FOR THE RECORD

Ca²⁺-Binding domain VI of rat calpain is a homodimer in solution: Hydrodynamic, crystallization and preliminary X-ray diffraction studies

HELEN BLANCHARD,^{1,4} YUNGE LI,^{1,4} MIROSLAW CYGLER,^{1,4} CYRIL M. KAY,^{2,4}
J. SIMON C. ARTHUR,^{3,4} PETER L. DAVIES,^{3,4} AND JOHN S. ELCE^{3,4}

¹ Biotechnology Research Institute, NRC, Montreal, Quebec H4P2R2,
Canada and Montreal Joint Centre for Structural Biology

² Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

³ Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

⁴ Protein Engineering Network of Centres of Excellence

(RECEIVED November 20, 1995; ACCEPTED December 13, 1995)

Abstract: The 21-kDa calcium-binding domain (VI) of the small subunit of rat calpain II has been expressed in *Escherichia coli*, purified, and crystallized. Two orthorhombic crystal forms have been obtained: space group P2₁2₁2₁ with $a = 50.3$, $b = 56.5$, $c = 141.3$ Å; and space group C222₁ with $a = 69.4$, $b = 73.9$, $c = 157.4$ Å. Diffraction data have been collected to 2.4 Å. Sedimentation equilibrium, dynamic light scattering, and gel-permeation chromatography indicate that domain VI exists as a homodimer in solution. In accordance with the protein's behavior in solution, each crystal form contains two molecules per asymmetric unit. Screening for heavy-atom derivatives is in progress. To decrease the sensitivity to mercurials and to aid in the search for useful derivatives, Cys-to-Ser mutants have been prepared, expressed, and crystallized.

Keywords: calcium-binding domain; calpain; crystallization; homodimer; small subunit

Calpains I and II (or μ -calpain and m-calpain, respectively) are intracellular, nonlysosomal, calcium-dependent cysteine proteinases with a neutral pH optimum. The names μ - and m-calpain reflect the approximately 50 μ M and 0.5 mM Ca²⁺ concentrations required for half maximal activity of the two purified enzymes in vitro. In contrast, both calpains are activated in vivo at low micromolar Ca²⁺ concentrations, by mechanisms that are not yet fully explained (Mellgren, 1987; Croall & DeMartino, 1991; Goll et al., 1992).

Activation by calcium may not very strictly require autolysis, because the active site has been shown to be functional before autolysis (Crawford et al., 1993). However, autolysis of the

small subunit follows very rapidly after addition of calcium, although autolysis of the large subunit differs between μ - and m-calpain (Saido et al., 1994). Once autolysis has occurred, the half-lives of the autolyzed forms are very short, limiting their time of action in vivo. The importance of calcium in activation is clear, and the physiological functions and regulation of the calpains are thought to be linked to Ca²⁺-regulated signaling pathways; however, because the physiological substrates of the calpains have not been unambiguously identified, these questions remain open. On much indirect evidence, it appears likely that the calpains play a regulatory role rather than simply a degradative role in cellular metabolism, because the latter function is supplied by the proteasome system (Goldberg, 1995).

The calpains are heterodimers, consisting of an 80-kDa catalytic subunit, (the large subunit), and a 30-kDa regulatory subunit, (the small subunit). The large subunit appears to contain four domains (I–IV) and the small subunit, two domains (V, VI) (Ohno et al., 1984). The C-terminal domains of both subunits, IV and VI, have been described as calmodulin-like because they each contain several EF-hand motifs, two of which have a close match to the consensus sequence for Ca²⁺-binding, (Minami et al., 1988; Nakayama & Kretsinger, 1994). It is not yet fully established which of the putative EF-hand motifs actually bind Ca²⁺, or how this binding regulates the activity of calpain (Minami et al., 1988). Because the small subunit is common to both of the calpains, it appears that the difference in Ca²⁺ requirements of the enzymes is determined mainly by domain IV in the large subunit (Kawasaki et al., 1986). It is clear that a detailed study of these Ca²⁺-binding domains is important for understanding the calpains.

