Crystalline Structure of Sarcoplasmic Reticulum from Scallop

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ABSTRACT Negatively stained sarcoplasmic reticulum from the scallop *Placopecten magellanicus* presented a variety of crystalline forms, the most common being tubular structures. These were characterized by paired rows of morphological units, spaced at $\sim$120 Å, running diagonally across the tubules. The orthogonal unit cell (120 $\times$ 55 Å) contained two units, related by a twofold axis, which probably represented the part of the Ca^{2+}-ATPase molecule projecting from the outer surface of the membrane.

Fragmented sarcoplasmic reticulum (FSR)[1] prepared from rabbit skeletal muscle and membranes reconstituted in vitro from SR Ca^{2+}-ATPase have been studied extensively by electron microscopy and x-ray diffraction. Negatively stained rabbit FSR shows vesicles of average diameter 0.15 μm (1) covered on their outer surface with projections $\sim$60 Å long and 30–40 Å diam which represent part of the Ca^{2+}-ATPase molecule (2–5). Freeze-fracturing studies show 85-Å-diam particles embedded in the concave fracture face, also interpreted as the Ca^{2+}-ATPase (6). A ratio of 3–4 surface projections per 85-Å inner particle has led to the suggestion that the ATPase has a tetrameric structure in the membrane (7). X-ray studies have confirmed the asymmetrical distribution of mass across the SR membrane and that the diameter of the projections is $\sim$35 Å (8–10). Rabbit SR vesicles do not, however, show sufficient order to allow a detailed structure analysis, although treatment with vanadate has recently been reported to cause an ordered surface array of subunits (11).

Scallop SR consists of cisternae connected by tubular elements, and is closely associated with the sarcolemma (12). We have prepared FSR from the cross-striated adductor muscle of *Placopecten magellanicus* and examined it by electron microscopy. Negatively stained vesicles showed a crystalline appearance, and images of one class of tubular SR have been analyzed by two-dimensional reconstruction techniques.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum Vesicles: Live sea scallops (*Pecten magellanicus*) were obtained from the Marine Biological Laboratory, Woods Hole, MA. Strips of the striated adductor muscle were excised from the animal and chemically skinned in a buffered solution containing 0.05–0.1% wt/vol saponin, 100 mM NaCl, 8 mM MgSO₄, 5 mM EGTA, 5

mM ATP, and 10 mM sodium phosphate, pH 7.0, for 3 h. The muscle bundles were then thoroughly washed with the same buffer without saponin (relaxing medium) and minced finely to give an approximately 7%-wt/vol suspension. (The saponin improves penetration by relaxing medium: It can easily be omitted if necessary, when the preparations have an identical appearance and activity but are in lower yield.) The procedure was carried out at 4°C. The suspension was then blended in a Sorvall omni-mixer (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT) (2 × 1 s at half speed). Gross debris was removed by centrifuging at 1–2,000 g in a clinical centrifuge for 3–4 min. The supernatant was spun twice at 8,000 g for 15 min in a Sorvall RC5 centrifuge and then at 44,000 g for ½ h. The pellet was extracted twice with 0.6 M NaCL at pH 7.2 and resuspended in 0.25 M sucrose, 1 mM ATP, 1 mM MgCl₂, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 7.2. This suspension was layered onto a discontinuous gradient composed of 8 ml 1.1 M sucrose, 8 ml 1.2 M sucrose, and 8 ml 1.45 M sucrose, in 1 mM ATP, 1 mM MgCl₂, 60 mM sodium phosphate, pH 7.2, and spun at 55,000 g (av.) for 1.25 h in a Beckman SW25.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). The band at the 1.1–1.2 interface was collected and diluted with 1 mM ATP, 1 mM MgCl₂, and 20 mM sodium phosphate at pH 7.2. After centrifugation at 44,000 g for ½ h, the pellet was resuspended in 0.25 M sucrose, 1 mM ATP, 1 mM MgCl₂, and 20 mM Na phosphate, at pH 7.2.

