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Association and Dissociation of the Calcium-Binding Domains of Calpain by Ca^{2+}

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The calmodulin-like domain of calpain is important for the association of the calpain large and small subunits. We expressed the calmodulin-like domains of the large subunits of rabbit μ- and m-calpains and their small subunits in *E. coli* and purified them to homogeneity. Unlike the full-length subunits, the calmodulin-like domains are soluble in buffer containing Ca^{2+}. We performed gel filtration chromatography of the purified proteins and found that all three calmodulin-like domains exist as homodimers in the absence of Ca^{2+} and dissociate into monomers upon the addition of Ca^{2+}. © 1999 Academic Press

Calpain is an intracellular cysteine protease whose activity is regulated by Ca^{2+} (1–4). Calpain is a heterodimer consisting of an 80kDa large subunit (CL) with four domains (domain I-IV) and a 30kDa small subunit (30K) with two domains (domain V and IV′). The large subunit is the catalytic subunit and the small subunit is thought to be essential for the regulation of activity. Domains IV and IV′ exhibit homology with calmodulin and contain several EF hand structures consisting of a calcium-binding loop lying between α-helices (5). Two isozymes, μ- and m-calpains, are expressed ubiquitously in mammalian cells. μ- and m-calpains differ in their Ca^{2+} sensitivities, high and low, respectively. Rabbit μCL and mCL show 50.3% amino acid sequence identity and share the small subunit (6,7).

The EF hand structures of the calmodulin-like domains are important for the association of the two subunits (8–10). The crystal structure of the calmodulin-like domain of the small subunit has been determined. The structure reveals that the molecule forms homodimeric crystals in the presence or absence of Ca^{2+} and that the EF hand structures are involved in the association of the two subunits (11–13).

It has been observed that calpain dissociates into subunits in the presence of Ca^{2+} (14). On the other hand, Mellgren et al. provided direct evidence that in the presence of Ca^{2+} concentrations that permit catalytic activity, both subunits of μ- and m-calpains are coprecipitated by a monoclonal antibody specific for the small subunit (15). To clarify this discrepancy, we performed gel filtration chromatography of the purified calmodulin-like domains of rabbit μCL, mCL, and 30K, all produced in *E. coli*, and found that the purified calmodulin-like domains exist as homodimers in the absence of Ca^{2+} and dissociate into monomers upon the addition of Ca^{2+}.

MATERIALS AND METHODS

Materials. Expression vectors pRU10, pRM15, and pDG301 encode C-terminal 221 residues of μCL, C-terminal 218 residues of mCL, and residues 69-266 of 30K, respectively, with N-terminal short sequences (11, 8, and 17 residues, respectively) of the N-terminal region of β-galactosidase derived from pUC vectors (16). Other reagents were of the highest grade obtainable.

Purification of calmodulin-like domains expressed in E. coli. We used *E. coli* strain YA21 to express the calmodulin-like domains of calpain. The transformants were cultured in 1 liter of LB broth containing 50μg/ml ampicillin at 37°C. When the absorbance at 550nm of the culture mixture reached 0.5-1.0, isopropyl-1-thio-β-D-galactoside (IPTG) was added at a final concentration of 1mM, and incubation was continued for a further 3h. Cells collected by centrifugation were washed and then suspended in buffer A (50mM Tris-HCl (pH 7.5), 5mM EDTA, and 5mM β-mercaptoethanol). The suspension was sonicated and the supernatant was separated from the precipitate by centrifugation at 17,000g for 15min at 4°C. The expressed products encoded by pRU10, pRM15, and pDG301 were referred to as U80K, M80K, and DG30K, respectively. All were soluble and extracted by sonication in buffer A. The extracted proteins were purified by DEAE-cellulose ion-exchange chromatography (DE-52, Whatman), gel filtration on Hiload 26/60 Superdex 75 (FPLC

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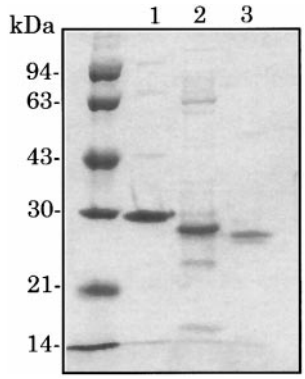

FIG. 1. SDS–polyacrylamide gel electrophoresis of calmodulin-like domains. The calmodulin-like domains of calpains were expressed in *E. coli* strain YA21 and purified to homogeneity. Lane 1: U80K. Lane 2: M80K. Lane 3: DG30K.

system, Pharmacia), and MonoQ ion-exchange chromatography (FPLC system, Pharmacia).

