Modulation of Erythrocyte Ca\textsuperscript{2+}-ATPase by Selective Calpain Cleavage of the Calmodulin-binding Domain*

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Peter James\textsuperscript{2}, Thomas Vorherr\textsuperscript{2}, Joachim Krebs\textsuperscript{2}, Alessandro Morelli\textsuperscript{3}, Giorgio Castello\textsuperscript{9}, Daniel J. McCormick\textsuperscript{4}, John T. Penniston\textsuperscript{11}, Antonio De Flora\textsuperscript{5}, and Ernesto Carafoli\textsuperscript{11}

From the \textsuperscript{1}Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland, the \textsuperscript{2}Institute of Biochemistry, University of Genoa, Genoa 16132, Italy, and the \textsuperscript{3}Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota 55905

The activity of the membrane-bound and the purified erythrocyte Ca\textsuperscript{2+}-ATPase in the absence of calmodulin was stimulated by calpain digestion but could be further increased to maximal levels by calmodulin (CaM). Thus, CaM sensitivity was retained by the digested ATPase, at least at short times of incubation. In membranes digested at higher temperatures and in the purified ATPase digested at higher calpain/ATPase ratios, the ATPase became fully activated.

The membrane-bound and the purified 138-kDa ATPase were converted by calpain to a fragment of approximately 124 kDa which still bound CaM and could be isolated on CaM columns when proteolysis occurred slowly but not when it occurred rapidly.

Carboxypeptidase digestion of the purified enzyme and of its fragment of about 124 kDa has shown that calpain attacked the CaM-binding domain near the C terminus of the ATPase. This has also been supported by digestion of the purified enzyme and of its fragment of about 124 kDa. A first cut occurred in the middle of the domain producing a fragment of about 14 kDa and a (CaM-binding) fragment of about 124 kDa. A second cut closer to the N terminus of the domain also produced a fragment of about 124 kDa, and accounted for the loss of CaM binding at prolonged times of incubation of the ATPase with calpain.

The messenger function of Ca\textsuperscript{2+} depends on its interaction with target enzymes. In some cases, the interaction is direct. Frequently, however, Ca\textsuperscript{2+} interacts first with calmodulin which then interacts with the target enzymes. The enzyme modulation by Ca\textsuperscript{2+} and CaM is normally reversible. Recently, it has been shown that Ca\textsuperscript{2+} produces irreversible, autolysis-mediated activation of calpain, a nearly ubiquitous Ca\textsuperscript{2+}-stimulated endopeptidase for recent reviews, see Refs. 1 and 2. It was already known that limited proteolysis of a number of CaM-dependent enzymes produces irreversible activation (2–5) and loss of CaM sensitivity. Recent studies have shown that calpain also causes limited proteolysis and activation of several CaM-modulated enzymes in cell-free systems (6–9) and in intact cells (10).

Calpain exists in two molecular forms characterized by different Ca\textsuperscript{2+} sensitivities (1–2, 11): calpain I and calpain II, also defined as micromolar and millimolar, respectively, due to their activity at micromolar and millimolar Ca\textsuperscript{2+} concentrations. In human erythrocytes calpain is present as an inactive proenzyme (procalpain) which can be converted through Ca\textsuperscript{2+}-dependent autoproteolysis to a mature form (calpain) fully active at micromolar Ca\textsuperscript{2+} (2). Procalpain is composed of a catalytic 80-kDa subunit and a 30-kDa subunit (2). The primary structures of both human subunits have been deduced from the nucleotide sequences of cDNA (1, 12) and found to contain distinct domains. The 80-kDa subunit is composed of four domains (I, II, III, and IV), whereas the 30-kDa subunit has two (V and IV'). Domains IV and IV' are homologous and have four consecutive EF hands, i.e. the helix-loop-helix Ca\textsuperscript{2+}-binding structures typical of CaM-like proteins (13).

