Identification and Primary Structure of a Calmodulin Binding Domain of the Ca$^{2+}$ Pump of Human Erythrocytes

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Exposure of the purified Ca$^{2+}$ pump of human erythrocytes to chymotrypsin led to the rapid loss of calmodulin activation. A fragment of about 12 kDa was removed from the ATPase in 1–2 min. Blotting experiments with $^{125}$I-labeled calmodulin showed that this fragment contains the calmodulin binding region. The remainder of the ATPase molecule was degraded to a number of fragments ranging from 3 to 120 kDa; none of them bound calmodulin.

To isolate the calmodulin binding domain, calmodulin which had been coupled to the Denny-Jaffe reagent (a cleavable radioactive photoaffinity cross-linker) was allowed to bind to the Ca$^{2+}$ pump. After illumination the couple cross-linker to the pump, the cleavable bond was split and the calmodulin removed, leaving the pump radioactively labeled. This pump was digested with chymotrypsin, and the products were separated by gel permeation chromatography. The only radioactive peak (migrating at about 12 kDa) was further purified on reverse-phase high pressure liquid chromatography (HPLC). Amino acid analysis showed the fragment to have a minimal molecular mass of 12.4 kDa and to contain a single methionine. After attempts to sequence the peptide directly failed, CNBr digestion was carried out on the labeled ATPase, producing both soluble and insoluble labeled material. After reverse-phase HPLC purification of the soluble material, a single radioactive peak was collected. Its sequence was

1 2 3 4 5 6 7 8 9 10
NH$_2$-Glu-Leu-Arg-Ary-Gly-Gln-Ile-Leu-Trp-Phe-
11 12 13 14 15 16 17 18 19 20 21 22
Arg-Gly-Leu-Asn-Arg-Ile-Glu-Thr-Gln-Ile-Lys-Val-
23 24 25 26 27 28 29 30 31 32 33
Val-Asn-Ala-Phe-Ser-Ser-Ser-Leu-His-Glu-Phe...

A portion of this peak was passed through a micro-calmodulin column; it bound in the presence of Ca$^{2+}$ and was eluted by EDTA, and by a mixture of EDTA and urea. Staphylococcal V8 protease digestion of the eluted peak produced the same sequence as shown above, but starting at Leu-2 and ending at Glu-32. Structural analysis of this peptide showed that it shares features with the calmodulin binding domains of other enzymes which are regulated by calmodulin.

The Ca$^{2+}$ pump of erythrocytes and other plasma membranes is activated by calmodulin (1, 2) by direct interaction rather than via stimulation of a calmodulin-dependent protein kinase. Previous work (3–8) has shown that in the absence of calmodulin the pump can be activated, i.e. its Ca$^{2+}$ affinity and its maximal rate increased, by a controlled treatment with trypsin. Trypsin splits the enzyme into a number of products, some of which, e.g. a particularly hydrophobic polypeptide of about 33 kDa (7), appear to be unrelated to the reaction cycle and to the interaction with calmodulin. Other products still behave as Ca$^{2+}$ transporting ATPases and retain the ability to interact with calmodulin (7–9). Among them there is a transient fragment of 90 kDa, which interacts with calmodulin and is activated by it (7), and one of 85 kDa, which is produced under special proteolysis conditions and has a greatly decreased response to calmodulin but normal ability to bind it (8). The 85-kDa polypeptide derives from that of 90 kDa and is degraded to fragments of 81 and 75 kDa if trypsin is applied for longer times under special conditions. Both the 81- and the 76-kDa fragments are fully active Ca$^{2+}$-stimulated ATPases; enriched preparations of the former have been shown to transport Ca$^{2+}$ in liposomal systems (9), but no reconstitution attempts have as yet been made with the latter. These last two fragments already have the high affinity for Ca$^{2+}$ and high activity which is achieved by the 90-kDa fragment when it interacts with calmodulin. The trypsin work has led to a proposal (7–9, 10) in which the protease acts first on one of the terminal domains of the ATPase, probably the N terminus, reducing it to a product of about 90 kDa and leaving intact the calmodulin interacting domain at the opposite end of the molecule. Further proteolysis is then visualized to occur within the calmodulin binding domain, removing first a 5-kDa fragment which is essential for the expression of full calmodulin stimulation of the ATPase, and then a 4-kDa domain which contains the calmodulin binding site proper. At this point the active site is visualized to have been completely accessible, and the ATPase activity is fully expressed even in the absence of calmodulin (9). Further proteolysis to the 76-kDa fragment has no extra effects on the V$_{max}$ of the ATPase, although it may increase somewhat its affinity for Ca$^{2+}$ (11).

