A catalytic subunit of calpain possesses full proteolytic activity

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Abstract Previous studies on the refolding of calpain, a heterodimer comprising a catalytic 80 kDa subunit and a regulatory 30 kDa subunit, indicate that both subunits are required for the expression of full protease activity. We reexamined the conditions for refolding of calpain and found that under optimized conditions the renatured 80 kDa subunit has full enzyme activity even in the absence of the 30 kDa subunit. The 30 kDa subunit stabilizes the 80 kDa subunit rather than enhancing its activity. The theory that calpain functions as a dimer requires reexamination.

Key words: Calpain; Calcium; Refolding; Protease

1. Introduction

Calpain is a cytosolic protease found at least in all animal tissues where it functions as one of the cellular receptors for calcium. It has been purified as a stable heterodimer consisting of a catalytic 80 kDa subunit (80K) and a regulatory 30 kDa subunit (30K) [1–3]. Previous studies on the dissociation and renaturation of calpain indicated that the isolated 80K is enzymatically active, but that its activity (< 5% of control) is significantly lower than that of the control containing both subunits, suggesting that 30K is essential for the expression of full proteolytic activity. In other words, 30K enhances the protease activity intrinsic to 80K [4]. In order to reconstitute calpain from subunits prepared by cDNA expression systems, we reexamined the conditions for reconstitution, because the maximum activity recovered from dissociated subunits is less than 50% of the original activity under the conditions currently used. Recent studies on the refolding of several proteins have revealed that polyethylene glycol (PEG), which is potentially similar to molecular chaperones such as GroE, strongly prevents aggregation during renaturation and enhances the renaturation rate and recovery of active proteins [5–8]. We therefore employed PEG to prevent the calpain subunits from forming the aggregates that usually appear during renaturation and succeeded in obtaining full enzyme activity after dissociation and reassociation. Under the same conditions, 80K showed full enzyme activity regardless of the presence of 30K.

2. Experimental

2.1. Materials

Guanidine hydrochloride (GuHCl) and polyethylene glycol 4,000 (PEG) were purchased from Wako Pure Chemicals. Bovine serum albumin (BSA) and ATP were from Sigma. Casein was from Merck. *Escherichia coli* heat shock proteins, GroE, were from Takara Shuzo. Calpain was purified as previously described [9] from rabbit and chicken skeletal muscle. Purified calpain samples were dialyzed before use against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol containing 150 mM NaCl (Buffer A).

2.2. Dissociation of calpain into subunits

Calpain in Buffer A was precipitated by the addition of 4 volumes of acetone, then dissolved and denatured in Buffer A containing 6 M GuHCl for 10 min at 4°C. The subunits were then separated by gel filtration through Superose 6 (Pharmacia Co) with Buffer A containing 6 M GuHCl [10].

2.3. Reconstitution of calpain subunits

The reconstitution of each subunit was carried out by dialysis. Subunits isolated as above were mixed at various molar ratios and dialyzed at various temperatures first against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 M NaCl, 5% (v/v) glycerol, containing 3 M GuHCl for 6 hrs and then against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 M NaCl, 10% (v/v) glycerol for 6 h. Polyethlene glycol (PEG) was added to the dialysis buffer at a PEG to calpain subunit molar ratio of 5 ~ 20 to 1. The dialyzed samples were kept at various temperatures and activities were measured at intervals. For renaturation using GroE, an 80K solution denatured with 6 M GuHCl was dialyzed against 50 mM HEPES, pH 7.8, containing 10 mM Mg $(CH_3COO)_2$, 50 mM KCl, and 2 mM DTT (Buffer B) for 6 h at 25°C. PEG was then added to the dialysis buffer. The concentration of GuHCl was reduced to less than 0.1 M by this dialysis procedure. GroE was then added to the refolding mixture at a molar ratio of 10 ~ 15 ([GroE]/[80K]). The mixtures were further incubated at 25°C for a few days in the presence or absence of 1 mM ATP [11].

2.4. Assay for calpain

Calpain was incubated at 30°C with casein (3 mg/ml) in 250 μl of 100 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and 5 mM 2-mercaptoethanol. After 20 min, the reaction was stopped by the addition of 250 μl of 10% (w/v) trichloroacetic acid. After cooling for 20 min at 0°C, the sample was centrifuged at 8,000 × g for 5 min and the A_{280} of the supernatant was recorded.

