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Crystallization and X-ray crystallographic analysis of m-calpain, a Ca^{2+}-dependent protease

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The absolute requirement of Ca^{2+} for proteolytic activity is a feature unique to the calpains, a family of heterodimeric cysteine proteases. Conditions are described which give rise to diffraction-quality crystals of m-calpain in two crystal forms, $P1$ and $P2_1$. Data have been collected from native crystals of m-calpain in both $P1$ and $P2_1$ forms, to 2.6 and 2.15 Å, respectively. Selenomethionine-containing crystals have been grown in both forms, and anomalous data from the $P2_1$ selenomethionine enzyme provided the location of 17 of the 19 Se atoms in the protein.

1. Introduction

The μ- and m-calpains are cytoplasmic cysteine proteases which are essentially ubiquitous in mammalian tissue (Croall & DeMartino, 1991; Suzuki *et al.*, 1995; Sorimachi *et al.*, 1997). They are of interest biochemically because they represent a unique combination of protease activity with Ca^{2+} regulation *via* EF-hands in one molecule. Their precise physiological roles are unknown, although their Ca^{2+}-dependence is likely to link them to signal-transduction events involving changes in intracellular Ca^{2+} concentration. They are also of interest in pathology, as it appears that inappropriate calpain activity may contribute to tissue damage following disturbance of Ca^{2+} homeostasis; for example, in the ischemia associated with stroke and heart attacks. These latter aspects have led to a search for highly specific calpain inhibitors for therapeutic purposes (Wang & Yuen, 1997).

The enzymes are heterodimers of an 80 kDa large subunit and a 28 kDa subunit. On the basis of amino-acid sequence homologies, the large subunit was originally described as consisting of four domains (I–IV), of which domain II contains the protease active-site residues and domain IV contains EF-hands which bind Ca^{2+} (Ohno *et al.*, 1984). The small subunit contains an N-terminal glycine-rich domain V and a C-terminal domain VI, which also contains EF-hands and has approximately 50% sequence identity with domain IV. On exposure to sufficient Ca^{2+}, calpain is activated and autolyzed, losing 9 and 19 N-terminal amino-acid residues from the m-calpain large subunit and 94 N-terminal residues (the whole of domain V) from the small subunit, before being more completely degraded (Elce, Davies *et al.*, 1997; Elce, Hegadorn *et al.*, 1997).

It is important to obtain the crystal structure of calpain for several reasons. No calpain structure has yet been reported and the structure will provide insights into its structure–function relationships. The protease domain of calpain, although originally called papain-like because of its typical Cys/His/Asn active-site residues, has very little primary sequence homology with other cysteine proteases (Berti & Storer, 1995; Arthur & Elce, 1996), so the structure may present novel or unusual features. Knowledge of the structure is also important for the development of highly specific calpain inhibitors. Finally, it may be possible to begin to understand the Ca^{2+} activation mechanism.

This work employed a recombinant rat m-calpain, referred to here as C105S-m-calpain, which differs slightly from the natural enzyme. The active-site Cys105 in domain II was mutated to Ser, which removes the activity of the enzyme and therefore prevents autodegradation during purification and crystallization (Arthur *et al.*, 1995). The large subunit also has a 14-residue C-terminal extension ending in a six-His tag, which facilitates purification and greatly increases the expression yield (Elce *et al.*, 1995). The small subunit consists of domain VI only, containing residues 87–268 of the wild-type sequence. This latter construct was chosen since the absence of domain V does not affect activity, autolysis or Ca^{2+} dependence (Berti & Storer, 1995; Arthur & Elce, 1996), while calpains containing domain V are poorly expressed and prone to degradation in *E. coli*. In this communication, we report the formation of diffraction-quality single crystals of C105S-m-calpain in two forms, $P1$ and $P2_1$.

2. Protein purification and storage

Rat m-calpain (C105S-m-80k-CHis$_6$/21k) was expressed in 4–8 l of *E. coli* strain BL21(DE3)

Table 1
Synchrotron diffraction data statistics.

Figures in parentheses refer to the highest resolution shell as given in the footnotes.