The work on trypsin has not permitted the isolation of the postulated small M$_i$ calmodulin binding fragment(s). In the present work the purified erythrocyte ATPase has been exposed to another proteolytic enzyme, chymotrypsin. Under the experimental conditions chymotrypsin did not activate
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the ATPase or activated it only marginally. However, it rapidly produced complete calmodulin desensitization, and we show here that the calmodulin domain was released from the ATPase as a peptide of about 12 kDa. The latter was labeled in the intact ATPase using a cleavable radioactive photophosphorylation cross-linker coupled to calmodulin. After removal of calmodulin the labeled ATPase was digested with chymotrypsin and the radioactive material collected in a peptide of about 12 kDa. CNBr splitting of the labeled ATPase produced a labeled fragment whose amino acid sequence contained the essential features of calmodulin binding peptides (12-14).

MATERIALS AND METHODS

Trypsin and chymotrypsin were purchased from Behring Diagnostics, trypsin soybean inhibitor from Sigma, and leupeptin from Fluka AG, Buchs, Switzerland. The HPLC solvents were from May and Baker, Dagenham, England, HPLC grade. The chemicals used for the sequencing work were purchased from Applied Biosystems, Foster City, CA. The *Staphylococcus aureus* V8 protease was purchased from Miles Laboratories Inc., Elkhart, IN. Molecular weight standards were purchased from Bio-Rad.

All other reagents were of the highest purity grade commercially available.

Preparation of the ATPase—The ATPase was isolated from ghosts as described in Refs. 6 and 15. However, the washing was extended from 2 to 24 h to ensure that contamination of the ATPase would be minimal, and 2 mM dithiothreitol was omitted in the final wash and elution buffers. Triton X-100 was used as the detergent and phosphate buffer, pH 7.0, 30% (v/v) glycerol, 7.5% (w/v) SDS, 10 mM dithiothreitol, and 0.05% (w/v) bromphenol blue (tracking dye for the samples) were then boiled for 5 min and applied to 15% Laemmli type (18) SDS-polyacrylamide gels.

Measurement of the ATPase Activity—The ATPase activity of the purified ATPase and of its proteolytic fragments was continuously monitored spectrophotometrically using the coupled-enzyme assay described in Refs. 6 and 15. The concentrations of free Ca²⁺ in the media were calculated with the help of a computer-generated program assuming a Keq in the Ca²⁺-EGTA complex of 0.21 μM at pH 7.0 (16, 17).

Digestion of the Purified Ca²⁺-ATPase by Chymotrypsin—Thirty-μl aliquots (about 5 μg of protein) of Triton X-100 solubilized purified Ca²⁺-ATPase prepared in the presence of phosphatidylcholine (15) were incubated on ice with 0.5 μg of chymotrypsin (1 mg/ml) for the times indicated in the legends for the figures. The reaction was stopped by the addition of 10 μl of a buffer containing 30 mM sodium phosphate, pH 7.0, 30% (v/v) glycerol, 7.5% (w/v) SDS, 10 mM dithiothreitol, and 0.05% (w/v) bromphenol blue (tracking dye for the fragments). The mixture was then boiled for 5 min and applied to 15% Laemmli type (18) SDS-polyacrylamide gels.

Identification of the Calmodulin Binding Polypeptide(s) Produced by Chymotrypsin—After separation on 15% SDS-polyacrylamide gel electrophoresis the samples were then blotted onto nitrocellulose filters (Bio-Rad) as described in Ref. 19. The filtration was then performed by the method of the manufacturer. The Cd₃⁺-ATPase was labeled with the Denny-Jaffe reagent (21) from Du Pont-New England Nuclear. The N-oxysuccinimide ester group allows conjugation of calmodulin to be pure and free of low molecular weight contaminations. The efficiency of labeling was 70%.

