Structural Membrane Proteins and Loosely Associated Proteins of the Sarcoplasmic Reticulum

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The protein composition of sarcoplasmic-reticulum vesicles, either unpurified or after fractionation on sucrose gradients, and with or without previous osmotic shock and sonication, was investigated by electrophoresis in acid polyacrylamide gels. The pattern of release of loosely bound proteins is discussed with respect to their localization in the interior of the vesicles.

Recent studies of the ATPase* of the sarcoplasmic reticulum from rabbit skeletal muscle are consistent with the view that this protein, with a mol. wt. of about 100 000, is an integral part of the membrane structure of the isolated sarcoplasmic-reticulum vesicles (MacLennan, 1970; MacLennan et al., 1971; Meissner et al., 1973). In extensively purified reticular membranes, the ATPase protein makes up about two-thirds of the total sarcoplasmic-reticulum protein (Meissner et al., 1973). The residual protein is accounted for mainly by two loosely bound components, with molecular weights between 65 000 and 50 000 (Duggan & Martonosi, 1970; Martonosi & Halpin, 1971; Meissner et al., 1973). Of these minor protein components, a protein with Ca2+-binding properties has been characterized by MacLennan & Wong (1971) and named 'calsequestrin'. This is similar to, and probably identical with, the Ca2+-precipitable protein of Ikemoto et al. (1972) and the M65 protein of Meissner et al. (1973). The Ca2+-binding protein(s) would have a role in the sequestration of Ca2+ after its inward transport across the sarcoplasmic-reticulum membrane.

With regard to the protein composition of the matrix of the intact sarcoplasmic reticulum in general, as well as in connexion with the intraluminal accumulation of Ca2+ under physiological conditions, the previous history of the preparation of sarcoplasmic-reticulum vesicles has, however, not been considered sufficiently critically. The origin of the vesicles from the intact sarcoplasmic reticulum by a generalized pinching-off process, and their ease of breakdown and re-formation are, on the other hand, well known. It is conceivable that the less attention that is given to maintaining the morphological integrity of the sarcoplasmic-reticulum vesicles during the isolation procedure, the greater will be the loss of the native protein contents to the supernatant fluid, particularly if the emphasis is on isolating the membrane component in a highly pure form, such as by preparative methods using KCl or NaCl solutions instead of sucrose media (see e.g., Martonosi, 1968; MacLennan, 1970).

The results reported here deal mainly with an electrophoretic study of the protein composition of the isolated sarcoplasmic reticulum from rabbit skeletal muscle, and with the effect on this composition of different methods of purification.

Experimental

The adductor magnus, a white fast-twitch muscle, from New Zealand rabbits was used for the isolation of sarcoplasmic-reticulum fragments by centrifugation of the mitochondria-free supernatant fraction (15 000g for 20 min) of the tissue homogenate in 0.3 M-sucrose or in 0.3 M-sucrose–5 mM-disodium EDTA, pH 8.0 (see the Results and Discussion section), by the general procedures reported previously (Margreth et al., 1970, 1972). The total sarcoplasmic-reticulum fragments were sedimented by centrifuging the supernatant at 150 000g for 60 min. A 'light' sarcoplasmic-reticulum fraction was isolated by centrifuging the supernatant at 80 000g for 30 min and re-centrifuging the supernatant from this step at 150 000g for 60 min. Purification of these fractions was achieved by centrifugation on sucrose density gradients, or by extraction with either KCl or potassium phosphate solutions, as reported in the Results and Discussion section. Linear 0.25–2 M-sucrose gradients (unbuffered) were obtained with a Buchler Universal Density Gradient Mixer and Polystaltic Pump. A portion (1 ml) of fragmented sarcoplasmic reticulum in 0.25 M-sucrose was layered over 10 ml of the gradient and centrifuged at 29 000 rev/min for 90 min in a Spinco SW41 rotor. The gradient was

