

Micromorphometric Evaluation of Changes in Symmetry of Sarcoplasmic Reticulum Membranes Induced by Vanadate

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Electron micrographs of light sarcoplasmic vesicles fixed with glutaraldehyde and osmium tetroxide followed by contrasting with uranyl acetate and lead citrate have been evaluated by registering their membrane profiles with a microdensitometer. The asymmetric arrangement of the two layers of the vesicular membrane could be ascertained by demonstrating a ratio of 1.5 for the thickness of the outer *versus* the inner membrane layer which is in general agreement with the proposed protein structure of the calcium transport enzyme. Treatment of the vesicles with low concentrations of vanadate (0.1 mM) results in a significant lowering of the symmetry ratio by 20% by reducing mainly the thickness of the outer membrane leaflet. Removal of the membrane lipids by treating the vesicles with phospholipase A₂ and bovine serum albumin diminishes the membrane surface by 50% resulting in a significant increase of both the membrane thickness and the asymmetry ratio by 30 and 12% respectively. The vanadate induced reduction of membrane asymmetry is accentuated after delipidation indicating that the membrane lipids are not essential for the asymmetric appearance of the native membrane. The stability of the spherical form of the vesicles to delipidation implies that the transport molecules are conically shaped allowing strong mutual interactions. At a measured height of the molecule of 80 Å in the membrane, the vanadate induced change in symmetry would be brought about by compensatory changes of less than 3 Å of the outer (35 Å) and the inner (25 Å) diameter of the cone.

Introduction

In recent years, the calcium transport system of the sarcoplasmic reticulum has been explored intensively and characterized with respect to its transport dynamics, its energetics as well as its protein structure [1–3]. In spite of the detailed picture which has evolved our knowledge concerning the energy-dependent changes connected with the vectorial ion movements has remained fragmentary. This problem has been analyzed most extensively by Blasie *et al.* [4–8] by time resolved X-ray studies on sarcoplasmic reticulum vesicles oriented in multilayers. During the short pulses of transport activity initiated by liberating ATP from its caged analog small quantities of calcium were moved from the extra into the intravesicular space. This transport activity was found to be related to temporal changes of the preparations' X-ray diffraction pattern which were interpreted as being

caused by distributing 8% of the total mass of the transport protein from the outer to the inner layer of the sarcoplasmic reticulum membrane. These changes lead to a reduction of the asymmetry of the sarcoplasmic reticulum membrane.

The asymmetric arrangement of the membrane constituents was quite early recognized by electron microscopy as well as by X-ray diffraction studies [9, 10]. The sarcoplasmic reticulum membranes are especially suited for this kind of studies due to their high content of functionally relevant protein [11, 12]. The complicated experimental set up of time resolved X-ray studies makes it difficult to realize the original aim of these studies to assign the registered structural changes to specific steps in the enzyme's reaction sequence. Different enzyme intermediates, such as the occluded calcium binding state and/or the ADP-sensitive phosphoenzyme EP₁ have been proposed tentatively to correspond to the observed structural changes [6, 8].

In this report we describe experiments which allow to assign changes in the molecular structure to the occurrence of a reaction intermediate analog, the vanadate enzyme complex. It has been demonstrated that this complex is formed when low concentrations of *ortho*-vanadate react with

Enzymes: calcium transport ATPase (EC 3.6.1.38); phospholipase A₂ (EC 3.1.1.4).

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the enzyme in the absence of calcium [13, 14]. Its correspondence to the EP₂ state of the enzyme is supported by the disappearance of the enzyme's high affinity binding sites for calcium [14]. In contrast to EP₂, the vanadate complex is quite stable. In a previous electron microscopic study we observed that the typical asymmetric structure of the vesicular bilayer was considerably reduced when the vesicles were treated with *ortho*-vanadate prior to glutaraldehyde and/or osmium fixation, and uranyl acetate and/or lead citrate staining [15]. We have extended this investigation by evaluating quantitatively the vanadate-induced morphological changes by scanning the obtained micrographs with a microdensitometer. To get an impression of the contribution of the membrane lipids to the vanadate-induced structural rearrangement, we included studies on delipidated preparations. It is well established that after lipid removal the asymmetric pattern of the sarcoplasmic reticulum membrane is even more evident than in control preparations [16, 17]. We could ascertain that the asymmetric distribution of the calcium transport protein in the vesicular membrane is reduced significantly when the protein binds vanadate. The change goes along with a considerable diminution of the membrane thickness, indicating a sizable shift of the protein mass in the direction to the luminal surface. These alterations remain evident even after enzymatic delipidation of the vanadate-treated membranes indicating that the lipid arrangement does not contribute essentially to the asymmetric structure of the native membrane.

Materials and Methods

Light sarcoplasmic reticulum vesicles were prepared as described by Hasselbach and Makinose [18] and modified by de Meis and Hasselbach [19] from rabbit skeletal muscle at 4 °C. A vesicular suspension of 20–30 mg·ml⁻¹ resulted which was used within 48 h.

Vanadate binding and delipidation

The vanadate complex of the transport enzyme was formed by incubating 0.1 mg·ml⁻¹ vesicular protein with 0.1 or 0.3 mM *ortho*-vanadate in media containing 40 mM KCl, 20 mM imidazole pH 7.0, 5 mM MgCl₂ and 1 mM EGTA for 12–14 h

as described by Medda and Hasselbach [14]. Saturation of the high affinity calcium-binding sites of the transport enzyme was achieved by incubating 0.2 mg·ml⁻¹ vesicular protein with 0.1 mM CaCl₂ in 0.1 M sodium cacodylate buffer pH 7.0. Calcium deprivation of the enzyme was performed in solutions containing 0.1 mM EGTA.