Cross-linking to the ATPase was performed as described in Ref. 22. The ATPase was used in the final elution buffer with the calcium concentration raised to 50 μM and that of magnesium to 2 mM. Trypsin experiments had shown that photolabeling of the ATPase (as visualized by gel electrophoresis and autoradiography) only occurred in the presence of calcium, and no cross-linking occurred in 2 mM Na-EDTA. Photolysis was carried out in a glass cuvette using a 350-watt medium pressure mercury lamp as a light source. The beam was filtered using a saturated copper sulfate solution (20-mm light path) to minimize damage. The high yield of trypan blue seen in the sequencing work (see "Results") showed that no destruction of the latter amino acid occurred. The labeled calmodulin was added in aliquots, incubated at room temperature for 10 min to allow binding, and then photolyzed for 1 min. The operation was repeated five times to the final cross-linking concentration.

Clavage of calmodulin away from the ATPase was achieved by 2 h of incubation at room temperature with 10 mM sodium dithionite, followed by dialysis against 10 mM phosphate buffer, 1% Triton X-100, and 100 mM dithionite in 50,000 M, cut-off dialysis tubing (Spectrapor, Los Angeles, CA) to remove calmodulin. The dialysis was continued for 3 days at 4°C and the buffer changed 12 times. The removal of calmodulin was checked both by gel electrophoresis (and autoradiography) and by HPLC gel permeation chromatography using aliquots from the dialyzed protein solution. Only labeled ATPase was found; no other radioactive material was observed. The sequencing of the labeling was confirmed by the observation that CNBr digestion only one water-soluble labeled peptide was isolated on HPLC and that another labeled peptide could be isolated from the insoluble material. The second peptide has so far remained intractable to sequence analysis (see "Results").

Chymotrypsin Digestion of Labeled ATPase—Five mg of labeled ATPase (prepared as described above) in 0.1% Triton X-100 was digested with 50 μg of chymotrypsin at 4°C for 30 min. The digestion was stopped by the addition of isopropanol alcohol to 20% final concentration and dithiothreitol to 10 mM final concentration and by boiling for 10 min. The mixture was then cooled down to 2°C.

The ATPase was solubilized completely by sonication in 70% formic acid and digested with CNBr under nitrogen at room temperature for 24 h in the dark. The reaction was then allowed to proceed for 10 min and then 1 mM for 1 h. The digestion was then performed as described above in 0.1% Triton X-100 with 100 mM dithiothreitol.

CNBr Digestion of Labeled ATPase—A large preparation of labeled ATPase (30 mg) was reduced and alkylated with iodoacetate in 6 M guanidinium chloride as described in Ref. 23. Excess reagent and guanidinium chloride were removed by dialysis against several changes of 1% Triton X-100. Subsequently, the ATPase was precipitated with trichloroacetic acid and then washed 10 times with acetone HCl and then with cyclohexane as described in Ref. 24 to remove lipids and detergents.

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the method of Lowry et al. (26). The protein was first precipitated with deoxycholate and trichloroacetic acid to avoid interference by Triton X-100 and HEPES (27).

RESULTS

Effects of Chymotrypsin on the Activity of the Purified Erythrocyte ATPase—Under the experimental conditions used in this work, chymotrypsin completely desensitized the ATPase to calmodulin in one to three min (Fig. 1). Unlike trypsin, which activated the ATPase substantially before removing calmodulin sensitivity (6, 7), under the experimental conditions chymotrypsin failed to activate the basal activity of the ATPase substantially.