Enzyme Assays: Ca^{2+}-ATPase was determined by a coupled assay (13) in the presence of 1 mM EGTA or 0.3 μM free Ca^{2+}. Oligomycin in ethanol was added to 10 μg ml⁻¹, and ethanol alone to 0.2% (vol/vol) in control assays.

Na^+, K^+-ATPase was measured as described previously (14), in the presence and absence of 1 mM ouabain. Calcium uptake was measured by the Millipore filtration method (15). Free Ca^{2+} concentrations were calculated using an iterative procedure that takes into account all possible metal ion-chelator intermediates (16). Succinic dehydrogenase was estimated by the succinate tetrazolium reductase method (17).

Protein concentrations were determined by the method of Bensodoun and Weinstein (18). Protein composition was analyzed by SDS gel electrophoresis on Weber Osborn gels (19). These were stained with Coomassie Blue R.250 and scanned at 560 nm in a linear scanner (Gilford Instrument Laboratories Inc., Oberlin, OH). Areas under the peaks were determined with an electronic planimeter (Numonics, Co. Lansdale, PA).

Electron Microscopy and Image Analysis: One drop of freshly prepared SR suspension was placed for 20 s onto a 400-mesh copper grid coated with a thin carbon film. The grid was washed dropwise with relaxing medium, followed by 100 mM ammonium acetate. The preparation was stained with three drops of 2% uranyl acetate aqueous solution. Excess stain was drained with filter paper. Low temperatures were maintained throughout this procedure. FSR unidirectionally shadowed with platinum was obtained, as described

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[1] *Abbreviation used in this paper:* FSR, fragmented sarcoplasmic reticulum.

by Castellani et al. (20), in an evaporator (Edwards High Vacuum, Grand Island, NY) at a 45° angle.

Electron micrographs were recorded in a Philips 301 electron microscope, fitted with an anticontamination device and calibrated using tropomyosin Mg^{2+} paracrystals. Micrographs were screened with a surveying optical diffractometer (21). Images giving the clearest diffraction patterns were selected (22) and digitized on a raster corresponding to ~6 Å in the image. Two-dimensional processing was carried out as described by Baker and Amos (22).

RESULTS

Membrane Composition and Activities

Polyacrylamide SDS gels of purified scallop FSR showed a major component with M_r = 106,000, the same as rabbit SR Ca^{2+}-ATPase (e.g., reference 7). This accounted for ~85% of the protein in the membrane preparations (Fig. 1). The other proteins present in significant amounts had molecular weights of 59,000, 26,000, and 20,000, although none of these accounted for >5% of the total protein. A small contamination of actin (M_r = 42,000; <1% total protein) was sometimes present. These preparations had a Ca^{2+}-ATPase activity 80–90% insensitive to oligomycin, with half-maximal activation at 0.1–0.2 μM free Ca^{2+}. Typical enzyme activities are shown in Table I. Ca^{2+}-ATPase activities of scallop FSR were high compared with those of rabbit. This may have been due to leakiness of the vesicles since calcium uptake, although present, was poor.

Electron Microscopy

Negatively stained preparations of FSR showed a variety of crystalline forms in the electron microscope, the most common being tubular structures (Fig. 2). These sometimes branched and often originated in sacs or cisternae. Spherical vesicles were also observed showing projections ~60 Å long emerging from the outer surface. Uncollapsed tubules were filled with stain and appeared to have a fringe of projections at the edges, similar to that of spherical vesicles. Their diameters ranged between 450 and 700 Å. Tubules collapsed onto
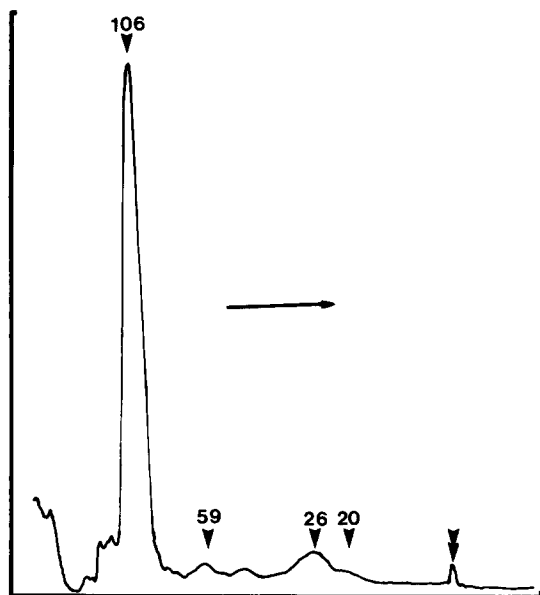