The domain structure of calpain, together with the CaM-binding property of the erythrocyte Ca\textsuperscript{2+}-ATPase (14, 15), suggest that calpain could be targeted to the ATPase, thereby producing its proteolysis. Two recent studies on the membrane-bound enzyme (16, 17) have shown that calpain indeed induces activation, thereby indicating proteolytic attack. One of the studies (17) showed that 0.64 \mu M calpain activated the ATPase to the maximal values obtained with optimal CaM concentrations: on the other hand, CaM protected the membrane-bound enzyme against the calpain-mediated activation. The other study (16) also showed activation of the ATPase but only in membranes previously treated with saponin or glycodeoxycholate. The same study also mentioned one experiment on a Triton X-100-solubilized Ca\textsuperscript{2+}-ATPase preparation, in which calpain, instead of activating the enzyme, apparently caused an inhibition. The lack of details in the latter experiment, which was evidently preliminary, makes any comment on the different effects on the membrane bound and the solubilized enzyme impossible. However, studies in our laboratories on the effects of various proteases on the purified human erythrocyte ATPase (18) have shown that calpain increased the basal activity of the purified enzyme, while leaving its interaction with CaM relatively unaffected: at the structural level this corresponded to the removal from the main body of the ATPase of a fragment of about 14 kDa. It was thus decided to study the functional and structural effects of calpain on the ATPase in greater detail and to do so on both the isolated and the membrane-bound ATPase.
The results have shown that the basal activity of the enzyme is indeed stimulated several-fold by calpain in both the membrane-bound and the purified state, whereas the maximal activity in the presence of CaM remains unchanged, resulting in a lowered stimulation ratio by CaM. The protease reduces the 138-kDa ATPase to a fragment of approximately 124 kDa, the initial attack occurring in the middle of the CaM-binding domain (19). Carboxypeptidase A worked on the purified ATPase and its fragment of about 124 kDa, also supported by work on a synthetic peptide corresponding to the CaM-binding domain. It has identified a second point of attack under more drastic conditions of calpain exposure. This second site is located at the beginning of the CaM-binding domain and accounts for the loss of CaM binding by the ATPase under these conditions. In the presence of calmodulin, the proteolysis of the synthetic CaM-binding domain is much slower and the pattern of fragmentation different. This probably accounts for the protection by CaM against calpain-mediated activation observed on the membrane-bound enzyme in Ref. 17.

**MATERIALS AND METHODS**

Leupeptin was purchased from Fluka AG, Buchs, Switzerland. Carboxypeptidase A and B were from Sigma. Calmodulin was isolated from bovine brain as described in Ref. 20 and radioiodinated using Enzymo-Beads from Bio-Rad, as described in Ref. 21. The Ca²⁺-ATPase was isolated from human erythrocytes and purified to homogeneity according to Ref. 22: Triton X-100 was the detergent used, and phosphatidylcholine the stabilizing phospholipid. Procalpain was purified from human erythrocytes to a homogenous form as in Ref. 23. Molecular weight standards were from Pharmacia, Uppsala, Sweden. All other reagents were of the highest purity commercially available.

Hemoglobin-free unsealed erythrocyte membranes devoid of endogenous CaM were prepared by hemolyzing freshly drawn human erythrocytes in the presence of EDTA, as described in Ref. 24.

**Measurement of the Ca²⁺-ATPase**—The purified enzyme and of its proteolytic (124 kDa) fragment was monitored continuously in a thermostatted spectrophotometer at 37 °C, using the coupled enzyme assay described in Ref. 22 and 24. The ATPase activity of erythrocyte membranes was assayed by measuring the amount of phosphate released in 30 min from 1 mM Na-ATP at 37 °C in the presence of 10 µM free Ca²⁺. One unit of activity is defined as the amount of enzyme that causes the release of 1 µmol of inorganic phosphate in 1 h at 37 °C. Additional details are given in the legends for the figures. The CaM activity was assayed according to Ref. 25, as described in Ref. 26. Calpain activity was monitored by following the liberation of acid-soluble peptide(s) with fluorescamine from human acid-denatured globin. One unit of activity corresponds to the amount of calpain which caused the release of 1 µmol of trichloroacetic acid-soluble (7%) NH₃·H₂O groups/h. When the ratio of calpain units to ATPase units is given, the ATPase activity refers to the conditions of maximal calmodulin stimulation. The free Ca²⁺ concentrations in the media were calculated using a computer program, assuming a Kd of the Ca²⁺-EGTA complex of 0.21 µM at pH 7.0 (27). However, for the experiments described in Fig. 8, the free Ca²⁺ concentrations in the buffers were calculated according to the program described in Ref. 38.

**Calpain Digestion of the Membrane-bound Ca²⁺-ATPase**—Procalpain purified from human erythrocytes was converted to calpain by incubation for 2 min at 25 °C in the presence of 0.5 mM Ca²⁺. Digestion of the purified Ca²⁺-ATPase was performed by incubation with calpain at the temperatures and under the conditions specified in the descriptions of the experiments, in the presence of 0.5 mM total Ca²⁺ with or without 20 mM CaCl₂. Aliquot of the reaction media were withdrawn at the times indicated, proteolysis was arrested by adding 10-fold molar excess of leupeptin, and assays for ATPase activity were performed as described above.