	Native	Native	Se-Met
Space group	*P*1	$P2_1$	$P2_1$
Beamline	CHESS-F1	CHESS-F1	BNL-X4A
Wavelength (Å)	0.910	0.910	0.979
Unit-cell parameters			
a (Å)	57.64	52.25	52.11
b (Å)	79.83	157.95	156.12
c (Å)	80.88	65.073	64.35
α (°)	60.47	90	90
β (°)	70.80	95.42	95.59
γ (°)	79.30	90	90
Solvent content (%)	~60	~45	~45
Resolution (Å)	25–2.6	25–2.15	25–2.8
Total number of reflections	227921	330258	228357
Number of unique reflections	35649	56269	25513
R_{sym}	0.041 (0.110)†	0.051 (0.272)‡	0.041 (0.157)§
Completeness (%)	97.7 (98.1)†	92.2 (85.5)‡	93.0 (88.2)§
$I/\sigma(I)$	17.1 (8.8)†	10.3 (2.0)‡	25.3 (4.2)§

† 2.69–2.6 Å, ‡ 2.23–2.15 Å, § 2.90–2.80 Å.

as previously described (Elce *et al.*, 1995). Briefly, the subunits were co-expressed from compatible plasmids and associate within *E. coli* to produce calpain with activity comparable to that isolated from natural sources. The selenomethionine derivative was similarly expressed in B834(DE3) cells (Hendrickson *et al.*, 1990). The proteins were purified by chromatography at 277 K successively on columns of DEAE–Sepharose, Ni–NTA agarose, sometimes also on Reactive-Red agarose, Ultra-gel AcA44 and finally by FPLC on Q-Sepharose 16/10. In general, the buffers contained 50 m*M* Tris–HCl pH 7.6 at room temperature, 2–5 m*M* EDTA, 10 m*M* 2-mercaptoethanol and appropriate NaCl concentrations. Reactive-Red agarose chromatography was carried out in 20 m*M* MOPS pH 7–7.4, 2 m*M* EDTA, 2 m*M* EGTA, 5 m*M* 2-mercaptoethanol and the appropriate NaCl concentrations (Croall & DeMartino, 1984). The yield of fully purified material was 10–15 mg of the methionine form and 4–8 mg of the selenomethionine derivative from 8 l of *E. coli*. The final product was concentrated to 10–12 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.6, 2 m*M* EDTA, 0.01% sodium azide and usually 10 m*M* dithiothreitol.

The concentrated protein sample, if not used immediately, was flash-frozen in 100 µl aliquots using liquid N_2 and stored at 203 K. On thawing, the protein retained its ability to crystallize and, in the case of active enzyme, retained protease activity, demonstrating that flash-freezing had not impaired the protein.

The relative importance of several factors influencing the crystallization behaviour of the product is not clear. Improvements in the purification procedure which seem to improve crystallization have included addition of fresh 10 m*M* 2-mercaptothanol and 0.01%(*w*/*v*) sodium azide in all buffers to minimize oxidation and bacterial degradation. In addition, acceleration of the protocol to minimize the time elapsed from sonication of the *E. coli* to the final column improved crystallization success. It is a measure of the difficulty of this crystallization that approximately 400 l of *E. coli* have been processed over the past three years for this purpose alone.

3. Crystallization and X-ray studies

Sparse-matrix screening was carried out using the hanging-drop method at room temperature (Jancarik & Kim, 1991). Several PEG-containing conditions yielded small crystals. After routine optimization, the best condition was determined to be 100 m*M* MES pH 6.5, 10–12% PEG 6000, 50 m*M* NaCl and ~10 mg ml^{-1} protein. At this stage, the only reducing agent in the drops was 2-mercaptoethanol remaining from protein purification. In early work, most crystals were small, tended to grow as multi-crystals or show surface defects and diffracted poorly using the in-house X-ray facility. In addition, reproducibility of crystallization was a major problem, since only 1% of drops contained diffraction-size crystals. The crystals were determined to belong to the triclinic space group *P*1 (Fig. 1). The best *P*1 crystals diffracted to 3.0 Å resolution at 100 K using the in-house X-ray facility.