Figure 1 Densitometry trace of SDS polyacrylamide gel (7.5%) of scallop adductor FSR. The peaks corresponding to M_r 106,000, 59,000, 26,000, and 20,000 are indicated (arrows). Double arrows mark dye front. Tail-less arrow indicates direction of migration in the gel.

TABLE I

Typical Enzyme Activities of Scallop FSR

	Ca^{2+}-ATPase (μmol/min/mg)		
	+1 mM EGTA	+0.3 μM free Ca^{2+}	Oligomycin-insensitive Ca^{2+} ATPase
+0.2% vol/vol EtOH	0.163	5.287	4.042
+10 μg/ml oligomycin	0.124	4.166	—
	Na^+,K^+-ATPase (μmol/min/mg)		
	No ouabain	+1 mM ouabain	Ouabain-sensitive ATPase
	0.118	0.023	0.095
	Succinic dehydrogenase (nmol/min/mg)		
	2.21		
	Ca^{2+} uptake (μmol/gm/15 min)*		
	No ATP	+5 mM ATP	Active uptake
	2.4 (av. 6)	60.7 (av. 6)	58.3

Some contamination with sarcolemma is indicated by the presence of ouabain-inhibitable Na^+,K^+-ATPase but cannot be quantitated because the specific activity of scallop adductor sarcolemma is not known. The succinic dehydrogenase activity is comparable to that of rabbit skeletal muscle FSR (e.g., Duggan [26]).
* Determined in 0.1 M KCl, 5 mM potassium oxalate, 5 mM $MgCl_2$, ± 5 mM ATP, 0.1 mM $^{45}CaCl_2$, 40 mM Tes, pH 7.0, at 25°C in a volume of 1.1 ml containing 100 μg of membrane protein.

the grid were uniformly stained and varied in width between 750 and 1,200 Å. This variability of diameters was probably due to intrinsic differences in tubule sizes as well as to a variable degree of flattening when they were laid on a grid. The superimposed image of the front and back half of the tubule, however, was similar in both uncollapsed and collapsed forms. All of them were characterized by a pattern of striations running diagonally across the tubules with a repeat of ~120 Å. There were two apparently identical rows of morphological units, 60 Å apart with different amounts of stain between alternate rows. Their size was comparable to that of the projections observed more easily on the surface of spherical vesicles and on negatively stained rabbit SR.

FSR unidirectionally shadowed with platinum showed features from only the front half (away from the support film) of the tubules (Fig. 3). Pronounced striations running diagonally across the tubules, seen more clearly in the collapsed forms, repeated at ~120 Å and wound in a right-handed manner. Under favorable orientation of the tubules to the direction of the metal stream, a finer midway striation was observed.