Native, delipidated (s. below) and vanadate-treated vesicles were fixed for 2 h at 4 °C with 2.5% glutaraldehyde solution in 0.06 M sodium cacodylate buffer pH 7.3 [20] or in 20 mM histidine-HCl buffer pH 7.0 [21], added or not with 5 mM CaCl₂. Following centrifugation at 50,000 rpm all pellets were washed twice with 20 mM histidine pH 7.0, 10 mM KCl and 5 mM CaCl₂ to allow digestion by phospholipase A₂ (Naja naja, EC 3.1.1.4, Koch-Light Laboratories Ltd., Colnbrook, Bucks, England).

The lipid constituents of native, glutaraldehyde fixed as well as vanadate-treated and glutaraldehyde-fixed vesicles were hydrolyzed with 0.2 mg phospholipase A₂·mg vesicular protein⁻¹ in the above medium at 20–22 °C for 90 min. The products of hydrolysis were removed from the vesicles by incubating the vesicular suspensions with lipid-free bovine serum albumin – 100 mg·ml⁻¹ incubation medium – for 2 h. BSA, pure 92%, from Serva, Heidelberg, F.R.G. was used. The vesicles were separated by centrifugation for 30 min at 50,000 rpm. The pellets were resuspended in the above incubation medium and repelleted. The phospholipid content of the vesicles was determined by measuring the residual organic phosphate and by thin layer chromatography as described earlier [21, 22].

Twin delipidation experiments were done with various samples of glutaraldehyde-fixed vesicles treated for 1–2 h at room temperature with 10 mM borohydrate in 0.1 M sodium cacodylate buffer pH 7.0, to stabilize the Schiff bases formed by the fixative [23].

Electron microscopy

Following fixation with glutaraldehyde as described above, various preparations were treated with 1% OsO₄ in distilled water for 90 min. In some cases, twin samples of native, delipidated and vanadate-treated vesicles were fixed with 2.5% glutaraldehyde solutions in 0.06 M sodium cacodylate buffer and postfixed with OsO₄ as above

[20]. The fixed preparations were pelleted by centrifugation. Part of the various specimens were block stained with uranyl acetate in distilled water for 1 h. Subsequently all pellets were dehydrated with ethanol followed by propylenoxide-epon mixtures, and finally with pure Epon 812. Polymerization was achieved at 60 °C for 20–24 h. Sections of 300 to 500 Å thickness were cut with a LKB ultramicrotome and stained with 2% uranyl acetate in distilled water [24] for 20–30 min and subsequently with lead citrate [25] for 5–10 min or with lead citrate alone. Electron micrographs were taken with a Siemens Elmiskop 101 equipped with a tilting device at a primary magnification of 40,000.

Morphometric analysis

Microdensitometric measurements of membrane bilayers were performed with the densitometer CD 50 (Desaga, Heidelberg, F.R.G.) by applying the reflexion modus on positive prints at a final magnification of 200,000. The circumference and the area of the vesicles were measured on the same photoprints using the semiautomatic image analyzer ASM 68 K (Leitz, Wetzlar, F.R.G.). The electron micrographs showing ideally cross-sectioned membranes were selected. They are recognized by the absence of electron dense material in the vesicular cavity on the one hand and by the presence of stretches of well separated bilayer structures on the other hand. The membrane profiles were scanned with a rectangular light beam at a wavelength of 600 Å measuring 50 µm in width and 0.1–0.2 cm in length. The width of the beam amounts to approximately 6% of the membrane thickness in the graphs thus allowing a sufficient resolution of the inner and the outer dense membrane layers. The trough separating the two layers becomes shallower when the slit width is increased. It disappears at a width of 400 µm. Concomitantly, the total thickness of the membranes increases by 20% as compared to the measurements with the narrow slit, as could be expected. Although the slit measures only 6% of the membrane, the density profiles are not trapezoidal as one should expect for a compact membrane layer; we, therefore, have to assume that there exist density differences of the electron dense material perpendicular to the plane of the membrane. Since the trough in the density profiles never reaches the base line, the three distances characterizing inner, middle and outer

membrane layers cannot be determined unambiguously. We, therefore, measured the corresponding distances in the density profiles in the middle in between the minimum of the trough and the peak of the lower, usually the second maximum. The measuring position is indicated by the line (*a*) in Fig. 2. These directly measured values are given in Table I as apparent values. They are smaller than the real ones which cannot be measured separately at the base of the profiles. Yet, the overall membrane thickness can be obtained by measuring the width of the base of the profile, line (*r*) in Fig. 2, which is by a factor of 1.29 ± 0.12 ($n = 20$) larger than the distance measured near the middle of the profile. The true dimensions of the membrane leaflets can be obtained by multiplying the three separately measured distances by this factor. The magnitude of the ratios between the thicknesses of outer and inner membrane layers which is used to characterize membrane asymmetry is not affected by this transformation.

These corrected values for the total membrane thickness are given in Table I to compare the results of our measurements with those obtained by X-ray studies [4]. The correction factor was obtained from measurements of 20 profiles, the total width of which could be measured reliably. All data are given in Tables I and II as means \pm S.D. of n measurements.

