CROSS-LINKING OF THE SARCOPLASMIC RETICULUM ATPASE PROTEIN

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SUMMARY: Treatment of rabbit sarcoplasmic reticulum vesicles with the cross-linking agent, cupric phenanthroline, causes production of high-molecular weight bands on SDS-gel electrophoresis. A plot of log mol wt vs mobility indicates that the main band produced from the ATPase (mol wt = 10^5) has a mol wt of 4 x 10^5 and thus suggests formation of a tetramer. Notably, bands corresponding to dimers, trimers, pentamers, etc., are absent. The bands attributable to calsequestrin and calcium binding protein are unchanged by cupric phenanthroline. With extended treatment, the tetramer itself is polymerized (mol wt>10^6). Partial disruption of the membranes with deoxycholate or Triton X-100 before cross-linking favors tetramer formation; the presence of sodium dodecyl sulfate, on the other hand, prevents intermolecular cross-linking. Our results suggest that the ATPase is at least partially associated within the membrane as a tetramer.

Though much evidence exists which shows that active transport proteins are intimately associated with a membrane (1), their arrangement within the membrane is unsettled. As has been found for numerous water-soluble proteins, association of polypeptide chains in a bilayer to form an oligomeric functional unit is a possibility which merits testing. Thus, for example, transmembrane channels between subunits might provide the pathway for energy-coupled pumping or excitation-coupled release in such systems as SR. Taking advantage of this relatively simple membrane system's protein sulfhydryl groups, we have used a cross-

Abbreviations used: SR, sarcoplasmic reticulum membranes; CuPA, cupric 1,10-phenanthroline; SDS, sodium dodecyl sulfate; MOPS, morpholinopropane sulfonic acid; CBP, calcium binding protein; CAL, calsequestrin.

linking catalyst, cupric phenanthroline (2), to form disulfide bridges between the ATPase protein chains. Results suggest that they exist within the bilayer as a tetramer.

MATERIALS AND METHODS: SR was prepared from rabbit hind leg muscles (3) and stored at 0° in 30% sucrose, 10 mM MOPS, pH 7.0. A stock solution of CuPA was made to 5 mM in $CuSO_4$ and 15 mM in 1,10-phenanthroline (Aldrich). Cross-linking was carried out at 25° in 1 mM $CaCl_2$, 0.1 M MOPS adjusted to pH 7.2 with triethanolamine and saturated with O_2. To this solution was added SR to 2 mg/ml, then CuPA (to 0.1 mM in Cu); other additions if any are mentioned in the figure legends. The reaction was stopped by adding EDTA to 1 mM, N-ethylmaleimide to 16 mM, and SDS to 10 mg/ml. The procedure for SDS-gel electrophoresis was adapted from Inesi et al. (4). Separating gels contained 5.1% acrylamide, 0.13% methylene bis acrylamide, 0.1% SDS, 0.07% ammonium persulfate, 0.03% tetramethyl ethylenediamine, 0.37 M Tris-HCl, pH 8.9. Stacking gels contained 4% acrylamide, 1% methylene bis acrylamide, 0.1% SDS, 0.06% tetramethyl ethylenediamine, 5 mg/ml riboflavin, 20% sucrose, 0.06 M Tris-HCl, pH 6.6. The running buffer was 0.1% SDS, 5 mM Tris, 38 mM glycine, pH 8.3. The tracking dye was pyronine Y, and the gels run at a constant current of 1 mA/tube. After fixing with trichloroacetic acid, the gels were stained with Coomassie Blue R250 (Ames).

RESULTS: As shown in Figure 1, SDS-gel electrophoresis patterns of SR treated for various times with CuPA reveal that the ATPase protein is altered. After 1 min a new band appears (band a) which migrates at about one-third the rate of the ATPase. At 5 min this band is intensified, another band appears (band b), while the intensity of the ATPase is decreased. After another 15 min band b is intensified while the ATPase and band a are attenuated. At one hr these three bands are almost gone, the protein having been cross-linked into large polymers which do not enter the gel. During this time little or no change occurs in the other SR proteins CBP and CAL.

