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Pressure-Induced Inactivation of Sarcoplasmic Reticulum Adenosine Triphosphatase during High-Speed Centrifugation†

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ABSTRACT: Sarcoplasmic reticulum vesicles were found to be highly sensitive to high-speed centrifugation in metal-deprived mediums at low temperature (4 °C). The irreversible modifications induced were easily detected from observation of the environment-sensitive spectrum of an iodoacetamide spin-label bound to the ATPase. Centrifugation also resulted in vesicle aggregation and inhibition of calcium transport, ATPase activity, and phosphoenzyme formation. These denaturation-like phenomena were prevented in the presence of sucrose, or by nucleotide binding, or, again, by cation binding to the ATPase high-affinity calcium binding sites and were only present when centrifugation was performed at low temperature. The crucial parameter during this process was found to be the hydrostatic pressure which developed in the centrifuge tube. SR vesicles exposed to 800 bars in a pressure bomb displayed the same features. It is suggested that irreversible denaturation takes place after one or both of the two following well-documented effects of pressure: a rise in the lipid order/disorder transition temperature or dissociation of the oligomeric structure of the calcium pump.

Although high hydrostatic pressure affects the structure and function of biological systems in many ways, little attention is generally paid to the pressure conditions prevailing during the centrifugation steps included in routine experimental procedures. A number of reports, however, point to the importance of this parameter [see, for instance, Wattiaux-De-Conninck et al. (1977); Formisano et al. (1978)]. In the course of our own spin-labeling studies with sarcoplasmic reticulum (SR)[1] vesicles, we found that the final bound label spectrum was dependent on the conditions prevailing during the free-label-removing centrifugation steps. The resulting enzymic

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[1] Abbreviations used: ATPase, adenosine triphosphatase; SR, sarcoplasmic reticulum; Mes, 2-[*N*-morpholino]ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, morpholinopropanesulfonic acid; SH, sulfhydryl group; ESR, electron spin resonance; ISL, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide (Syva); EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

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activity also proved crucially sensitive to high-speed centrifugation.

We studied this denaturation-like phenomenon in order to characterize its dependency on solvent conditions and temperature. We showed that the high hydrostatic pressure generated by centrifugation was mainly responsible for the changes experienced by the protein.

Apart from the practical implications of these observations, we discuss the possible significance of our results in terms of protein–lipid interaction and of the hypothetical oligomeric structure of the calcium pump.

Experimental Procedures

Sarcoplasmic reticulum fragments derived from rabbit skeletal muscle were prepared and tested as previously described (Champeil et al., 1978). The phospholipid to protein ratio was around 0.8 μmol/mg of protein for our preparation. Vesicles were labeled with an iodoacetamide spin-label (ISL) in a medium which, except for the experiment depicted in Figure 1, contained 100 mM Mops, 100 mM KCl, residual sucrose from the stock SR suspension, 5 mM $MgCl_2$, and 0.3 mM EGTA (pH 7); protein and label concentrations were 3–20 mg/mL and 0.4–0.6 mM; incubation time at room temperature varied from 10 min to 2 h without altering the effect of the centrifugation described below (0.6–2 mol of label reacted with each ATPase polypeptide). At the end of the incubation period, the free label was washed out by dialysis or centrifugation in a calcium-containing medium (see below). Vesicles were resuspended in a small volume of a standard buffer. ESR spectra were recorded at 20 °C with a Bruker ER 200tt spectrometer. Most of the bound label displayed a strongly immobilized spectrum.

Results

Labeled vesicles were diluted in various media and submitted to high-speed centrifugation before resuspension in a standard buffer and ESR spectrum examination (Spinco 50 Ti rotor, 45000 rpm, 1 h at 4 °C). Very different spectra were obtained depending on the ionic conditions prevailing during the centrifugation step. Figure 1 shows that chelation of calcium ions during centrifugation at pH 6[2] allowed a weakly immobilized component to dominate the spectrum (spectrum 2 vs. spectrum 3); magnesium chelation only had a slight further influence (spectrum 1). At pH 7.5, on the other hand, not only calcium and magnesium but also potassium had to be removed from the centrifugation medium to obtain an almost pure weakly immobilized signal (spectra 9, 8, and 7 vs. spectrum 10). Potassium, magnesium, and calcium, therefore, apparently compete with protons for binding to a critical site.

