cDNA for the C-terminal Ca\(^2+\)-binding domain of rat calpain small subunit was cloned by means of the polymerase chain reaction. The encoded protein (21 kDa), which corresponds closely to the natural autolysis product of the small subunit, was produced in soluble form in *Escherichia coli* at a level of 20 mg/liter of cell culture. Rat calpain II large subunit (80 kDa) was produced from a cDNA clone in *E. coli* in soluble form at a level of approximately 1 mg/liter. The 80-kDa subunit alone had no protease activity, with or without Ca\(^{2+}\), but Ca\(^{2+}\)-dependent protease activity was obtained following association of the two subunits, which was achieved either by co-expression of the two subunit cDNAs in *E. coli*, or by mixing the two partially purified subunits in the presence of 1 M NaSCN followed by dialysis. The heterodimeric (80 + 21 kDa) protease had a Ca\(^{2+}\) requirement for 50\% activity of 0.35 mM and a specific activity at 2 mM Ca\(^{2+}\) of approximately 1 unit/\mu g, values essentially identical to those of natural (80 + 30 kDa) calpain II. The results establish association and biological activity of the bacterially produced subunits and provide a system for studying structure-function relationships in calpain by means of mutagenesis.

Calpains I and II (\(\mu\)-calpain and \(m\)-calpain) (EC 3.4.22.17) are cytoplasmic Ca\(^{2+}\)-dependent cysteine proteinases, found in most tissues of vertebrates (1, 2). Both enzymes are heterodimers, consisting of a large, 80-kDa, catalytic subunit, and a small, 30-kDa, regulatory subunit. The 80-kDa subunit occurs as one of two closely related but genetically distinct polypeptides, corresponding to calpain I or calpain II, and the 30-kDa subunit is identical in the two enzyme forms. When assayed in vitro, calpain I requires approximately 50 \(\mu\)M Ca\(^{2+}\) for full activity, and calpain II requires approximately 500 \(\mu\)M Ca\(^{2+}\), but they are clearly both active in vivo at <1 \(\mu\)M Ca\(^{2+}\) (1, 2). The natural (80 + 30 kDa) enzymes are pro-enzymes, which in the presence of Ca\(^{2+}\) undergo autoproteolysis of both subunits; this generates active proteinases (approximately 76–78 kDa') which have a lower Ca\(^{2+}\)-requirement for subsequent hydrolysis of substrate proteins. Calpain large subunits have been cloned from chicken, cow, and rat, and variant large subunit genes have been detected at the cDNA level but not as proteins (3–5). Much research has been carried out on the calpain proteins obtained from chicken, cow, and pig (6–10), but the large subunits from cow and pig have not yet been cloned. Small subunit cDNA sequences have been reported from numerous species (11, 12), but not yet from rat. A yield of 5–10 mg of calpain/kg of tissue can be obtained from chicken or bovine skeletal muscle or pig kidney (6–10). The process is laborious, involving four or five column steps, and it is very difficult to eliminate all contamination with partially activated or degraded forms of calpain, which may be present in the original tissue or formed during purification (8, 9). To obtain larger amounts of pure subunits for structural and mechanistic studies it is necessary to develop expression systems. These studies will also involve the use of site-directed mutagenesis as a means to produce altered subunits, for which an efficient expression system is again essential. In the small subunit, the N-terminal glycine-rich regions are removed during autolysis and were thought likely to cause difficulties both in cloning and in expression. A cDNA coding for a 21-kDa C-terminal fragment of the rat calpain small subunit has therefore been cloned and expressed; this protein corresponds closely to the natural autolysis product. For the large subunit, we reported recently the cloning and bacterial expression of cDNA for the large subunit of rat calpain II (4). The product was found entirely in inclusion bodies, but by altering some aspects of the expression system it has been found possible to obtain soluble large subunit. Active rat calpain II has been generated by association of these two subunits, thus demonstrating the biological relevance of these subunit clones. Association occurred both in vivo during coexpression of the genes in *E. coli*, and in vitro on mixing of the partially purified subunits under appropriate conditions.

