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The Purified Calcium-Pumping ATPase of Plasma Membrane
Structure–Function Relationships

Ernesto Carafoli, Mauro Zurini, and Gustavo Benaim

The Ca^{2+}-pumping ATPase of plasma membrane, known to exist since 1966 [13], has now been characterized as a high Ca^{2+} affinity enzyme, present in all likelihood in all eukaryotic plasma membranes (for a recent review, see Ref. 11). The essential properties of this enzyme, as they can be extracted from a very large number of studies, are summarized in Table I. The ATPase is perhaps less well characterized than other members of the E_1, E_2 class of transport ATPases like the Na^+/K^+-ATPase and the Ca^{2+}-ATPase of sarcoplasmic reticulum. For example, it is still not known whether the acyl phosphate formed during the enzyme cycle is an aspartyl phosphate. It is also not established to the satisfaction of all specialists whether the Ca^{2+}/ATP stoichiometry is, as indicated in Table I, always and invariably 1. Recent reviews on the ATPase have appeared, the most comprehensive being perhaps the one by Schatzmann [14].

One interesting property of the plasma membrane Ca^{2+}-ATPase is its sensitivity to calmodulin which was discovered in 1977 [3,4]. The stimulation is due to the direct interaction of calmodulin with the enzyme [7,10] and corresponds to a shift of the affinity for Ca^{2+} toward lower K_m values. However, the liver plasma membrane Ca^{2+}-pumping ATPase is insensitive to calmodulin [5,6]. It now appears probable that this ATPase belongs to a distinct class of plasma membrane Ca^{2+}-ATPases.

The interaction of the ATPase with calmodulin was utilized by Niggli *et al.* [9] to devise a successful method to isolate and purify the enzyme from membranes. Previous attempts [see e.g. Ref. 12] based on conventional protein purification procedures had met with formidable difficulties, the most serious being the very minute amounts of enzyme present in plasma membranes (about 0.02% of the total membrane protein). Niggli *et al.* [9] used a calmodulin affinity chromatography column, on which a Triton X-100 solubilizate of EDTA-

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Table I. Properties of the Plasma Membrane Ca^{2+}-Pumping ATPase

Type of enzyme	E_1, E_2, forms acyl phosphate
Affinity for Ca^{2+} (K_m)	<1 μM
V_{max} of Ca^{2+} transport	0.5 nmole/mg membrane protein/sec
Ca^{2+}/ATP stoichiometry	1:1?
Inhibition	Vanadate, $K_{1/2} < 1$ μM

washed erythrocyte membranes was applied. After extensive washing of the column with Ca^{2+} removed most of the unbound protein, elution with EDTA produced a sharp protein peak that contained a very high Ca^{2+}-ATPase activity. The EDTA-eluted peak possessed essentially a single protein of M_r 138,000, which formed acyl phosphate upon incubation with [γ-^{32}P]-ATP and Ca^{2+}, and was completely inhibited by low concentrations of vanadate.

The purified ATPase has now been characterized in the laboratories of Carafoli and Penniston (for a comprehensive review see Ref. 2). Such studies reproduce the properties of the enzyme *in situ* (Table II) and have helped settle some of the issues which *in situ* studies had left undecided. These issues include the ATP/Ca^{2+} stoichiometry, which now appears to be almost certainly 1, and the charge balance during the transport cycle: the enzyme functions as an electroneutral Ca^{2+}–$2H^+$ exchanger.

One of the particularly important observations made on the purified enzyme is that calmodulin as an activator can be replaced by a limited proteolytic treatment of the enzyme [8]. As is the case with activation by calmodulin, that produced by proteolysis is also essentially a K_m effect, i.e., the activated enzyme has a much higher affinity for Ca^{2+} (K_m about 0.5 μM) than the enzyme in the absence of activating treatments (K_m 10–20 μM). Controlled proteolysis, coupled to activity measurements and to a variety of affinity-label procedures, has recently been used to map functional domains in the isolated enzyme molecule. In the following pages, a brief account of this work will be presented.