**Gel Electrophoresis Analysis of the Digested Purified Ca²⁺-ATPase**—The samples for gel electrophoresis were boiled for 5 min in a sample buffer containing 7.5% SDS in 30 mM sodium phosphate, pH 7.0, 3% 2-mercaptoethanol (w/v), 0.2% (w/v) bromophenol blue, to stop proteolyis. Electrophoresis was carried out on Laemmli-type gels (28). Samples to be used for blotting experiments were loaded in duplicate on the gels, so that one-half of each gel was used for silver staining (29) and half for electrobllotting. The gels were electrophorontoed onto nitrocellulose sheets (30), the nitrocellulose was incubated overnight at 4 °C with blocking buffer (0.14 M NaCl, 10 mM sodium phosphate, pH 7.2, 2% defatted milk powder). The change of buffer on the nitrocellulose at room temperature with 125I-labeled CaM (50 nM, specific activity 50 mCi/nmol) in the same buffer but with either 0.1 mM CaCl₂ or 2 mM Na-EGTA. After two washes with the respective buffers, the sheets were removed, air-dried, and exposed to Kodak XAR-5 x-ray film at −70 °C for 2–3 days.

**Isolation of the CaM-binding 124-kDa Fragment of the Ca²⁺-ATPase**—Preparative digestion of the ATPase with calpain for the isolation of the fragment of about 124 kDa was carried out as described above, in the absence of CaM. After 30 min, the entire digest was percolated through a CaM column at 4 °C in the presence of 0.5 mg/ml phosphatidylcholine or phosphatidylserine as described previously for native Ca²⁺-ATPase (22). The fragment was eluted from the column with 2 mM Na-EGTA in a buffer containing 130 mM NaCl, 20 mM HEPES-NaOH, pH 7.2, 1 mM MgCl₂, 2 mM dithiothreitol, 0.05% (w/v) Triton X-100, and 5% (v/v) glycerol. The peak fractions, identified by activity measurements and SDS-PAGE, were pooled and stored at −70 °C.

**For the experiments on carboxypeptidase digestion, the 124-kDa CaM-binding fragment was isolated on a CaM column as described above. Following long times of digestion without CaM, no intact 138-kDa ATPase was present: the pure 124-kDa fragment unable to bind CaM was obtained by collecting the Ca²⁺ flow-through from the column used to prepare the CaM-binding 124-kDa fragment, loading it directly onto an erythrocyte Ca²⁺-ATPase monoclonal antibody (5F10) column, and washing with the same buffer (approximately 15 bed volumes). Monoclonal antibody 5F10 has an epitope towards the middle of the ATPase molecule so it will interact with only the 124-kDa fragment unable to bind CaM allowing the small C-terminal fragments to be washed out. The fragment was then removed from the column with 2 M ethanolic acid.

**Carboxypeptidase Digestion of the Membrane-bound Ca²⁺-ATPase**—Digestion of Ca²⁺-ATPase in erythrocyte membranes was carried out by incubating various amounts of pre-converted calpain at 25 °C with 1.3 mg of protein of membranes prepared as described above. The medium of incubation (0.6 ml final volume) was 10 mM HEPES-KOH, pH 7.4, containing 65 mM KCl, 0.25 mM MgCl₂, and 0.15 mM CaCl₂. Control experiments were also performed without CaCl₂ by following the proteolysis of the Na⁺/K⁺-ATPase. Other control experiments without calpain were run in parallel. Aliquots containing 150 µg of membrane protein were withdrawn at the times indicated, treated with 0.35 mM leupeptin, and assayed for Ca²⁺-ATPase activity as described under "Materials and Methods." Samples to be used in the blotting experiments for analysis of the acyl phosphate complex and for 125I-labeled CaM overlay were run in duplicate on the gels as described for the purified Ca²⁺-ATPase. Briefly, 70-µl aliquots of the incubation mixtures containing 150 µg of membrane proteins were treated with a Ca²⁺ solution (0.62 mM EGTA, 6.25 mM CaCl₂, 0.3 mM lanthanum nitrate). Five µl of [γ-32P]ATP (0.17 µmol, specific activity 3,5Ci/µmol), stabilized in 5 mM β-mercaptoethanol, were then added on ice, followed 30 s later by a solution containing 0.6 µl of 6% (w/v) trichloroacetic acid, 10 mM phosphate, and 1 mM Na-ATP. Aliquots were used for electrophoretic experiments followed by 125I-labeled Ca²⁺ overlay were treated in the same way but without addition of radioactive ATP. In both types of experiments, pellets obtained by centrifugation for 5 min at 12,000 × g were resuspended in 0.6 µl of 6% trichloroacetic acid, recovered as above, and dissolved in 0.15 µl of a solution containing 0.25 M Tris-HCl, pH 6.8, 10 mM EDTA, 0.3 M β-mercaptoethanol, an 1% (w/v) SDS. The solubilized material was then treated with 0.05 µl of a solution containing 4% (w/v) sucrose and 0.04% (w/v) bromophenol blue. Electrophoresis on an acrylamide gradient was carried out using a mini-Protean II apparatus from Bio-Rad, 10% acrylamide thick gel, at 40 mA, 100 V, and 4°C.