Results

In agreement with previous studies [21, 22] the phospholipid content of the vesicular membranes was reduced by the delipidation procedure applied to less than 10% from 0.6 to 0.04 µmol·mg protein⁻¹. Preparations fixed with glutaraldehyde were somewhat less susceptible to the action of phospholipase A₂ resulting in 80% delipidation. Treating the vesicles with vanadate before glutaraldehyde fixation and phospholipid digestion does not interfere with delipidation. Preparations treated with borohydrate to stabilize the Schiff bases which are formed by glutaraldehyde [23] did not differ in their appearance from unfixed material.

The electron micrographs of ideally cross cut sections of native as well as of lipid deprived membranes shown in Fig. 1 and 2, and respectively, in Fig. 4 and 5a clearly reveal that the membrane's constituents are asymmetrically arranged. The fact

that the two layers of the membranes remain clearly separated from one another after the bulk of the membrane lipids (approximately 80%) was removed definitely demonstrates that the lipids are not essential for the asymmetric appearance of the membranes (Fig. 4 and 5a). The degree of apparent asymmetry, which had been defined as the ratio between the thickness of the outer and the inner membrane layer as described in Materials

and Methods, is not reduced but rather significantly augmented by delipidation. This persistence is most remarkable because at the same time a striking change of the structure of the vesicles takes place. Their mean diameter of 800 Å shrinks to 550 Å in agreement with previous observations [16, 17]. This shrinkage corresponds to a diminution of the vesicular surface by approximately 50% (Table II). The densely packed protein gives

Fig. 1–5. Electron micrographs of ultrathin sections through sarcoplasmic reticulum preparations fixed with glutaraldehyde and osmium tetroxide, embedded in epon, and contrasted with uranyl acetate and lead citrate as described in Materials and Methods. Bars in the figures mean 0.1 µm. The density profiles of membrane bilayers shown in Fig. 2–5 were registered with a microdensitometer at a slit width of 50 µm from the outside (O) to the inside (I) of the vesicles on electron micrographs at a final print enlargement of 200,000.