3. Results

The conditions for reconstituting calpain from isolated 80K and 30K were examined (Table 1). The recovery of protease activity was significantly low in the absence of 30K, but increased gradually upon the addition of 30K, reaching a maximum recovery at a molar ratio of 1 : 1. These results suggest that the existence of a stoichiometric amount of 30K is essential for the expression of full enzyme activity of 80K. A significant difference between previous studies and the present work is the method of renaturation and hence the recovery of activity [4,12]. The addition of PEG kept the subunits in solution and enhanced the recovery of protease activity of the reconstituted samples. The effect of 30 K on the activity recovery of 80K is seen more clearly in Fig. 1. Fig. 1 shows the time course of the activity recovery of 80K in the presence and absence of 30K (molar ratio = 1 : 1). Almost complete recovery of activity was observed after 20 days at 4°C in the presence of 30K added prior to the first dialysis step (see section 2). On the other hand, the recovery of activity by 80K alone was much slower without

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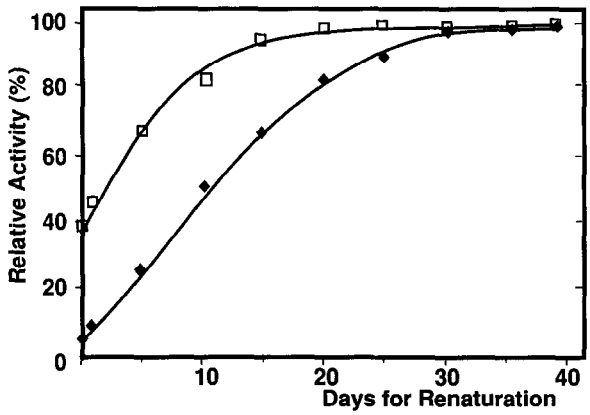

Fig. 1. The recovery of protease activity by 80K in the presence of PEG at 4°C. The protein concentration of 80K and the amount of PEG are shown in Table 1. The recovery of protease activity was measured during incubation at 4°C after overnight dialysis against Buffer A in the presence of PEG. The activity of native calpain measured with 2 mM Ca^{2+} was taken as 100%. Renaturation of 80K with (□) and without (♦) equimolar 30K is shown.

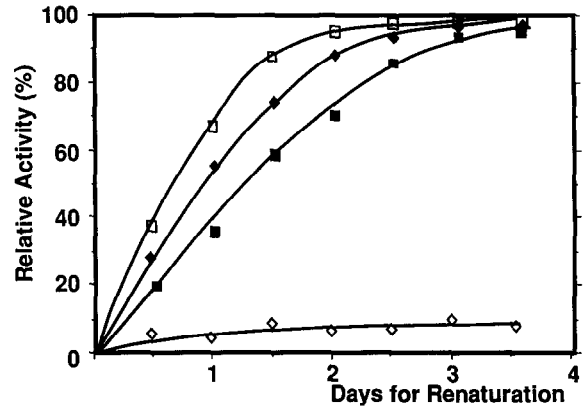

Fig. 2. Effects of GroE and temperature on the recovery of activity of 80K. The concentrations of protein and PEG are shown in Table 1. Renaturation was performed at 25°C in the presence of PEG. Samples were assayed for activity every 12 h after dialysis. □: renaturation of 80K with 30K, ♦: renaturation of 80K with GroE plus 1 mM ATP, ■: renaturation of 80K alone, ◇: renaturation of 80K in the absence of PEG.

30K, but finally reached the same level as the native control after 30 days. This result suggests that 30K acts as a chaperone but contradicts the idea that 30K is essential for the full expression of protease activity. The effect of PEG, 30K, and a chaperone, GroE, on the activity recovery was further examined at 25°C (Fig. 2). While the activity recovery of 80K alone is very low (less than 10%), the addition of 30K, GroE, or PEG significantly enhanced recovery in this order. GroE without ATP and BSA (cf. Table 2), however, had no effect (data not shown), which strongly suggests that GroE and 30K facilitate the refolding of 80K as a chaperone. Further, the rate of recovery at 25°C was significantly accelerated over that at 4°C, reaching a plateau after a few days. Presumably, a molecular chaperone, GroE or 30K, associates with 80K during refolding and prevents its aggregation. PEG has an effect similar to GroE and enhances the activity recovery of 80K. Complete recovery of 80K activity was successfully achieved even at high protein concentrations (≥ 1.0 mg/ml) in the presence of PEG (molar ratio of PEG to 80K : 10). The effects of some factors on the activity recovery of 80K were examined as shown in Table 2. In the absence of PEG, the activity recovery of 80K was 5% or 60% with or without 30K, respectively. The addition of BSA or NaCl had no significant effect in the absence of PEG, whereas in the presence of PEG, full protease activity was observed both with and without 30K. Interestingly, in the absence of NaCl, no activity was recovered even in the presence of PEG and 30K. The presence of NaCl is essential for the complete recovery of activity of 80K.

Table 1
Reconstitution of calpain from subunits

Subunit molar ratio [80K]:[30K]	Activity (%)*
1:0	10.5
1:0.5	48.5
1:1.0	89.5
1:1.25	89.0
1:1.5	89.6

Isolated 80K and 30K in 6 M GuHCl were mixed at various molar ratios at a protein concentration of 1.0 mg/ml and dialyzed at 10°C overnight against Buffer A in the presence of PEG (PEG : 4,000 [PEG]/[Protein] = 10). Means of three independent measurements are shown. *Activity relative to untreated calpain.

