Vanadate induces the formation of two-dimensional crystalline arrays of Ca\textsuperscript{2+}-ATPase molecules in sarcoplasmic reticulum. The Ca\textsuperscript{2+}-ATPase membrane crystals are evenly distributed among the terminal cisternae and longitudinal tubules of sarcoplasmic reticulum, but very few crystals were observed in the T tubules.

Tryptic cleavage of the Ca\textsuperscript{2+} transport ATPase into two major fragments (A and B) did not interfere with the vanadate-induced formation of membrane crystals. The ability of Ca\textsuperscript{2+}-ATPase to crystallize was lost after further cleavage of the A fragment into the A\textsubscript{1} and A\textsubscript{2} subfragments that is known to be accompanied by loss of Ca\textsuperscript{2+} uptake. Vanadate (0.1–5 mM) inhibited the secondary cleavage of Ca\textsuperscript{2+}-ATPase by trypsin suggesting that the susceptibility of the tryptic cleavage sites is influenced either by the conformation of the enzyme or by the formation of ATPase crystals.

Treatment of sarcoplasmic reticulum vesicles with vanadate induces the formation of two-dimensional crystalline arrays of the Ca\textsuperscript{2+} transport ATPase, which can be seen by negative staining with uranyl acetate (1) or by freeze-fracture (2). The effect of vanadate is attributed to the stabilization of the E\textsubscript{2} conformation of the Ca\textsuperscript{2+} transport ATPase. These observations clearly indicate that Ca\textsuperscript{2+}-ATPase molecules possess specific sites for interactions and suggest a dynamic equilibrium between ATPase monomers and oligomers in the membrane.

Here we report data on the formation of membrane crystals in subfractions of muscle microsomes enriched in vesicles originating from the terminal cisternae and the longitudinal elements of sarcoplasmic reticulum; Ca\textsuperscript{2+}-ATPase crystals were only rarely found in the T tubule fraction. Observations are also presented on the effect of limited proteolysis upon the formation and stability of Ca\textsuperscript{2+}-ATPase membrane crystals and on the effect of vanadate upon the susceptibility of Ca\textsuperscript{2+}-ATPase to cleavage by trypsin.

**MATERIALS AND METHODS**

Sarcoplasmic reticulum vesicles prepared from rabbit skeletal muscle were separated by sucrose gradient centrifugation into fractions enriched in T tubules, terminal cisternae, or the longitudinal tubules of the sarcotubular system (3).

For crystallization of the Ca\textsuperscript{2+}-ATPase the membrane preparations (1 mg of protein/ml) were incubated at 2°C in 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, and 5 mM vanadate for times ranging from a few minutes to several days. The vesicle suspensions were placed on carbon-coated parlodion films, stained with freshly prepared 1% uranyl acetate (pH 4.3), and viewed with a Siemens Elmiskop I electron microscope at 60 kV accelerating voltage. Magnification was calibrated using catalase crystals negatively stained with uranyl acetate (4).

**RESULTS AND DISCUSSION**

The formation of two-dimensional membrane crystals of Ca\textsuperscript{2+} transport ATPase was observed in sarcoplasmic reticulum or purified Ca\textsuperscript{2+} transport ATPase preparations treated with 5 mM vanadate (1, 2). The crystal lattice covers the surface of a major portion (40–60%) of the vesicles present in rabbit skeletal muscle microsomes (Fig. 1). Vesicles with extensive crystallization frequently assume a cylindrical shape with an average diameter of about 600–700 Å, but crystals are also present on spherical membrane profiles. The lattice lines are oriented diagonally across the surface of the cylinder-shaped vesicles (Fig. 2), and frequently the rows of negatively stained surface particles run in pairs, separated from neighboring pairs by wider bands of negative stain. The unit cell dimensions are consistent with ATPase dimers.

Interestingly even under optimum conditions for crystallization about one-fourth to one-third of the vesicles did not develop ATPase crystals. Therefore, the question arises whether the Ca\textsuperscript{2+} ATPase molecules in vesicles derived from different regions of the sarcotubular system show differences in their propensity for the formation of membrane crystals.

Sarcoplasmic reticulum vesicles in Subfractions of Muscle Microsomes—The sarcoplasmic reticulum of skeletal muscle is divided into two morphologically distinct regions, the terminal cisternae and the longitudinal tubules (5). The Ca\textsuperscript{2+} transport ATPase is the major protein component of both types of membranes, and vesicles derived from the two regions show similar Ca\textsuperscript{2+} transport activity. However, the terminal cisternae contain large amounts of calsequestrin, and there are differences between the two membranes with respect to other proteins as well.