Images of negatively stained collapsed tubules and their computed diffraction patterns are shown in Fig. 4. The pattern of reflections (Fig. 4, b and d) could be indexed by two orthogonal lattices (1/120 × 1/55 Å^{-1}) related by a vertical mirror line. The two lattices were produced by the superimposed front and back halves of the tubules. The variation in width of the tubules led to different relative orientations of the front and back lattices, and this sometimes caused some of their reflections to overlap. An example of a diffraction pattern where reflections from the two lattices overlap is shown in Fig. 4b. The reflections from the two lattices are well resolved in Fig. 4d. Diffraction patterns from images of
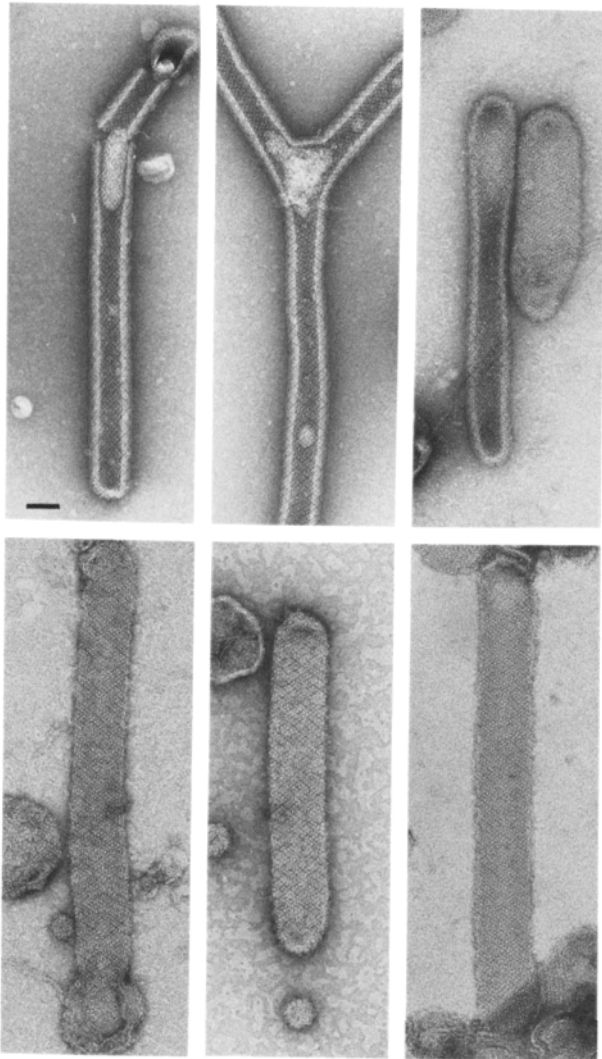
FIGURE 2   Electron micrographs of negatively stained scallop FSR. The top three images show tubules filled with stain. The bottom row shows tubules collapsed onto the grid. Dark striations (stain) alternate with rows of morphological units (white) running diagonally across the tubules. Bar, 0.5 μm. × 82,000.

substantial morphological similarity to rabbit SR.

The scallop muscle was initially disrupted in a relaxing medium containing EGTA in order to minimize the amount of homogenization that may disrupt the membranes. Duggan and Martonosi (23) showed that treatment of rabbit SR with EGTA increases the Ca^{2+}-ion permeability of the vesicles, and this may explain the high ATPase activities and low Ca^{2+} uptake shown by scallop FSR preparations. The crystalline appearance of these negatively stained membranes did not seem to be induced by the staining since thin sections of fixed preparations showed features similar to those of negatively stained images (data not shown). In addition, unidirectionally shadowed vesicles showed a highly ordered surface structure very similar to that in negatively contrasted images.

The question arises as to the relationship of the morphological units seen in the reconstructed image and the Ca^{2+}-ATPase. The 106,000-mol-wt protein seen on SDS gels of purified scallop FSR represented ~85% of the membrane protein and appeared to correspond to the Ca^{2+}-ATPase. Like rabbit SR, scallop SR had a fringe of projections 30–40 Å wide and ~60 Å long: only the 106,000-mol-wt protein was present in sufficient quantity to account for these. The units seen in the reconstructed image had an approximate diameter of 36 Å. If it were assumed that they corresponded to the 60-Å-long projections and penetrate across the membrane, they would have an approximate cylindrical shape of 36 × 100 Å with a volume of 1.02×10^5 Å^3, quite close to the anhydrous volume of a protein with molecular weight 106,000 and partial specific volume 0.73 ml/gm, 1.28×10^5 Å^3. Thus, the units seen in the reconstructed image corresponded to single ATPase molecules, rather than a domain of the ATPase. The arrangement of staggered pairs of units related by a twofold axis might suggest a possible dimeric functional grouping of ATPase molecules; however, the relationship of the strong and weak contacts between the morphological units to a possible oligomeric organization of the ATPase molecules will require further studies, now in progress, using low-dose electron microscopy.

