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Two-dimensional Arrays of Proteins in Sarcoplasmic Reticulum and Purified Ca^{2+}-ATPase Vesicles Treated with Vanadate*

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Two-dimensional crystalline arrays of the Ca^{2+} transport ATPase were observed by negative staining in sarcoplasmic reticulum membranes and purified Ca^{2+} transport ATPase vesicles treated with Na_3VO_4. The formation of the Ca^{2+}-ATPase crystals was inhibited by Ca^{2+}.

The Mg^{2+} + Ca^{2+} activated ATPase of sarcoplasmic reticulum can be visualized by negative staining with uranyl acetate or K^+-phosphotungstate in the form of 40 Å diameter surface particles (1), and by freeze fracture as 85 Å diameter intramembranous particles (2), which are more numerous in the cytoplasmic than in the luminal fracture face.

In sarcoplasmic reticulum and in reconstituted ATPase vesicles, the average density of the 40 Å surface particles is greater than that of the 85 Å intramembranous particles; this observation led to the suggestion that the 85 Å particles represent oligomers of several (probably four) Ca^{2+}-ATPase molecules (3-5). While the assessment of the functional significance of ATPase-ATPase interactions is not yet completed (6), the existence of these interactions is now generally accepted.

We now report the regular formation of extensive two-dimensional "crystalline" arrays of 40 Å surface particles in sarcoplasmic reticulum vesicles treated with vanadate, under conditions similar to those described by Skriver *et al.* (7) and Hebert *et al.* (8) on Na^+,K^+-ATPase. The crystalline arrays cover the entire surface of a major portion (30-50%) of the vesicles present in sarcoplasmic reticulum or purified Mg^{2+} + Ca^{2+} activated ATPase preparations, and are assumed to reflect interactions between ATPase molecules. The observations provide the basis of a new approach to the study of the structure of Ca^{2+} transport ATPase within its native environment.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles prepared as described by Nakamura *et al.* (9) were used for the isolation of purified Mg^{2+} + Ca^{2+} activated ATPase (10). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the assay of ATPase activity were carried out as described earlier (9, 11).

For negative staining, the vesicle suspensions (1 mg of protein/ml) were placed on carbon-coated parlodion films and stained with freshly prepared 1% uranyl acetate (pH 4.3) or K^+-phosphotungstate (pH 4.3 or 7.0). The specimens were viewed with a Siemens Elmiskop I electron microscope at 60 kV accelerating voltage. For magnification calibration, catalase crystals negatively stained with 1% uranyl acetate were used (12).

RESULTS

Two-dimensional arrays of protein crystals develop upon treatment of rabbit skeletal sarcoplasmic reticulum vesicles at 2 °C with 5 mM Na_3VO_4 in the presence of 0.1 M KCl, 10 mM imidazole, pH 7.4, and 0.5 mM EGTA[1] (Figs. 1, 2). Mg^{2+} (5 mM) promoted the crystallization. The crystal lattice is best visualized by negative staining with uranyl acetate at pH 4.3 (Fig. 1). A slightly disorganized pattern was observed by negative staining with K^+-phosphotungstate at pH 4.3 or 7.0 (Fig. 2).

The development of crystalline arrays is apparent on portions of the membrane surface already after a few hours of incubation with 5 mM Na-vanadate, and within 1-2 days the entire surface of a large fraction (30-50%) of the vesicles is covered with protein crystals (Fig. 1). At 0.1 mM vanadate concentration, substantial crystallization required several weeks of incubation, although the inhibition of ATPase activity by 0.1 mM vanadate was essentially complete after 15 min (Fig. 3).

A similar two-dimensional array of protein crystals was induced by 5 mM Na_3VO_4 in purified Ca^{2+} transport ATPase vesicles (Fig. 4), and in sarcoplasmic reticulum vesicles washed with 0.1 mg of deoxycholate/mg of protein to reduce extrinsic proteins (not shown). The rate of appearance of the crystal lattice is slower in purified ATPase and in deoxycholate washed vesicles than in native sarcoplasmic reticulum membranes.