Ca^{2+} binding assay. Samples (ca 10-20μg) were spotted onto nitrocellulose membranes. The membranes were soaked in 5mM $CaCl_2$, 20mM Tris-HCl, pH7.5, and 1mM dithiothreitol (DTT) containing 74kBq/ml $^{45}CaCl_2$ for 30min and washed twice with buffer B (20mM Tris-HCl, pH7.5, 1mM DTT) for 5min. Autoradiography was performed with a BAS1500 autoradiography analytical system (Fuji-Film).

Dissociation of homodimers. Purified proteins were dialyzed against buffer B (20mM Tris-HCl (pH 7.5) and 1mM dithiothreitol), and applied onto a gel filtration column (G3000SW (0.8×60cm), HPLC system, Hitachi) that had been equilibrated and then eluted with buffer B containing different concentrations of $CaCl_2$. Molecular weights of the purified proteins were estimated from the elution volumes of bovine serum albumin (67kDa), ovalbumin (43kDa), and chymotrypsin (25kDa).

Other methods. Emission spectra were recorded in a spectrofluorometer at excitation 280nm using a slit width of 10nm. Protein concentrations were determined with a DC protein assay kit (Bio-Rad) using bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% slab gels.

RESULTS AND DISCUSSION

Recombinant plasmids (pRU10, pRM15, and pDG301) produce the calmodulin-like domains of rabbit μCL, mCL, and 30K (U80K, M80K, and DG30K, respectively) as fusion proteins with short sequences of β-galactosidase derived from pUC vectors at their N-termini. These N-terminal extensions are short (8-17 residues) and the sequences of the N-terminal seven residues are the same. Therefore, these extensions do not have a significant influence on the interactions of the calmodulin-like domains. The final calmodulin-like domain preparations were very pure as shown in Fig. 1. The molecular masses of U80K, M80K, and DG30K as estimated by SDS-PAGE are consistent with the calculated values (27.1, 26.0, and 25.1kDa, respectively).

The purified calmodulin-like domains bind Ca^{2+}, while bovine serum albumin does not (data not shown), indicating that the Ca^{2+} binding of the calmodulin-like domain is specific. The specific binding of the calmodulin-like domain to Ca^{2+} suggests that the purified proteins are not denatured. Moreover, the emission spectra at excitation 280nm of the purified proteins changes dramatically in both the intensity of fluorescence and the λ_{max} of emission when urea is added to 8M (data not shown). Since the addition of a denaturant causes a conformational change, the purified proteins should maintain the tertiary structure.

Figure 2 shows the gel filtration chromatograms of the calmodulin-like domains at various Ca^{2+} concentrations. It is natural to think that the peaks with calculated molecular masses of 26-32kDa represent the monomer proteins while the peaks at 42-55kDa are those of the dimer proteins. Almost all of the purified protein eluted as dimers in buffer without Ca^{2+}. In buffer containing 500μM of Ca^{2+}, most of the U80K dissociated into monomers (Fig. 2a), while only a small portion of M80K (Fig. 2b) and DG30K (Fig. 2c) existed as monomers. We could not discriminate between 30kDa and 26kDa by SDS gel electrophoresis. In 5mM Ca^{2+}, more of the M80K and DG30K dissociated into monomers.

The apparent molecular mass of the M80K homodimers differed at different Ca^{2+} concentrations, with the molecular mass decreasing as the Ca^{2+} concentration increased. Thus, the M80K homodimer might undergo structural changes in the presence of Ca^{2+}.

In the presence of Ca^{2+}, purified full length calpain forms aggregates and self-precipitates, making various physicochemical analyses of calpain in the presence of Ca^{2+} very difficult. Therefore, in a previous study (14) of the molecular properties of calpain, the detergent Triton X-100 was added to the buffer to prevent the formation of aggregates and precipitates in the presence of Ca^{2+}. It is possible that the presence of detergent would have some influence on the association of the large and small subunits. In addition, calpain autolyzes quickly in the presence of Ca^{2+}. Therefore, to prevent proteolysis, we expressed the calmodulin-like domains of calpains in *E. coli*, and obtained proteins that are soluble and do not form aggregates in the presence of Ca^{2+} concentrations sufficient for activation.

