Further Characterization of Calpain-Mediated Proteolysis of the Human Erythrocyte Plasma Membrane Ca\textsuperscript{2+}-ATPase\textsuperscript{1}

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The membrane-bound form and a solubilized and purified form of the Ca\textsuperscript{2+}-ATPase from human erythrocyte have been proteolysed under controlled conditions by highly purified Ca\textsuperscript{2+}-dependent neutral cysteine-protease, calpain 1, in the absence and in the presence of the calmodulin-calciuim complex. In the absence of calmodulin the 136-kDa enzyme was transformed into a group of fragments of 125–124 kDa, followed by the slower formation of a second group of fragments of 82–80 kDa. These heterogeneous fragments were capable of forming an acylphosphate intermediate. The 125– and 82-kDa minor components of each heterogeneous group of fragments (125–124 and 82–80 kDa) were capable of binding calmodulin, whereas the 124- and the 80-kDa major components did not. In the presence of calmodulin, however, the native enzyme was transformed into a 127-kDa fragment followed by the slower formation of an 85-kDa fragment. Both fragments (127 and 85 kDa) formed an acylphosphate intermediate and were capable of binding calmodulin. The presence of calmodulin during calpain action effectively protected the Ca\textsuperscript{2+}-ATPase from proteolytic activation (K. K. W. Wang, A. Villalobo, and B. D. Rougogalis 1988) Arch. Biochem. Biophys. 266, 696–704) and prevented the formation of the calmodulin-insensitive 124- and 80-kDa fragments. Smaller fragments not capable of forming the acylphosphate intermediate were also produced, in particular a 39–37 kDa doublet band retaining the capacity to bind calmodulin. In contrast to the membrane-bound form, the purified form of the Ca\textsuperscript{2+}-ATPase was proteolysed by calpain at a slower rate.\textsuperscript{2}

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\textsuperscript{2}Calpain-mediated activation of the Ca\textsuperscript{2+}-ATPase could occur in vivo in response to an increase in the intracellular Ca\textsuperscript{2+} concentration (for review on calpain, see (4–6)).

In our earlier studies we found that calpain-mediated activation of the membrane-bound Ca\textsuperscript{2+}-ATPase was accompanied by the loss of its sensitivity to calmodulin (2). In addition, we observed that calmodulin protected the Ca\textsuperscript{2+}-ATPase against calpain-mediated activation (2). As (3) has also reported that calpain from erythrocyte activated the membrane-bound Ca\textsuperscript{2+}-ATPase but inactivated the solubilized enzyme. In the present study,
we further investigate the molecular mechanism of calpain-mediated activation of the Ca\(^{2+}\)-ATPase and the protective effect of calmodulin by utilizing the membrane-bound form and a solubilized and purified form of the Ca\(^{2+}\)-ATPase. We describe the effect of calpain on the membrane- 
ATPase activity, the fragmentation pattern as visualized by the formation of the F\(^{39}\)phosphoenzyme intermediate, the peptide pattern of the fragmented purified enzyme, and the identification of the fragments conserving the capacity to bind cal-
modulin.

MATERIALS AND METHODS

Materials. Leupeptin, trypsin (bovine pancreas, type III), trypsin inhibitor soybean, trypsin IV), PMSE. TLCK, ATP (disodium salt), enal, Triton X-100, phosphotoluenesulphonic acid, N-ethylmaleimide-activator-
agarose (calmodulin-agarose), and \(\alpha\)-toyolamine-
agarose were products from Sigma Chemical Co. (DEAE-Phosphatase, phenyl-Sepharose, and Sephyseryl G-300 were from Pharmacia, ultrafiltration mem-
branes (PM-30) were from Amicon, and dichotrophic resin was from Bioresources-Mans February, calmodulin (bovine brain) was obtained from Calbiochem, anion-exchange from MCB Manufacturing Chemicals, Inc. and X-O-MAT AR X-ray blue sensitive film from Kodak. All other reagents used (in this work were of analytical grade.