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*Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethyamine)tetra-acetate.
collected from the bottom of the centrifuge tube by a Buchler Densi-flow apparatus and peristaltic pump and continuously monitored at 280 nm in the IEC swinging-bucket flow cell of a Gilford recording spectrophotometer, with the outlet connected to a fraction collector. Centrifugation on discontinuous sucrose gradients (see the Results and Discussion section) was at 25 000 rev./min for 150 min in a Spinco SW-25.1 rotor. Polyacrylamide-gel electrophoresis [after solubilization of the protein in an acid–phenol mixture containing the anionic detergent Nonidet P.40 (Panet & Selinger, 1970)] and determinations of enzyme activities were done as described by Margreth et al. (1972). The steady-state concentration of 32P-labelled protein in the isolated sarcoplasmic reticulum was determined after 15 s at 0°C, by using the assay conditions and conditions for purification of the protein described by Inesi et al. (1970). After electrophoresis, the gel was cut into 2 mm slices for counting of radioactivity in Bray's (1960) solution by liquid-scintillation spectrometry. Protein was determined by the method of Lowry et al. (1951), as modified by Campbell & Sargent (1967).

Results and Discussion

The protein electrophoretic pattern of total sarcoplasmic-reticulum fragments isolated in 0.3 M-sucrose is shown in Plate 1 (gel I) and appears to be characterized by a main band of low mobility (component 1) below some aggregated material at the origin, by two prominent bands (components 2 and 5), and by two fainter bands with intermediate mobilities (components 3 and 4).

Centrifugation of the fragments on a continuous sucrose gradient provides an enriched membrane subfraction which separates as a broad layer, at sucrose densities between 0.5 M and 1.5 M-sucrose (Plate 2a). Disc-gel electrophoresis of the pooled fractions from two adjacent regions in this area of the gradient (Plate 2, gels II and III) shows a protein pattern which differs from that of the sarcoplasmic-reticulum fragments before purification by a selective loss of com-

Fig. 1. Densitometric records after staining with Amido Black, of electrophoretic gels of sarcoplasmic-reticulum protein

(a) Membrane subfraction separated by centrifugation of untreated sarcoplasmic-reticulum fragments on a continuous sucrose gradient; (b) membrane subfraction isolated as in (a) but from fragments after osmotic shock and sonication; ——— represents the pattern of distribution of the radioactivity incorporated into the protein from [γ-32P]-ATP (see the text); (c) total sarcoplasmic-reticulum fragments, washed with 0.3 M-sucrose and extracted once with 0.6 M-KCl–5 mM-imidazole, pH 7.4. The peaks corresponding to the several electrophoretic bands are numbered as in Plate 1. For further details see the text.
Polyacrylamide-gel electrophoresis patterns of the proteins of sarcoplasmic-reticulum fragments

Polyacrylamide-gel electrophoresis patterns of: (I) unpurified, total fragments; (II) membrane subfraction from sucrose density gradient after hypo-osmotic treatment and sonication; (III) unpurified 'light' fragments (3); (IV) same preparation as in (III), after one extraction with 20mm-potassium phosphate buffer, pH8.0. For details see the text.
EXPLANATION OF PLATE 2

Polyacrylamide-gel-electrophoresis patterns of subfractions from total sarcoplasmic-reticulum fragments obtained by centrifugation on a continuous sucrose density gradient

The pattern of distribution of the protein in the gradient after centrifugation of the fragments is indicated by the $E_{280}$ records. The electrophoretic gels are exactly positioned at the areas in the gradient from which the subfractions were derived (see the text). (a) Untreated fragments (6.9mg of protein). (b) Fragments (10.4mg of protein), previously exposed to hypo-osmotic treatment and sonication, under conditions based on those used by Sottocasa et al. (1967), for separation of the outer mitochondrial membrane. The fragments were suspended in a small volume of 10mm-Tris-HCl, pH7.5. After 10min, an equal volume of 0.5M-sucrose–2mm-ATP–2mm-MgSO$_4$ solution was added and the suspension was sonicated for 15s (300W). For details of centrifugation and electrophoresis see the Experimental section.

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components 2 and 5, particularly from the vesicles distributing at the higher sucrose densities. The released proteins are recovered in the upper portion of the gradient (Plate 2, gel I).