Controlled Proteolysis of the Purified Plasma Membrane Ca^{2+}-ATPase by Trypsin

Trypsin splits the purified plasma membrane ATPase according to a pattern which is considerably more complex than that of the Ca^{2+}-ATPase of sarcoplasmic reticulum. However, under controlled experimental conditions the pattern is reproducible and presents a

Table II. The Purified Ca^{2+}-ATPase of the Erythrocyte Membrane

M_r	138,000
Affinity for Ca^{2+} (K_m)	0.4–0.5 μM
V_{max} of Ca^{2+} transport	180–1200 nmoles/mg protein/sec
Ca^{2+}/ATP stoichiometry	1
Inhibition	Vanadate, $K_{1/2} < 1$ μM
Activation	Calmodulin, acidic phospholipids, fatty acids, limited proteolysis

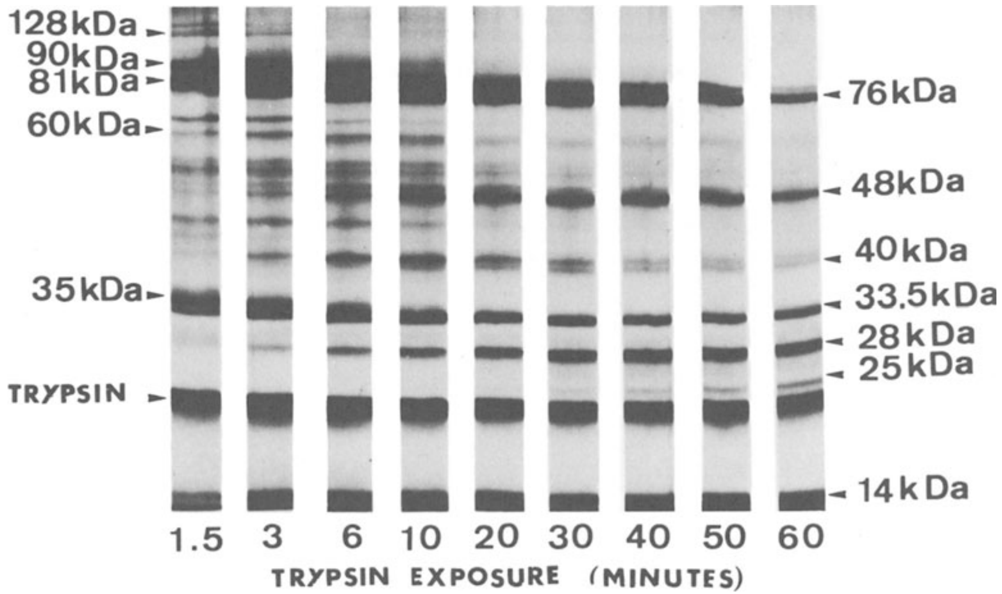

Figure 1. Controlled proteolysis and activation of the purified Ca^{2+}-ATPase. Aliquots (50 μl) of Triton X-100-solubilized, purified Ca^{2+}-ATPase (corresponding to about 7 μg of protein) were exposed at 0°C to 1 μg of trypsin for the times indicated. The reaction was stopped by the addition of threefold concentrated electrophoresis buffer followed by 5 min boiling, and 40 μl of each sample was submitted to 7% SDS-polyacrylamide slab gel electrophoresis. The gels were stained with a silver impregnation method. Standards: myosin (200K); β-galactosidase (116.25K); phosphorylase B (92.5K); bovine serum albumin (66.2K); ovalbumin (45K); carbonic anhydrase (31K).