That this site is actually the calcium-specific site involved in SR-ATPase activation was demonstrated by experiments in which the free calcium concentration during centrifugation was varied by means of a Ca–EGTA buffer. Protection of the strongly immobilized component was attained in the same range of low calcium concentrations as those allowing phosphoenzyme formation, both at pH 6 in the presence of Mg^{2+} and K^+ and at pH 7 in the absence of these cations (not shown). Furthermore, at pH 5.5, i.e., under conditions where enzyme affinity for calcium is so low that endogenous or

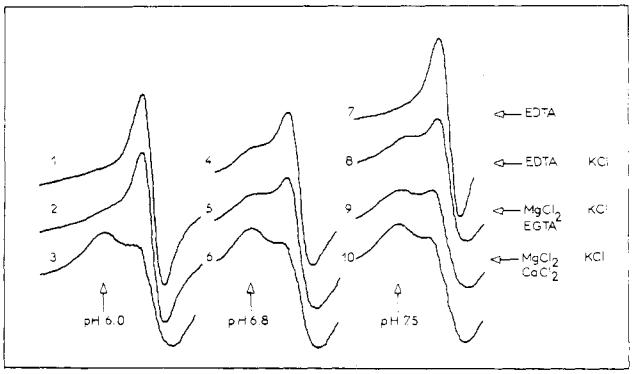

FIGURE 1: Spectra of labeled vesicles after centrifugation in different mediums. In this experiment, vesicles (3 mg of protein/mL) were labeled for 1.75 h at 4 °C in 0.1 M KCl, 50 mM Tris–maleate, 25 mM residual sucrose, 0.3 mM EDTA, and 0.4 mM ISL (pH 6.8). Aliquots of the vesicle suspension were then taken, from which free label was removed by dilution in various mediums, high-speed centrifugation (rotor 50 Ti, 45000 rpm, 1 h at 4 °C), resuspension, and a second centrifugation. All pellets were then resuspended at a high protein concentration in a standard buffer, and their spectra, of which only the low field part is shown, were recorded. All centrifugation media were buffered with 50 mM Tris–maleate; pH and cations were as shown in the figure. When present, indicated species were at concentrations of 0.2 mM Ca^{2+}, 5 mM Mg^{2+}, 0.3 mM EGTA, 0.5 mM EDTA, and 100 mM KCl.

contaminating calcium in the preparation does not bind to the "high-affinity" site, label mobilization during centrifugation was found to occur even in the absence of EGTA (but not in the presence of 0.5 mM calcium); this excludes the possibility that EDTA- or EGTA-induced denaturation might explain our results.

We also noticed that, in the absence of calcium, the presence of 5 mM ATP had a protective effect on the strongly immobilized signal, although we did not check the extent of ATP hydrolysis to ADP. The same protective effect was true for high concentrations of sucrose (0.3 M) or glycerol (30%).

We tested the temperature dependence of the centrifugation effect between 2 and 25 °C and found it was maximal at low temperature. On the other hand, centrifugation at 18 °C had virtually no effect on the label's ESR spectrum (spectra not shown).

Figure 2 shows that the high-speed centrifugation effect was in fact due to the hydrostatic pressure generated in the centrifuge tube. In this experiment, the hydrostatic pressure exerted on membranes centrifuged in a metal-deprived medium was varied from 100 to 600 bars by means of different sample volume and rotation speed combinations. After centrifugation, the vesicles displayed spectra well correlated with the estimated pressure at the bottom of the centrifuge tube: 100, 200, and 600 bars for spectra 22, 23, and 24 (Wattiaux, 1974). Figure 2 further shows that the pressure-induced phenomenon was irreversible, even under a second exposure to pressure (see spectra 1 and 12).