By contrast, prior hypo-osmotic treatment and sonication of the isolated sarcoplasmic-reticulum fragments before centrifugation on the sucrose gradient leads to both a sharper separation of a membrane layer in the 0.7–1.4 M-sucrose region (Plate 2b) and to a larger and relatively indiscriminate release of loosely bound proteins from the sarcoplasmic-reticulum vesicles (gels IV and V in Plate 2). Consequently the electrophoretic pattern of the proteins of the sarcoplasmic-reticulum membrane subfraction is considerably simplified (Fig. 1b) with respect to the corresponding subfraction from the untreated preparation (Fig. 1a), somewhat similarly to the results obtained after extraction of the sarcoplasmic-reticulum fragments with 20 mM-potassium phosphate (Plate 1, gel IV), or 0.6 M-KCl (Fig. 1c).

From measurements of succinate-cytochrome c reductase activity (see Margreth et al., 1972), it was ascertained that the sarcoplasmic-reticulum fragments after purification on sucrose gradients were essentially devoid of mitochondrial contaminants. In a few experiments the rotenone-insensitive NADH–cytochrome c reductase activity was assayed in the purified membranes and found to be similar to that of the original sarcoplasmic-reticulum fragments, i.e. very low, which is a characteristic of fast-muscle sarcoplasmic reticulum (Margreth et al., 1971). The membrane ultrastructure, seen after negative staining with ammonium molybdate, showed the characteristic presence of subunit particles of about 3 nm diameter attached to the outer surface.

The identification of the several protein components in the electrophoretic gels of the isolated sarcoplasmic reticulum was undertaken as follows. After incubation of the sarcoplasmic-reticulum fragments at 0°C with [γ-32P]ATP (Inesi et al., 1970, and legend to Fig. 1), the distribution of radioactivity was determined in the electrophoretic gel of the protein. It was found to overlap completely with the area corresponding to component 1 (Fig. 1b) (see also Panet & Selinger, 1970); this allows its identification with the ATPase protein.

On account of the relative rates of migration, components 3 and 4 were, on the other hand, tentatively identified with the C components found by

Fig. 2. Densitometric records of soluble proteins from unpurified sarcoplasmic-reticulum fragments

Gel electrophoresis of the soluble protein separated by sucrose-density-gradient centrifugation of sarcoplasmic-reticulum fragments as in Plate 2(a), gel I. (a) Co-electrophoresis with 20 μg of crystalline lactate dehydrogenase; (b) soluble protein alone; (c) co-electrophoresis with 20 μg of crystalline phosphofructokinase. For further details see the text.
Martonosi & Halpin (1971), which probably correspond to the M₆₅ and M₄₅ proteins of Meissner et al. (1973). The identification of bands 2 and 5 was helped by evidence for the presence of phosphofructokinase (EC 2.7.1.11), as well as lactate dehydrogenase activity (EC 1.1.1.27), in the sarcoplasmic-reticulum fragments from rabbit muscle (see below), in agreement with earlier work on the corresponding fraction from leg muscle (see Margreth et al., 1967). From co-electrophoresis with crystalline phosphofructokinase and with lactate dehydrogenase from rabbit muscle (both Boehringer products), it was found that the purified enzymes moved as single bands coinciding with sarcoplasmic-reticulum components 2 and 5 respectively (Figs. 2a, 2b and 2c). It was assumed that under the conditions used for solubilization of the protein and for gel electrophoresis, the lactate dehydrogenase monomer (mostly M₄) would dissociate into subunits of 35000 daltons, and phosphofructokinase into protomers of about 80000 daltons (see Darnall & Klotz, 1972).