The Mg^{2+} + Ca^{2+} activated ATPase represents 70-80% of the protein content of sarcoplasmic reticulum and about 95% of the protein content of purified ATPase. Therefore, it is reasonable to assume that the two-dimensional crystals are formed by interaction between Ca^{2+}-ATPase molecules, although some contribution by other proteins (for example proteolipids) cannot be entirely excluded.

The crystallization of the Ca^{2+}-ATPase was prevented by 0.45 mM $CaCl_2$ in an incubation medium containing 0.5 mM EGTA and 5 mM Na_3VO_4 (Fig. 5*A*); therefore, μM free [Ca^{2+}] inhibits the induction of protein crystals by vanadate. This is in accord with earlier findings (13-15) that low concentration of Ca^{2+} protects the Ca^{2+}-ATPase of sarcoplasmic reticulum from inhibition by vanadate (Fig. 3). Addition of Ca^{2+} (0.45-4.5 mM) to sarcoplasmic reticulum, previously incubated for 4 days with 5 mM vanadate in a calcium-free medium, caused within 4 h the "cracking" and disappearance of the previously formed crystal lattice (Fig. 5*B*). At lower [Ca^{2+}], small clusters of negatively stained particles are still present (Fig. 5*B*), which

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[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

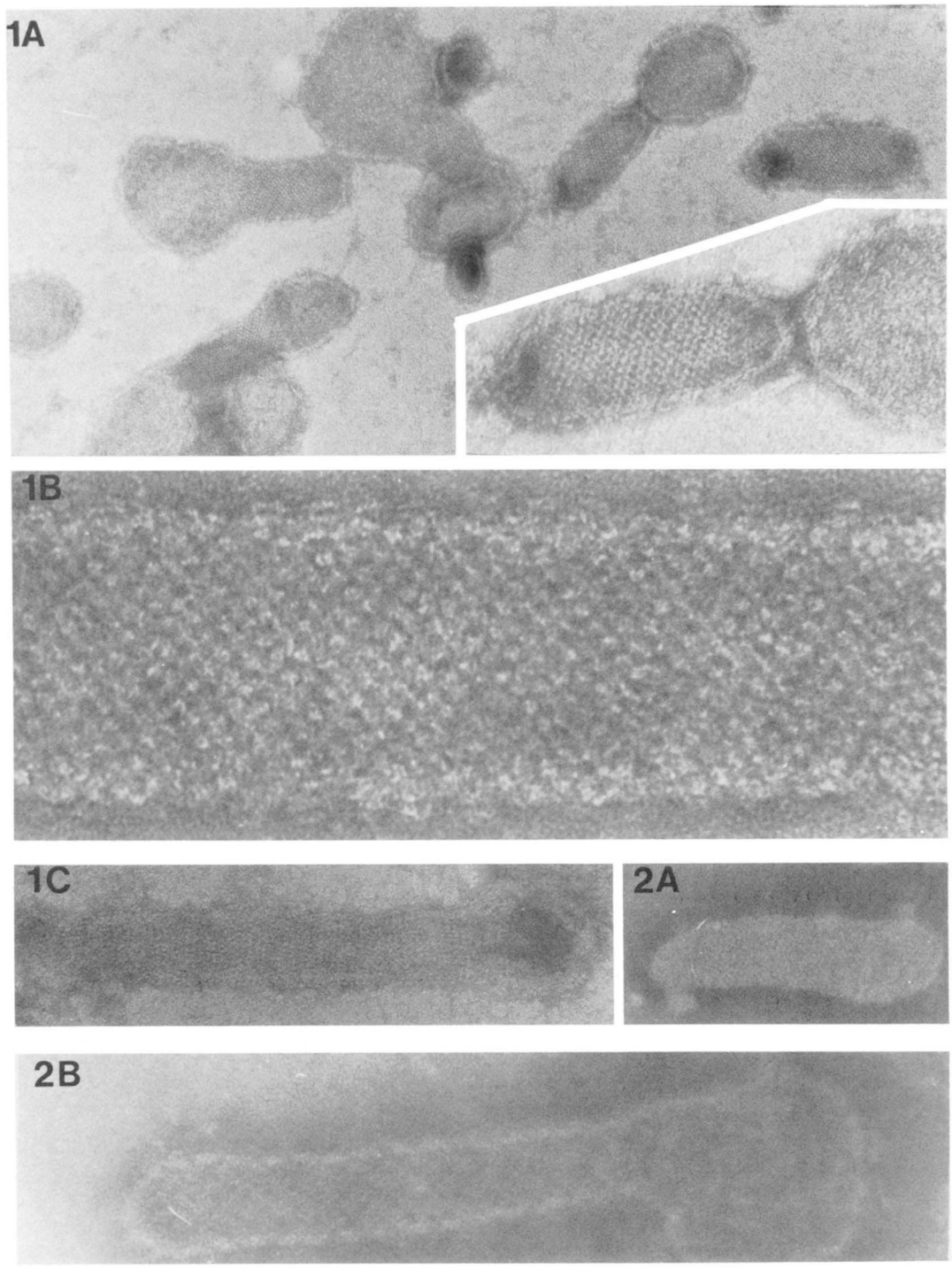