The high-speed centrifugation effect on SR enzymic and transport activities was tested under conditions defined in the preceding paragraphs, i.e., at pH 6 (20 mM Mes), 4 °C, and in the presence of 100 mM KCl and 5 mM $MgCl_2$. We performed these experiments with both unlabeled and labeled vesicles and obtained similar results irrespective of the presence of the protein-bound iodoacetamide label. We also compared vesicles centrifuged in the presence or absence of calcium to uncentrifuged samples left in the same media. All our results (Table I) show the following: (i) Vesicles which were either kept in an EGTA or a calcium medium or centrifuged in the calcium medium had similar properties; only the EGTA-centrifuged sample was distinctly different. (ii) Phosphorylated

[2] The actual pH during centrifugation might be slightly more acid than the nominal value. The pH of the most pressure-sensitive buffer known (phosphate buffer) would, however, only decrease by less than 0.2 unit over a pressure range of 600 bars (Neuman et al., 1973; Schade et al., 1980).

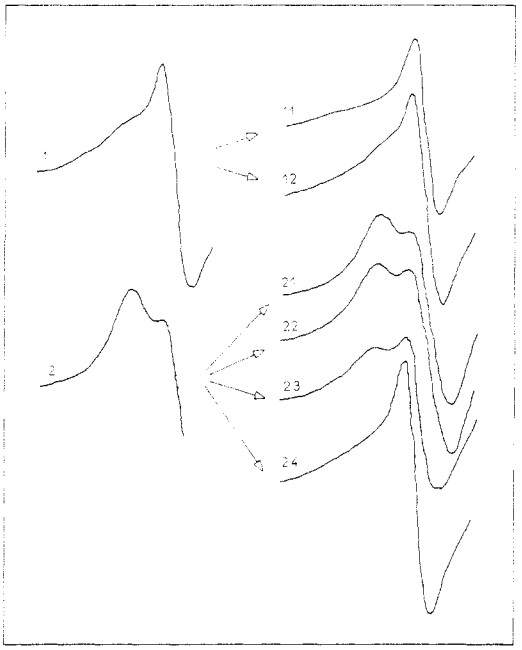

FIGURE 2: Effect of the pressure generated in the centrifuge tube. Labeled vesicles were submitted to a first centrifugation in either an EDTA or a calcium medium, and the resulting spectra are shown (1,2). A second centrifugation was then applied to both types of samples. Vesicles already centrifuged in the calcium medium (2) were spun down a second time in either the calcium (21) or the EDTA medium (22–24). Vesicles already centrifuged in EDTA (1) were spun down a second time in either the EDTA (11) or the calcium medium (12). Samples were centrifuged in the Spinco 50 Ti rotor. Sample volume and centrifugation speed and time varied as follows: small volume with high speed (0.5 mL, 50 min at 50 000 rpm, spectrum 22), large volume with low speed (8 mL, 3 h at 30 000 rpm, spectrum 23), or large volume with high speed (8 mL, 50 min at 50 000 rpm, all other spectra). Estimated pressures at the bottom of the centrifuge tube were respectively about 100 (22), 200 (23), and 600 bars (all other samples; Wattiaux, 1974). The mediums referred to as "EDTA" or "calcium" contained 50 mM KCl, 150 mM Mes (pH 6, 4 °C), and either 0.3 mM EGTA + 0.6 mM EDTA or 5 mM $MgCl_2$ + 0.3 mM EGTA + 0.5 mM $CaCl_2$.

Table 1: Effect of Centrifugation on SR Functional Parameters[a]

	EP (nmol mg^{-1})	ATPase (μmol mg^{-1} min^{-1})	Ca transport (μmol mg^{-1} min^{-1})
after centrifugation in an EGTA medium	0.9 ± 0.1	0.17 ± 0.02	0.08 ± 0.01
after centrifugation in a Ca medium	3.2 ± 0.4	0.36 ± 0.04	0.5 ± 0.05
after incubation in an EGTA medium	3.5 ± 0.4	0.46 ± 0.05	0.8 ± 0.1
after incubation in a Ca medium	4.2 ± 0.4	0.41 ± 0.04	0.9 ± 0.1

[a] Labeled vesicles were centrifuged or simply incubated 1 h at 4 °C in a medium containing 80 mM KCl, 5 mM $MgCl_2$, 0.3 mM EGTA or 0.2 mM $CaCl_2$, 20 mM Mes at pH 6. Assays were performed at pH 7 and 20 °C as previously described (Champeil et al., 1978); 5 mM oxalate was present for the ATPase activity and calcium transport measurements.

