ULTRASTRUCTURE AND CALCIUM TRANSPORT IN CRUSTACEAN MUSCLE MICROSONES

R. J. BASKIN

From the Department of Zoology, the University of California, Davis, California 95616

ABSTRACT

Fragmented sarcoplasmic reticulum (FSR) from crustacean muscle was examined following preparation by a variety of electron microscopic techniques. The 30-40 A particles which appeared on the outer surface of FSR vesicles following negative staining were not observed following preparation by freeze-drying, freeze-etching, thin sectioning, or critical-point drying. Crustacean FSR exhibited high values of calcium uptake and extensive nodular formation in the presence of oxalate. 80-90 A diameter membrane particles were seen in freeze-etch preparations of both intact lobster muscle and FSR vesicles. Thin sections of FSR vesicles revealed a membrane thickness of 60-70 A. The membrane appeared to be triple layered, each layer having a thickness of 20-25 A.

INTRODUCTION

The ability of microsomal fractions, isolated from lobster muscle, to take up calcium when provided with an energy source has been demonstrated and measured (12). It has been assumed, in analogy to other work on vertebrate skeletal muscle, that these microsomal fractions contain vesicles derived from the sarcoplasmic reticulum (3). An interesting anomaly is that lobster muscle microsomal fractions also show the ability to concentrate strontium, whereas vertebrate sarcoplasmic reticulum apparently does not have this ability (12).

Microsomal fractions showing calcium uptake ability are readily isolated from lobster muscle and would seem to provide an excellent test system for a functional analysis of isolated sarcoplasmic reticulum. We have examined the ultrastructure of isolated lobster and barnacle sarcoplasmic reticulum before and after calcium uptake. Adenosine triphosphatase (ATPase) activity in these preparations has been measured. We have also examined the ultrastructure of isolated and intact sarcoplasmic reticulum by the method of freeze-etching. The suitability of lobster and barnacle microsomal preparations in studies of both the ultrastructure and calcium uptake properties of fragmented sarcoplasmic reticulum has become apparent.

Previous studies on skeletal muscle microsomes (1, 3) have shown the presence of 30-40 A particles surrounding isolated microsomal vesicles following preparation by the method of negative staining. We have found that these particles are also present on lobster and barnacle microsomal vesicles and we have studied the conditions necessary for their appearance.

METHODS

Preparation of FSR and Measurement of Calcium Transport

The abdominal muscles from a lobster (Homarus americanus) were homogenized with four volumes of ice-cold 10 mM Tricine (California Biochemical Corp., Los Angeles, Calif.) for 40 sec in a Waring Blender. Myofibrils were removed by centrifuging at 3000 g for 20 min. The supernatant was filtered through glass wool to remove lipids and centrifuged at 8000 g for 20 min. The supernatant was centrifuged...
for 1 hr at 28,000 g. The final pellet was suspended in 10 ml of 0.6 M KCl in a Potter-Elvehjem homogenizer. After a 1 hr incubation at 4°C, the suspension was again centrifuged for 1 hr at 28,000 g. This pellet was resuspended in a 0.3 M sucrose-10mM Tricine solution (final concentration was about 4.0 mg S-R protein per ml solution).

A microsomal preparation was also obtained from the scutal-tergal adductor and depressor muscles of the barnacle (Balanus nubilus). The procedure was the same as that described above for lobster microsomes.

Measurements of calcium uptake were performed in the manner described by Fanburg and Gergely (4), but with Tricine as a buffer. Microsomal protein was measured by a Folin method with bovine serum albumin as a standard.

ATPase activity was expressed as the amount of inorganic phosphate liberated, as measured by the method of Fiske and SubbaRow (5), following termination of the reaction with 100% trichloroacetic acid.