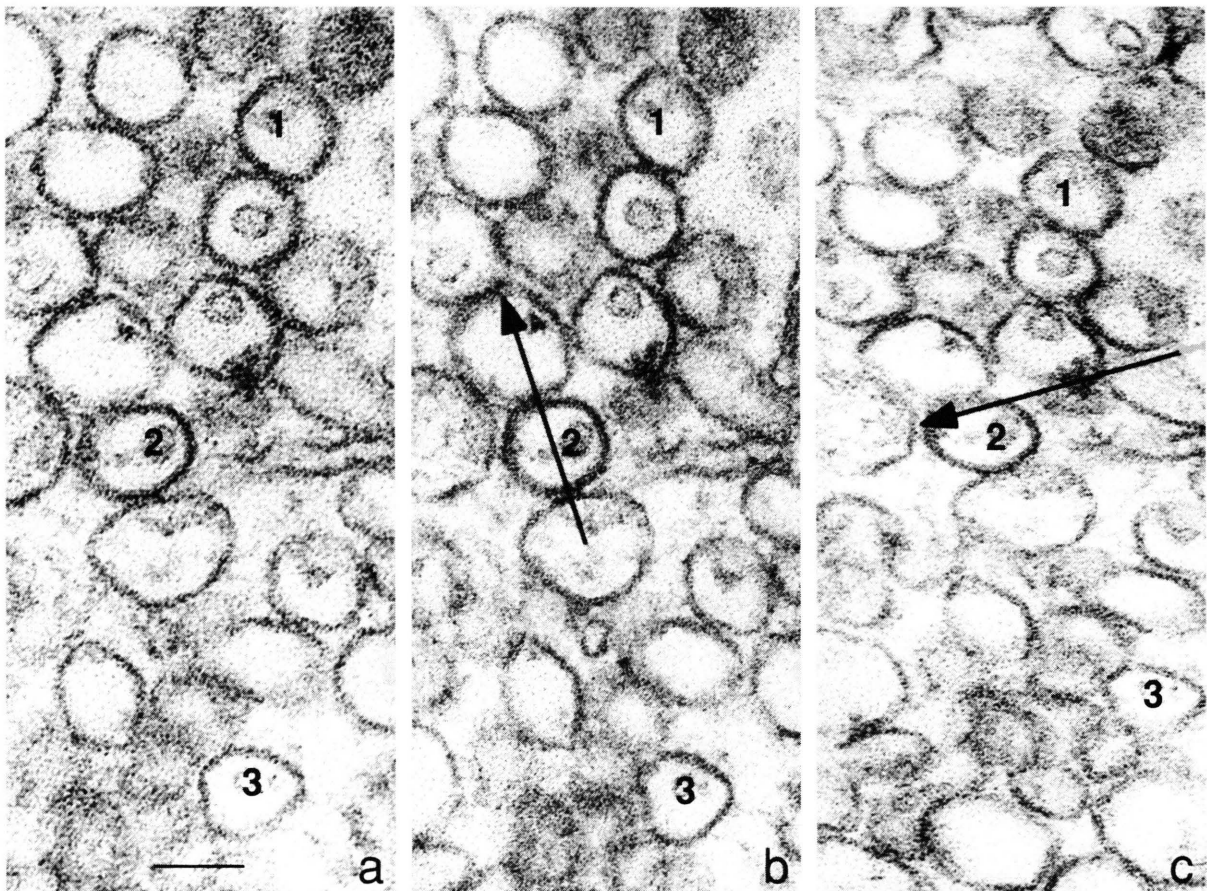


Fig. 1. Photographs of the same section through sarcoplasmic reticulum vesicles taken at different projections after tilting the specimen plane in the microscope. Arrow in b and c indicates the tilting direction, in both cases about 30° against the horizontal plane of the specimen represented in a. 1, 2 and 3 indicate the same vesicle in the different micrographs. Vesicle shape and membrane bilayer pattern vary to a great degree with tilting the specimen plane. However, the characteristic asymmetric pattern of the membrane bilayer can be seen in the same vesicle at various projections. × 120,000.

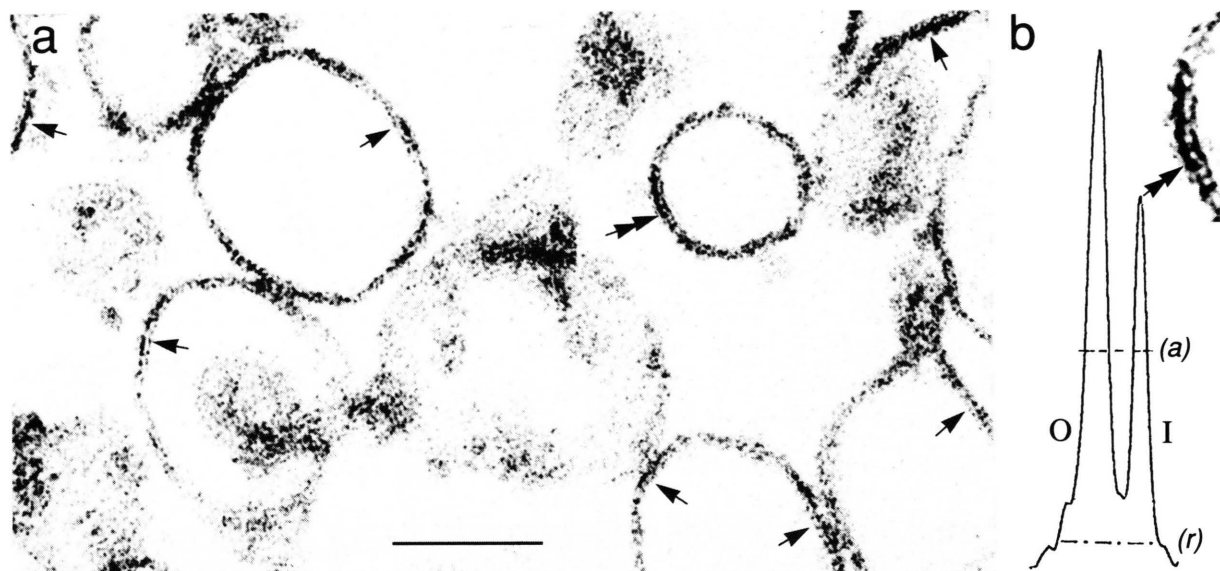


Fig. 2. Membrane bilayer profiles of native sarcoplasmic reticulum vesicles routinely fixed with glutaraldehyde and osmium tetroxide as control.

a. Survey electron micrograph used for microdensitometry. Single arrows indicate the characteristic asymmetric feature of cross cut vesicular membranes. The double arrow indicates the vesicle used for the scan represented in b. $\times 200,000$.

b. Asymmetric density profile registered with the microdensitometer. (a) and (r) indicate the apparent and respectively the real membrane thickness (*cf.* Table I). The double arrow on the enclosed inset electron micrograph indicates the place of the scan. $\times 400,000$.