Pattern of Fragmentation of the Purified ATPase by Chymotrypsin—The rapid calmodulin desensitization of the ATPase by chymotrypsin corresponded to a pattern of fragmentation which was more rapid and more extensive than that seen with trypsin (6, 7). In the experiment shown in Fig. 2, the intact ATPase had already completely disappeared after 1 min of exposure. The time of degradation shown in the figure is representative of a number of experiments with chymotrypsin but cannot be taken as absolute, since in some cases remnants of the intact ATPase were still visible at 2 min. In the experiment shown, the largest M, fragments visible at 1 min were 120 and 105 kDa and were accompanied by a large number of fragments ranging from 85 to 12 kDa. The heavier fragments were evidently transient products since they had already disappeared after 2 min of chymotrypsin exposure. At this time, the heaviest fragments were 78 and 70 kDa and were accompanied by a number of polypeptides ranging between 50 and 3–4 kDa. After 10 min, the fragments of 78 and 70 kDa had also disappeared, whereas the remainder of the pattern had not changed significantly. Most of the products in the range between 50 and 3–4 kDa thus apparently were limit polypeptides, since they remained visible and at approximately constant concentrations for the entire duration of the experiment shown in the figure (60 min). No detailed investigation has as yet been performed on the distribution of all functional domains (e.g. the ATP binding site, the site of formation of the phosphoenzyme) among the fragments produced by chymotrypsin. However, the calmodulin binding domain was removed immediately from the ATPase by the treatment, as shown by the 125I-calmodulin gel overlay experiment shown in the middle panel of Fig. 2. Unlike the case of trypsin (7), where the calmodulin binding ability remained associated with high M, degradation products, chymotrypsin evidently removed the calmodulin binding domain from the ATPase in the form of a low M, fragment. The experiment shown in Fig. 2 indicates that this fragment indeed was a peptide of about 12 kDa, which was visible at 1 min, became stronger at 2 min, and disappeared at longer times of chymotrypsin exposure (in some experiments, however, it persisted past the 2-min limit, see above). As the radioactive band at 12 kDa became stronger between 1 and 2 min, radioactive bands faintly visible at 1 min at 33 and 26 kDa became weaker suggesting that products of about 26 and 33 kDa containing the calmodulin binding domain were further degraded to the 12-kDa fragment. However, the 12-kDa region contained other products unrelated to the calmodulin binding domain, since the stained band at this molecular size persisted in the gels long after the calmodulin binding had disappeared. The control experiment in the right panel of Fig. 2 shows that the calmodulin binding to the 12-kDa fragment had the expected Ca2+ dependence.

Labeling of the Calmodulin Binding Domain of the ATPase and Preparation of Labeled Chymotryptic Fragments—Calmodulin labeled with the Denny-Jaffe reagent was incubated with 5 mg of purified ATPase and cross-linked to its calmodulin-binding domain(s) by photoactivation. The efficiency of cross-linking was about 10%. Calmodulin was then cleaved off as described under “Materials and Methods,” leaving the radioactive label on the calmodulin binding domain of the ATPase. The specificity of the cross-linking procedure was shown by the absence of radioactivity in the ATPase if the labeling was performed in the presence of EDTA. Radioactive bands were found in autoradiograms of SDS gels at about 155 and 172 kDa (data not shown) in agreement with previous findings with azido-modified calmodulin (7). This indicated the presence of more than one calmodulin binding site/ATPase molecule. Although the matter was not pursued further, it is possible that in addition to the well known high affinity calmodulin binding site the ATPase contains a second site, possibly with a lower calmodulin affinity.

The labeled ATPase was digested with chymotrypsin for 30 min using a chymotrypsin to ATPase ratio of 1:100, w/w, as described under “Materials and Methods,” except that the reaction was stopped by boiling in 10 mM dithiothreitol, 20% propanol. The digest was then separated by gel permeation chromatography; two Waters (Milford, MA) 1204I columns and

![Figure 1](image1.png)  
**Fig. 1.** Stimulation of the Ca2+-ATPase by proteolytic digestion. The purified erythrocyte Ca2+-ATPase was digested on ice by chymotrypsin as indicated under “Materials and Methods.” At the times indicated in the figure, aliquots of the reaction medium were withdrawn and the digestion was stopped by adding a 10-fold (w/w) excess of 15 mM β-mercaptoethanol. At zero time, the inhibitor was added before the protease. The ATPase activity was determined at 30 °C by using the coupled enzyme assay described in Refs. 13 and 14 either in the absence (open squares) or presence (closed squares) of 20 mM calmodulin.

