviously desirable to solve the calpain structure in the presence of Ca^{2+} as the most direct approach to understanding the mechanism of Ca^{2+} activation. This has not yet been achieved and may even be impossible because Ca^{2+} causes subunit dissociation and aggregation (Yoshizawa *et al.*, 1995; Dutt *et al.*, 1998; Elce *et al.*, 1997; Pal *et al.*, 2002). The structure of the protease core fragment, domains I and II of μ-calpain, has however been solved in the presence of Ca^{2+}, revealing previously unsuspected Ca^{2+}-binding sites which are essential for calpain activity (Moldoveanu *et al.*, 2002).

It therefore remained of interest to determine the crystal structure of the entire μ-calpain molecule in the search for significant differences related to the K_d. This has however been impeded by the lack of good-quality protein. It is difficult to purify μ-calpain from natural sources such as human platelets in amounts adequate for crystallography and in our hands both human and rat μ-calpains were very poorly expressed in *E. coli*. It was found that a hybrid form of calpain that we call m-Bam-μ-DraI-m-80k/21k (termed μ-like calpain for short), containing 85% of the μ-calpain sequence, was fully active and provided a useful (and so far unavoidable) compromise between expression level and proportion of μ-type sequence (Dutt *et al.*, 2002). The large subunit of this calpain contains rat m-calpain residues 2–48, μ-calpain residues 59–649 and m-calpain residues 638–700, together with a 14-residue C-terminal His

tag. Here, we describe the crystallization and preliminary X-ray data of the inactive Cys115Ser form of this hybrid calpain in the absence of Ca^{2+}.

2. Materials and methods

2.1. Purification and crystallization

The expression and purification of recombinant rat calpains in *E. coli* has been described previously (Elce *et al.*, 1995). Introduction of the required *Bam*HI and *Dra*I restriction sites and construction of the hybrid m-Bam-μ-Dra-m large-subunit construct has also been described, together with characterization and Ca^{2+} titration of the active m-Bam-μ-Dra-m-80k/21k calpain (Dutt *et al.*, 2002). For crystallography, the enzyme was purified successively on columns of DEAE-Sepharose, phenyl-Sepharose, Ni–NTA-agarose and Q-Sepharose. The yield of highly purified enzyme was approximately 2 mg per litre of original *E. coli* culture. The final product was concentrated to 10–15 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.6, 100 m*M* NaCl, 0.2 m*M* EDTA, 10 m*M* DTT.

For crystallization of C115S-m-Bam-μ-Dra-m-80k/21k calpain, 5 µl of the protein solution was mixed with 1–2 µl of 6.0 m*M* *n*-nonyl-β-D-maltoside (Hampton Research, USA) and 3–4 µl of reservoir solution containing 8% polyethylene glycol 6000, 100 m*M* MES pH 6.25, 50 m*M* NaCl; the mixtures were set up in sitting drops for crystallization at room temperature against 1 ml of the reservoir solution. Crystals appeared within 1–2 d and required another 4–5 d for complete growth. Crystals were usually obtained as clusters of plates. In attempts to improve the quality of the crystals, both microseeding and macroseeding were used.

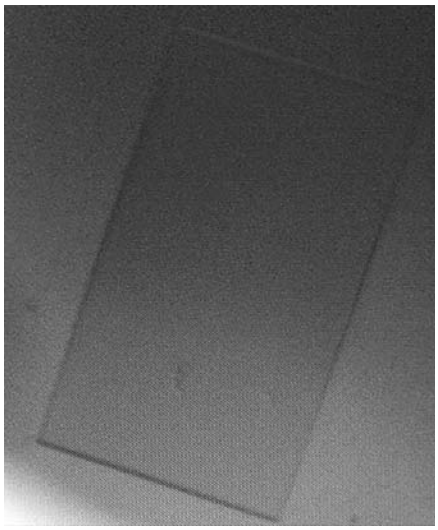

Figure 1
A typical crystal of μ-like calpain (~1.0 × 0.5 × 0.1 mm) obtained by macroseeding.