For further examination of the bonds involved in the association of the glycolytic enzymes with the isolated sarcoplasmic reticulum from rabbit skeletal muscle, additional experiments were done with a 'light' sarcoplasmic-reticulum fraction, isolated by differential centrifugation in a 0.3 M-sucrose medium, buffered at pH 8.0 with 5 mM-disodium EDTA to preserve full activity of phosphofructokinase (see Margreth et al., 1967). Under these conditions the activities of phosphofructokinase and of total lactate dehydrogenase (measured as reported by Margreth et al., 1967) were [expressed in μmol of product formed/min per mg of protein (±S.E.M., number of determinations in parentheses)] 7.96 ± 0.85 (8) and 3.40 ± 0.62 (5) in the unwashed sarcoplasmic-reticulum fraction, with values of 2.64 ± 0.49 (7) and 11.67 ± 1.59 (6) in the 150000 g supernatant fraction respectively. The phosphofructokinase activity associated with the sarcoplasmic reticulum, which was about three times more concentrated than that in the soluble fraction, accounted for about 15% of the activity in the original extract.

The suspension of sarcoplasmic-reticulum vesicles in 0.3 M-sucrose-EDTA solution was further fractionated by layering 5 ml (about 20 mg of protein) over a discontinuous sucrose gradient consisting of 10 ml of 0.5 M-sucrose and 10 ml of 1.2 M-sucrose, both containing 5 mM-disodium EDTA, pH 8.0. A lightly

Fig. 3. Gel electrophoresis of the fractions separated by centrifugation of ‘light’ sarcoplasmic-reticulum fragments on a discontinuous sucrose gradient

For details see the text. (a) Supernatant fraction; (b) 0.5 M-sucrose layer; (c) 0.5–1.2 M-sucrose interface.
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opalescent band which occupied the lower two-thirds of the 0.5M-sucrose layer, and a thick band at the 0.5–1.2M-sucrose interface, were visible after centrifugation. Electron microscopy of negatively stained samples from the different layers showed the largest concentration of vesicles at the interface.

Almost none of the lactate dehydrogenase activity entered the gradient. On the other hand, there was a varying degree of inactivation of phosphofructokinase under these conditions which prevented us from establishing its distribution along the gradient by activity measurements. However, densitometric records of the electrophoretic gels of the several protein fractions (Fig. 3) show that lactate dehydrogenase (component 5) is virtually absent from the membrane fraction, in agreement with the kinetic data, whereas component 2, with the same mobility as phosphofructokinase (Fig. 2), is a major component of this fraction (Fig. 3c), along with the ATPase protein (component 1). It is noteworthy that components 3 and 4 have a pattern of distribution in the sucrose gradient similar to that of lactate dehydrogenase, and are almost completely released into the supernatant fraction under the prevailing conditions. Duggan & Martonosi (1970) have reported that the C components (Martonosi & Halpin, 1971) of purified sarcoplasmic-reticulum fragments are removed by treatment with EDTA or EGTA solutions at pH 8–9.

Therefore the present results indicate that several proteins are present in the isolated sarcoplasmic-reticulum fragments for which the operational definition of loosely associated proteins applies. These include not only the two proteins investigated by Duggan & Martonosi (1970) and Meissner et al. (1973), but also proteins with unrelated functions, such as phosphofructokinase in particular (see also Margreth et al., 1967). Some of the experimental evidence reported here supports the interpretation that these proteins are, in part at least, associated with the matrix in the interior of vesicles. The different rates of efflux of these proteins from the vesicles after their morphological integrity had been altered by osmotic shock and ultrasonication, as well as the differential patterns of elution according to the composition of the isolation and extraction media, may be dependent on a number of variables, e.g. the bonds involved in the association with the membrane and possibly, with phosphofructokinase, its state of molecular aggregation (see Leonard & Walker, 1972) under the prevailing conditions.

In fact white muscles of the rabbit only the ATPase protein conforms to the requirements for a structural membrane protein of the sarcoplasmic reticulum (see also MacLennan et al., 1971; Meissner et al., 1973). However, our findings outline some inherent limitations of the information gained from the study of highly purified membrane preparations, with respect to the identification and study of loosely associated proteins such as Ca2+-binding proteins (MacLennan & Wong, 1971; Ikemoto et al., 1972; Meissner et al., 1973) which, in the intact sarcoplasmic reticulum, presumably operate in series with the Ca2+-transport ATPase.

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References