Adjustments were made both to the purification procedure and to the crystallization conditions in attempts to improve the reproducibility of crystal formation. After improvements in the purification, a new crystal form, $P2_1$, was unexpectedly obtained (Fig. 2). The crystallization conditions were very similar to those which produced the original *P*1 crystals, except for the presence of fresh 10 m*M* dithiothreitol. The quality of the $P2_1$ crystals was much better; they typically diffracted to 2.8 Å resolution at 100 K. Most crystallization drops contained crystals, but the number of drops containing well formed single crystals large enough for in-house X-ray analysis remained low (5–8%). The switch from *P*1 to $P2_1$ was potentially very useful, since it may permit the use of multi-crystal averaging to solve the structure. It is now possible to obtain crystals of the methionine and selenomethionine derivatives of C105S-m-calpain in both *P*1 and $P2_1$ forms.

X-ray diffraction data have been collected for calpain crystals of both *P*1 and $P2_1$ forms and the synchrotron data statistics are summarized in Table 1. There is one molecule per asymmetric unit in both forms. The triclinic *P*1 form has a higher solvent content and lower diffraction resolution than the monoclinic $P2_1$ form. The anomalous signal resulting from data collected at the absorption peak wavelength of the Se atom

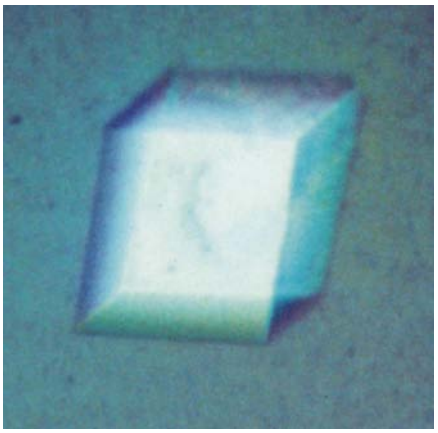

Figure 1
*P*1 crystal of C105S-m-calpain. The approximate size is 0.2 × 0.2 × 0.2 mm.

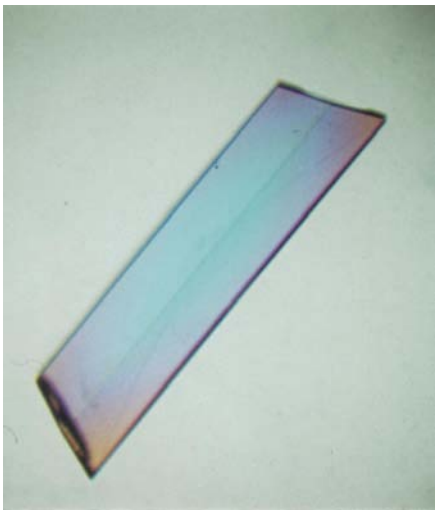

Figure 2
$P2_1$ crystal of C105S-m-calpain. The approximate size is 1.0 × 0.3 × 0.25 mm.

(0.979 Å) has enabled us to locate 17 out of the expected 19 Se atoms using the direct method in *Shake-and-Bake* (Miller *et al.*, 1994).

Both crystal forms decayed very fast at room temperature upon exposure to X-rays, making it essential to collect data at cryogenic temperatures. For $P1$ crystals, serial transfer to crystallization solutions supplemented with 10, 20 and 30% glucose for 2 min at each concentration followed by immediate flash-cooling in liquid propane gave the best conditions for data collection. For $P2_1$ crystals, serial transfer through solutions supplemented with 5, 10, 20, 30 and 40% ethylene glycol for ~5 min at each concentration followed by flash cooling in liquid propane was found to be optimal.

A number of data sets have been collected at 100 K for both crystal forms of calpain, using both in-house and synchrotron X-ray sources. The in-house facility consists of a MAR Research imaging plate and a Rigaku RU-200 X-ray generator operated at 50 kV and 100 mA. Higher resolution data were collected using a Quantum IV CCD at the F1 station of the Cornell High Energy Synchrotron Source (CHESS). Anomalous data from the $P2_1$ selenomethionine calpain were collected using an R-AXIS IV imaging plate at the X4A beamline of Brookhaven National Laboratory (BNL). All data were processed using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997).

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