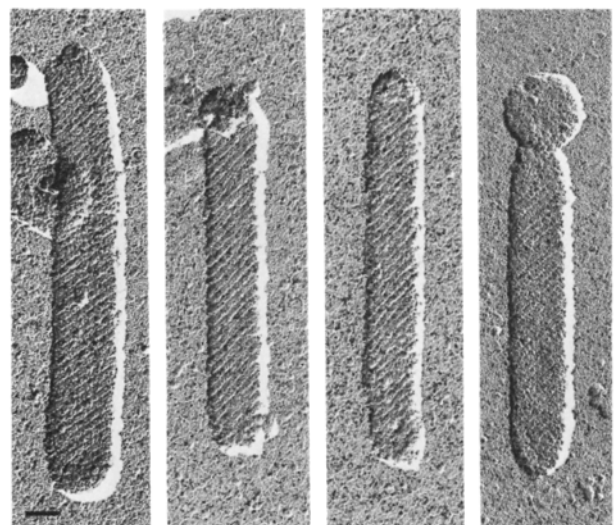Scallop FSR membranes strongly resemble vanadate-

unidirectionally shadowed tubules determined the assignment of reflections to the front or back side.

Negatively stained tubules that showed nonoverlapping diffraction patterns from front and back sides have been used for two-dimensional reconstruction (an example is shown in Fig. 5). The two sides, analyzed separately, appeared very similar. The back side, however, seemed consistently better preserved, probably owing to the support provided by the stain. The reconstructed image (Fig. 5c) shows the unit cell oriented with the short axis at an angle of ~55° to the tubule axis. It contained two morphological units ~36 Å diam related by a twofold axis. The strongest contact between units gave rise to rows in a direction parallel to that of the short unit-cell axis. Adjacent rows were staggered by about half a unit. Pairing of the rows appeared to be due to a weaker contact between staggered units.

DISCUSSION

The membranes described in this paper can be identified as SR on the basis of enzyme activity, composition, and their