number of well-recognizable basic features (Fig. 1). Some of the fragments produced are limit polypeptides, like those of M_r 14,000, 28,000, 33,500, 48,000. Others, such as those of M_r 124,000 and 90,000, are transient. A group of fragments in the 85,000–75,000 region gradually condenses into a doublet of M_r 81,000–76,000. But it seems evident that the latter products, although certainly less transient than, for example, the 90,000-M_r polypeptide, tend to be degraded further after prolonged proteolysis. The pattern shown in Fig. 1 can be

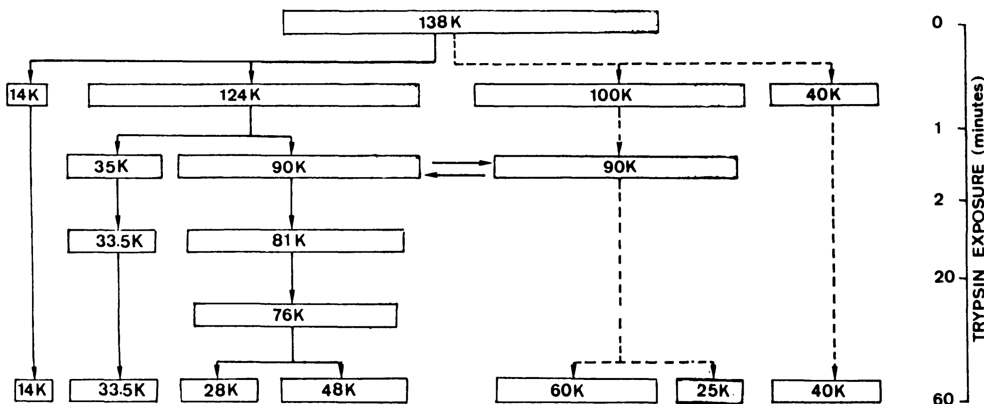

Figure 2. Model for trypsin proteolysis. It is proposed that attack by trypsin at a few points, with some molecules being resistant at some points, will give rise to the observed patterns. The cleavage points shown and the pattern indicated by the solid lines will produce the main features seen by protein staining. The dashed lines indicate the minor proteolysis pathway (see text).

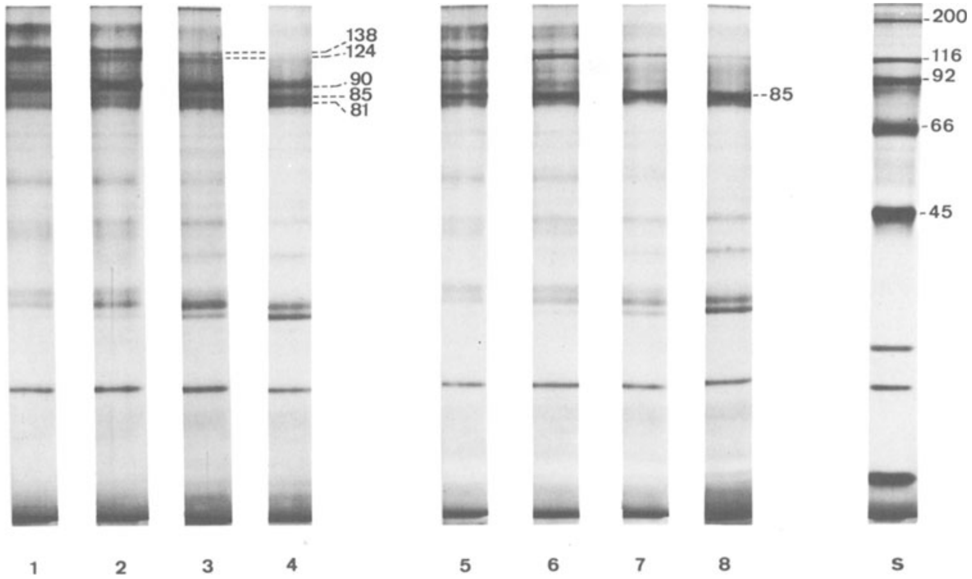

Figure 3. Effect of calmodulin (plus Ca^{2+}) on the pattern of trypsin proteolysis of the purified erythrocyte Ca^{2+}-ATPase. The basic conditions of the experiments were those of the experiment shown in Fig. 1, except that the temperature of the proteolysis medium was 37°C, and the trypsin/ATPase ratio was much lower (0.25 μg trypsin, about 7 μg ATPase). Ca^{2+} was 100 μM, calmodulin 1 μg. Concentration of polyacrylamide in the gels, 10%.