FIG. 1. **Membrane crystals of Ca^{2+} transport ATPase in sarcoplasmic reticulum vesicles stained with uranyl acetate.** Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.5 mM EGTA and 5 mM Na_3VO_4 for 72 h at 2 °C, followed by negative staining with 1% uranyl acetate (pH 4.3). Magnification: *A*, × 111,636; *A insert*, × 279,089; *B*, × 624,713. *Sample C* was incubated with 1 mM Na_3VO_4 and 0.02% Na azide for 6 weeks. Magnification, × 196,454. Most vesicles had the regular diagonal crystal lattice and their protein composition did not differ significantly from fresh sarcoplasmic reticulum.

FIG. 2. **Membrane crystals of Ca^{2+} transport ATPase stained with K^+-phosphotungstate.** Sarcoplasmic reticulum vesicles were treated with 5 mM Na_3VO_4 as described in Fig. 1, followed by negative staining with 1% K^+-phosphotungstate at pH 7.0 (*A*) or 4.3 (*B*). Magnification: *A*, × 160,605; *B*, × 304,240.

are similar to the particle clusters seen occasionally in reconstituted ATPase vesicles (5). The inhibition of crystallization by μM [Ca^{2+}] constitutes further evidence that the lattice is formed by interaction between transport ATPase molecules. The cracking of vanadate-induced ATPase crystals by Ca^{2+} was not accompanied by a reversal of the inhibition of Ca^{2+}-ATPase activity.

Vesicles with extensive crystallization frequently assume an elongated cylindrical shape of about 500–700 Å in diameter with variable length; these emerge from spherical membrane