The transverse or T tubules are invaginations of the surface membrane. The Ca\textsuperscript{2+} transport ATPase of T tubules (3, 6) is immunologically (7–9) and kinetically (6) distinct from the Ca\textsuperscript{2+} transport ATPase of sarcoplasmic reticulum.

In crude sarcoplasmic reticulum preparations the vesicles derived from the three types of membranes are mixed in varying proportions but can be separated by sucrose gradient

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1 The abbreviation used is: EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N',N'\(^{-}\)-tetraacetic acid.
Vanadate (5 mM) induced massive crystallization of the Ca\(^{2+}\) transport ATPase in the two sarcoplasmic reticulum fractions. In preparations enriched in terminal cisternae the crystallization occurred most frequently on spherical membrane profiles (Fig. 3), while in the putative longitudinal tubule fraction the crystals were usually seen on the surface of elongated cylinders (Fig. 4).

The T tubule vesicles contained only few poorly developed crystalline regions, and even these may be attributable to a slight admixture of sarcoplasmic reticulum membranes (Fig. 5).

As the amount of the 100,000-Da protein, which is tentatively identified as the Ca\(^{2+}\) transport ATPase, does not differ widely between the various membrane fractions, the absence of significant Ca\(^{2+}\)-ATPase crystal formation in the T tubule vesicles suggests the following possible explanations.

1. The Ca\(^{2+}\) transport ATPase of T tubules, although similar in apparent molecular weight, may be structurally different from the sarcoplasmic reticulum enzyme. This possibility is supported by the immunological (7-9) and kinetic (6) differences between the Ca\(^{2+}\) transport ATPases of the T tubule and the sarcoplasmic reticulum membranes.

2. Structural constraints in the T tubule vesicles may prevent the formation of Ca\(^{2+}\)-ATPase crystals even if the ATPase has the propensity for crystallization. Such constraints may arise from the presence of proteins unique to T tubules, such as the 80,000- and 30,000-Da components (3), that may interact with the Ca\(^{2+}\) transport enzyme. Alternatively the absence of crystallization may be related to the small diameter...
of the spherical vesicles derived from T tubules. Even in sarcoplasmonic reticulum tubules covered with extensive arrays of Ca\(^{2+}\)-ATPase crystals, the lattice is usually absent on the two hemispherical ends of the tubules, as if the sharp curvature of the membrane would be incompatible with the formation of a regular crystal lattice. The crystals seen in spherical terminal cisterna membranes are usually in larger vesicles that may provide a more accommodating surface contour.

A choice between these and other alternatives requires purification and reconstitution of the T tubule Ca\(^{2+}\)-ATPase.

The Effect of Trypsin Treatment on the Formation of Membrane Crystals—As shown in Scheme 1 (10) the Ca\(^{2+}\) transport ATPase of sarcoplastic reticulum is first cleaved by trypsin at a highly susceptible site (T1) into two major fragments (A and B) (Fig. 6A). This cleavage occurs without inhibition of ATPase activity or Ca\(^{2+}\) transport (11). Fragment A has an approximate molecular weight of 57,000 and contains the active site aspartyl group that is phosphorylated by ATP (12). The apparent molecular weight of fragment B is about 52,000; its function is unknown. Further cleavage of fragment A at a vanadate concentration as low as 0.1 mM. Vanadate is an analog of inorganic phosphate that inhibits the enzyme. The 63,000-Da Ca\(^{2+}\) binding protein, which is present in sarcoplastic reticulum without vanadate treatment, B fragments are present even after 480-min exposure to trypsin (Fig. 6B), and the Ca\(^{2+}\)-ATPase crystals are also fully preserved (Fig. 7, D, E, and F). Vanadate (5 mM) does not inhibit the activity of trypsin with Azocoll as substrate (13) while the hydrolysis of phosphorylase (94,000-Da band) is actually activated (Fig. 6B).

Vanadate (5 mM) slightly decreases the rate of cleavage of the Ca\(^{2+}\)-ATPase at the T1 site (Fig. 6B) but completely inhibits the cleavage of the A fragment into the A1 and A2 subfragments at the T2 site (Fig. 6A). As a result the A and B fragments are present even after 480-min exposure to trypsin (Fig. 6B), and the Ca\(^{2+}\)-ATPase crystals are also fully preserved (Fig. 7, D, E, and F). Vanadate (5 mM) does not inhibit the activity of trypsin with Azocoll as substrate (13) while the hydrolysis of phosphorylase (94,000-Da band) is actually activated (Fig. 6B).