intermediate formation after the addition of ATP in a standard assay medium (Champeil et al., 1978) was severely inhibited by previous centrifugation in an EGTA-containing medium. The extent of inhibition was found to vary between 50% and 80%; when the experiment was performed with labeled preparations, this proportion correlated well with the extent of label mobilization allowed by the centrifugation step. (iii) ATPase activity and calcium transport velocity in the presence of oxalate were also found to be inhibited by centrifugation in an EGTA medium. These parameters were, however, respectively less and more inhibited than phosphoenzyme formation; the reason for this is probably that centrifugation in EGTA not only inactivated certain ATPase molecules (inhibition of phosphoenzyme formation) but also made the vesicles more leaky, with resultant acceleration of ATP hydrolysis by the remaining active molecules.

Table I also suggests that calcium-deprived vesicles may experience a slow inactivation even under atmospheric pressure. Pressure would greatly accelerate this process.

With respect to the general morphological effects of the EGTA centrifugation step, we noticed that the turbidity of the vesicle suspension increased after spinning, suggesting final vesicle aggregation (hence the increased leakiness mentioned above); with respect to sulfhydryl groups, we found by DTNB titration that centrifugation had virtually no oxidizing effect on SR SH groups [cf. Coan & Inesi (1977)]. We tested the possibility of EGTA-induced cross-linking of ATPase polypeptides via a disulfide bridge by running electrophoresis gels of centrifuged samples in the presence or absence of mercaptoethanol and found no evidence for such cross-linkage nor, incidentally, did we find any specific release of proteins induced by the centrifugation step.

Preliminary experiments were performed with a pressure bomb to test the effect of pressure per se on SR vesicles (1 mg/mL) suspended in the above-mentioned mediums. We found that a 1-h incubation at 800 bars and 4 °C had the same effect as centrifugation from the point of view of both ESR spectrum mobilization and turbidity increase. These experiments with the pressure bomb therefore support the conclusions drawn from the results in Figure 2.

Discussion

In an earlier paper we studied the chemical reactivity toward iodoacetamide spin-labels of sarcoplasmic reticulum ATPase SH groups. We reported that the presence of the enzyme's ligands influenced both the labeling rate—and we confirm this observation—and the bound label spectrum shape (Champeil et al., 1978). Further investigation, however, showed us that the final bound label spectrum was not dependent on the labeling conditions, but rather on the conditions prevailing during the centrifugation steps. These last results therefore contradict part of our initial interpretation.

The first practical conclusion to be drawn from the present study is that high-speed centrifugation of SR vesicles must be performed with some care. From both centrifugation and pressure bomb experiments, we found that SR vesicle exposure to high hydrostatic pressure under ionic conditions where calcium sites were not protected by cations was followed by an irreversible modification of the calcium pump; this was evidenced by activity inhibition, SH environment modification, and a tendency to aggregation. These denaturation-like phenomena were prevented by the presence of ATP or of sucrose or glycerol at high concentrations and were well developed at 2 °C, but not at 20 °C. In practise, the conditions leading to denaturation are fairly common (for example, pH 6.8, 5 mM $MgCl_2$, 100 mM KCl, and 0.3 mM EGTA, 4 °C, and 45 000 rpm in the Spinco 50 Ti rotor, as in our Figure 1). Fortunately, however, the presence of sucrose at relatively high concentrations (0.3 M) protects the vesicles, and sucrose is often present in the various protocols described in the literature. It might, however, be significant, in relation to our results, that various authors described irreversible modifications following metal deprivation of vesicles (Inesi & Scales, 1974; Nakamura et al., 1977).

Turning now to the discussion of our results, the first point which we wish to emphasize is that the pressure-induced inactivation of calcium-deprived vesicles cannot be explained by a denaturing effect of chelating agents. On the contrary, the extent of denaturation was inversely correlated with saturation of the ATPase high affinity calcium binding sites, even in the absence of any chelating agent under suitable pH conditions (see Results). This rules out any trivial effect of EGTA or EDTA. In addition, our observations are probably not directly related to the calcium removal induced inactivation of calcium transport described by McIntosh and Berman (1978) since both our observations and theirs have opposite temperature dependencies. Besides, phosphoenzyme formation and ATPase activity were not inhibited in their experiments, in contrast to ours.[3]

The second point which calls for comment is the fact that the modifications we observed were irreversible, whereas the effects of pressure are generally believed to be reversible. Evidently, therefore, pressure-induced reversible events are followed by an independent irreversible transition, so that irreversibility is not the result of the pressure increase in itself. Numerous examples of this situation can be found in the literature (Wattiaux, 1974; Visser et al., 1977; Schade et al., 1980). Pressure behaves in a similar way to temperature, since temperature-induced changes are a priori reversible, although irreversible thermal denaturation may also occur.