The stacking gel contained 15 mM Tris phosphate, pH 5.8, 4.3% (w/v) acrylamide, 0.11% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.07% (w/v) ammonium persulfate, and 1 µl TEMED/ml. The resolving gel was a linear downward gradient of 5% to 10% acrylamide in 25 mM Tris-phosphate, pH 6.8, containing 0.1% SDS, 0.1% ammonium persulfate and 1.2 µl TEMED/ml.

1. J. T. Penninston, unpublished data.
After electrophoresis, the portion of the gel containing samples not treated with \(^{32}\)P-ATP was cut and submitted to the electrophoretic procedure followed by \(^{125}\)I-labeled calmodulin overlay exactly as for the purified \(\text{Ca}^{2+}\)-ATPase (see above). The portion of the gel loaded with \(^{32}\)P-ATP treated samples was stained with Coomassie Blue, air-dried, and scanned with a Trinitrox 3M screen at -70°C for at least 12 h. Standard proteins of known molecular size were \(eta\)-spectrin, ankyrin, bands 2, 3, 4, 1, 6, and 7 of erythrocytes.

**Isolation of the Initial Peptide Released from the Purified ATPase upon Calpain Attack**—1 nmol of purified ATPase was digested on ice for 5 min with 1 nM of calpain. The supernatant was centrifuged and the pellet washed twice with acetone containing 0.1% HCl (v/v). The pellet was then sonicated in 1 ml of water and centrifuged. The supernatant containing the released peptide was concentrated under a dry nitrogen stream and applied onto the sequence.

**Determination of the C-Terminal Sequences of ATPase Fragments by Carboxypeptidase Digestion**—Three samples were used for carboxypeptidase digestion: (a) pure 138-kDa intact ATPase prepared using fresh blood to ensure the absence of any 124 kDa contamination (27); (b) the pure 124 kDa CaM-binding fragment isolated on a CaM column as described above; and (c) the pure 124-kDa fragment unable to bind CaM as described above.

The three samples were prepared for digestion in identical manners. The probes were precipitated with acetonitrile to 10% final concentration and centrifuged. The pellets were washed with water four times and redissolved in 0.1% TFA, 0.1 M N-ethylmorpholine acetate, pH 8.5. Approximately 20 nmol of each sample was digested with 20 μg of carboxypeptidase at 37°C. Samples for analysis were removed at 1 and 30 min and 1, 2, 5, and 10 h. The intact ATPase and CaM-binding 124 kDa fragment were digested with carboxypeptidase A treated with phenylmethylsulfonyl fluoride, the 124-kDa non-CaM-binding fragment was digested with phenylmethylsulfonyl fluoride-treated carboxypeptidase B.

The samples were then precipitated with 10% trichloroacetic acid and centrifuged. The supernatant was analyzed using an Applied Biosystems 430A (Foster City, CA) using on-line F-TOF detection with a model 130A analyzer.

**Synthesis of the Calmodulin-binding Domain Peptide**—The synthesis was carried out using an Applied Biosystems 430 A peptide synthesizer using tertiary butoxycarbonyl derivatives and the program as supplied. Double coupling was performed after cycle 14. The peptide cleaved from the support was purified to homogeneity by reverse-phase HPLC.

**Digestion of the Synthetic Peptide by Calpain**—Typically, 10 nmol of the peptide were digested using either 1 or 15 IU of calpain for varying times at room temperature in the presence of 50 μM Ca2+. Parallel experiments under identical conditions were carried out in the presence of 10 nmol of calmodulin. The digestion was stopped by injecting the mixture directly into the reverse-phase HPLC system.

**HPLC Analysis and Amino Acid Sequencing of Separated Peptides**—The peptides were separated by reverse-phase HPLC. A Machery and Nagel (Oensingen, Switzerland) C18, 3 μm, 100 Å reverse-phase column was developed using a gradient of 0.2 min 100% A, 2-18 min to 20% B, 18-28 min 20% B, 28-48 min to 30% B, 48-52 min to 50% B. A is 0.1% trifluoroacetic acid in water and B is 50% acetonitrile, 0.1% trifluoroacetic acid in water.

**Chromatography was carried out using LKB equipment (LKB, Uppsala, Sweden). Sequencing was performed using an Applied Bio-system 470A Sequencer and 120A phenylthiohydantoin derivative analyzer.**

**Protein Determination**—The protein concentrations of the purified ATPase and its fragments were determined by a modification of the method of Lowry et al. (31). The protein was first precipitated with deoxycholate and trichloroacetic acid to avoid interference by Triton X-100 and HEPES (31, 32).