provisionally interpreted according to a scheme in which a major proteolytic pathway produces in sequence most of the (major) fragments seen (Fig. 2). A second pathway, probably reflecting a minor conformer of the ATPase, is postulated to produce a number of other minor products.

The fractionation scheme in Fig. 1 reflects the splitting of the enzyme at 0°C, in a

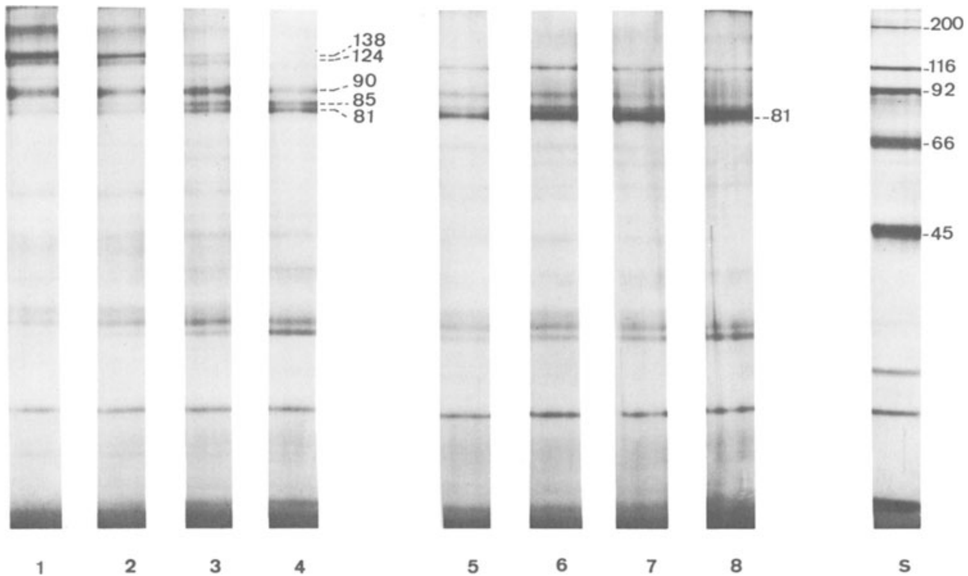

Figure 4. Effect of vanadate on the pattern of trypsin proteolysis of the purified erythrocyte ATPase. Conditions as in the experiment of Fig. 3, except that calmodulin and Ca^{2+} were replaced by 10 μM vanadate and 10 mM Mg^{2+}. Concentration of polyacrylamide in the gels, 10%.

medium containing 130 mM KCl, 20 mM Hepes, pH 7.2, 1 mM $MgCl_2$, 100 mM $CaCl_2$, 2 mM dithiothreitol, 0.05% Triton X-100, 0.05% phosphatidylcholine, 1 μg trypsin, and 7 μg purified ATPase. Presumably, under the conditions chosen, the ATPase was not "activated" and neither of its two configurations (E_1 and E_2) was privileged. On the other hand, very evident differences in the fragmentation scheme were visible when the enzyme was exposed to trypsin in the activated (presumably E_1), as compared to the E_2 configuration [1]. The former conformation was obtained by adding Ca^{2+} and calmodulin under conditions of limiting trypsin at 37°C, and produced a pattern that proceeded until a major 85,000-M_r polypeptide was formed and accumulated (Fig. 3). In the E_2 configuration, which was obtained by the addition of vanadate and Mg^{2+}, the proteolysis proceeded instead to a fragment of M_r 81,000, which under the experimental conditions was relatively protected from further digestion (Fig. 4). This suggests conformational changes were induced by Ca^{2+}-calmodulin, or by vanadate, which make different portions of the ATPase accessible to the attack by trypsin.