Preparation of calmodulin-depleted cytoplasmic membranes. Calmodulin-depleted human erythrocyte membranes were prepared as described in a previous report (1) except that the hemoglobin buffer contained 10 mM TLCK, 100 mM PMSE, and 1 mM t-butane trypsin inhibitor, and the washing buffers (II and III) contained 50 mM PMSE. The final coupled mem-
brane suspensions were resuspended in 180 mM KCl, 2 mM dithio-
treitol, 1 mM EDTA, 50 mM PMSE, and 2 mM potas-
sium-HEPES (pH 7.4) buffers (IV-VII).

Solubilization and purification of the Ca\(^{2+}\)-ATPase. The solubilization and purification of the Ca\(^{2+}\)-ATPase from calmodulin-depleted membranes were carried out as described in (7) with the following mod-
ifications: concentrated membranes were solubilized in a Triton X-100/glycerol ratio of 1:1 (w/w) or occas-
onally 1:5 (w/w) in the presence of 500 \(\mu\)g PMSE and 100 \(\mu\)g leupeptin. 

Isolation and purification of calpain I. Calpain I was prepared from membrane-free hemolysate of human erythrocyte as previously described (3) with the fol-
lowing modifications: the pH of the hemolysate was adjusted to 6.4, the hemolysate buffer contained 5 mM 
EDTA, and the DEAE-Phosphatase column elution gradi-
te was from 0 to 500 mM NaCl. An additional final 
step using a gel filtration column (Shandon Sephacryl 320, 1.5 × 60 cm) was also included. These preparations of calpain (5–20 mg/ml protein) contain both the large and small subunits of calpain (50 and 29 kDa), which constitute more than 90–95% of the total protein us-
ing electrophoretic criteria, and did not have Ca\(^{2+}\)-in-
dependent proteolytic activity.

Determination of the phosphoenzyme-bound Ca\(^{2+}\)- 
ATPase activity. Calmodulin-depleted membranes (50–100 \(\mu\)g protein/ml) were incubated at 37°C for 
30 min in a total volume of 0.4 ml containing 55 mM 
Tris-maleate, 66 mM KCl, 43 mM ouabain, 85 mM 
MgCl\(_2\), 120 mM calmodulin (when added), 2 mM 
ATP, 0.1 mM EDTA, and varying concentrations of 
CaCl\(_2\) to obtain the free Ca\(^{2+}\) concentration indicated in the legend to the figure (pH 7.2). The iMM\(^{2+}\)-ATPase ac-
tivity (assayed in the absence of added CaCl\(_2\) and in 
the presence of 2.5 mM EDTA) was subtracted from the total activity assayed in the presence of calcium.

Determination of the activity of the purified Ca\(^{2+}\)- 
ATPase. Purified Ca\(^{2+}\)-ATPase (1 to 2 \(\mu\)g protein) 
was assayed as described in (7) for 30 min in a total 
volume containing 55 mM Tris-maleate, 66 mM KCl, 
43 mM ouabain, 85 mM MgCl\(_2\), 120 mM calmodulin 
(when added), 2 mM ATP, 2 mM dithiothreitol, 0.1% 
(w/v) Triton X-100, 0.1% (w/v) nondenatured 
soybean, 100 mM EDTA, and varying concentrations of 
CaCl\(_2\) to obtain the free Ca\(^{2+}\) concentration indicated in the legend to the figure (pH 7.2).

Determination of the phosphoenzyme-activated 
Ca\(^{2+}\)-ATPase. Plasma membranes (50–100 \(\mu\)g protein) were incubated at 4°C for 15 or 30 min 
in 50 mM KCl, 5 mM potassium-Heps, 40 mM Tris-male-
ate, 0.1 mM ouabain, 0.1 mM EDTA, 0.2 mM MgCl\(_2\), 
0.25 mM CaCl\(_2\), 100 mM LaCl\(_3\), 200 mM calmodulin, and 2 \(\mu\)M ATP containing 6 mM \(\alpha\)-32-
phosphate. After the partic-
ulate Ca\(^{2+}\)-ATPase (5–20 \(\mu\)g protein) was incubated at 4°C for 15 or 30 min of 50 mM KCl, 40 mM Tris-
maleate, 0.1 mM EDTA, 0.1 mM EDTA, 50 mM MgCl\(_2\), 2.5 
Mm CaCl\(_2\), 100 mM LaCl\(_3\), 300 mM calmodulin, 0.025 
(w/v) Triton X-100, 0.1% (w/v) nondenatured 
soybean, and 4 \(\mu\)M ATP containing 6 mM \(^{32}\)P-cyclic 
AMP. The reaction was initiated by the addition of 
\(^{32}\)P-cyclic AMP and terminated by the addition of ice-cold
10% (w/v) trichloroacetic acid. The precipitated pro-
teins was removed for acid gel electrophoresis at pH 6.8 as described below.