**RESULTS**

**Calmodulin Stimulation of the Purified ATPase under Different Experimental Conditions**—The results of the kinetic measurements on the \(\text{Ca}^{2+}\)-ATPase digested with calpain refine and extend those previously published from these laboratories on the stimulation of the ATPase by trypsin (33, 34). An extensive search of the most appropriate experimental conditions to study the stimulation by CaM has resulted in a greater initial quantitative response to the activator. Previous standard preparations of purified ATPase were stimulated by CaM about 2.5-4-fold, whereas the stimulation ratios measured in the present work were between 6 and 7. The preparations used here had the same \(V_{\text{max}}\) in the presence of CaM, as those used previously; however, the basal ATPase rate was much lower. The difference was traced back to the handling of the purified enzyme: although several factors are likely to have played a role, it was observed that the procedure of thawing the frozen purified ATPase greatly influenced its response to CaM (Fig. 1).

The rapid thawing procedure used in previous work (22, 24, 33, 34) produced stimulation ratios which seldom exceeded 3.0. If the frozen ATPase was allowed to thaw slowly on ice, the basal activity was markedly reduced, and the stimulation factor became accordingly higher (3.0-4.0). ATPase preparations thawed rapidly, but then allowed to stand on ice for 1 h, had a similar low basal activity and a stimulation factor that routinely exceeded 4.0. The lowest basal activity and the highest CaM stimulation factor (but not the highest maximal activity) were obtained with frozen enzyme preparations allowed to thaw slowly on ice (1-2 h), refrozen, and allowed to thaw slowly on ice once more (Fig. 1). In the experiments described here, the ATPase was thawed rapidly and then allowed to stand on ice 1 h before use. Occasional SDS gel electrophoresis controls showed that the preparations used were essentially represented by the 138-kDa component (about 98%) before and after the thawing and standing on ice procedure. Thus, the effect of the treatment on the (basal) activity was not due to proteolysis and preferential association of potentially inhibitory small peptides with the 124-kDa contaminant.

**Effect of Calpain on the Purified Erythrocyte ATPase**—Calpain has been shown to degrade a number of membrane proteins once the activation of procalpain has been triggered by a variety of conditions (2). Fig. 2A (left panel) shows the effect of calpain on the intact purified \(\text{Ca}^{2+}\)-ATPase in the absence of CaM. During digestion the intact ATPase (138 kDa) was degraded to a fragment of approximately 124 kDa, but the expected smaller fragment of approximately 14 kDa could not be observed.
was not seen on the 7% gels (Fig. 2A, left panel). Control experiments (data not shown) have indicated that CaM was not degraded by calpain and that the presence of CaM did not alter the pattern of proteolysis in a way which could be revealed by the gel system used (however, see the section below on the two different products of approximately 124 kDa). The 124-kDa fragment was heterogeneous, see below) retained CaM-binding properties. The middle panel shows the nitrocellulose sheet after electroblotting and incubation with 125I-labeled CaM in the presence of 0.1 mM Ca2+ and the right panel after incubation in the presence of 1 mM EGTA + 125I-labeled CaM. Evidently, the CaM-binding region of the ATPase was preserved in the 124-kDa fragment. In this experiment the radioactive band at about 124 kDa was visible for up to 30 min and then was degraded until a 30–43-kDa radioactive fragment became visible near the front of the gel. Further supporting evidence for the maintenance of the CaM-binding region in the 124-kDa fragment obtained under conditions of mild calpain proteolysis was the demonstration that the latter could be purified by CaM affinity chromatography (see below).

When the digestion was carried out with substantially higher amounts of calpain at room temperature, the rate and the extent of activation of the basal ATPase activity were considerably higher (data not shown). Under these conditions, (e.g. 5.0 units of calpain/1.0 unit of Ca2+-ATPase), the stim-
ulation varied between 5- and 6-fold, reaching the levels of the CaM-stimulated ATPase activity in 20 min. Thus, the ATPase digested under these conditions had become desensitized to CaM. The structural counterpart of the desensitization to CaM was the rapid conversion of the native 138-kDa Ca2+-ATPase to a 124-kDa fragment which was apparently indistinguishable from that produced by the lower calpain concentration, but which failed to bind 125I-labeled CaM in gel overlay experiments (not shown). The results indicate that substantial differences in CaM binding were evidently produced by subtle changes in the 124-kDa fragment not visible by the PAGE analysis system used here. This might indicate either a distinctive attack of calpain depending on its levels relative to the ATPase, or more likely, sequential cuts at two sites of the ATPase, resulting in the maintenance or in the loss of the CaM-binding properties, respectively. The two sites would be very close to each other, since in both cases a fragment of approximately 124 kDa is produced. This second possibility has in fact been validated by work on the synthetic CaM-binding domain described below.