Functional Domains in the Plasma Membrane Ca^{2+}-ATPase

Among the domains of functional interest in the ATPase molecule are the calmodulin-binding region and the region of ATP-binding and/or acyl phosphate formation. Experiments on the trypsinized enzyme have now identified the calmodulin receptor in the (transient) 90,000-M_r polypeptide [15]. The conclusion rests on affinity labeling studies with azido-modified, radioactive calmodulin (not shown), and especially on the isolation of the pure 90,000-M_r fragment from calmodulin affinity columns. The 90,000-M_r fragment had calmodulin-stimulated, Ca^{2+}-dependent ATPase activity, and yielded the 81,000-M_r polypeptide upon further proteolysis (Fig. 5). It is of great interest that the 90,000-M_r calmodulin receptor could be reconstituted into liposomes, and shown to pump Ca^{2+} inwardly by a calmodulin-sensitive process (15; Fig. 6). This raises the question as to the role of the ~ 50,000-M_r portion of the ATPase molecule, which is lost as proteolysis progresses to the 90,000-M_r polypeptide. Possibly, this region, which evidently is not involved in the reaction mechanism proper, could be responsible for some regulatory function.

The second domain of interest in the ATPase molecule is the site where ATP is bound and where the acyl phosphate bond is formed. Work is now in progress on the identification of the fragment which bears the acyl phosphate function [15]. So far, it can be concluded from experiments with [γ-^{32}P]-ATP that acyl phosphate is formed on all fragments of M_r higher than 76,000–81,000. Association of radioactivity from [γ-^{32}P]-ATP with lower M_r products has not yet been demonstrated, but this could be due to difficulties relative to the experimental system. Indeed, separation of the lower M_r fragments under polyacrylamide gel conditions that would not be harmful to the acyl phosphate bond has proven very difficult.

As for the ATP-binding site, it need not be the same as the acyl phosphate site, although the two sites, even if separated by a (long) sequence of amino acids, may be very close in space due to the folding of the polypeptide chains. The Ca^{2+}-ATPase of plasma membranes, unlike other membrane ATPases, apparently bears the acyl phosphate and the ATP-binding function in the same fragments produced by the proteolysis (Fig. 7). For this experiment, radioactive dialdehyde ATP, produced by oxidation of [^{14}C]-ATP by periodic acid, was used. The autoradiograph shows radioactivity in the intact enzyme and in the same fragments of M_r down to 76,000–81,000 which also became labeled by ^{32}P radioactivity from [γ-^{32}P]-ATP. In addition, the experiment shows that one of the two fragments which the scheme in Fig. 1 depicts as deriving from the splitting of the 81,000–76,000-M_r product, that having M_r of 48,000, also becomes labeled. By contrast, no radioactivity is visible in the region