Polyacrylamide gel electrophoresis and autoradi-
graphy. SDS-polyacrylamide gradient (5-20%, w/v) slab gel electrophoresis under alkaline condition (pH 9.0) was performed essentially following the protocol of Laszlo and Ferré (9). For determination of the phosphorylated intermediate, no gel electrophore-
sis was performed as described. SDS-polyacrylamide gradient gels were prepared as above except that the pH of the separating and stacking gels were 7.0 and 6.8, respectively. The gel was then run overnight (16-
18 h) at 30 mA in 30 mM Mops and 0.1% (w/v) SDS (pH 6.8) at 4°C. The gels stained with Commassie brill-
ant blue R250 were dried on Whatman No. 1 paper and the X-ray film was exposed to the gel for 34-72 h at -70°C.

Treatment of calf thymus-depleted membranes with calpain. Calmodulin-depleted plasma membranes (1-
2 mg protein) were treated with 0.42 unit ml-1 cal-
pain (0.5 μg protein) at 35°C in an appropriate volume of 40 mM KC1, 30 mM potassium-Hepes, 6.5 mM MgCl2, 50 mM Tris-maleate, 10 mM EDTA, 300 mM calmodulin (when added), and a combi-
nation of EGTA and CaCl2 to attain a free Ca2+ con-
centration of 200 μM (pH 7.4). At the desired times, aliquots were taken (160-190 μl) and transferred to 1 ml of buffer I (pH 7.4) containing 200 mM sucrose. Subse-
sequently, the treated membranes were washed four times with the same buffer. Finally, the 1.000 g sup-
ernatants were resuspended in buffer I and to the origi-
nal volume before assaying for ATPase activity at the

Humphries intermediate.

Treatment of the purified Ca2+-ATPase with calf-

polyacrylamide gel (60-80 μg protein) was incu-
bated with 0.82 unit ml-1 calpain (8.0 μg protein) at 35°C in an appropriate volume of 50 mM Tris-maleate, 3 mM potassium-Hepes, 0.5 mM MgCl2, 0.5 mM EDTA, 10 mM dithiothreitol, 300 mM calmodulin (when added), 30 mM KC1, and a combination of EGTA and CaCl2 to obtain a free Ca2+ concentration of 300 μM (pH 7.4). At the desired times, aliquots were taken for ATPase assay and determination of the phosphorylated intermediate in the presence of 200 mM sucrose and subjected to gel electrophoresis. Analytical procedure Inorganic phosphate was de-
terminated colorimetrically (9). Protein concentra-
tions were determined by the method of Lowry et al. (10). Free Ca2+ concentrations were determined by the computer program described by Goldin et al. (11), modified as previously described (7), and calpain was assayed as previously described (2).

RESULTS

Calpain Digestion of the Membrane-Bound Ca2+-ATPase

A time course of the effect of calpain on

FIG. 1. Activity of the membrane-bound Ca2+-
ATPase treated with calpain in the presence and ab-
sence of calmodulin. Membranes (1.2 mg protein) were treated with calpain in the absence (dotted) or in the presence (squares) of calmodulin as described under Materials and Methods. Aliquots (30 μg mem-
brane protein) were taken at the indicated time and the membranes were washed and thereafter assayed for Ca2+-ATPase activity with 4.3 μM free Ca2+ in the absence (open symbols) or in the presence (filled sym-
ols) of calmodulin (see Materials and Methods). Re-
sults presented here are representative of three sepa-
rate experiments.

the membrane-bound Ca2+-ATPase activ-
ity is presented in Fig. 1. In the absence of calmodulin the protase progressively ac-
ivated the Ca2+-ATPase and decreased its capacity to be stimulated by calmodulin. However, in the presence of calmodulin the proteolytic activation of the Ca2+-ATPase was significantly reduced and the calmod-
ilin stimulation was preserved.