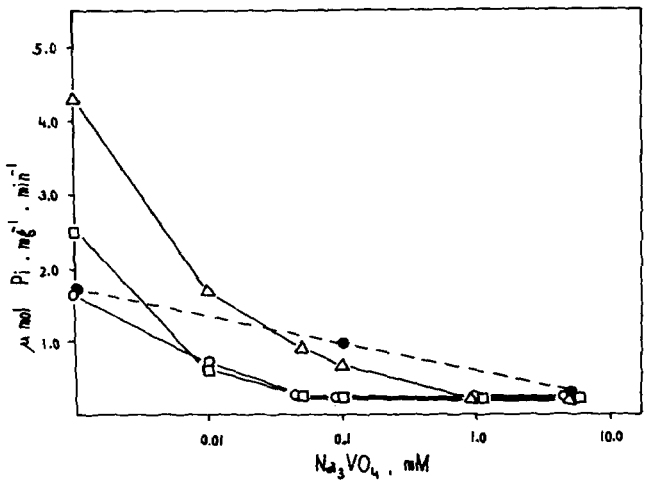

FIG. 3. **Effect of vanadate on ATPase activity.** The preparations were incubated in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.5 mM EGTA, 5 mM $MgCl_2$, 5 μg/ml of A23187, and Na_3VO_4 at concentration indicated in the *abscissa* at 25 °C for 15 min. Protein concentration, 50 μg/ml. ATPase reaction was started with the addition of 0.45 mM $CaCl_2$ and 5 mM ATP; aliquots were taken after 10 and 20 min for the assay of inorganic phosphate. △——△, purified ATPase; ○——○, rabbit sarcoplasmic reticulum; □——□, rabbit sarcoplasmic reticulum washed with 0.1 mg of deoxycholate/mg of protein; ●——●, rabbit sarcoplasmic reticulum with 0.45 mM Ca throughout incubation with Na_3VO_4.

profiles with a random distribution of surface particles (Fig. 1*A*). The crystallization of the Ca^{2+} transport ATPase may confer the cylindrical shape upon the vesicles. In those instances where crystallization occurred in ellipsoidal vesicles (Fig. 1*A*, *inset*), the regular lattice was usually confined to the central portion of the surface, and the two ends, with maximum curvature, showed random distribution of surface particles.

Usually the lattice lines run diagonally across the cylinder-shaped vesicles with an inclination of about 45° to the longitudinal axis of the cylinder (Fig. 1, *A* and *B*, and Fig. 4, *C* and *D*). The approximate dimensions of the lattice, based on visual inspection of electron micrographs, are similar to the unit cell dimensions of the Na^+,K^+-ATPase crystals described by Hebert *et al.* (8). Optical diffraction studies are in progress to determine the actual spacing of the unit cell.

In one case, the lattice lines were perpendicular to the cylinder axis and the unit cells were approximately square shaped (Fig. 1*C*). This isolated pattern may arise from distortion of the vesicle during negative staining, but could indicate multiple crystal forms, contaminating membranes, or one sided staining.

Frequently, rows of negatively stained particles are arranged in pairs forming ladder-like structures which are separated from neighboring pairs by wider bands of negative stain (Figs. 1*B* and 4*D*). As a result, the distance of separation between rows of particles alternates. Isolated "ladder"-like linear polymers frequently occur on spherical vesicles (Fig. 6) and later associate laterally to form the extended lattice with the appearance of cylindrical profiles. Therefore, the different patterns of crystallization reported here probably reflect stages of development of the mature crystal lattice. We are now attempting to demonstrate the existence of distinct crystal forms by forcing the ATPase molecules into different conformations.

So far, all crystalline preparations are enzymatically inactive. The transport activity of the Ca^{2+}-ATPase with the associated changes in enzyme conformation may be incompatible with the formation of an extended crystal lattice.

DISCUSSION

Although small regular arrays of 40 Å particles were occasionally observed in sarcoplasmic reticulum fragments and reconstituted ATPase vesicles negatively stained with K^+-phosphotungstate (5, 16, 17), the formation of extended sheets of two-dimensional crystals of the Ca^{2+} transport ATPase requires treatment with relatively high concentration of Na_3VO_4.

Vanadate apparently binds to the low affinity phosphate-binding site of the enzyme, which is exposed only in the absence of Ca^{2+} (14); as a result, the stable E_2-vanadate form accumulates,with inhibition of the ATPase activity and of the phosphorylation of the enzyme by inorganic orthophosphate (14).

The same crystal lattice was observed after vanadate treatment of three types of preparations: (*a*) native sarcoplasmic reticulum vesicles; (*b*) sarcoplasmic reticulum vesicles washed with 0.1 mg of deoxycholate/mg of protein to remove extrinsic membrane proteins; and (*c*) purified ATPase vesicles which contained only the Ca^{2+} transport ATPase, together with a small amount of proteolipid.