**EXPERIMENTAL PROCEDURES**

**Rat Calpain Small Subunit C-terminal Domain, cDNA Cloning**—Calpain small subunit amino acid sequences from several species are very highly conserved (11, 12). On this basis, and in order to maintain a homogeneous rat system, primers were designed for polymerase chain reaction amplification of cDNA coding for most of the C-terminal two-thirds of rat calpain small subunit (see legend to Fig. 1). An amplified DNA band of the correct size was obtained from a rat liver cDNA library in Agt11 kindly supplied by Dr. C. Mueller, Queen's University, and was cloned by means of *NdeI*/*PstI* digestion into the ampicillin-resistant vector pT7-\(\phi\)F (4). A synthetic linker DNA was then inserted at the *PstI* site, providing sequence for the six C-terminal amino acids (which are totally conserved in bovine, human, pig, and rabbit calpain small subunits), together with a stop codon, and yielding a plasmid which is referred to as pT7-\(\phi\)F-21k (see Fig. 1).

**Rat Calpain II Large Subunit, Modification of the cDNA**—The natural second codon in rat calpain II, GCC (Ala) (4), was altered by site-directed mutagenesis to GCT (Ala), in an attempt to increase the level of expression (13); in practice, this had no significant effect on expression but subsequent constructs carry the GCT second codon. To delete most of the 3' untranslated region, a *HindIII* site was introduced 20 base pairs downstream of the 80-kDa stop codon by site-directed mutagenesis, and the coding region was then transferred by means of *Neol*/*HindIII* digestion from pT7-\(\phi\)F into the kanamycin-resistant expression plasmid pET-24a(+) (Novagen, Madison, WI). The resultant construct is referred to as pET24a-80k.
Expression of Rat Calpain Subunit Genes—Plasmids encoding rat calpain sequences were transfected into E. coli BL21(DE3) with or without pLysS (Novagen), and the cells were plated onto LB/agar containing ampicillin (100 μg/ml) and/or kanamycin (50 μg/ml) as appropriate. The presence of pLysS had no marked effect on expression of single clones, but reduced the yield of active calpain containing plasmids for both calpain subunits. Isolated colonies were grown to an A<sub>600</sub> of 0.7 at 30 °C, and expression was induced by addition of IPTG to a final concentration of 0.4 mM. The cultures were grown for a further 3 h at 30 °C, and the cells were harvested by centrifugation and washed once in 0.15 M NaCl. The cells were lysed by sonication (five times for 45 s, on an ice-ethanol bath) in buffer A (5 mM EGTA, 10 mM β-mercaptoethanol, 20 mM Tris-HCl, pH 7.8) with addition of glycercol (10%, v/v) and phenylmethylsulfonyl fluoride (50 μg/ml). All subsequent steps were carried out at 4 °C or on ice. The lysate was centrifuged at 15,000 × g for 45 min, and the supernatant was applied to a column of 150 ml of DEAE-Sepharose or Whatman DE52, and eluted with a linear gradient of 0–750 mM NaCl in a total volume of 700 ml of buffer A. The small subunit (21 kDa) was eluted at about 125 mM NaCl; large subunit (80 kDa) or heterodimeric calpain (80 + 21 kDa) were eluted at about 0.25 M NaCl, as expected also for natural calpain (80 + 20 kDa). None of the column matrices had been exposed to natural rat calpain.

Calpain Assay—Calpain activity was measured in a final volume of 0.4 ml 50 mM Tris-HCl, pH 7.8, 10 mM β-mercaptoethanol, containing 2 mg of casein, 5 mM EDTA or CaCl<sub>2</sub>, and enzyme sample. The tubes were incubated at 25 °C for 30 min, and the reaction was terminated by addition of 0.3 ml 10% (w/v) trichloroacetic acid. After standing on ice for 15 min, the tubes were centrifuged at 10,000 × g for 15 min, and the A<sub>280</sub> of the supernatant was measured. The amounts of 80-kDa subunit in crude extracts and column fractions were estimated visually from immunoblots by comparison with a native rat calpain large subunit (SO kDa) or heterodimeric calpain (25 kDa). None of the column matrices had been exposed to natural rat calpain.