Binding of calmodulin to the membrane-bound Ca2+-ATPase could present its pro-
etolysis or induce a different pathway of fragmentations. We confirmed that the fragment formation of the membrane-bound ATPase was indeed different in the absence versus that in the presence of cal-
modulin as demonstrated by following the

32P-phosphorylated intermediate of the enzyme (see Fig. 2).

In the absence of calmodulin, the native enzyme (135 kDa) was rapidly trans-
formed into a 124-kDa fragment within 1-2
min. This is followed thereafter by the progressive formation of a second frag-
ment of lower molecular mass (90 kDa). In
the presence of calmodulin, however, the relative molecular masses of the $^{32}$P-labeled fragments were 125 and 80 kDa, respectively. The phosphorylation of the bands was dependent on the presence of Ca$^{2+}$ and they were sensitive to hydroxy-
amine treatment (results not shown), demon-
strating that they indeed represented fragments of the Ca$^{2+}$-ATPase capable of forming the acylphosphate intermediate.

**Calpain Digestion of the Purified Ca$^{2+}$-ATPase**

The purified Ca$^{2+}$-ATPase was also sub-
ject to calpain digestion. Figure 3A shows a time course of calpain digestion on the activity of the purified Ca$^{2+}$-ATPase. Calpain proteolysis in the absence of calmodulin produces a slight activation of the Ca$^{2+}$-ATPase in the first 15 min while the activity in the presence of calmodulin re-
mained approximately the same during this time period. The basal and the calmo-
dulin-stimulated activities declined pro-
gressively thereafter. Calpain digestion, in the presence of calmodulin also produced a progressive decrease of the Ca$^{2+}$-ATPase activity (Fig. 3A).

We then looked at the effect of different calpain activity on the Ca$^{2+}$-ATPase after a prolonged period of incubation (2 h). Fig-
FIG. 4. Fragmentation of the purified Ca⁺⁺-ATPase by calpain and formation of the phosphorylated intermediate. (A) Purified calpain (50 μg protein) (Unit 1) was incubated at 37°C for 30 min in 50 mM Tris-HEPES (pH 7.4), 10 mM dithiothreitol, 0.5 mM EDTA, and 0.5 mM CaCl₂ (lane 2). The calpain reaction was stopped with 20% (v/v) ice-cold trichloroacetic acid and the precipitated protein was processed for gel electrophoresis at pH 8.6. (B) Purified Ca⁺⁺-ATPase (47 μg protein) was treated with calpain (5 μg protein) in the absence (--) or in the presence (+) of calmodulin (CaM) in cerebral cortex Materials and Methods. At the indicated time, aliquots were taken and the protein was precipitated with 10% (v/v) ice-cold trichloroacetic acid. Protein was then subjected to electrophoresis (pH 6.3). Calpain fragments are indicated by △ and the band of calmodulin is indicated by ▽. (C) Purified Ca⁺⁺-ATPase (50 μg protein) was either untreated (--) or incubated with calpain (50 μg protein) for 60 min (•) in the absence (--) or in the presence (+) of calmodulin as indicated under Materials and Methods. Proteolysis was stopped with 20% (v/v) trichloroacetic acid. The samples were then subjected to phosphorylation with [γ-³²P]ATP (see Materials and Methods). The reaction was stopped with 10% (v/v) ice-cold trichloroacetic acid and the precipitated protein was subjected to gel electrophoresis under acidic conditions (pH 6.3) and autoradiography. Results presented here are representative of three separate experiments.