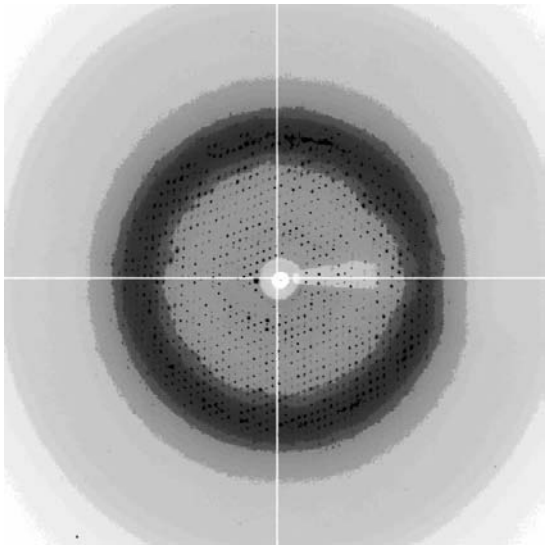

Figure 2
Diffraction pattern of μ-like calpain.

2.2. X-ray diffraction analysis

Several data sets were collected using both a home source (MAR imaging plate equipped with Rigaku RU-200 X-ray generator operated at 50 kV and 100 mA) and synchrotron sources, namely the F1 station of Cornell High Energy Synchrotron Source (CHESS) and the X8C beamline of Brookhaven National Laboratory, using a Quantum IV CCD detector. In all cases, data collections were carried out at 100 K after treating the crystals with a series of cryoprotectant solutions containing 10, 20, 30 and 40% glycerol in the crystallization buffer for 2–3 min in each solution, followed by flash-cooling in the gas stream of liquid nitrogen. All data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997)

3. Results

The crystallization condition found to be successful for C115S-m-Bam-μ-Dra-m-80k/21k calpain is very close to that used for rat m-calpain (Hosfield, Ye *et al.*, 1999), except that a detergent was found to be essential. Initial screening with various detergents (Hampton Research Detergent Screening Kits) showed that crystals could be obtained with several detergents, but the best results were achieved with *n*-nonyl-β-D-maltoside. Crystals were almost always obtained as clusters of plates, from which single crystals of dimensions ~0.4 × 0.2 × 0.1 mm could be separated for X-ray work. To avoid the difficult task of separating single crystals from clusters, we used both microseeding and macroseeding. Macroseeding yielded larger crystals of 1–2 mm in their longest dimension (Fig. 1); however, these crystals showed poor diffraction.

The best available single crystals separated from the clusters showed diffraction to 3.2–3.5 Å resolution at the in-house X-ray source and to 2.7 Å resolution at the synchrotron source (CHESS) (Fig. 2). The crystals belong to space group $P2_1$, with unit-cell parameters $a = 72.7$, $b = 184.6$, $c = 86.3$ Å, $\beta = 100.7^\circ$. The 2.7 Å data set is 90.6% complete, with an $R_{\rm sym}$ of 0.05. Assuming two molecules per asymmetric unit, the $V_{\rm M}$ value was calculated to be 2.89 Å^3 Da^{-1}, which corresponds to a solvent content of 57.1% (Matthews, 1968). In comparison, in both rat and human m-calpain crystals (Hosfield, Elce *et al.*, 1999; Masumoto *et al.*, 2000) there is only one molecule in the asymmetric unit and they are not isomorphous to μ-like calpain. In a self-rotation function calculation using the program *POLARRFN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994), a strong peak was found (at $\psi = 12.8$, $\varphi = 180$, $\kappa = 180^\circ$) with 62.4% of the magnitude of the origin peak, in comparison with the next highest peak of 33.2%. The structure determination of μ-like calpain is currently in progress.

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