![Figure 2](image2.png)  
**Fig. 2.** Pattern of fragmentation of the purified ATPase by chymotrypsin and identification of the calmodulin binding domain. The proteolysis conditions and the procedures for the identification of the calmodulin binding fragment(s) are described under “Materials and Methods.” 15% silver-stained Laemmli type gels (18) are shown in the left panel and autoradiograms of the same gels (2 days of exposure) in the middle and right panels. The lanes in the middle panel refer to incubations in the presence of 0.5 mM Ca2+ and those in the right panel in the presence of 2 mM Na-EGTA. The silver-stained gel on the left and the figures in the margin refer to M, protein standards.
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an LKB (Uppsala, Sweden) GPC 2000 column were connected in series and fractions collected. The only radioactive peak, corresponding by calibration to about 12 kDa, was collected and run on reverse-phase HPLC using a Brownlee (Applied Biosystems, Foster City, CA) 300A C8 2.2 × 4.6-mm column with a gradient running from 0 to 100% B in 60 min. The labeled peak was divided into two 500-pmol fractions, of which one was used for amino acid analysis and one for amino acid sequencing. Table I shows the amino acid composition of the fragment. However, it proved impossible to obtain sequencing information, possibly due to internal cyclization of the N-terminal residue of the fragment. Thus, since the analysis in Table I showed a single methionine, it was decided to carry out CNBr digestion.

**CNBr Fragmentation of the ATPase after Labeling of the**

**TABLE I**

| Amino acid composition of the purified 12-kDa fragment produced by the chymotrypsin digest |
| Amino acid | nmol | Composition |
|------------|------|-------------|
| 1          | Asx   | 5.466       | 11         |
| 2          | Thr   | 2.180       | 4          |
| 3          | Ser   | 5.719       | 11         |
| 4          | Glx   | 6.615       | 13         |
| 5          | Gly   | 4.510       | 9          |
| 6          | Ala   | 3.766       | 8          |
| 7          | Cys   | 0.411       | 1          |
| 8          | Val   | 2.287       | 6          |
| 9          | Met   | 0.462       | 1          |
| 10         | Ile   | 2.598       | 5          |
| 11         | Leu   | 3.959       | 8          |
| 12         | Tyr   | 1.517       | 3          |
| 13         | Phe   | 1.590       | 3          |
| 14         | Lys   | 2.427       | 5          |
| 15         | His   | 2.140       | 4          |
| 16         | Arg   | 1.901       | 4          |
| 17         | Pro   | 4.000       | 8          |

**FIG. 3.** Preparative separation of the labeled CNBr peptide on reverse-phase HPLC. After digestion and centrifugation, about 20 mg of peptides were injected onto the C18 30A self-packed column described under "Materials and Methods." The gradient was run for 5 min at 0% B and then to 100% B in 1 h at 1 ml/min. Detection was at 206 nm (solid line), and tubes were measured for radioactivity (dashed line) in a Kontron Gamamatic Counter. The radioactive peak eluting between 45 and 55 min was collected and the fractions pooled.

**TABLE II**

| Amino acid sequence of the labeled CNBr fragment after HPLC purification |
|--------------------------|-----------------|-----------------|
| NH2-Glu-Leu-Arg-Arg-Gly-Gln-Ile-Leu-Trp-Phe-Arg-Gly-Leu-Aan-Arg-Ile-Gln-Thr-Gln-Ile-Lys-Val-Val-Aan-Ala-Phe-Ser-Ser-Ser-Leu-His-Glu-Phe |

**FIG. 4.** Rechromatography of pooled fractions from the preparative HPLC separation. The pool of radioactive material from the experiment shown in Fig. 3 was reduced in volume to 1 ml by flushing with nitrogen and then injected onto the CB Brownlee column (see "Materials and Methods"). A gradient was run for 5 min at 0% C and then to 80% C in 1 h. The radioactivity (dashed line) was associated with only 1 peak. Detection was at 206 nm, and the radioactivity was measured in a Kontron Gamamatic Counter.