ure 3B shows that in the absence of calmodulin, increasing concentrations of calpain activate the basic Ca⁺⁺-ATPase activity (assayed in the absence of calmodulin). Furthermore, increasing calpain activity produced a gradual decline of the calmodulin-stimulated Ca⁺⁺-ATPase activity when the purified enzyme was treated in either the absence or the presence of calmodulin. We also studied the fragmentation pattern of the purified Ca⁺⁺-ATPase produced by calpain (Fig. 4B). In the absence of calmodulin, the purified Ca⁺⁺-ATPase band (135 kDa) was quickly transformed into a diffuse 125–124 kDa band. This was followed by the formation of another diffuse band of 92–90 kDa, and smaller fragments of 55, 39, 37, and 32 kDa. In contrast, in the presence of calmodulin the most prominent fragments have relative molecular masses of 127 and 85 kDa, and smaller fragments of 55, 39, 37, and 32 kDa. Figure 4A shows the fragments produced by calpain autoxidation (36, 46, 36, 27, and 18 kDa) as a control. Figure 4C shows the fragments of the purified Ca⁺⁺-ATPase capable of forming [γ-³²P]-labeled phosphorylated intermediate (125–124 and 92–90 kDa in the absence of calmodulin, and 127 and 85 kDa in the presence of calmodulin), Therefore, the [γ-³²P]-labeled acyclic phosphate-forming fragments of the purified Ca⁺⁺-ATPase were similar to those of the membrane-bound enzyme. It was of interest to establish the effect of calmodulin on the ATP hydrolytic activity of the fragmental form of the Ca⁺⁺-ATPase. Therefore, we treated the purified Ca⁺⁺-ATPase with calpain in the absence
of calmodulin and passed the resulting heterogeneous fragments (Fig. 5B, lane 1) through a calmodulin-agarose column in the presence of calcium. Figure SA presents the elution profile of that preparation.

A first peak of ATPase activity (not stimulated by calmodulin) was obtained in the flowthrough fractions and electrophoretic analysis of that peak demonstrated that it contained 125- to 124-kDa protein bands (Fig. 5B, lane 2). A second ATPase peak (stimulated by calmodulin) was obtained after elution of the calmodulin-agarose column with EGTA (Fig. 5A). Electrophoretic analysis of this second peak demonstrated that it contains a prominent 125-kDa and a fainter 82-kDa protein band (Fig. 5B, lane 3). Therefore, the heterogeneous 125-124 kDa and 82-80 kDa bands previously identified could be further resolved into calmodulin-dependent fragments and calmodulin-independent fragments.

**Purification of the Calmodulin-Binding Fragments of the Ca\(^{2+}\)-ATPase**

In these experiments, two batches of calmodulin-depleted membranes were treated with calpain, one in the absence and one in the presence of saturating concentration (5 μg mg prot\(^{-1}\)) of exogenously added calmodulin. After treatment, both batches of membranes were washed with an EDTA-containing buffer to remove calmodulin and/or calpain and solubilized with Triton X-100, and the Ca\(^{2+}\)-ATPase fragments were purified with two individ-
FIG. 6. Purification of the calmodulin-binding frag-
mants of the Ca\textsuperscript{2+}-ATPase. Calmodulin-depleted
membranes were incubated during 60 min with 0.05
unit ml\textsuperscript{-1} calpain in the absence or presence of cal-
modulin as described under Materials and Methods.
Membranes without treatments were used as controls.
(A) Autoradiography showing [\textsuperscript{32}P]ADP/ATP
formation of the membrane-bound Ca\textsuperscript{2+}-ATPase (18
\mu g protein) either untreated (lane 1), or treated
with calpain in the absence (lane 2) or in the presence
of calmodulin (lane 3). (B) Coomassie blue staining of
the native or fragmented Ca\textsuperscript{2+}-ATPase purified from
1.5 mg protein membranes mentioned in A using a
calmodulin-agarose column. Lanes 1, 2, and 3 corre-
spond to the purified Ca\textsuperscript{2+}-ATPase from the mem-
brane preparations shown in lanes 1, 2, and 3 of A,
respectively. (C) Autoradiogram showing [\textsuperscript{32}P]
phosphate formation by the purified native or frag-
mented Ca\textsuperscript{2+}-ATPase illustrated in B. Lanes 1, 2,
and 3 correspond to the purified ATPase preparation
shown in lanes 1, 2, and 3 of B, respectively. Results
presented here are representative of two separate ex-
periments.