The cDNA sequence of the 21-kDa C-terminal domain of rat calpain small subunit is shown. Portions of the sequence shown in upper case letters were specified by synthetic oligonucleotides. The deduced amino acid sequence is shown below the cDNA sequence, and for comparison the amino acid sequence of the corresponding portion of rabbit calpain small subunit (11) is shown where it differs from that of rat. The primers used for polymerase chain reaction to obtain this clone were: (sense) 5'-catagctagctagtytccaaacattgagc-3' (terminating at nt 23 in the coding sequence); (antisense) 5'-gcagctgagcagctgctagc-3' (terminating at nt 523 in the coding sequence), and cloning was achieved by means of NdeI/PstI digestion. The sequence 3' of the underlying PstI site was inserted by means of synthetic oligonucleotides. The sequence is available from the authors and under GenBank<sup>TM</sup> accession no. U10861.
9% polyacrylamide gel is shown, after staining with Coomassie Brilliant Blue. Tracks are recovered from the extract following chromatography on Whatman column. A colony of BL21(DE3) cells containing the plasmids for could be detected, with or without Ca^{2+}, either in the 80-kDa peak fractions or elsewhere in the column eluate.

In Vivo Association of Subunits to Form Active Calpain—An isolated colony of BL21(DE3) cells containing the plasmids for the 80- and 21-kDa subunits was grown in 6 liters, and expression was induced with IPTG. Active calpain (3,500 units) was reconstituted from the AcA44 column shown in Fig. 3A. Fig. 3B shows the results of an immunoblot of fractions eluted from the latter column. Some inactive and presumably aggregated calpain subunits were eluted in the void volume, but the peak of calpain activity (fractions 40–44) correlated with the presence of both large and small calpain subunits. The positions of molecular mass markers used to calibrate the column are also shown in Fig. 3B, and the peak of proteinase activity was calculated to have a molecular mass of 100 kDa.

In Vitro Association of Subunits to Form Active Calpain—Calpain subunits, when separately produced, could be recombined by mixing in the presence of 1 M NaSCN, followed by dialysis (see “Experimental Procedures”). A sample of calpain reconstituted in vitro from 4 liters of each of the expressed subunits was chromatographed on a column of Q-Sepharose, and 2,250 units of calpain activity were eluted at 0.25 M NaCl (data not shown).

Ca^{2+} Requirement for Proteinase Activity—Peak activity fractions from the AcA 44 column of in vivo reconstituted calpain (Fig. 3B) were pooled and their proteinase activity was measured as a function of free Ca^{2+} concentration. The results (Fig. 4) showed that 50% of maximum activity was reached at a free Ca^{2+} concentration of 0.35 mM, with a maximum at 2–4 mM Ca^{2+}, values which are consistent with those normally reported for calpain II (1, 2).

**DISCUSSION**

The small subunit C-terminal domain was expressed in soluble form at levels of 20 mg/liter of cell culture and was readily purified. This protein is seven N-terminal amino acid residues longer than the terminal autolysis product of natural calpain small subunits (9, 17, 19), although only the N-terminal could be detected, with or without Ca^{2+}, either in the 80-kDa peak fractions or elsewhere in the column eluate.

Met residue differs from the natural consensus sequence. The natural small subunit terminal autolysis product is normally referred to as 18 kDa because of its mobility on SDS gels, but its
size was established in the case of pig calpain as 20.3 kDa, by electrospray mass spectrometry (9).