**Calmodulin Binding Domain**—A large preparation of purified ATPase (30 mg) was labeled with the radioactive Denny-Jaffe reagent coupled to calmodulin. After removal of calmodulin, the preparation was treated as indicated under "Materials and Methods" and subjected to CNBr digestion (24 h, see...
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“Materials and Methods”). After centrifugation, about 50% of the radioactivity remained associated with soluble products. The soluble digest was injected onto a 10 × 256-mm RP-300 C18 self-packed column (packing material, Toyo Soda, Tokyo, Japan) with a gradient from 0 to 100% B in 60 min. The radioactivity eluted as a broad peak between 45 and 55 min (Fig. 3); other radioactive peaks, indicated as A, B, and C on Fig. 3, contained no peptides. The broad peak was pooled and run using a gradient of 0–80% C in 1 h on a 2.2 × 4.6-mm Brownlee 300A C8 column. The single radioactive peak shown in Fig. 4 was collected, and one-quarter of it (corresponding to 5 nmol) was subjected to sequence analysis, yielding the 34-amino acid-long structure shown in Table II. Three-quarters of the radioactive peak were loaded onto a microcalmodulin column, packed at the tip of a Pasteur pipette, in the presence of 0.01% Trition X-100 and excess Ca2+; all of the radioactivity bound to the column and remained bound as the column was washed with Ca2+-containing buffer (20 mM HEPES, 130 mM NaCl, 1 mM MgCl2, 0.01% Trition X-100, 0.05 mM Ca2+). About 30% of the radioactivity was removed at this point with 2 mM EDTA (20 mM HEPES, 130 mM NaCl, 1 mM MgCl2, 0.01% Trition X-100); the rest was eluted with the same buffer with 2 M urea. The radioactive eluates were rerun separately on the HPLC system (Fig. 5) and shown to produce one single identical peak. One-quarter (2 nmol initial yield) of the purified eluate was used for sequence analysis, producing this structure: NH2-Glu-Leu-Arg-Arg-Gly-Gln-Ile-Leu-Trp-Phe-Arg-Gly-Gln-Ile-Leu-Trp-Phe-Arg-Gly-Leu-Asn-Arg-Arg-Gly-Gln-Ile-Lys-Val-Val-Asn-Ala-Phe-Ser-Ser-Ser-Ser-

DISCUSSION

The labeling of the CNBr fragment by the Denny-Jaffe reagent shows that this domain of the Ca2+-pump is involved in interaction with calmodulin. This interaction was confirmed for the isolated peptide by its binding to calmodulin-Sepharose and elution with EDTA. That the peptide came off in two separate fractions, one eluted with EDTA and the other with EDTA plus urea, is easily explained in terms of the variations in the environment of the calmodulin binding region. Calmodulin may bind to CNBr-activated Sepharose via any of a number of lysines, and calmodulin bound in different ways would be expected to have different affinities for calmodulin binding peptides. Thus, those calmodulins which had low affinities might release a peptide in EDTA alone, while those which had higher affinities would require EDTA plus urea. That urea was required to elute this peptide is not surprising, since the isolated calmodulin binding domain may well have a higher affinity for calmodulin than does this same domain when surrounded by other portions of the molecule, probably including regions of negative charge.

The initial cyanogen bromide fragment was probably not sequenced to its end, so that there remained some doubt as to whether the calmodulin binding region was in the sequenced portion of this peptide or in a portion more toward the carboxyl terminus. Because of this uncertainty, the V8 protease digestion was carried out. The label remained in the sequenced portion of the peptide after this cleavage, demonstrating that this was indeed the calmodulin binding region. The amino acid sequences of the domains of proteins and peptides which bind calmodulin show only a limited degree of strict sequence homology (12, 28). Only the sequences from closely related proteins (such as smooth and skeletal muscle myosin light chain kinases) are truly homologous. However, the sequences of calmodulin-activated enzymes (the myosin light chain kinases, the calmodulin-dependent protein kinase, phosphofructokinase, and the Ca2+-ATPase) share several structural features: 1) a strong predominance of lysine or arginine residues in clusters; 2) a preponderance of hydrophobic residues, especially in the first half of the domain; 3) a tryptophan in the first half of the domain; and 4) the presence in the second half of the domain of one or more serines or threonines. In some cases this last feature has been demonstrated to be a target sequence for phosphorylation, and it has been further shown that phosphorylation affects calmodulin binding and vice versa.