Citic calmodulin-agarose columns. As a con-
trol, a batch of untreated membranes was used and
the native Ca\textsuperscript{2+}-ATPase was puri-
ified as above. Figure 6 A illustrates the formation of the \textsuperscript{32}P-labeled phosphorilated intermedi-
ate of the native membrane-bound Ca\textsuperscript{2+}-
ATPase (138 kDa) (lane 1), the 161- and
80-kDa, membrane-bound fragments pro-
duced by calpain in the absence of calmod-
ulin (lane 2), and the 127- and 85-kDa
membrane-bound fragments produced by
calpain in the presence of calmodulin (lane
3). Figure 6 B shows the protein pattern of
the purified native enzyme (138 kDa) plus
a smaller contaminant of 100 kDa (lane 1),
and the fragments purified with the cal-
modulin-agarose column from membranes
acted with calpain in the absence (lane 2)
or in the presence (lane 3) of calmodulin.
The major fragments purified from the calmodulin-depleted, calpain-treated
membranes were the 125-kDa fragment, a small amount of the 82-kDa fragment, and the
39-37 kDa doublet. No fragment of 80-
kDa was observed. However, both the 127-
and 85-kDa fragments were purified from
the calmodulin-saturated, calpain-treated
membranes. In addition, larger amounts of
the 39-37 kDa doublet were also observed.

When the above-mentioned purified native
and fragmented enzymes were incubated
with Ca\textsuperscript{2+}-ATPase, only the intact en-
zyme (138 kDa) (Fig. 6C, lane 1) and
the 127-, 125-, 85-, and 82-kDa fragments (Fig.
6C, lanes 2 and 3) form the \textsuperscript{32}P-phosphory-
lated intermediate. We therefore recon-
firm that the 80-kDa fragment formed in the
absence of calmodulin (Fig. 6A, lane 2) was
not retained by the calmodulin-agarose
column.

Table I presents the recovery of the total
Ca\textsuperscript{2+}-ATPase activity in the experiment
shown in Fig. 6. The untreated membranes
show a 5.8-fold stimulation of the Ca\textsuperscript{2+}-
ATPase activity induced by calmodulin,
and the Ca\textsuperscript{2+}-ATPase purified from these
membranes also show a 3.1-fold stimula-
tion. However, the calpain-treated mem-
branes (in the absence of calmodulin) had
increased Ca\textsuperscript{2+}-ATPase activity when
assayed in the absence of calmodulin and
the stimulation induced by calmodulin was
reduced to 1.5-fold. However, the Ca\textsuperscript{2+}-
ATPase fragments purified from these
membranes (most prominently the 125-
kDa fragments) still show a 25-fold stimu-
lation of the ATPase activity induced by
calmodulin compared to a 5.1-fold stimula-
tion of the purified intact Ca\textsuperscript{2+}-
ATPase. On the other hand, membranes treated with
calpain in the presence of calmodulin show
only a slight increase in the basal Ca\textsuperscript{2+}-
ATPase activity (assayed in the absence of
calmodulin) and a 4.4-fold stimulation
induced by calmodulin. The Ca\textsuperscript{2+}-ATPase
fragments (127 kDa plus 85 kDa) purified
**TABLE I**

<table>
<thead>
<tr>
<th>Membrane bound activity (μmol min⁻¹mg⁻¹)</th>
<th>Purified</th>
<th>Fold stimulation by calmodulin</th>
<th>Recovery of purified Ca⁺⁺-ATPase activity from the membranes (%)</th>
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</thead>
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<tr>
<td>Addition</td>
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<td>CaM + CaM</td>
<td>CaM + CaM</td>
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<td></td>
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</tr>
<tr>
<td>None</td>
<td>2.1</td>
<td>12.2</td>
<td>2.6</td>
</tr>
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<td>Calpain</td>
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<td>19.6</td>
<td>0.4</td>
</tr>
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<td>Calpain-phospho-calmodulin</td>
<td>2.6</td>
<td>11.4</td>
<td>3.1</td>
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</tbody>
</table>

*Note: Membranes (150 mg protein) were treated with calpain (150 units ml⁻¹) in either the absence or presence of 20 μM calmodulin as indicated. Unstimulated membranes were used as control. The native or fragmented Ca⁺⁺-ATPase was then solubilized and purified using three separate calmodulin-agarose columns. The total activity of both the membrane-bound and the purified Ca⁺⁺-ATPase activities were determined using aliquots assigned at 4.5 and 2 μM free Ca⁺⁺, respectively, in the absence or in the presence of calmodulin (CaM) as indicated. The fold stimulation by calmodulin was calculated as the ratio of the Ca⁺⁺-ATPase activity in the presence of calmodulin versus the Ca⁺⁺-ATPase activity in the absence of calmodulin. Percentage recovery of Ca⁺⁺-ATPase activity from the membranes was calculated using the Ca⁺⁺-ATPase activities assayed in the presence of calmodulin. Results presented here are representative of two separate experiments.*

from these membranes show a 2.5-fold stimulation of activity by calmodulin. When we looked at the recovery of the total Ca⁺⁺-ATPase activity, also shown in Table 1, it became clear that in the case of the membranes treated with calpain in the absence of calmodulin only a small portion of the fragmented Ca⁺⁺-ATPase was retained by the calmodulin-agarose column, 10% compared to 50 and 60% in the case of membranes treated with calpain in the presence of calmodulin or without calpain treatment, respectively.