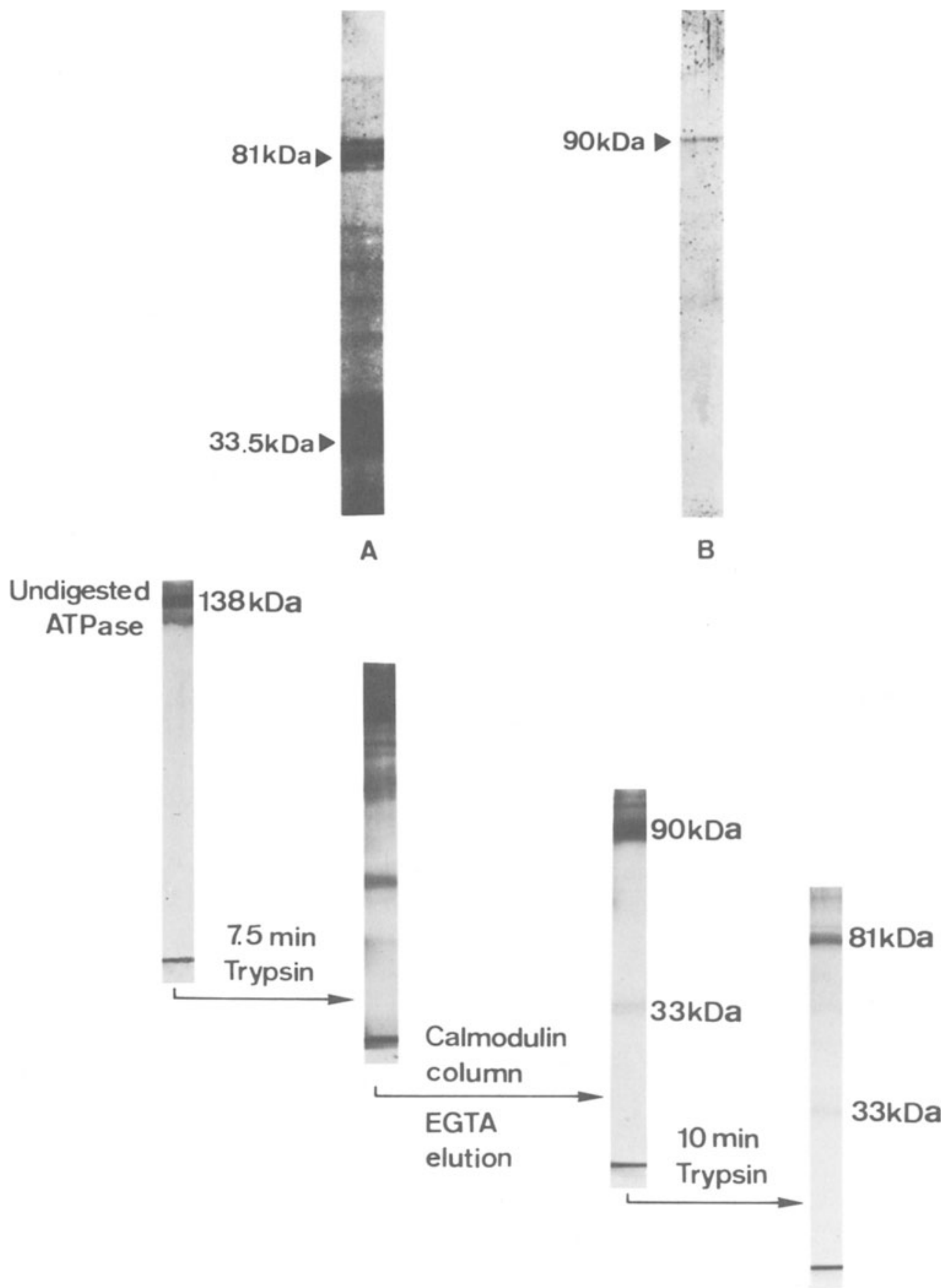

Figure 5. Calmodulin affinity chromatography of the trypsinized purified Ca^{2+}-ATPase, and proteolytic degradation of the purified 90,000-M_r fragment.

(Top panel) Trypsinized purified Ca^{2+}-ATPase was passed through a calmodulin affinity column in the presence of 50 μM $CaCl_2$. After several washes with a Ca^{2+}-containing buffer, the column was eluted with 2 mM EDTA. Details are given in Ref. 15. Fractions of the Ca^{2+}-wash and of the EDTA-eluate were submitted to 7% SDS-polyacrylamide slab gel electrophoresis. The gels were stained with a silver impregnation method. (A) Ca^{2+}-wash; (B) EDTA-eluate.