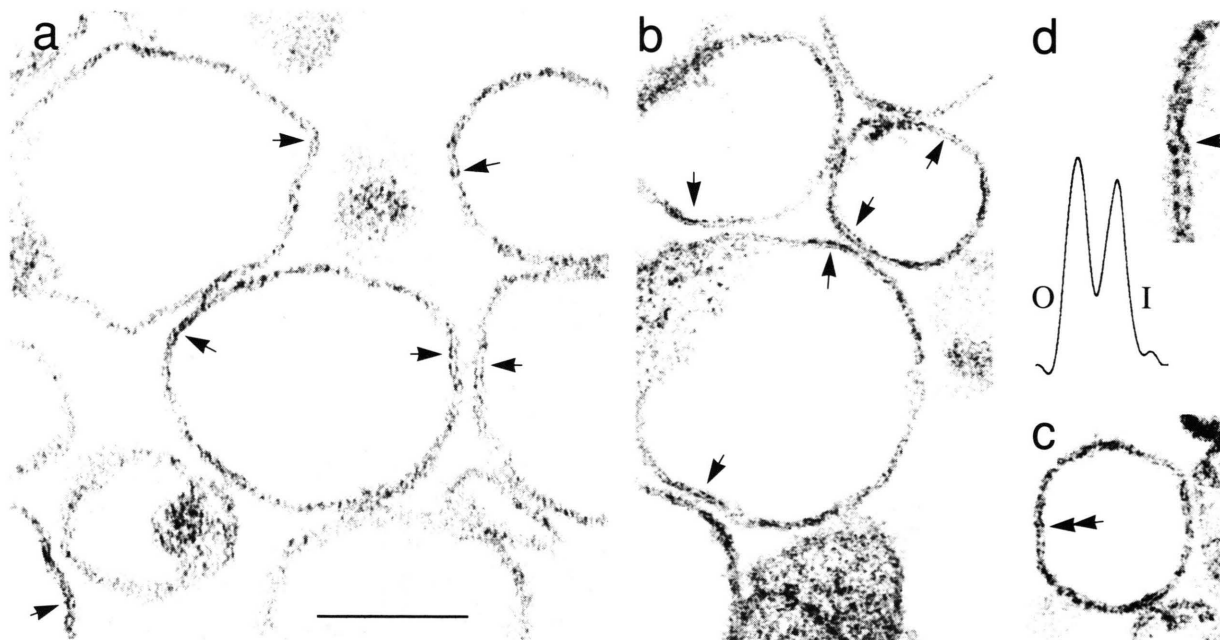


Fig. 3. Membrane bilayer profiles of native sarcoplasmic reticulum vesicles treated with 0.1 mM vanadate and fixed with glutaraldehyde and osmium tetroxide.

a–b. On comparison with the native preparation represented in Fig. 2 the tripled layered structure of the cross cut vesicular membranes displays a rather symmetric arrangement (arrows); the total membrane thickness appears somewhat reduced (*cf.* Table I).

c. Vesicles used for measurement represented in d. The double arrow indicates the place of the scan. a–c $\times 200,000$.

d. Density profile registered with the microdensitometer confirming the reduction of asymmetry of the membrane bilayer after treatment with vanadate. The double arrow on the enclosed inset micrograph indicates the place of the scan (*cf.* c). $\times 400,000$.

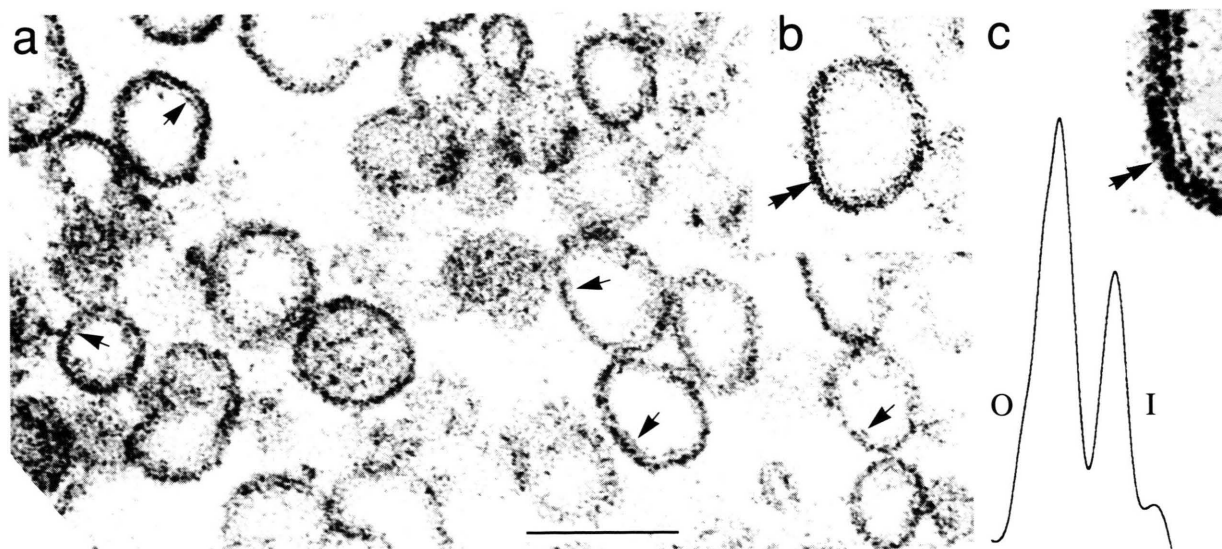


Fig. 4. Membrane bilayer profiles of native vesicles of sarcoplasmic reticulum delipidated with phospholipase A_2 in combination with BSA, and subsequently fixed with glutaraldehyde and osmium tetroxide as described in Materials and Methods.

a–b. Following delipidation vesicle size is strongly reduced and the asymmetric pattern of the cross cut membrane bilayer of most vesicles (single arrows) is even more evident than in those of the control preparations represented in Fig. 1 and 2. The membrane thickness is augmented (*cf.* Table I). The vesicle in b with well outlined asymmetry of the membrane bilayer (double arrow) was used for the scan represented in c. $\times 200,000$.

c. Pronounced asymmetric pattern of a density profile registered on the membrane of the vesicle represented in b. The double arrow on the enclosed inset electron micrograph indicates the place of the measurement (*cf.* b). $\times 400,000$.