For the large subunit, the original pT7-7fn-80k construct yielded only the insoluble large subunit following induction at 42 °C in K38 cells (4). The 80-kDa coding sequence was then transferred to pET-24d (+); this plasmid contains the same promoter and ribosome binding sequences as the pT7 plasmids, but the transfer removed the 900-base pair calpain large subunit 3'-untranslated region (which contains several eukaryotic mRNA instability sequences) and provided a T7 transcription termination signal. In BL21(DE3) cells grown at 30 °C and induced with IPTG, this construct yielded about 50% soluble yield yielded only the insoluble large subunit following induction at low temperatures in BL21(DE3) cells grown at 30 °C and induced with IPTG, this construct yielded about 50% soluble yield yielded only the insoluble large subunit following induction at 42 °C in K38 cells (4). The 80-kDa coding sequence was then transferred to pET-24d (+); this plasmid contains the same promoter and ribosome binding sequences as the pT7 plasmids, but the transfer removed the 900-base pair calpain large subunit 3'-untranslated region (which contains several eukaryotic mRNA instability sequences) and provided a T7 transcription termination signal. In BL21(DE3) cells grown at 30 °C and induced with IPTG, this construct yielded about 50% soluble product.

When expressed alone, the 80-kDa subunit had no detectable proteinase activity, either with or without Ca2+; the bacterial system has the advantage that there is no possibility of contamination with the small subunit. The natural pig calpain II large subunit isolated by chromatography in 1 M NaSCN was also recently reported to have no activity (9). In some earlier reports, the large subunit, isolated from natural calpain by chromatography in 1 M NaSCN or 6 M urea, was found to have from 3-30% of the activity of intact calpain (16, 20, 21). It appears likely that incomplete separation of the subunits was responsible for these findings of partial activity.

Reconstitution of calpain from natural intact or autolysed subunits has been described previously, as also has reconstitution from a natural large subunit together with a portion of the rabbit calpain small subunit cloned and expressed as a fusion protein (16, 20, 21). The results presented here confirm and extend these earlier findings; they demonstrate that the large- and small recombinant calpain subunits, obtained by expression of cDNA clones which we have prepared, can associate to generate active rat calpain II, both within a bacterial cell, and also in vitro as a result of incubation in 1 M NaSCN followed by dialysis. No activity was obtained on simple mixing of the two subunits. Association of the two subunits was equally efficient in vivo in bacteria and in vitro following 1 mM NaSCN treatment and dialysis, as indicated by the yield of active calpain from the two procedures. The recombinant calpains were not pure at this point, but their specific activities were estimated from immunoblot intensities of the large subunit, to be approximately 1 unit/mg, equal to that of natural rat calpain II (22).

The Ca2+ requirements of 0.35 mM for 50% activity and 2-4 mM for maximum activity of the recombinant (80 + 21 kDa) calpain are equal to those reported for intact natural (80 + 30 kDa) calpain II from several species (1, 2). Since the recombinant rat calpain contains a pre-activated small subunit, it is clear, as previously reported (16, 23), that the fall in Ca2+ requirement which characterizes calpain activation is caused only by autolysis of the large subunit.

It is highly probable that the active calpain produced here is a heterodimer, since it behaves identically to native calpain II in terms of column chromatography and Ca2+ sensitivity, and because the position of elution of calpain activity from the Aca 44 column was as expected for the molecular mass of 80 + 21 kDa. The subunits are non-covalently associated, since they can be separated in 1 M NaSCN and on SDS gels, but subunit stoichiometry could not be further corroborated from the immunoblots because of differing responses of the subunits to the available antibodies.

These results demonstrate biological activity of the bacterially expressed rat calpain subunit clones and show that association of the subunit proteins can take place both within E. coli and in vitro. It is therefore practicable to purify the subunits separately, in order to study their activities and interactions. Finally the results make it possible to assay the activity of calpains containing modified large and small subunits generated by mutagenesis, so that structure-function relationships of calpain can be studied in detail.

**REFERENCES**