The third point to notice is the fact that SR ATPase inactivation was observed for moderate pressure values (600 bars), much lower than those reported to significantly modify ionization constants, to modify protein–ligand interactions, or to induce denaturation of proteins [1–10 kbars; see Neuman et al. (1973), Johnson et al. (1974), Li et al. (1976), and Torgerson et al. (1979)]. On the other hand, such moderate pressure values are known from the membrane and enzymology literature to give rise to two well-documented effects, not mutually exclusive. The first is a pressure-induced shift in membrane lipid order–disorder transition temperature [2 °C for each 100 bars; see Liu & Kay (1977), Wattiaux de Conninck et al. (1977), Ceuterick et al. (1978), Kamaya et al. (1979), and de Smedt et al. (1979)]. The second effect is a pressure-induced dissociation of oligomeric proteins (Josephs & Harrington, 1967; Jaenicke & Koberstein, 1971; Penniston, 1971; Formisano et al., 1978; Schade et al., 1980).

Insofar as speculation is allowed, our observations on the pressure dependence of the ATPase inactivation rate might therefore tentatively be explained as follows. A first hypothesis would be that pressure affects the ATPase by stiffening the aliphatic chains of nearby phospholipids. This hypothesis has been proposed for a number of membrane proteins, including SR-ATPase (Wattiaux de Connink et al., 1977; Ceuterick et al., 1978; de Smedt et al., 1979; Heremans & Wuytack, 1980). However, in the case of this enzyme, a breakpoint in the activity vs. pressure curve was only found for temperatures of 20 °C or more [Figure 2 in Heremans & Wuytack (1980)], so that the effect of pressure at low temperature remains in this case to be investigated. Anyway, the probable rigidification of the aliphatic chains at high pressure would only be sufficient to explain our results if the ATPase were rapidly inactivated in the presence of EGTA when embedded in a rigid lipid matrix. Although this is only indirect evidence, no such EGTA-dependent cold lability has been described yet for ATPase preparations reconstituted with dipalmitoyllecithin for instance (Warren, 1974; Nakamura et al., 1976; Kleeman & McConnell, 1976). A second hypothesis would be that pressure might dissociate an oligomeric complex. In the light of the present knowledge of the SR Ca^{2+} pump's quaternary structure, this hypothesis is particularly attractive, since a number of reports are in favor of an oligomeric structure for the ATPase (le Maire et al., 1976, 1978; Vanderkooi et al., 1977; Scales & Inesi, 1976) and since the ATPase monomer, as opposed to the oligomer, was recently reported to be especially sensitive to calcium chelation, which induced irreversible inactivation and modified SH reactivity; the monomer activity was furthermore found to be protected by sucrose or glycerol [Møller et al. (1979) and personal communication from J. P. Andersen]. These are the parameters which, in our experiments, were also found to be important for recovery of the SR native state after pressurization. Some of these parameters might in fact also affect the actual dissociation of the oligomeric ATPase, since it was recently demonstrated with lactic dehydrogenase that specific solvent conditions are of crucial importance with respect to the dissociation equilibrium under pressure (Schade et al., 1980).

From our analysis, we therefore suggest that the study of the SR ATPase under pressure might allow further insight into the calcium pump's protein–lipid interactions and/or quaternary structure. It might be possible to establish experimentally whether one of the two above hypotheses is correct by checking freeze–fracture pictures after glutaraldehyde fixation or freezing of the vesicles under pressure or by testing the fluorescence transfer between ATPase monomers in a pressurized cell (Vanderkooi et al., 1977).

[3] Note that we performed experiments under their conditions at 37 °C and found an inactivation time for calcium transport more than 3 times slower than the one they report.

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