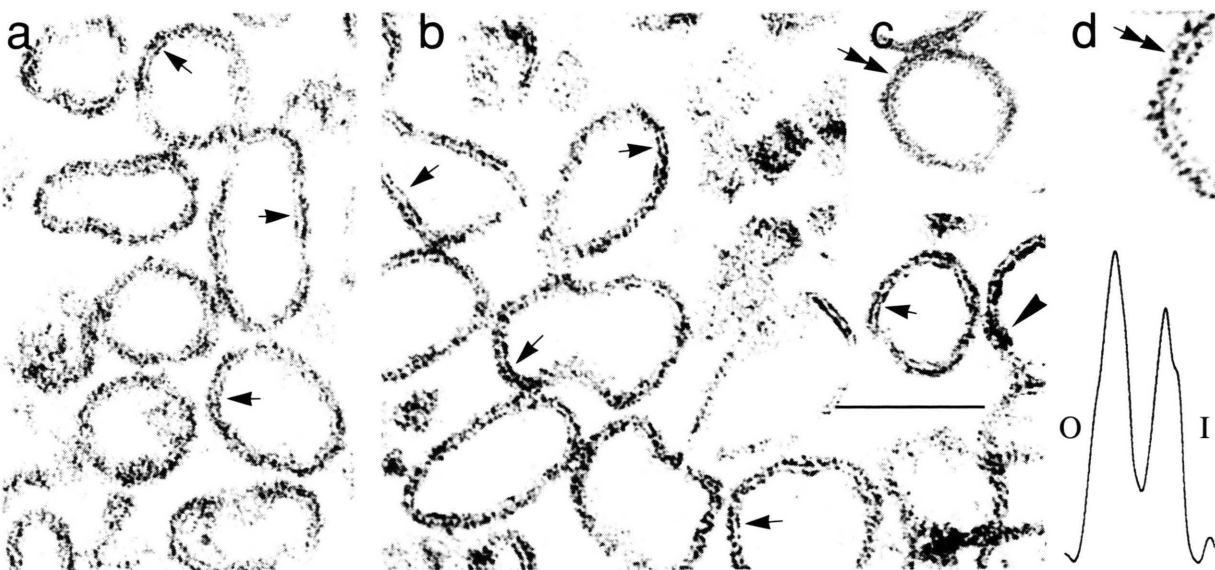


Fig. 5. Membrane bilayer profiles of native (a) and vanadate-treated (b–c) sarcoplasmic reticulum membranes delipidated after fixation with glutaraldehyde as described in Materials and Methods.

a. Alteration of the vesicular membrane observed after delipidation of native vesicles in Fig. 4 is also seen when membrane phospholipids are removed from vesicles previously fixed with glutaraldehyde. Also in this case the asymmetric

Table I. Changes in thickness of outer and inner leaflet of sarcoplasmic reticulum membrane bilayer induced by vanadate as seen in control and delipidated preparations. *n* = number of scans.

Preparations	total bilayer	Apparent* outer leaflet Å [m ± S.D.]	inner leaflet	Membrane thickness	
				Real* total bilayer Å [m ± S.D.]	Ratio outer/inner leaflet % [m ± S.D.]
(1) Untreated vesicles fixed with glutar- aldehyde – control <i>n</i> = 163	62.54 ±7.76	28.46 ±7.55	16.18 ±3.17	80.60 ±7.80	1.76 ±0.52
(2) Vesicles treated with 0.1–0.3 mM vanadate and fixed with glutar- aldehyde <i>n</i> = 160	56.68 ±7.92	23.00 ±6.05	16.17 ±3.45	73.10 ±7.94	1.42 ±0.51
Difference: (1)–(2)	5.86	5.46	0.01 n.s.**	–	0.34
(3) Vesicles fixed with glutaraldehyde and delipidated – control <i>n</i> = 171	81.85 ±12.75	35.59 ±12.30	19.43 ±2.27	105.00 ±13.00	2.04 ±0.66
(4) Vesicles treated with 0.1–0.3 mM vanadate and fixed with glutar- aldehyde before delipidation <i>n</i> = 165	77.41 ±9.28	30.62 ±8.24	21.75 ±4.53	99.80 ±9.40	1.41 ±0.47
Difference: (3)–(4)	4.44	4.97	–2.32	–	0.63

* The apparent distances were obtained by measuring the width of the two peaks of the density profile in the middle between the trough and the lower maximum as described in Materials and Methods. The real bilayer thickness is derived from the width of the profile measured at its base.

** n.s., not significant; all other differences are significant at a level of $p < 0.001$.

rise to sharper contrast of the membranes, but perfect cross cuts were obtained less frequently. The apparent thickness of the membrane of the shrunken vesicles appears to have considerably increased from 62 to 82 Å (Table I).

The vanadate concentrations of 0.1–0.3 mM, which we applied to modify the calcium transport

enzyme, are considerably lower than those required to induce aggregation and crystallization of the enzyme in the membranes but suffice to completely saturate the enzyme's binding sites [14, 26]. The results of densitometric measurements of the membrane profiles of control and vanadate-treated preparations (*cf.* Fig. 2 and 3) are compiled in

pattern of the membrane bilayer profile (arrows) is more pronounced than in the control vesicles represented in Fig. 1 and 2.

b–c. Reduction of total membrane thickness and the symmetric arrangement of the membrane bilayer profile induced by vanadate on native vesicular membranes (*cf.* Fig. 2 with 3 and Table I) are also evident when the vanadate-treated vesicles are delipidated subsequently to glutaraldehyde fixation (*cf.* also a). Single arrows in b and double arrow in c indicate membrane bilayer profiles with rather symmetric pattern. Arrowhead in b indicates a vesicular membrane fragment the inner leaflet of which appears very thick at one place, probably due to artifactual stain precipitation (*cf.* 15). $\times 200,000$.

d. Almost symmetric density profiles of vanadate-treated and subsequently delipidated vesicles measured on the vesicle represented in c. The double arrow on the enclosed inset electron micrograph indicates the place of the scan (*cf.* c). $\times 400,000$.