Therefore, it is reasonable to assume that the observed crystals arise from interaction between Ca^{2+}-ATPase molecules. Surprisingly, the rate of crystallization was definitely slower in deoxycholate washed sarcoplasmic reticulum vesicles and in purified ATPase preparations than in native sarcoplasmic reticulum membranes.

This observation may have several implications. 1) Washing of the membranes with deoxycholate solutions or the solubilization of the ATPase with deoxycholate may sufficiently alter the disposition of the ATPase molecules in the membrane to hinder their insertion into the crystal lattice. It is known that the asymmetric distribution of intramembranous particles detectable by freeze fracture is lost during solubilization of the Ca^{2+} ATPase. 2) Changes in the lipid composition or the presence of residual detergent may hinder the association between ATPase molecules. 3) Extrinsic proteins of the sarcoplasmic reticulum, which are removed during the washing or purification process, may play some ordering function during the crystallization. There is no evidence so far that the Ca^{2+} transport ATPase interacts with any of the extrinsic proteins of sarcoplasmic reticulum.

The formation of two-dimensional crystals apparently requires that most of the ATPase molecules assume the E_2 conformation. Ca^{2+} in a concentration sufficient to saturate the high affinity Ca^{2+} binding site of the enzyme prevents the formation of ATPase crystals and "cracks" the crystals that were formed previously. It is well established that Ca^{2+} in low concentration protects the Ca^{2+} transport ATPase activity against inhibition by vanadate (13, 14), presumably by stabilizing the E_1 enzyme form. Surprisingly, the cracking of ATPase crystals by calcium is not accompanied by reactivation of the ATPase activity. Furthermore, inhibition of ATP hydrolysis by 0.1 mM Na_3VO_4 is essentially complete in 15–30 min, while the appearance of ATPase crystals requires several weeks. By raising the vanadate concentration to 5 mM, the time required for crystallization is reduced to 1–2 days without further inhibition of ATPase activity. These observations imply that inhibition of ATPase activity by vanadate is a required but not sufficient condition for crystallization to occur. Vanadate binding to additional low affinity sites may promote the stabilization of the conformation that is required for ATPase-ATPase interaction.