**The Effect of Calpain on the Ca2+-ATPase in the Erythrocyte Membrane**—Fig. 3 shows the effect of calpain on the Ca2+-ATPase present in CaM-free erythrocyte membranes. Control experiments (not shown) ruled out kinetic changes in the ATPase activity when membranes were incubated in the absence of calpain. The basal ATPase activity was progressively stimulated at two different concentrations of calpain (1.0 and 2.0 units, respectively, per 1.0 unit of Ca2+-ATPase) to the same levels reached with the addition of optimal CaM concentrations, i.e. nearly 6-fold. This activation was more extensive than that observed with low amounts of calpain and the pure ATPase. Perhaps this was due to the higher temperature used for the digestion.

Structurally, the digestion of the membrane-bound ATPase resulted in the rapid conversion of the native 138-kDa form to the fragment of about 124 kDa (Fig. 4). The left panel shows the acyl phosphate intermediate of the Ca2+-ATPase (1st lane) and of its digestion product(s) (2nd–5th lanes). Since the visualization of the ATPase, which is present in the membrane in minute amounts, would have been impossible with conventional gel staining procedures, the phosphorylated enzyme was visualized instead, as routinely done for the enzyme in situ. Under the experimental conditions, the conversion of the 138-kDa ATPase to the 124-kDa fragment was almost complete after 2.5 min of incubation, i.e. at a time when the activation of the basal activity was not complete (see Fig. 3). Moreover, a smaller fragment of 82–83 kDa still able to form the acyl phosphate intermediate was also formed (peak at 5 min). The high Mf band seen in the gel above the ATPase was hydroxylamine-resistant, probably indicating phosphorylation of the β-subunit of spectrin (35).

The right panel of Fig. 4 shows the results of the 125I-labeled CaM overlay experiment. The conversion of the 138-kDa ATPase to the fragment of about 124 kDa was apparently slower with respect to the results shown in the left panel of the figure. Although no conclusive explanation for the finding can be offered, this effect is likely to be related to the different CaM affinity of the 138- and 124-kDa forms. The former, naturally, had the expected high affinity for CaM, as also indicated by the intensity of the 138-kDa band, whereas the affinity of the 124-kDa fragment produced under these conditions was likely to be lower (see Fig. 3). The digestion of the native, membrane-bound ATPase is thus probably more rapid than it would appear on the basis of the CaM-binding overlay experiments.

Some residual CaM binding to the fragment of approximately 124 kDa could be observed at 20 min even though at this time no stimulation of the ATPase activity by CaM could be seen. However, when compared to the binding seen to the same amount of undigested protein at zero time this residual amount of CaM binding would represent a negligible fraction of the fragment of about 124 kDa present (probably less than 1%) and would thus not influence the activity measurement. As for the fragment of about 14 kDa which was expected to result from the transition of the ATPase to the fragment of about 124 kDa, it was not detected near the front of the gel in parallel experiments in which the gels were stained with Coomassie Blue (not shown). Possibly, further degradation of the putative 14-kDa fragment had taken place.

**Site of Calpain Attack on the Ca2+-ATPase**—When a large
preparation of purified ATPase was treated with calpain under the conditions specified under "Materials and Methods," at short times of digestion a single polypeptide was released from the ATPase. Electroblotting onto polyvinylidene difluoride membranes after SDS-PAGE electrophoresis has shown the size of the peptide (by Ponceau S staining) to be about 14 kDa. Given the uncertainties in the determination of M, on SDS gels (the Ca^{2+}-ATPase, which runs on gels as a 138-kDa molecule is instead only about 134 kDa when sequenced, Refs. 36 and 37), and the figure of 14 kDa given here is only indicative. The fragment could be easily separated from the remaining body of the ATPase, since after trichloroacetic acid precipitation, it was preferentially solubilized in water. The partial sequence obtained for the N-terminal of the water-extracted 14-kDa fragment was as follows

R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S-(S).

Indeed, recent work (19) has shown this sequence to be contained in the calmodulin binding domain of the ATPase, which is located at the C-terminal end of the molecule (36, 37). On the other hand, previous experiments have shown that the intact ATPase has a blocked N terminus. At the short times of incubation with calpain used for these experiments, only this sequence was found. At longer times of incubation with calpain, other sequences appeared, all of them C-terminal to this.

To further define the site(s) of calpain cutting, carboxypeptidase digests of the native enzyme and the two putative fragments of about 124 kDa, one CaM-binding and one non-binding, were carried out.