Vanadate is an analog of inorganic phosphate that inhibits the ATPase and Ca\(^{2+}\) transport activities of sarcoplastic reticulum by stabilizing the E2 enzyme conformation (14, 15). The inhibition of the tryptic cleavage of Ca\(^{2+}\)-ATPase at the T2 site by vanadate suggests that conversion of the enzyme from the E1 into the E2 conformation decreases the sensitivity of the T2 site to trypsin. An analogous difference between the proteolytic cleavage pattern of the E1 and E2 forms of the (Na,K)-ATPase was observed earlier by Jorgensen (16). The protection of Ca\(^{2+}\)-ATPase against trypsin was observed even at a vanadate concentration as low as 0.1 mM. Vanadate increases the rate of hydrolysis of phosphorylase by trypsin, suggesting that in this case the conformation favored by vanadate is more sensitive to proteolysis. It is reasonable to assume that vanadate alters the conformation of phosphorylase by forming a stable complex at the phosphate binding site of the enzyme. The 63,000-Da Ca\(^{2+}\) binding protein, which is a usual component of sarcoplastic reticulum (17), was not digested by trypsin under either condition.

Sucrose (1 M) partially protects the Ca\(^{2+}\)-ATPase against inhibition by 5 mM vanadate and under similar conditions prevents the vanadate-induced crystallization of the enzyme. The protection by sucrose is not attributable to contaminating calcium as it is observed at free Ca\(^{2+}\) concentrations less than 10\(^{-7}\) M; sucrose obtained from four different manufacturers gave similar results. Sucrose (1 M) also slowed the rate of

**FIG. 6. Tryptic hydrolysis of sarcoplastic reticulum.** Sarcoplastic reticulum vesicles (1 mg of protein per ml) were digested with trypsin (0.05 mg/ml) in a medium of 0.1 M KCl, 10 mM imidazole, 0.5 mM EGTA, and 5 mM MgCl\(_2\) without (A) or with 5 mM Na\(_2\)VO\(_4\) (B) at 25 °C, for times ranging from 15 s to 480 min. The digestion was stopped by the addition of soybean trypsin inhibitor (0.1 mg/ml). To samples labeled 0 trypsin and trypsin inhibitor were added together. SH, trypsin-free control sarcoplastic reticulum. M, molecular weight marker. A, sarcoplastic reticulum without vanadate treatment. B, prior to trypsin digestion the preparation was exposed to 5 mM Na\(_2\)VO\(_4\) in 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.5 mM EGTA, and 5 mM MgCl\(_2\) at 2 °C for 36 h. The samples were solubilized in a solution of 10% sodium dodecyl sulfate, 20 mM Tris-Cl pH 8.0, 2% β-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue, and applied to sodium dodecyl sulfate-polyacrylamide gradient gels (6–18%) for electrophoresis. The Coomassie blue stained gels were analyzed with an LKB Ultrascan laser densitometer coupled with a Hewlett-Packard integrator plotter (3390A). KD, kilodalton.

![Scheme 1](AC-N COOH)

![Scheme 1](AC-N COOH)

![Scheme 1](AC-N COOH)

![Scheme 1](AC-N COOH)

![Scheme 1](AC-N COOH)
FIG. 7. Effect of trypsin digestion on the formation of Ca\textsuperscript{2+}-ATPase membrane crystals. Sarcoplasmic reticulum vesicles were digested with trypsin for 30 s (left column, samples A, D, G), 30 min (middle column, samples B, E, H), or 480 min (right column, samples C, F, I), in the absence of vanadate (top and bottom rows, samples A, B, C, G, H, I) or after pretreatment with 5 mM Na\textsubscript{3}VO\textsubscript{4} (middle row, samples D, E, F) as described for
hydrolysis at the $T_1$ site of the Ca$^{2+}$-ATPase and provided significant protection of the phosphorylase against tryptic cleavage (Fig. 8). The vanadate inhibition of the tryptic cleavage at the $T_2$ site remained essentially complete even in the presence of 1 M sucrose (Fig. 8).

The mechanism of the effect of sucrose is unknown, but it is likely to involve changes in the conformation of the enzyme either by interaction of sucrose with the hydrophilic portion of the ATPase molecule or by reducing the range of motions available to the enzyme due to increase in medium viscosity.

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Fig. 8. Effect of trypsin on sarcoplasmic reticulum in the presence of 1 M sucrose. The experiment was carried out as described in Fig. 6, A and B, except that 1 M sucrose was added to the digestion medium. A, no vanadate; $B$, 5 mM Na$_2$VO$_4$ was added 36 h (2°C) prior to trypsin digestion to induce crystallization of the Ca$^{2+}$-ATPase. KD, kilodalton.
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