The purified calmodulin-like domains expressed in *E. coli* form homodimers in buffer without Ca^{2+}, and these homodimers dissociate into monomers when Ca^{2+} is added. The Ca^{2+} concentrations required for the dissociation of the homodimers are different for U80K, M80K, and DG30K, with M80K requiring higher Ca^{2+} concentrations than U80K. The Ca^{2+} concentrations required for 50% activation of μ- and m-calpain are 50μM and 700-800μM, respectively (17). Even though

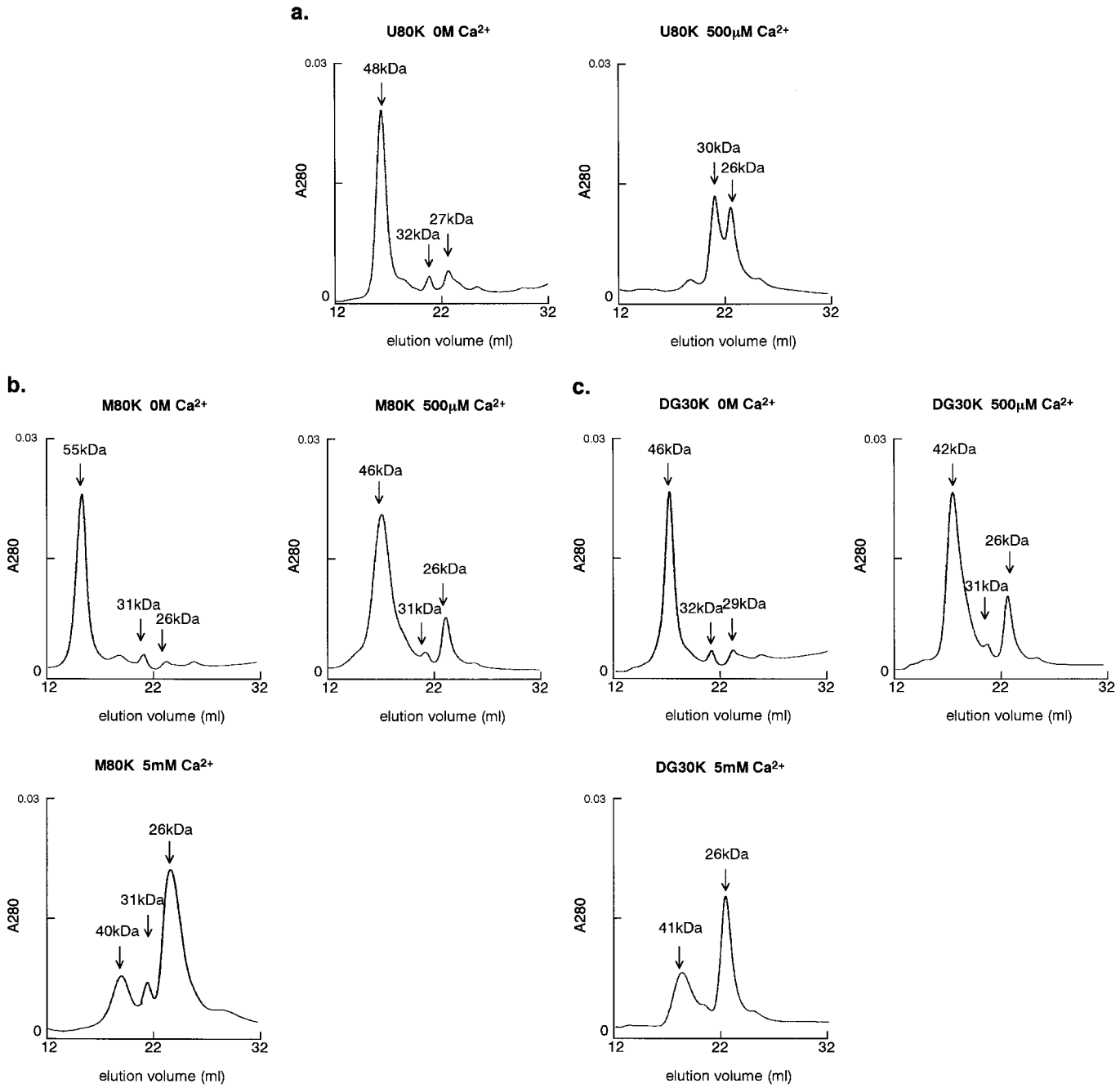

FIG. 2. Gel filtration chromatography of U80K (a), M80K (b), and DG30K (c) in various concentrations of Ca^{2+}. Purified calmodulin-like domains of rabbit μCL, mCL, and 30K were applied to a gel filtration column in buffer containing various concentrations of $CaCl_2$. Molecular masses were estimated based on the elution volumes of bovine serum albumin, ovalbumin, and chymotrypsin (14.4, 15.8, and 23.9 ml, respectively).

our observations were made with a homodimer that differs from an active calpain heterodimer, the result is intriguing because the calcium sensitivity and concentrations required for dissociation are of the same order as for native calpains.

Our observation that homodimers of the calmodulin-like domain of calpain dissociate into monomers in the presence of Ca^{2+} suggests a calcium-dependent conformational change in the calmodulin-like domain. However, this does not necessarily reproduce the behavior of calpain heterodimers in the presence of Ca^{2+}. Since the amino acid sequences of the calmodulin-like domains of the large and small subunits of calpain are similar to each other (6,7), the association and dissociation observed here could occur in heterodimeric calpain, supporting our previous observation that calpain

dissociates in the presence of Ca^{2+}. Further studies using heterodimers composed of the calmodulin-like domains of μCL or mCL and 30K are now in progress.