Electron Microscopy: Positive Staining

Aliquots of the FSR suspensions were fixed by the addition of 10% glutaraldehyde (pH 7.0) to the cuvette (final concentration, 2%). After 20 min of fixation at room temperature, the suspension was centrifuged at 10,000 g and the resulting pellet was postfixed with 1% osmium tetroxide for 1 hr. The pellet was dehydrated in acetone and embedded in a specially prepared resin which was a mixture of Araldite (Ciba Products Co., Summit, N.J.) and Epon 812 (Shell Chemical Co., New York). Sections were prepared with a diamond knife. They were poststained in lead citrate.

Freeze-Drying and Critical-Point Drying

In order to prepare microsomes for freeze-drying, a drop of the FSR suspension was applied to a carbon-coated grid. Most of the suspension was removed by gentle blotting with filter paper, such that only a thin layer of solution remained. The grid was frozen in liquid Freon (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.) and placed on the cooled (−150°C) stage of a vacuum chamber. The frozen suspension was allowed to sublime in a high vacuum (2 × 10⁻⁶ torr) for 6 hr. The grid was removed and negatively stained with 2% phosphotungstate.

The method used for critical-point drying has been previously described (13). Prior to drying, the vesicles were fixed by the addition of glutaraldehyde (final concentration, 2%) to the incubation solution. After fixation for 20 min at room temperature the vesicles were dried by the critical-point technique. The dried specimens were shadowed with platinum at an angle of about 30°.

Negative-Staining and Freeze-Etch Microscopy

Microsomal suspensions were prepared for viewing with a Hitachi HU 11E electron microscope by negative-stain and freeze-etch techniques as described in a previous investigation (1, 3).

RESULTS

Calcium Uptake and ATPase Activity

Calcium uptake in the presence of oxalate averaged 16.3 μmoles Ca²⁺ per mg S-R protein for a 10 min incubation. (The range for 10 preparations was from 10.8 to 24.0 μmoles Ca²⁺ per mg S-R protein.) A comparison of calcium uptake measurements in FSR from a variety of muscle is shown in Table I. The lobster FSR preparation is the most active, showing the most calcium uptake.

| Muscles type                      | μmoles Ca²⁺/mg protein |
|----------------------------------|------------------------|
| Lobster abdominal muscles        | 17.0                   |
| Lobster abdominal muscles        | 11.2                   |
| Lobster abdominal muscles        | 19.5                   |
| Lobster abdominal muscles        | 17.7                   |
| Lobster abdominal muscles        | 24.0                   |
| Lobster abdominal muscles        | 21.7                   |
| Lobster abdominal muscles        | 11.0                   |
| Lobster abdominal muscles        | 14.7                   |
| Lobster abdominal muscles        | 10.8                   |
| Lobster abdominal muscles        | 14.9                   |
| Rabbit heart (average of four prepa-| 0.43                   |
| rations [1])                      |                        |
| Rabbit skeletal (average of five preparations [1]) | 2.04                  |
FIGURE 1 Lobster abdominal muscle microsomes negatively stained with 2% phosphotungstate. Vesicles show formation of tails and tadpole shape. Mitochondrial fragments (arrows) are visible. $\times 70,000$. 
uptake per milligram of S-R protein. This result is also supported by the results of the electron microscope negative-stain studies in which nearly all the vesicles show evidence of calcium oxalate nodules. (See Discussion below.)

In spite of the very high values of calcium uptake, the ATPase activity was comparable to that previously reported in vertebrate preparations (1, 3), i.e. having an average value (for 12 preparations) of 3.3 µmoles P₁/mg S-R protein following a 15 min incubation.

**Negative Staining**

A preparation of lobster muscle microsomes negatively stained with 2% phosphotungstic acid

![Image](2).