Table II. Size reduction of control and vanadate-treated sarcoplasmic reticulum vesicles resulting from lipid removal. *n* = number of vesicles measured.

Preparations	Circumference [nm] (<i>m</i> ± S.D.)	Differences* [%]	Surface area [nm ²] (<i>m</i> ± S.D.)	Radius** [nm]
Untreated vesicles fixed with glutar- aldehyde – control <i>n</i> = 177	400 ± 143	–	55.960 ± 42.200	65.2
Delipidated vesicles fixed with glutar- aldehyde <i>n</i> = 182	256 ± 83	36.0	22.600 ± 14.800	41.3
Vesicles fixed with glutaraldehyde before delipidation <i>n</i> = 223	256 ± 73	36.0	19.870 ± 10.370	39.8
Vesicles treated with 0.1–0.3 mM vanadate and fixed with glutar- aldehyde before delipidation <i>n</i> = 189	247 ± 83	38.3	20.300 ± 14.500	39.7
Vesicles fixed with glutaraldehyde and treated with boro- hydrate before delipidation <i>n</i> = 240	249 ± 67	37.8	19.844 ± 11.09	39.7

* All differences between the delipidated preparations and their respective controls are significant at a level of $p < 0.001$.

** The mean vesicles radii were obtained from the circumference and the cross section assuming that the cross sections are circular shaped.

Table I. It shows that vanadate treatment causes a considerable change in the distribution of the electron dense material in the membranes. The thickness of the outer layer in proportion to that of the inner layer of native vesicles, the asymmetry ratio, is reduced by approximately 20% (Table I, last column). This vanadate-induced rearrangement is not changed when the native vesicles treated with vanadate are delipidated subsequently to glutaraldehyde fixation (*cf.* Fig. 3 and Fig. 5b–d). It results in an even larger decrease of the asymmetry ratio by 30%. The relative change in asymmetry appears to be brought about in native vesicle by a reduction of the thickness of the outer layer alone while in delipidated vesicles a thickening of the inner layer also contributes to the diminution of the density differences between the outer and the inner membrane layer (*cf.* Fig. 4 and 5 and Table I).

Our supposition that the electron dense material represents the calcium transport enzyme is

strongly supported by the fact that the basic features of the bilayer structure remains unchanged after lipid removal. During lipid removal and the subsequent washing procedures most of the accessory proteins are removed from the vesicles showing that these constituents do not contribute essentially to the appearance of the preparation in the electron microscope [27].

The vanadate-induced changes in the protein distribution in the vesicular membrane does not lead to significant changes in the lateral spacing of the membrane protein as must be concluded from the observation that the vesicular diameter is not measurably affected by vanadate binding (Table I). The total thickness of the membrane of native preparations as well as after delipidation, however, are reduced significantly by vanadate by 9% and 5% respectively (Table II). The described structural changes were not observed when the vesicles were treated solely with EGTA assumed to

convert the protein into its E_2 conformation [2]. Hence we must suppose that the observed transformation is not related to the formation of the calcium-free protein alone. It is rather the binding of vanadate which causes the change in the arrangement of the calcium transport enzyme in the membrane [28, 29].

Discussion

The results of this electron microscopic evaluation of thin sectioned glutaraldehyde-osmium acid fixed and uranyl acetate and lead citrate stained light sarcoplasmic reticulum vesicles demonstrate that considerable structural changes occur when vanadate reacts with the calcium-free transport enzyme. It should be noted that the asymmetric appearance of the native vesicular membrane does not depend on the deposition of electron dense osmium or uranyl before embedding. We have previously shown that vesicular membranes solely fixed with glutaraldehyde exhibit asymmetry although at a lower contrast [16, 17]. Since calcium removal alone had no discernible effect, we conclude in agreement with previous vanadate-binding studies that vanadate binding is required to force the enzyme into its low affinity calcium binding state being accessible for calcium only from the luminal surface of the vesicles [14]. The magnitude of the observed change in membrane asymmetry appears to be considerably greater than that reported by Blasie *et al.* based on time-resolved X-ray diffraction studies on oriented multilayers of light sarcoplasmic reticulum vesicles [5]. This numerical disagreement requires consideration of the respective experimental procedures. The ATPase molecules in the multilayer are not regularly ordered as shown in electron microscope controls of pelleted and dehydrated preparations [31]. This problem has been downgraded by applying corrections for several kinds of membrane disorder [32]. Yet, a most severe problem concerns the difficulty in defining the distribution of the various reaction intermediates of the calcium transport cycle after the reaction has been started by light-induced ATP liberation. In order to affect the distribution of the reaction intermediates experiments at different magnesium concentrations were performed [7]. Under all applied conditions,

however, the ADP-sensitive EP_1 intermediate rapidly transits to the ADP-insensitive EP_2 intermediate [33, 34]. Another complication concerns the accumulation of ADP when the transiently liberated ATP is exhausted. ADP drives the first reaction sequence backwards and prevents the occlusion of calcium [34]. Consequently, neither the size of the unphosphorylated nor that of the phosphorylated enzyme fraction can hardly be estimated. Therefore, the degree of transposition of protein mass deduced by the authors and related to the EP_1 intermediate must be considered to represent a minimal value. The main advantage of using vanadate as phosphate analog is the fact that vanadate forms a quite stable intermediate which already at low concentrations reaches a binding maximum of $8 \text{ nmol} \cdot \text{mg}^{-1}$, *i.e.* the transport enzyme is completely trapped as its protein vanadate complex [14, 35]. This is in contrast to the binding of inorganic phosphate or to the phosphoryl transfer reaction from ATP. Under both conditions only a fraction resides in the low affinity EP_2 state of the enzyme [1, 30, 36]. We could not detect any structural change when we used 10 mM phosphate instead of vanadate for enzyme modification. Evidently, under these conditions the structural changes are considerably smaller than those induced by vanadate binding.