The intact ATPase yielded the following C-terminal sequence

T - S - L

the CaM-binding fragment of about 124 kDa the sequence

L - N

and the non-CaM-binding fragment of about 124 kDa the sequence

L - R

The sequence for the intact ATPase agreed with the published clones (36, 37). The dipeptide sequence of the CaM-binding fragment of about 124 kDa occurs only in the middle of the CaM-binding domain (19) where a β turn is predicted.

That of the fragment of about 124 kDa unable to bind CaM occurs seven times in the ATPase, three times in the N-terminal 5-kDa portion (hence it cannot occur in the fragment of about 124 kDa discussed here), and three times in regions which would produce fragments between 50 and 100 kDa. These three fragments would lack part of the ATP-binding domain (36, 37) and would thus be inactive as ATPases.

The last occurrence of the sequence is at the beginning of the CaM-binding domain, nicely explaining the existence of a fragment unable to bind CaM of about 124 kDa (11 amino acids shorter than the CaM-binding 124-kDa fragment). At variance with the CaM-binding 124-kDa fragment, whose ATPase activity was stimulated by CaM, the 124-kDa fragment which did not bind CaM was fully active in its absence (data not shown).

Calpain Attack on the Synthetic CaM-binding Domain—To further support the points discussed in the preceding section, a peptide corresponding to the calmodulin-binding domain (19) of the ATPase was used as a model for investigating the mode of action of calpain on the enzyme. Its sequence was

L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S

Caution is obviously necessary in relating work on synthetic peptides to the situation in the native protein, but in this case it was felt that the synthetic peptide would nevertheless provide useful information. Routinely, 10 nmol of peptide were digested using 1 or 10 units of calpain at room temperature in the presence and absence of 10 nM CaM. The splitting products were separated by HPLC and sequenced. In the absence of calmodulin, the peptide was degraded very quickly (1-2 min) through the same cut seen with the native ATPase. Sequence analysis of the degradation products after 2 min of digestion indicated that a first cut occurred as shown in Fig. 5. The N-terminal half of the synthetic domain was further degraded by calpain under more drastic proteolysis conditions (10 units of calpain, 60 min) to loose the first two amino acids (second cut in Fig. 5). This is assumed to correspond to the complete loss of calmodulin binding by the 124-kDa fragment of the ATPase produced under the more drastic digestion conditions described above, in agreement with the carboxypeptidase work. As discussed above, the difference between the 124-kDa CaM-binding fragment and the fragment produced by this second cut, which did not bind calmodulin, was evidently too small to be resolved on the gels used.

The fragmentation pattern of the synthetic peptide was slower and qualitatively altered if the digestion was performed in the presence of calmodulin. In this case the first cut at the site indicated in Fig. 5 occurred only after 10 min of incubation, irrespective of the calpain concentration used.

CaM present or absent

CaM absent

CaM present

SECOND CUT FIRST CUT FIRST CUT

LRRGQILWFRLGRNLRIQTQIKVVNAFSSS

Fig. 5. Splitting pattern of the synthetic calmodulin-binding peptide by calpain in the absence or presence of calmodulin.

Fig. 6. Measurement of the $K_m$(Ca^{2+}) of the 124-kDa fragment of the ATPase. 2 nmol of ATPase were incubated with 0.1 IU of calpain for 15 min on ice. The free Ca^{2+} concentration was adjusted to 50 nM and the sample loaded onto a mini-CaM column. The column was washed with 10 bed volumes of the calcium buffer and then eluted with the EDTA buffer as described under "Materials and Methods." The protein concentration of the active fractions was determined and the ATPase measured with the coupled enzyme assay at various free calcium concentrations. The open squares represent the activity of the purified 124-kDa fragment alone, the closed squares in the presence of 2 nM calmodulin, and the closed diamonds the activity of the intact ATPase in the absence of calmodulin.
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agrees well with the preservation of the binding of CaM by the ATPase when calpain attack occurred in the presence of calmodulin and also with the recent finding that calmodulin protects the membrane-bound ATPase against the activation by calpain (17). A second cut in the presence of CaM took place at the same location seen in the absence of CaM and occurred only after protracted times of incubation (i.e. approximately 60 min at high calpain concentrations).

\(K_m(Ca^{2+})\) of the Fragment of about 124 kDa—The 124-kDa fragment (or rather, one of the two, see above) could be purified on a CaM column (see "Materials and Methods") as a component that did not contain associated C-terminal peptides released by calpain. Evidently, the hydrolyzed peptides did not remain associated with the main body of the protein (i.e. to the 124-kDa fragment(s)) through noncovalent forces.