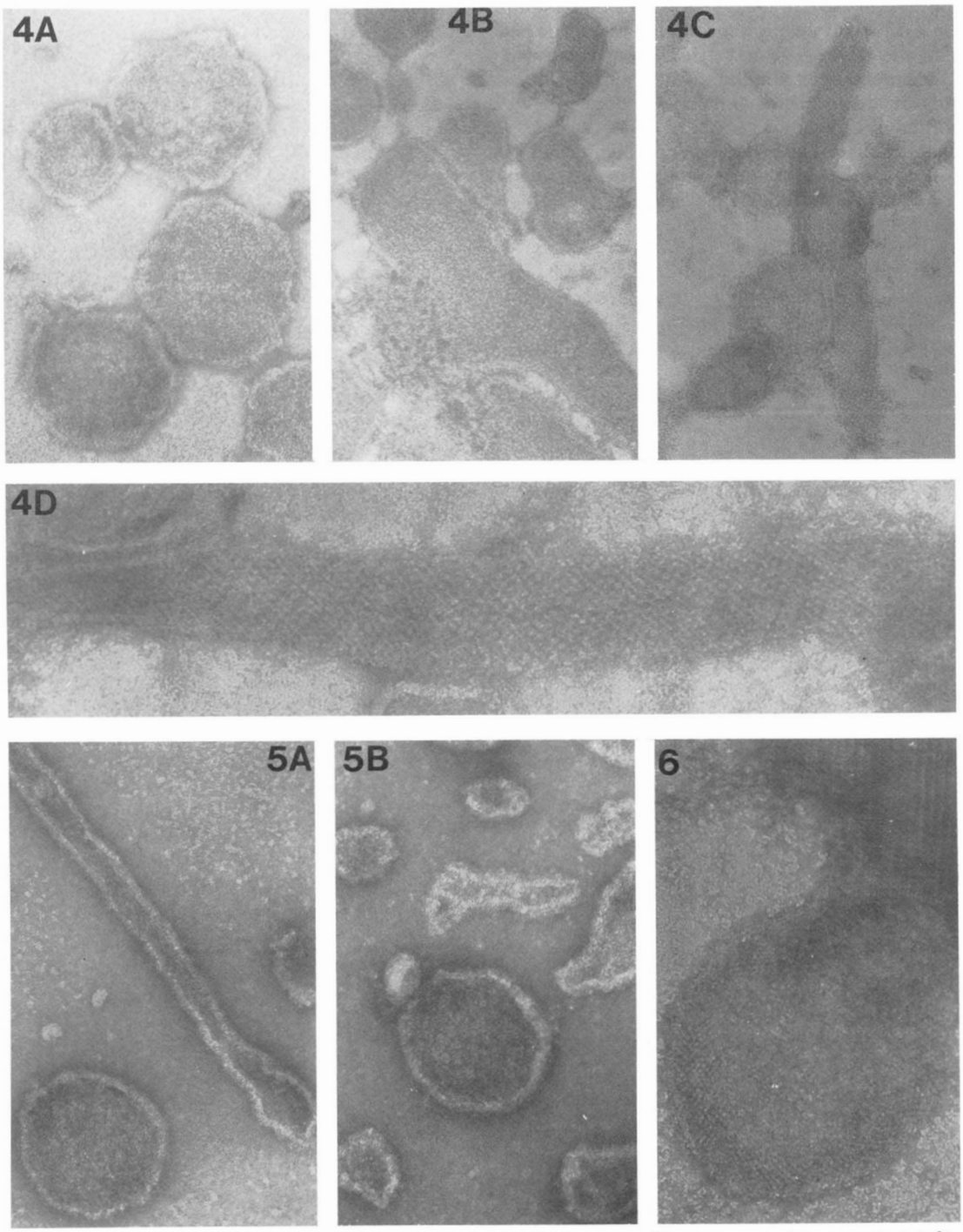

FIG. 4. **Membrane crystals of Ca^{2+} transport ATPase in purified Ca^{2+}-ATPase vesicles.** Purified Ca^{2+}-ATPase vesicles were incubated in 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.5 mM EGTA, and 5 mM Na_3VO_4 for 4 h (*B*), 20 h (*C*), and 96 h (*D*) at 2 °C. *Sample A* served as control without vanadate. Magnification: *A*, × 160,605; *B*, × 119,695; *C*, × 119,695; *D*, × 274,872. Negative staining with uranyl acetate.

FIG. 5. **Effect of Ca^{2+} on Ca^{2+}-ATPase membrane crystals.** Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.5 mM EGTA, and 5 mM Na_3VO_4 for 4 days at 2 °C, with Ca^{2+} added as follows. In *A*, 0.45 mM Ca was present from the outset. To *sample B* Ca^{2+} was added to a final concentration of 0.45 mM during the final 4 h of incubation. At the time of Ca^{2+} addition, membrane crystals like those shown in Fig. 1 were present. In sample *A*, crystals never formed, while in *B* the crystalline arrays were destroyed by Ca^{2+} within 4 h. Magnification: *A*, × 119,695; *B*, × 119,695. Negative staining with uranyl acetate.

FIG. 6. **The formation of Ca^{2+}-ATPase crystals.** Sarcoplasmic reticulum vesicles were incubated with 5 mM Na_3VO_4 for 20 h otherwise under conditions like in Fig. 1. Prior to staining with uranyl acetate, catalase crystals were added to the sample as magnification standard (*upper right corner*; line spacing 88 Å). Magnification, × 160,605.

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