**Trypsin Fragmentation of the Ca⁺⁺-ATPase**

We also compared the fragmentation patterns of the Ca⁺⁺-ATPase produced by calpain and trypsin.

Calpain treatment of the purified Ca⁺⁺-ATPase in the absence of calmodulin produced the characteristic 124-, 89-, 55-, and 39-37 kDa fragments (Fig. 7B, lane 1). On the other hand, short time exposure to trypsin (3 min) in the absence of calmodulin produced a 124-kDa fragment and smaller fragments of 86, 82, 31, and 32 kDa (Fig. 7B, lane 2). Longer exposure to trypsin (30 min) resulted in the accumulation of the 82-, 77-, and 32-kDa fragments (Fig. 7B, lane 3).

Calpain treatment in the presence of calmodulin led to the formation of 124-, 88-, 55-, and 39-37 kDa fragments (Fig. 7C, lane 1), whereas short time exposure to trypsin in the presence of calmodulin produced a 127-kDa fragment and smaller fragments of 86, 82, 34, and 32 kDa (Fig. 7C, lane 2). Longer exposure to trypsin in the presence of calmodulin led to the accumulation of the 82- and 32-kDa fragments (Fig. 7C, lane 3). Figure 7A shows the purified native Ca⁺⁺-ATPase as a control.

**DISCUSSION**

We have recently reported that the plasma membrane-bound Ca⁺⁺-ATPase could be irreversibly activated by calpain, and its capacity to be stimulated by calmodulin was subsequently lost (2). In addition, calmodulin protected the Ca⁺⁺-ATPase against these processes (2). In the
**Fig. 7.** Comparison of the fragmentation pattern of the purified Ca⁺⁺-ATPase by calpain and trypsin. (A) Untreated purified Ca⁺⁺-ATPase (60 μg protein). Purified Ca⁺⁺-ATPase (60 μg protein) was treated at 30°C with calpain (0.25 unit ml⁻¹) for 30 min (lane 1) or with trypsin (0.4 μg ml⁻¹) for 3 min (lane 2) or 30 min (lane 3) in the absence (B) or the presence (C) of 300 μM calmodulin (CaM) in a medium containing 50 mM Tris-maleate, 5 mM potassium-HEPES, 6.5 mM MgCl₂, 0.5 mM EDTA, 10 mM dithiothreitol, 45 mM KCl, and 200 μM free Ca⁺⁺ (pH 7.4). The proteolysis was stopped with 10% (v/v) ice-cold trichloroacetic acid. The precipitated protein was subjected to gel electrophoresis (pH 8.5). Calpain fragments are indicated by (Ⅰ) and trypsin is indicated by (Ⅱ). Results presented here are representative of three separate experiments.

In the present study, we further investigated the action of calpain on the Ca⁺⁺-ATPase molecule. A scheme of the fragmentation sequence is suggested in Fig. 8. In the absence of calmodulin, the Ca⁺⁺-ATPase was sequentially proteolyzed to a 125–124 kDa heterogeneous fragment and thereafter to a 82–89 kDa heterogeneous fragment both capable of forming the acylphosphate intermediate (Fig. 1B). However, only the major 125– and 82–89 kDa components lost that capacity (Fig. 5).

When we studied the fragmentation pattern of the membrane-bound Ca⁺⁺-ATPase we had to rely on the formation of the [³²P]acylphosphate intermediate to visualize the different fragments. With this technique it becomes difficult to identify whether the 124- and 80-kDa fragments also contain the minor 125- and 82-kDa components (Figs. 2 and 6A). However, the Ca⁺⁺-ATPase fragments purified from membranes pretreated with calpain in the absence of calmodulin have reactive molecular masses 125 and 82 kDa (Figs. 6B and 6C); therefore, it appears that the 124- and 80-kDa fragments are the calmodulin-sensitive, proteolytically activated form of the Ca⁺⁺-ATPase.