FIGURE 3   Electron micrographs of SR tubules undirectionally shadowed with platinum. Prominent 120-Å striations are seen. The image on the far right also shows finer striations lying between the major striations, caused by exclusion of the metal. Bar, 0.5 μm. × 82,000.
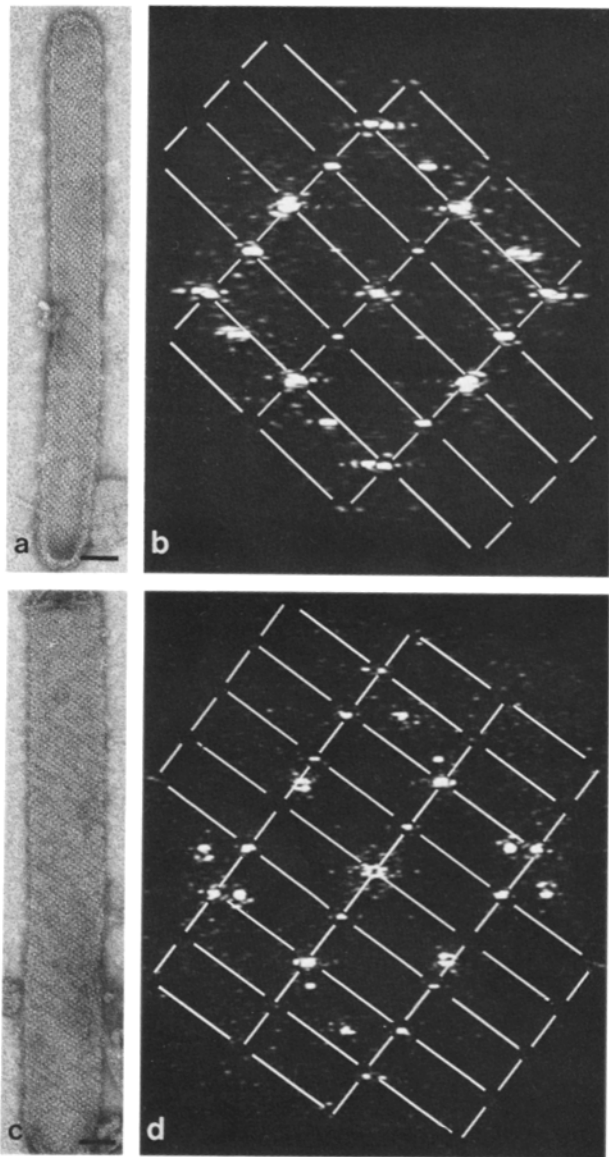
FIGURE 4 Electron micrographs of collapsed SR tubules with width of 700 Å (a) and 1,200 Å (c) and their computed diffraction patterns (b and d). The overlay in b and d shows the orthogonal lattice which fits the reflections from the back side of the tubules. Note that in b some of the reflections from near and far sides superimpose whereas in d they are well resolved. Bar, 0.5 μm. × 102,000.
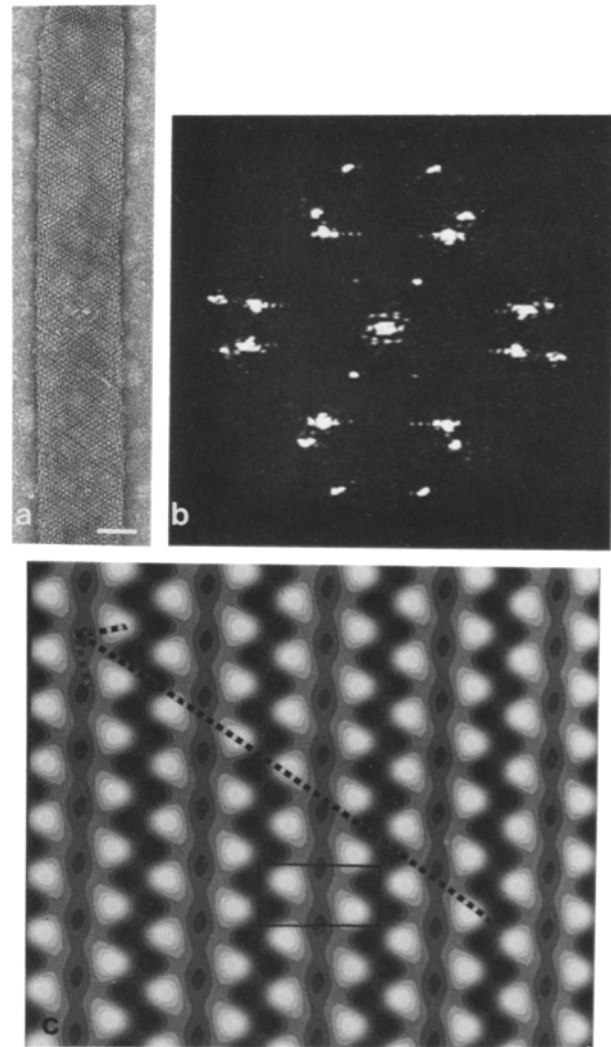

FIGURE 5 Electron micrograph (a) and its computed diffraction pattern (b) of a collapsed tubule. c shows the two-dimensional reconstructed image of near and far side averaged. The broken arrow indicates the tubule axis. The unit cell (boxed) measures 120 × 55 Å. The angle between the tubule axis and the short unit cell axis is ~55°. The protein is white as in the original micrograph (a). Bar, 0.5 μm. × 102,000.

treated rabbit SR (11), although the scallop preparation is enzymatically active and is not exposed to vanadate at any point. The same highly ordered membranes are seen in homogenates of the muscle made in the absence of ATP (e.g. 1 mM EGTA, 45 mM Tes, pH 7.5), so that vanadate contamination of the ATP is not a factor. Interestingly, the scallop SR also shows resemblances to two-dimensional crystalline arrays of Na^+, K^+-ATPase induced by vanadate (24, 25), suggesting that this type of ordered array of subunits may be a general feature of membrane transport enzymes.

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