The registered reduction of the symmetry ratio of the bilayer obtained from the microdensitometric evaluation of the sectioned vesicles and derived as described in Materials and Methods needs some additional consideration to assign it to the magnitude of the true extent by which the transport molecule undergoes redistribution of its mass. First of all, it depends on the model adopted for the shape of the molecule. Nearly all attempts of defining the molecule's shape result in the proposal of a more or less elongated cylinder [5, 37]. If we consider this model as applicable for the enzyme's shape in the membrane the registered change in membrane thickness would correspond to a change of the distribution of its mass in the bilayer. In the native membranes the change in mass distribution appears to be restricted to the outer segment. No compensatory change either in the inner membrane layer or in the inner membrane space could be seen. In contrast, however, after delipidation a significant increase of the protein mass in the inner layer became visible revealing that the molecules

are shifted perpendicularly to the plane of the membrane. The apparent absence of this shift in native membranes suggests that a rearrangement of membrane lipids from inside to outside might compensate for the inward movement of the protein. Since the shrinkage of the outer protein layer is only partially – 50% – compensated by an expansion of the inner layer we must assume that a considerable volume shrinkage takes place in the molecule's outer segment. Such a large volume change appears to be highly unlikely. It is generally assumed that proteins only endure small volume changes [38]. The recently observed quite large volume expansion of $200\text{--}300\text{ ml}\cdot\text{mol}^{-1}$ which the calcium transport enzyme undergoes when it binds vanadate has the wrong sign, on the one hand, and is negligible in size compared to the occurring shrinkage of approximately $10,000\text{ ml}\cdot\text{mol}^{-1}$ on the other hand. Most likely, the protein loops in the outer membrane segment in the native membranes are loosely folded and get more closely packed by vanadate binding [3]. This can give rise to changes in the electron microscopic pattern of the membrane appearing much larger than the mass shift observed in X-ray diffraction studies. The vanadate-induced movement of the calcium transport enzyme in the lipid bilayer is closely related to the protein's interaction with its lipid surrounding, as demonstrated previously by Hasselbach and Medda [39]. They showed that the delipidated enzyme does not react with vanadate but that vanadate binding can be restored by relipidation.

Lipid removal appears to lead to a significant increase of the thickness of the double layer structure of the native as well as the vanadate-treated preparation. It mainly concerns the outer and the middle layer. Delipidation appears to induce a structural change in the sense of an elongation of the enzyme molecule, mainly of its two outer segments. Structural changes occurring after lipid removal have been reported repeatedly [16, 40]. The fact that the enzyme solubilized in detergents appears to be much longer – 120 Å instead of 65 Å – than in the membrane may be due to the removal of the constrain which is exerted by the native lipids in the membrane. Negative staining of native vesicles also appears to induce an extension of the molecule giving rise to a lollipop like structure [41–43]. In this context the given numbers for the

various dimensions need some comments. The definition given in Materials and Methods allows to obtain well reproducible values for the thickness of the three layers of the membranes. They are, however, somewhat smaller than the true dimensions because of the bellshaped or nearly triangular profiles of the densitograms, the broadness of which were measured as apparent membrane dimensions in the middle between their intermediate minima and their lower maxima. The true width of the two layers has been obtained from the values measured near the middle of the profiles and the total width measured near to the base of the profile. It is 1.29 ± 0.12 times greater than the width measured at the reference line near the middle of the profile. The assumptions underlying this procedure have been checked by measuring the width of black bars of known thickness with different openings of the measuring slit. The corrected dimensions for the total thickness are given in brackets in Table I. It is evident that the total length of the molecule remains considerably smaller than that deduced for the molecule dissolved in the presence of detergents. Our estimated values, however, well agree with the data deduced from X-ray diffraction studies [4, 5, 10]. The finding that delipidation does only reduce the size of the vesicles but does not destroy their shape indicates that the spherical shell is the most favorable arrangement of the lipid-deprived transport molecules. This, however, requires that the transport molecules are conically shaped.

From the dimensions given above and a volume of the molecule of $130,000\text{ ml}\cdot\text{mol}^{-1}$ calculated from the known molecular weight, one can easily deduce that the outer and the inner diameter of the truncated cone measure 68 and 44 Å , respectively, at a height of 80 Å . Changes of $4\text{--}6\text{ Å}$ of the outer diameter are sufficient to produce the vanadate-induced symmetry change. The comparison of the magnitude of native and delipidated vesicles on the one hand and of the size of the outer diameter of the transport molecule on the other hand, allows to calculate that the transport molecules in the native membrane are separated by a distance of approximately 20 Å filled by membrane lipids. This dense packing was already apparent in electron microscopic pictures after labeling the surficial thiol residues of the transport molecules with thiol reactive electron dense ferritin [9].

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Notes

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