On the other hand, in the presence of calmodulin, the enzyme was proteolyzed to different higher molecular mass fragments of 127 and 85 kDa, respectively (Figs. 2 and 4B). Both of these fragments were capable of forming a [³²P]-phosphorylated acylphosphate intermediate and were retained by the calmodulin-Sepharose column in the presence of calcium (Figs. 4C and 6). This is consistent with the fact that calmodulin protects the proteolytic activation and the
loss of calmodulin stimulation of the enzyme.

Calpain also produces some other fragments of the purified Ca\(^{2+}\)-ATPase with relative molecular masses of 65, 20, 37, and 32 kDa (Fig. 4B). In contrast to the larger fragments discussed above, none of these smaller fragments form a phosphorylated intermediate (Fig. 4C). Therefore it seems that the 80-kDa fragment is the smallest form of the Ca\(^{2+}\)-ATPase still capable of forming the acyl phosphosphate intermediate after proteolysis by calpain. This is similar to the smallest 2P-labeled fragments (81 and 26 kDa) obtained by trypticinactivation of both the membrane-bound (12, 13) and the purified Ca\(^{2+}\)-ATPase (14). Interestingly, the Ca\(^{2+}\)-ATPase activity of both of these tryptic fragments were reduced to be insensitive to stimulation by calmodulin (12, 15).

In light of these results, we also studied the comparative fragmentation patterns obtained by calpain and trypsin (Fig. 3). We could observe transient formation of 154- and 157-kDa fragments produced by trypsin in the absence and presence of calmodulin, respectively, as observed in the case of calpain. This suggests that both proteases recognize similar or identical sites of cleavage in the Ca\(^{2+}\)-ATPase molecule. Our earlier studies demonstrated that calmodulin failed to prevent proteolytic activation of the Ca\(^{2+}\)-ATPase by trypsin in contrast to the case of calpain (2). In agreement with these results Fig. 7 shows that even in the presence of calmodulin, trypsin produced large amounts of an 82-kDa fragment, previously reported to be of high activity and insensitive to calmodulin (11-13). The small difference in relative molecular mass of the tryptic fragments reported by us and others (12-13) is probably due to the different gel system used.

In the case of calpain-mediated fragmentation, the fact that the 82-kDa fragment was still able to bind calmodulin whereas the 80-kDa fragment did not bind calmodulin suggests that a 2-kDa region is critical for this function. Since the 80-kDa fragment was not formed to any significant extent in the presence of calmodulin, it appears that calmodulin protects this 2-kDa region of the enzyme from the proteolytic action of calpain. Sarkadi et al. (12) have suggested that the calmodulin-binding domain of the Ca\(^{2+}\)-ATPase encompassed a region about 10 kDa in length and resided at the C-terminal end of the enzyme, based on their limited proteolysis studies of the membrane-bound enzyme. Zurini et al. (14) also identified a calmodulin-binding region of about 8-10 kDa.

It is of interest to point out that the 89-95 kDa doublet fragments were also purified by the calmodulin-agarose column (Fig. 56, lanes 2 and 3). Therefore, it appears that they are obtained from further fragmentation of the calmodulin-binding 85- and/or 82 kDa fragments.

As (0) has recently claimed that the calpain-mediated proteolytic activation of the membrane-bound Ca\(^{2+}\)-ATPase was best seen after the membranes were first treated with the detergent saponin or glycodeoxycholate. This could have been because calmodulin was not exclusively removed from the membranes used in that work and therefore the remaining calmodulin could considerably protect the Ca\(^{2+}\)-ATPase against proteolytic activation. The subsequent treatment of the membranes with the detergent caused the further removal of the remaining calmodulin and enhanced the susceptibility of the Ca\(^{2+}\)-ATPase to calpain.

The present study not only documents the molecular basis of the calpain-mediated proteolysis of the plasma membrane Ca\(^{2+}\)-ATPase from erythrocite, but also illustrates how calpain can be used as a tool to understand the molecular organisation of the Ca\(^{2+}\)-ATPase molecule.

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