As an initial step in understanding the function and regulation of calpain by calcium, we have crystallized the C-terminal portion of the rat calpain small subunit (domain VI). This domain was cloned, expressed, and purified as described previously (Graham-Siegenthaler et al., 1994). The sequence of the cDNA

Reprint requests to: Mirosław Cygler, Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Quebec H4P2R2, Canada; e-mail: mirek.cygler@bri.nrc.ca.

encodes a protein of 184 residues with a calculated molecular mass of 21,257 Da. The predicted amino acid sequence is 91% identical to that of the corresponding portion of human calpain small subunit.

During purification by gel permeation chromatography, domain VI eluted well ahead of its monomeric molecular mass position (not shown). Because electrospray mass spectrometry gave a molecular weight of $21,267 \pm 5$ Da, the oligomerization state of domain VI was examined by sedimentation equilibrium centrifugation (Zhu et al., 1993) and by light scattering using the procedures of Farrow et al. (1994). In the sedimentation equilibrium experiment, the plot of the natural logarithm of the fringe displacement as a function of the square of the radial distance from the rotor center was fairly linear (Fig. 1). From the slope of this plot, the apparent weight average molecular weight was calculated to be $42,180 \pm 2,530$ Da.

In laser light-scattering experiments, dilutions of the domain VI stock solution ranging from 5.22 to 2.37 mg/mL were injected directly into the flow cell of a Dawn F multiangle laser light-scattering photometer (Wyatt Technology) using a manual injector. The molecular weight extrapolated from a Zimm plot was $46,300 \pm 3,000$ Da. When light scattering was used in conjunction with gel permeation chromatography, a consistent

Fig. 1. Sedimentation equilibrium study of rat calpain domain VI. Domain VI was loaded at a concentration of 5.19 mg/mL in 20 mM potassium phosphate, pH 7.5, containing 100 mM KCl and 0.01% NaN_3 and ultracentrifuged at 12,000 rpm at 20 °C. Sedimentation was monitored by interference optics, and the apparent molecular weight was derived from linear regression analysis of the plot of logarithm of the fringe displacement, $\ln y$, versus the square of the radial distance from the rotor center, r^2 .

molecular weight (average $40,760 \pm 2,600$ Da) was observed across the peak of eluted domain VI. All three methods of molecular weight analysis are consistent with the occurrence of domain VI as a dimer (predicted molecular weight 42,514 Da) with no indication of dissociation into monomers, or of higher oligomerization. Because domain VI is known to remain bound to the highly homologous domain IV even after extensive autolysis of calpain (Nishimura & Goll, 1991; Crawford et al., 1993), it was not unexpected that domain VI should form a stable homodimer.

Sparse matrix crystal screening (Jancarik & Kim, 1991) identified two sets of useful crystallization conditions, polyethylene glycol (PEG) and high salt, providing two crystal forms. Crystals were grown at 20 °C by the hanging drop vapor diffusion method. The hanging drops consisted of 3 μL of protein solution, at a concentration of 14 mg/mL, and 3 μL of reservoir solution. Although a low concentration (0.1 mM) of calcium chloride was initially present in the crystallization medium, later the same crystal forms were obtained in the absence of calcium and we proceeded with calcium-free crystallization. Subsequently, seeding was used to initiate nucleation and, where necessary, for crystal enlargement.

Form 1 was grown using reservoir solutions containing 14% PEG 8000 and 50 mM cacodylate buffer, pH 6.5. These crystals grew to approximately 0.8 mm $\times$ 0.25 mm $\times$ 0.25 mm. They are orthorhombic, space group $P2_12_12_1$, with $a = 50.3$, $b = 56.5$, and $c = 141.3$ Å, and contain two molecules in the asymmetric unit, with $V_m = 2.4$ Å³/Da. A native data set was collected on a R-Axis IIC area detector mounted on a Rigaku RU-300 rotating anode generator. A total of 38,050 observations with $I/\sigma(I) > 1.0$ were merged to 12,432 unique reflections, $R_{\text{merge}} = 5.4\%$. The data set is 87% complete to 2.5 Å.