(Bottom panel) After trypsinization and calmodulin affinity chromatography, 50 μl of column eluate was digested for 10 min at 0°C with 0.5 μg trypsin. A small amount of the 33,500-M_r component was found after the second trypsinization but this was not due to proteolysis at this step, since the same amount appeared as a contaminant of the 90,000-M_r fragment. [From Ref. 15.]

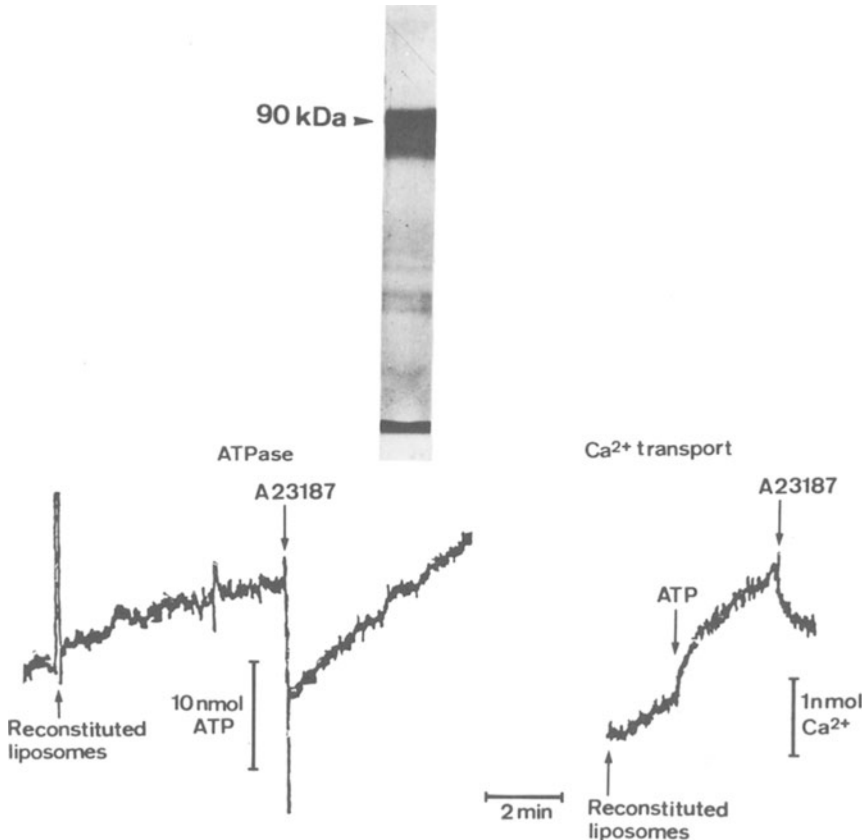

Figure 6. Ca^{2+}-ATPase and Ca^{2+} uptake by the 90,000-M_r fragment reconstituted into liposomes. The 90,000-M_r fragment shown in the gel was reconstituted and its ATPase and Ca^{2+} uptake measured. The ATPase reaction was measured by a coupled enzyme assay [15]. The reaction was started by adding 50 μl of reconstituted vesicles containing 0.2 μg of ATPase protein. The Ca^{2+} uptake was monitored using a Ca^{2+}-selective electrode; the total reaction volume was 1 ml and contained 130 mM KCl, 20 mM Tris-Cl, pH 6.6, 0.5 mM $MgCl_2$, 10 μM $CaCl_2$ 1 mM dithiothreitol, and 50 μl of reconstituted vesicles. The reaction was started by adding ATP to a concentration of 50 μM. Ionophore A23187 concentration was 2 μM. [From Ref. 15.]

corresponding to the polypeptide of M_r 28,000. The label in the 48,000-M_r product increases with trypsinization time, whereas the label in the 81,000–76,000-M_r doublet decreases (Fig. 7). This supports the proposal (see the scheme of Fig. 1) that the limited trypsin products of M_r 28,000–48,000 arise from the degradation of the 81,000–76,000-M_r polypeptides (the derivation of the 81,000-M_r product from the 90,000-M_r calmodulin-binding region has been directly demonstrated by the experiment of Fig. 5).