**Figure 2** Lobster microsomes negatively stained with 2% uranyl acetate. Particulate fringe around vesicles is visible. × 100,000. The particles (inset arrow) are approximately 30-40Å in diameter. *Inset* × 160,000.
is shown in Fig. 1. The appearance of the preparation is similar to that seen in vertebrate skeletal muscle microsomes. Tadpole-shaped vesicles and vesicles with "tails" are commonly seen among the lighter vesicles (i.e. those that the stain has not penetrated). The darker vesicles do not show these characteristics. They are generally round or ovoid in shape. Mitochondrial fragments surrounded by 80–90 A particles are also seen (arrows) but they are not common.

The vesicles showed a rough outer particulate layer (Fig. 2). Particles were 30–40 A in diameter and were visible on a majority of vesicles. They are similar to those seen in microsomal preparations from vertebrate skeletal muscle (7). A lightly stained "membrane" region approximately 60–70 A thick surrounds each vesicle.

An examination of the vesicles following 15 min of calcium oxalate accumulation (Fig. 3) shows the presence of extensive nodule formation.

Figure 3  Barnacle muscle microsomes following a 15 min incubation period in a medium containing calcium and oxalate. The majority of the vesicles show nodular deposits which presumably represent calcium oxalate precipitates. X 60,000.
FIGURE 4 Positively stained and sectioned lobster microsomes. Vesicles were spherical or ovoid in cross-section. Tubules or tadpole configurations were not found. Membrane was triple layered (arrows), each layer being approximately 20–25 Å thick. × 235,000.
Particularly striking is the large number of vesicles (more than 80%) showing the presence of nodules. These nodules presumably represent deposits of calcium oxalate and indicate the extraordinary activity of the lobster microsomal preparations.

**Thin Sections**

Thin sections of microsomal vesicles revealed a triple-layered membrane (Fig. 4) consisting of an outer dark layer, a middle light layer, and an inner dark layer. Each layer was 20–25 Å thick, the total thickness of the three layers being 60–70 Å. All the vesicles were round or ovoid in shape and no tadpole-shaped vesicles were seen. The vesicles ranged in diameter from 250 to 2400 Å.

**Critical-Point Drying**

A suspension of lobster muscle microsomes was examined following critical-point drying and shadowing with platinum. This preparation method avoids the distortion caused by the movement of a liquid-air interface such as occurs during drying following negative staining. The vesicles observed were generally round and exhibited a smooth surface (Fig. 5). No evidence of a particulate fringe was seen.

**Freeze-Drying**

Lobster muscle microsomes that were prepared by freeze-drying followed by staining with phosphotungstate (Fig. 6) showed no evidence of “tadpole” formation. The vesicles were round or oval in shape. A lightly stained “membrane” layer, 60–70 Å in thickness, surrounded each vesicle. The outer surface of the vesicles appeared smooth and showed no evidence of particles. Most of the vesicles were positively stained, indicating that at the time of application of the

![Figure 5](image-url)
FIGURE 6 Lobster microsomes following freeze-drying on a grid and the application of 4% phosphotungstate. Vesicles appear smooth, showing no evidence of a particulate fringe. × 140,000.

FIGURE 7 Freeze-etch image of barnacle muscle microsomes. Microsomal suspensions were centrifuged to a pellet in a medium containing 50% glycerol. The pellet was frozen and fractured as previously described (2). Vesicles appeared spherical and some were covered with particles 80–90 Å in diameter. × 36,000. Double arrows indicate direction of shadow. *Inset* × 60,000.
stain (2% phosphotungstate) they were quite permeable to it. This is in contrast to the appearance of vesicles which have been negatively stained without prior freeze-drying (Fig. 1). These vesicles generally show less permeability to this stain.

**Freeze-Etching**

A freeze-etch micrograph of a pellet resulting from centrifugation at 120,000 g is shown in Fig. 7. Pellets were prepared from both lobster muscle and barnacle muscle microsomes. These pellets were more loosely packed than pellets prepared in a similar manner with rabbit skeletal muscle microsomes (3). In general the results were similar to those obtained with rabbit skeletal muscle microsomes. The vesicles were spherical and did not show tails or a tadpole configuration. Vesicles that had cleaved both concavely and convexly are seen. 80-90 A particles are visible on the surface of the concavely cleaved vesicles. The convexly cleaved vesicles generally appeared smooth; however, occasional 80-90 A particles are also seen on these surfaces. Freeze-etched microsomes from lobster muscle appear similar to freeze-etched microsomes obtained from barnacle muscle. The density of particles varied considerably from vesicle to vesicle. On some vesicles, particles covered less than 1% of the surface; on others, the particles covered as much as 20% of the surface.