4. Discussion

Since calpain is isolated as a dimer, it is quite natural to think that calpain functions as a dimer. Previous results demonstrating that the presence of 30K significantly enhances the activity recovery of 80K supported this idea [12]. However, maximum recovery of activity after dissociation and reassociation of calpain was less than 50%. In order to obtain fully active calpain by reconstitution of the two subunits produced by a recombinant DNA technique, we reexamined the conditions for the reassociation of calpain from subunits. First we examined the conditions for the reassociation of the two subunits isolated by dissociation of native calpain and found that significant subunit aggregation occurred during the dialysis to remove GuHCl for renaturation. To keep the dissociated subunits in solution during renaturation, various reagents were tested. As a result,

Table 2
Effect of PEG, NaCl, and BSA on the renaturation of 80K

PEG (#4000)	NaCl (0.1 M)	BSA (0.2 mg/ml)	Relative activity (%)	
			80K	80K + 30K
−	−	−	0	0
−	+	−	5	60
−	+	+	5	62
+	−	−	0	0
+	−	+	0	0
+	+	−	99	99
+	+	+	98	99

The recovery of protease activity by 80K in the presence or absence of 30K was measured after dialysis at 10°C in the presence (+) or absence (−) of PEG, NaCl, or BSA. The activity of native calpain measured with 2 mM Ca^{2+} was taken as 100%. Means of three independent measurements are shown.

significant effects of PEG and NaCl were found. The effect of NaCl is not sufficiently clear, but PEG presumably interacts with exposed hydrophobic sites on the dissociated subunits and keeps them in solution during association [13–15]. It is astonishing that 80K, which has been regarded as a catalytic subunit because of the presence of a cysteine protease domain, shows full activity even in the absence of 30K. This indicates that 30K is not essential for protease activity, at least in vitro, and suggests that the calpain monomer (80K) is fully active in vivo. A monomeric calpain species has been described previously [16–18]. The monomer, however, seems to be unstable under the conditions used. Further, apparent loss of 30K is often observed when the preparation is partially degraded during purification. It is therefore difficult to conclude whether the 'monomer' described corresponds to 80K itself or not.

Since it has been believed that both subunits are essential for protease activity and that 30K functions to enhance the proteolytic activity of 80K, the structure–function relationship of calpain must be reexamined.

References

[1] Saido, T.C., Sorimachi, H. and Suzuki, K. (1994) FASEB J. 8, 814–822.
[2] Suzuki, K. (1990) in: Intracellular Calcium dependent Proteolysis (Mellgren, R.L. and Murachi, T., Eds.) p. 25–35, CRC Press, Boca Raton, FL.
[3] Sorimachi, H., Saido, T.C. and Suzuki, K. (1994) FEBS Lett. 343, 1–5.
[4] Tsuji, S. and Imahori, K. (1981) J. Biochem. 90, 233–240.
[5] Hartl, F.U., Martin, J. and Neupert, W. (1992) Annu. Rev. Biophys. Biomol. Struc. 21, 293–322.
[6] Gething, M.J. and Sambrook, J. (1992) Nature 355, 33–44.
[7] Jeffrey, L.C., Stuart, E.B., James, R.S., Marjorie, W., Judy, Y.C. and Daniel, I.C.W. (1992) Bio/Technology, 10, Sep. 1013–1019.
[8] Jeffrey, L.C. and Theodore, W.R. (1992) J. Biol. Chem. 267, 3147–3153.
[9] Inomata, M., Nomoto, M., Hayashi, M., Nakamura, M., Imahori, K. and Kawashima, S. (1984) J. Biochem. 95, 1661–1670.
[10] Kikuchi, T., Yumoto, N., Sasaki, T., and Murachi, T. (1984) Arch. Biochem. Biophys. 234, 639–645.
[11] Mizobata, T., Akiyama, Y., Ito, K., Yumoto, N. and Kawata, Y. (1992) J. Biol. Chem. 267, 17773–17779.
[12] Imajoh, S., Kawasaki, H. and Suzuki, K. (1987) J. Biochem. 101, 447–452.
[13] Cleland, J.L. and Wang, D.I.C. (1990) Biotechnology 8, 1274–1278.
[14] Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. and Hartl, F.-U. (1991) Nature 352, 36–42.
[15] Friberger, P. (1982) J. Clin. Lab. Invest. 42, 15–98.
[16] Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Melloni, E. (1985) Biochem. Int. 11, 35–44.
[17] Pontremoli, S. and Melloni, E. (1986) Annu. Rev. Biochem. 55, 455–481.
[18] Fukui, I., Toyohara, H., Ito, K., Hamakubo, T. and Murachi, T. (1988) Biochemistry 27, 3260–3267.