A summary of the results discussed above on the definition of some of the functional domains of the ATPase is presented in Fig. 8; only the relevant trypsin proteolysis fragments are shown. Work now in progress includes the splitting of the ATPase by proteolytic agents different from trypsin, the identification of the acyl phosphate-forming site among the low-M_r products of proteolysis, and the identification of the domain(s) of the ATPase which interacts with effectors of activity different from calmodulin.

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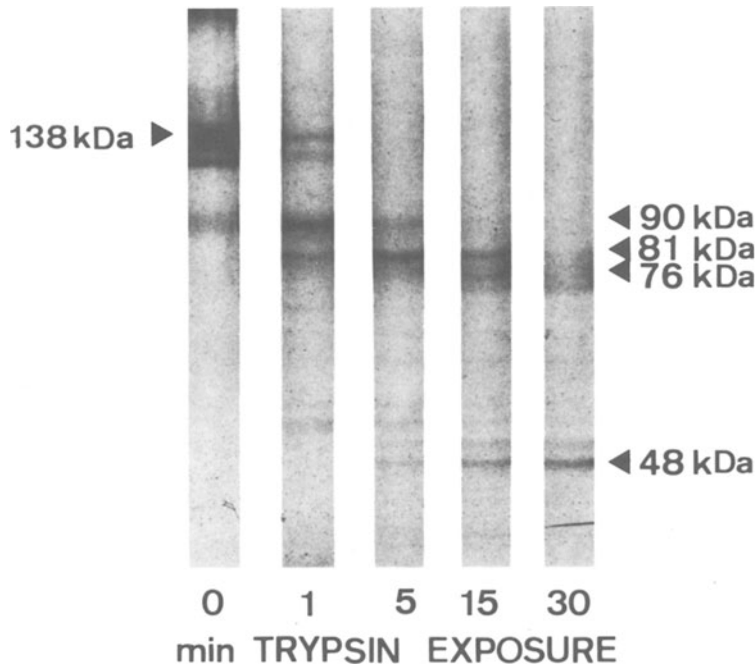

Figure 7. Localization of ATP-binding sites by labeling with ATP-dialdehyde. Autoradiogram showing binding of ATP dialdehyde to ATPase and to selected fragments. 200 μl of 100 μM [U-^{14}C]-ATP (specific activity 0.55 Ci/nmole) was oxidized for 1 hr on ice and in the dark with 10 μl of a 2.2 mM $NaIO_4$ solution. At the end of the incubation, excess $NaIO_4$ was removed by addition of 1 μl 100% ethylene glycol. Samples of purified ATPase were incubated for 1 hr at 37°C with 6–7 M *o*-ATP, transferred to ice, and submitted to trypsin treatment for times varying from 0 to 30 min. Electrophoresis was carried out on 7% gels. The gels were dehydrated in 100% Me_2SO, and immersed for 3 hr in 20% 2,5-diphenyloxazole (PPO) in Me_2SO (w/w), washed 1 hr with several changes of water, dried, and autoradiographed prior to staining by a silver impregnation procedure [From Ref. 15.]

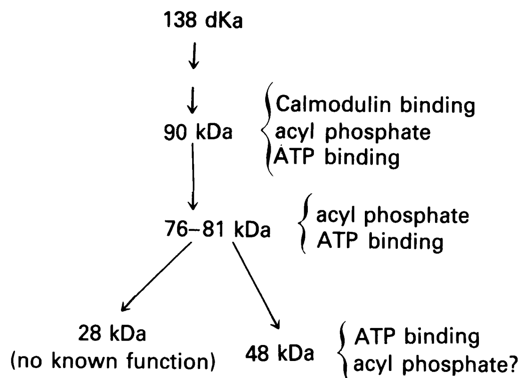

Figure 8. Functional domains in the Ca^{2+}-ATPase as revealed by controlled trypsin proteolysis.

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