In order to estimate the molecular weights of the CuPA-generated bands, plots of log mol wt vs relative mobility (to dye) were done. In addition to utilizing the known apparent molecular weights of the ATPase, CBP and CAL of 105, 67 and

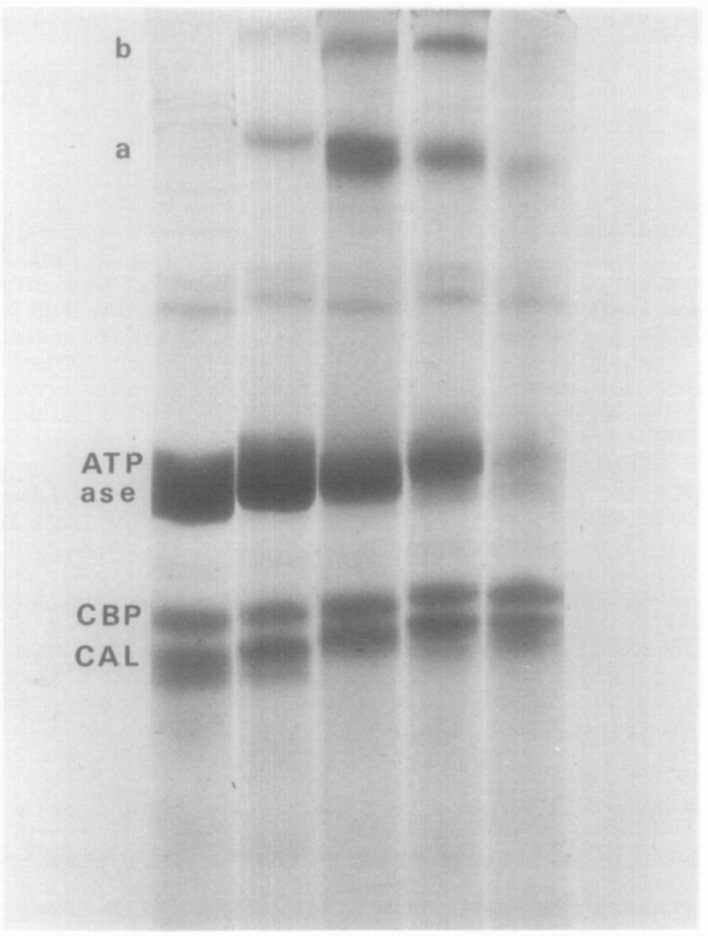

Figure 1. SDS-gel electrophoresis patterns of SR incubated for various times with CuPA. From left to right, samples incubated for 0,1,5,20 and 60 min, respectively.

55 x 10^3, respectively (5), calibration was aided by the use of rabbit myosin heavy chains (210 x 10^3), bovine serum albumin (67 x 10^3) and rabbit aldolase (40 x 10^3). As seen in Fig. 2, bands a and b have apparent molecular weights of about 400 and 700 x 10^3, respectively.

In the presence of deoxycholate, production of oligomers is more rapid, so that afer one minute more than half of the monomeric ATPase is gone (Fig. 3). The rate of formation of

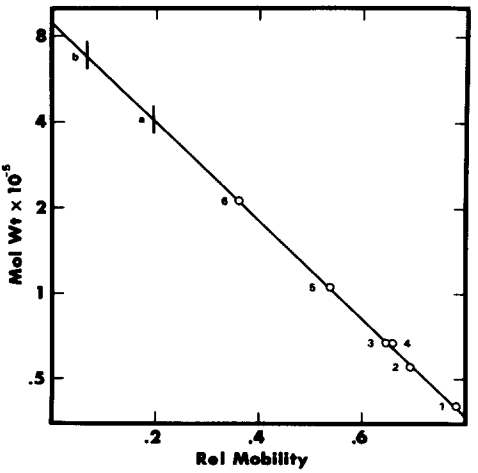

Figure 2. Semi-logarithmic plot of the apparent molecular weight vs the relative mobility of SR proteins and selected standards. 1, aldolase; 2, CAL; 3, bovine serum albumin; 4, CBP; 5, ATPase; 6, rabbit myosin heavy chain; a and b are the bands produced by CuPA treatment of SR.