Form 2 was obtained from a reservoir containing 38% saturated ammonium sulfate and 25 mM Pipes buffer, pH 6.8, and these crystals grew to a size of 0.7 mm $\times$ 0.3 mm $\times$ 0.2 mm. They belong to an orthorhombic system, space group $C222_1$, with unit cell dimensions $a = 69.4$, $b = 73.9$, and $c = 157.4$ Å, and also have two molecules in the asymmetric unit. Although data to only 2.9 Å have been collected using a conventional X-ray source, these crystals diffracted to higher resolution at the synchrotron. Native diffraction intensity data were collected at the Photon Factory, Tsukuba, Japan, from two crystals on a Weissenberg camera with Fuji image plates (Sakabe, 1991) and processed and merged with the Weis program (Higashi, 1989). The total number of observations collected was 45,537, which merged to 12,088 unique reflections, $R_{\text{merge}} = 6.6\%$ giving a data set 75% complete to 2.4 Å. When the C-centered cell is converted to a primitive cell, one obtains cell dimensions of $a = 50.7$, $b = 50.7$, $c = 157.4$ Å, and $\beta = 93.6^\circ$. The β angle, being so close to 90°, indicates a possible pseudo-tetragonal symmetry. Indeed, the self-rotation function (Fig. 2) reveals a peak on the $\kappa = 90^\circ$ section, near the c -axis. The peak height is 67% of the crystallographic symmetry peak, and its symmetry equivalent occurs on the $\kappa = 180^\circ$ section at $\phi = 45^\circ$. Interestingly, we have found that some soak conditions cause a rearrangement of molecules that transforms this noncrystallographic symmetry into a crystallographic fourfold axis.

Heavy-atom screening was undertaken initially on form 1. However, because it became experimentally more difficult to obtain these crystals, we proceeded to scan for heavy-atom derivatives using crystals of form 2, which are more reliably repro-

Fig. 2. Two sections of the self-rotation function for form 2 crystals. **A:** The $\kappa = 90^\circ$ section showing a strong peak corresponding to a pseudo fourfold rotation along the c -axis. **B:** The $\kappa = 180^\circ$ section with crystallographic and noncrystallographic twofold rotations. Contours start at 2.5σ level and increase in 0.5σ intervals.

duced and do not visibly deteriorate upon storage. Heavy-atom soaks were carried out at 20°C in reservoir solutions with a gradual increase of heavy-atom concentration. In cases where heavy-atom reagents were relatively insoluble in ammonium sulfate, soaking experiments have also been undertaken after transfer of the crystals into PEG solutions. Extensive screening for heavy-atom derivatives has proved unsuccessful. Attempts to bind lanthanides into the calcium-binding sites resulted in severe cracking of the crystals at concentrations as low as 0.1 mM , suggesting a rearrangement of the molecules. Even at low concentrations ($<0.3\text{ mM}$) and short soaking times ($<2\text{ h}$), various mercury compounds caused loss of diffraction, sometimes associated with crystal cracking. The 21-kDa fragment contains only two cysteine residues, Cys 60 and Cys 148, which were considered the probable cause of the observed sensitivity of the crystals toward mercurials. Each or both of the cysteines have been mutated to serines and the mutants have been expressed, purified, and crystallized. All these mutants are fully active when reconstituted with the large, catalytic subunit as heterodimers. The crystallization conditions to obtain form 2 crystals and their cell dimensions are comparable to those of the wild-type protein. The double mutant crystals retained internal order after soaking with mercury compounds and appear not to bind these heavy-atom reagents, confirming that the sensitivity of crystals to mercurials was due to the presence of cysteines. Both of the single mutants remain sensitive to mercurials although the Cys 60 Ser is less sensitive than the Cys 148 Ser mutant. Using crystals of the former, we have been able to obtain a weak but useful

derivative with HgCl_2 . Further screening for heavy-atom derivatives is in progress with all crystals.