The \(K_m\) of the fragment of approximately 124 kDa for Ca\textsuperscript{2+} in the absence of CaM was determined exactly as for the ATPase, as described in Ref. 22 (see legend for Fig. 1) (Fig. 6). The Ca\textsuperscript{2+} concentrations in the buffer used for the experiments were calculated according to Ref. 38. The figure shows that the fragment of about 124 kDa purified on the CaM column had a lower \(K_m\) for Ca\textsuperscript{2+} than the 138-kDa intact ATPase in the absence of CaM and was still stimulated by the latter. Other experiments have shown that in the presence of CaM the \(V_{max}\) of the intact ATPase and of the fragment of 124 kDa isolated from the CaM column were nearly identical.

**DISCUSSION**

The work presented here provides compelling evidence that the erythrocyte Ca\textsuperscript{2+}-ATPase is a specific substrate for calpain (experiments not shown under "Results" have shown that the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is insensitive to calpain). The observation that a fragment of about 124 kDa was routinely present in standard Ca\textsuperscript{2+}-ATPase preparations suggests that calpain indeed attacks the ATPase in intact erythrocytes or soon after their hemolysis. This was also suggested by the substantial decrease of the amount of the fragment of about 124 kDa in preparations in which the membranes and the detergent solubilize were treated with leupeptin (not shown).

The principal effect of the controlled calpain proteolysis of the Ca\textsuperscript{2+}-ATPase in purified preparations and in the intact membranes was an evident increase of the basal activity. The \(V_{max}\) of the intact enzyme and of its 124-kDa fragment were nearly identical, but the \(K_m(Ca^{2+})\) of the former in the absence of CaM (6-15 \(\mu\)M) was much higher: the \(K_m(Ca^{2+})\) of the fragment of about 124 kDa isolated from CaM columns was about 0.5 \(\mu\)M. In the presence of CaM, it declined to about 0.3 \(\mu\)M, whereas that of the intact enzyme declined to about 0.5 \(\mu\)M.

Calpain is a cysteine protease which contains calmodulin-like domains (see the Introduction section), and unlike other unspecific proteases, e.g. those of the lysosomes, it shows substrate specificity and is found free in the cytoplasm. Unlike calpain, calpain often attacks protein substrates yielding few discrete fragments as cleavage products. Despite this the enzyme does not show as striking an amino acid sequence preference as that of trypsin or thrombin. The general requirements are generally assumed to be satisfied by the sequence Leu-X-Arg-Y, the cut occurring between the residues X and Arg (39). However, the requirement is not absolute, even if a strong preference is shown for Leu or Val as the penultimate residue, N-terminal to the cut. The residue C-terminal to the cut can be Arg or Lys, although many other residues can substitute at this position. Whereas the sequence does not appear to be critically important, a conformational specificity also seems to be very important. Although the site of attack by calpain has already been documented for a number of small peptides (for a review see Ref. 39), it is appropriate to point out that information on the site of attack in the primary structure of large enzyme proteins is virtually absent (as this work was being handled by the reviewers an article describing the site of attack by calpain on fodrin appeared, Ref. 40).

Previous work has shown (41) that the purified ATPase may be activated in the absence of calmodulin, i.e. its \(K_m(Ca^{2+})\) decreased to the levels reached with saturating CaM concentrations, by acidic phospholipids. Separate experiments not presented here have shown that the \(K_m(Ca^{2+})\) of the fragment of about 124 kDa isolated from CaM columns was nearly identical in the same way by acidic phospholipids. Although the site and mechanism of interation of acidic phospholipids with the ATPase are not known, they are evidently preserved in the 124-kDa fragment.

The role of calpain in the messenger function of Ca\textsuperscript{2+} may be of general importance. It has been shown that limited proteolysis of many CaM-dependent enzymes such as myosin light chain kinase (42-44), calcineurin (44), the Ca\textsuperscript{2+}-dependent cyclic nucleotide phosphodiesterase (45), and the Ca\textsuperscript{2+}-ATPase of plasma membranes from various sources (33, 46) by various proteases leads to activation and to the loss of CaM dependence. On the other hand, it has been demonstrated that calpain degrades in a controlled way various proteins "in vivo" as well as in cell-free systems (39, 47-50).

The cut(s) produced by calpain on the erythrocyte Ca\textsuperscript{2+}-ATPase occurs close to the C-terminal portion of the molecule, where the major CaM-binding domain of the Ca\textsuperscript{2+}-ATPase is located (19), and it has been shown above that calpain splits the ATPase in the middle of this domain. Given the characteristic domain structure of calpain (1), it is tempting to speculate that the highly specific attack of calpain at the two distinct sites of the CaM-binding domain is brought about by the targeting of the CaM-like domains of calpain (IV and/or IV') to the CaM-binding region of the Ca\textsuperscript{2+}-ATPase. This would confer to erythrocyte calpain relative specificity toward the Ca\textsuperscript{2+}-ATPase and would provide a potentially selective mechanism of regulation of the Ca\textsuperscript{2+}-ATPase activity in the erythrocyte.

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