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REFERENCES

1. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) *Biochem. J.* **328,** 721–732.
2. Suzuki, K., and Sorimachi, H. (1998) *FEBS Lett.* **433,** 1–4.
3. Ono, Y., Sorimachi, H., and Suzuki, K. (1998) *Biochem. Biophys. Res. Commun.* **245,** 289–294.
4. Kinbara, K., Sorimachi, H., Ishiura, S., and Suzuki, K. (1998) *Biochem. Pharmacol.* **56,** 415–420.
5. Kretsinger, R. H., and Barry, C. D. (1975) *Biochem. Biophys. Acta* **405,** 40–52.
6. Emori, Y., Kawasaki, H., Imajoh, S., Kawashima, S., and Suzuki, K. (1986) *J. Biol. Chem.* **261,** 9472–9476.
7. Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S., and Suzuki, K. (1986) *J. Biol. Chem.* **261,** 9465–9471.
8. Imajoh, S., Kawasaki, H., and Suzuki, K. (1987) *J. Biochem.* **101,** 447–452.
9. Minami, Y., Emori, Y., Imajoh-Ohmi, S., Kawasaki, H., and Suzuki, K. (1988) *J. Biochem.* **104,** 927–933.
10. Elce, J. S., Davies, P. L., Hegadorn, C., Maurice, D. H., and Arthur, J. S. (1997) *Biochem. J.* **326,** 31–38.
11. Blanchard, H., Li, Y., Cygler, M., Kay, C. M., Simon, J., Arthur, C., Davies, P. L., and Elce, J. S. (1996) *Protein Sci.* **5,** 535–537.
12. Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S. C., Davies, P. L., Elce, J. S., and Cygler, M. (1997) *Nature Struct. Biol.* **4,** 532–538.
13. Lin, G., Chattopadhyay, D., Maki, M., Wang, K. K. W., Carson, M., Jin, L., Yuen, P., Takano, E., Hatanaka, M., DeLucas, L. J., and Narayama, S. V. L. (1997) *Nature Struct. Biol.* **4,** 539–547.
14. Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) *Biochem. Biophys. Res. Commun.* **208,** 376–383.
15. Zhang, W., and Mellgren, R. (1996) *Biochem. Biophys. Res. Commun.* **227,** 890–896.
16. Minami, Y., Emori, Y., Kawasaki, H., and Suzuki, K. (1987) *J. Biochem.* **101,** 889–895.
17. Suzuki, K., Tsuji, S., Ishiura, S., Kimura, Y., Kubota, S., and Imahori, K. (1981) *J. Biochem.* **90,** 1787–1793.