The importance of these features has also been extensively studied in the binding of calmodulin to peptide hormones (e.g., glucagon, glucagon-like peptide, zrenoicorticotrophic hormone), neuropeptides (e.g., β-endorphin, the gastric inhibitory peptide, the vasoactive intestinal peptide), and proteins (e.g., the γ subunit of phosphorylase kinase and tropomin I), as well as several small synthetic peptides and naturally occurring peptides in venoms (e.g., melittin and mastoparan). The calmodulin binding domains of these latter peptides all share at least features 1) and 2) listed in the previous paragraph. This matter has been covered in several recent articles (28–30).

Secondary structure predictions using a variety of methods (Chou-Fasman, Garnier, Osathorpe and Robson, and others in the University of Wisconsin sequence analysis software package) show the peptide to have a high probability of helix formation for 5–6 amino acids at the N and C terminals. The remainder shows a strong probability of β sheet with a β-turn in the middle separating the two halves of the domain. Since many calcium-dependent calmodulin-binding peptides form amphiphilic helices upon binding calmodulin (31), the moments program of the University of Wisconsin software package was used to predict the region of the peptide which had the highest propensity for forming such a structure. The region is depicted using the helix wheel representation in Fig. 6. As can be seen in the figure, the five positively charged residues lie on one side of the helix while the opposite side is predominantly hydrophobic. The presence of a tryptophan residue in the first half and the three serines in the second half shows that the calmodulin binding region of the Ca2+-ATPase fits in clearly with the structural features common to the other calmodulin binding peptides and proteins.

By contrast, the degree of homology with the deduced structure of a domain of the ATPase (from a putative clone recently suggested by Brandt et al. (32) was rather poor. It is possible, however, that the latter structure corresponds to the portion of the calmodulin interacting domain of the ATPase which has been proposed to be essential for the expression of calmodulin stimulation (8) rather than to that of the portion
which contains the calmodulin binding site proper.

The location of this calmodulin binding in the Ca2+-pump molecule has been determined primarily by proteolysis studies utilizing trypsin, chymotrypsin, and carboxypeptidase. The first study of this sort (7), carried out with trypsin, showed that trypsin cleaved off a very large portion of the ATPase (about 50 kDa) without any noticeable effect on the enzymes' properties. The enzyme was still responsive to calmodulin and required calmodulin for full activity. Further trypsin degradation of the remaining 90-kDa polyptide to 81 (7-9) or 76 (11) kDa caused the complete loss of calmodulin stimulation and resulted in a fully activated enzyme. This showed that the calmodulin binding domain was relatively small, but it remained uncertain whether it was in the middle of the molecule and exposed by removal of the 50-kDa moiety or whether it was at an end of the molecule, in which case it would have to be at the opposite end from the end initially attacked by trypsin. A detailed analysis of all of the possibilities suggested that the most logical explanation of the data was that the calmodulin binding site was at the opposite end of the molecule from the large 50-kDa portion (7). This point of view was reinforced by the initial studies on chymotrypsin digestion (33) which showed that activation of the molecule occurred at a time when substantial amounts of very large products (120 and 105 kDa) were still present. This showed that the calmodulin binding region was indeed at one end of the molecule. Independently, Sarkadi et al. (34) found similar results with chymotrypsin and also showed that carboxypeptidase digestion led to some activation of the ATPase. This suggests that the binding domain is situated at the carboxy-terminal end of the Ca2+-ATPase. The present study reinforces the previous results with chymotrypsin and demonstrates the actual sequence of the calmodulin binding site.

It is possible that there are two calmodulin binding sites present in the Ca2+-pump molecule. Zurini et al. (7) showed that two molecules of calmodulin could bind to the enzyme both by gel electrophoresis of azidocalmodulin cross-linked to the pump and by stoichiometric studies of the binding of iodinated calmodulin. Since full activation of the pump requires the binding of only one calmodulin/pump molecule (35) it seems likely that any second calmodulin binding site is not biologically functional. The 50% of the radioactivity which was associated with the insoluble fraction after cyanogen bromide cleavage might represent the second binding site for calmodulin. Work on the purification and sequencing of the labeled insoluble fraction is currently underway.

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