Acknowledgments: We thank Les Hicks for superb technical assistance with the sedimentation and light scattering analyses. This work was funded in part by the Protein Engineering Network of Centres of Excellence. This is NRCC publication no. 39910.

References

- Crawford C, Brown NR, Willis AC. 1993. Studies of the active site of m-calpain and the interaction with calpastatin. *Biochem J* 296:135–142.
- Croall DE, DeMartino GN. 1991. Calcium activated neutral protease (CALPAIN) system: Structure, function and regulation. *Physiological Reviews* 71:813–847.
- Farrow NA, Muhandiram R, Singer A, Pascal SM, Gish G, Shoelson SE, Pawson T, Forman-Kay JD, Kay LE. 1994. Backbone dynamics of a free and phosphopeptide complexed Src homology 2 domain studied by ^{15}N NMR relaxation. *Biochemistry* 33:5984–6003.
- Goldberg AL. 1995. Functions of the proteasome: The lysis at the end of the tunnel. *Science* 268:522–523.
- Goll DE, Thompson VF, Taylor RG, Zalewska H. 1992. Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin. *Bioessays* 14:549–556.
- Graham-Siegenthaler K, Gauthier S, Davies PL, Elce JS. 1994. Active recombinant rat calpain II. *J Biol Chem* 269:30457–30460.
- Higashi T. 1989. The processing of diffraction data taken on a screenless Weissenberg camera for macromolecular crystallography. *J Appl Crystallogr* 22:9–18.
- Jancarik J, Kim SH. 1991. Sparse matrix sampling: A screening method for crystallization of proteins. *J Appl Crystallogr* 24:409–411.
- Kawasaki H, Imajoh S, Kawashima S, Hayashi T, Suzuki K. 1986. The small subunits of calcium dependent proteases with different calcium sensitivity are identical. *J Biochem* 99:1525–1532.
- Mellgren RL. 1987. Calcium-dependent proteases: An enzyme system active at cellular membranes. *FASEB J* 1:110–115.
- Minami Y, Emori Y, Imajoh-Ohmi S, Kawasaki H, Suzuki K. 1988. Carboxy-terminal truncation and site-directed mutagenesis of the EF hand structure-domain of the small subunit of rabbit calcium-dependent protease. *J Biochem* 104:927–933.
- Nakayama S, Kretsinger RH. 1994. Evolution of the EF-hand family of proteins. *Annu Rev Biophys Biomol Struct* 23:473–507.
- Nishimura T, Goll DE. 1991. Binding of calpain fragments to calpastatin. *J Biol Chem* 266:11842–11850.
- Ohno S, Emori Y, Imajoh S, Kawasaki H, Kisaragi M, Suzuki K. 1984. Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? *Nature* 312:566–570.
- Saido TC, Nagao S, Shiramine M, Tsukaguchi M, Yoshizawa T, Sorimachi H, Ito H, Tsuchiya T, Kawashima S, Suzuki K. 1994. Distinct kinetics of subunit autolysis in mammalian m-calpain activation. *FEBS Lett* 346:263–267.
- Sakabe N. 1991. X-ray diffraction data collection system for modern protein crystallography with a Weissenberg camera and an imaging plate using synchrotron radiation. *Nucl Instr Meth Phys Res A* 303:448–463.
- Zhu BY, Zhou NE, Kay CM, Hodges RS. 1993. Packing and hydrophobicity effects on protein folding and stability: Effects of B-branched amino acids, valine and isoleucine, on the formation and stability of two-stranded α -helical coiled-coils/leucine zippers. *Protein Sci* 2:383–394.