In order to study the appearance of intact sarcoplasmic reticulum, a thin slice of lobster remotor muscle was prepared by the method of freeze-etch. This muscle has been reported to contain over 60% sarcoplasmic reticulum (10), and, for this reason, is especially suitable for the present study. The resulting preparation is shown in Fig. 8. A series of thick filaments projecting from the surface of the preparation is seen in proximity to the vesicles of the sarcoplasmic reticulum. Many of the vesicles show the presence of 80-90 A particles similar to those seen in the vesicles of the microsomal preparations.

**DISCUSSION**

Lobster and barnacle muscle microsomes isolated in solutions of low ionic strength show ultrastructural characteristics similar to those seen in vertebrate skeletal muscle microsomes. In particular:

(a) Microsomes prepared by negative staining show the presence of 30-40 A particles surrounding most vesicles. (b) Tadpole-shaped vesicles and vesicles with tails are seen only in microsomal preparations negatively stained with phosphotungstic acid. (c) Particles 80-90 A in diameter are seen in preparations of freeze-etched vesicles. Smooth (nonparticle-containing) vesicles are also seen.

**Small Particles**

The 30-40 A particles which are observed in negatively stained preparations of microsomes may be related to calcium transport (7, 8). They are not, however, seen with techniques other than negative staining. These particles, if present within the membrane, might be expected to be visible in freeze-etch micrographs, since membranes are considered to split along inner hydrophobic regions as a result of the freeze-etch technique (2). Vesicles prepared by the method of freeze-etching show no evidence of 30-40 A particles, either on their outer rim or in the region of the membrane exposed by the fracture. However, the deep-etching techniques recently described by DaSilva and Branton (2) would seem to offer a better approach to this question, since the method used in this investigation probably could not reveal particles near the membrane surface.

If these particles are structurally distinct from the rest of the membrane, it would be expected that they would be visible in preparations of sectioned, freeze-dried, or critical-point-dried microsomes. A particulate outer layer was not seen in microsomes prepared with any of these techniques.

In the present investigation, as well as in the work of Hasselbach and Elfvin (6), the thickness of sectioned vesicle membranes was about 60-70 A. The freeze-dried vesicles showed 50-60 A thick "membranes." The lighter "membrane" layer in negatively stained microsomes was 60-70 A as found in this investigation and 60-80 A in the work of Martonosi (9). (These values for negatively stained vesicles do not include the 30-40 A thick particle layer.)

The "membrane" and particle layers in negatively stained preparations therefore have a total thickness of 90-120 A. This is considerably greater than the thickness of sectioned vesicle membranes or of the "membrane" layer seen in freeze-dried vesicles. This suggests that the process of negative staining and the drying of the vesicles on the
Figure 8  Freeze-etch image of lobster remotor muscle prepared as in Fig. 6. 80-90 A particles are visible on vesicle surfaces. Double arrows indicate direction of shadow. X 130,000.
surface of a grid are required for the appearance of the 30–40 A particles. Particles may, for example, result from shrinkage during vesicle drying after a granular constituent of the membrane had adhered to the surface film of the grid. It must also be pointed out that the membrane regions observed in sectioned preparations (11) may not correspond to the “membrane” or “lighter surrounding layers” observed in this and other investigations (9) following negative staining.

The thin sections of microsomal pellets studied in this investigation exhibited a 60–70 A membrane thickness. This is in agreement with the work of Hasselbach and Elfvin (6). Both investigations also showed this membrane to be triple layered, with outer dark layers and an inner light layer. In the present investigation, however, in contrast to the work of Hasselbach and Elfvin, we observed that each of the three membrane layers was approximately 20–25 A thick. This may represent a difference in structural make-up between microsomal vesicle membranes from crustacean muscle and those from vertebrate skeletal muscle. This possibility, however, requires further investigation, and is not established on the basis of present evidence.

Vesicle Shape

The presence of tadpole-shaped vesicles in phosphotungstic acid–stained microsomes, and their absence in microsomes prepared by any other method (i.e. thin sectioning, freeze-etching, freeze-drying), supports the argument (3) that this shape is the result of osmotic forces developed during vesicle drying in the presence of a non-penetrating stain. This effect may be used to determine the integrity of vesicle membranes following isolation of the microsomes. Preparations with intact vesicle membranes will show extensive tadpole formation upon application of phosphotungstic acid and subsequent drying. Poorly prepared or damaged membranes will allow the stain to penetrate into the vesicle interior, and tadpole formation will not occur.

Formation of Nodules

The extensive nodular formation observed in the lobster and barnacle microsomes when incubated with calcium, oxalate, and ATP correlates with the high values of calcium uptake that were measured. This also is in agreement with the conclusion that extensive tadpole formation is indicative of intact vesicle membranes. Crustacean muscle microsomes appear, therefore, to offer a more active preparation than vertebrate skeletal muscle microsomes because they have a lesser amount of nonvesicular contamination and give a greater yield of intact vesicles. A third possibility, while unlikely, is not excluded by this investigation. Crustacean muscle microsomal vesicles may be inherently more active due to the composition of their calcium uptake machinery. Arguing against this, however, are the measurements of ATPase activity in crustacean microsomes. This activity is not elevated as is the calcium uptake activity, but is comparable to the ATPase activities measured in vertebrate skeletal microsomes.

Freeze-Etch Particles

The 80–90 A membrane particles observed in microsomes prepared by the freeze-etch method may lie within the vesicle membrane (3). The demonstration, in this investigation, of the presence of these particles, both in preparations of isolated microsomes and in intact muscle sarcoplasmic reticulum, indicates that they are not merely the result of the microsome isolation procedure. The previously advanced (3) possibility that they are the site of ATPase enzymes is supported by their extensive distribution in crustacean microsomes and the level of ATPase activity in these preparations.

This investigation was supported in part by National Institutes of Health grant No. HE 12978-01.

Received for publication 20 April 1970, and in revised form 19 June 1970.

REFERENCES

1. BASKIN, R. J., and D. W. DeAMER. 1969. J. Cell Biol. 43:610.
2. DASilVA, P., and D. BRANTON. 1970. J. Cell Biol. 45:598.
3. DeAMER, D. W., and R. J. BASKIN. 1969. J. Cell Biol. 42:296.
4. FANBURG, B., and J. Gergely. 1965. J. Biol. Chem. 240:2721.
5. FISKE, C. H., and Y. SUBBARow. 1925. J. Biol. Chem. 66:375.
6. HASSELBACH, W., and L. ELFVIN. 1967. J. Ultrastruct. Res. 17:598.
7. Ikemoto, N., A. Steeter, and J. Gergely. 1966. Fed. Proc. 25:465.
8. Inesi, G., and H. Asai. 1968. Arch. Biochem. Biophys. 126:469.
9. Martonosi, A. 1968. Biochim. Biophys. Acta. 150:694.
10. Rosenbluth, J. 1969. J. Cell Biol. 42:534.
11. Stoeckenius, W., and D. Engelman. 1969. J. Cell Biol. 42:513.
12. Van Der Kloot, W. 1965. Comp. Biochem. Physiol. 13:547.
13. Zobel, C. R., R. J. Baskin, and S. L. Wolfe. 1967. J. Ultrastruct. Res. 18:537.