oligomers relative to that of higher polymers would appear to be increased. Since the detergent partially disrupts the vesicular SR, decreased formation of multiples of a tetramer might be expected. Similar experiments carried out in the presence of the non-ionic detergent Triton X-100 yield essentially the same results. By contrast, CuPA treatment in the presence of SDS produces no oligomers (Fig. 3; gel 6); instead the ATPase band is broadened, probably as a result of intramolecular cross-links.

These results were obtained with a PA:Cu ratio of 3:1, so that the predominant complex is $Cu(PA)_3$, based on the stability constants in the literature (6). For PA:Cu ratios of 2:1, as used on erythrocytes (7), oligomerization of the SR ATPase was qualitatively similar but slower. For low molecular weight mercaptans, Kobashi (2) found the rate of disulfide formation

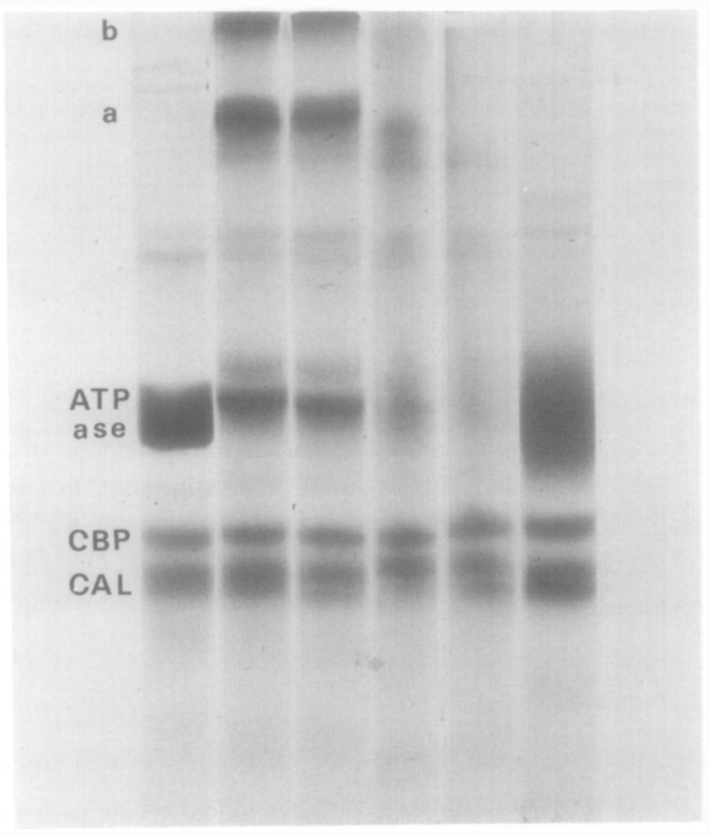

Figure 3. SDS-gel electrophoresis patterns of SR incubated for various times with CuPA in the presence of detergents. Gel on far right: 2 mg/ml SDS present; incubation time, 60 min. Other gels: 1 mg/ml deoxycholate present; from left to right, samples incubated for 0,1,5,20 and 60 min, respectively.

to be the same for the 2:1 and 3:1 complexes. The latter's greater effectiveness in cross-linking SR sulfhydryls may be attributable to its somewhat greater solubility in non-polar media; thus we find the buffer:octanol partition coefficients to be 3.7 and 2.5 for the 2:1 and 3:1 complexes, respectively. As might be expected (2), the rate of oligomer formation was greater at the higher pH of 7.8.

DISCUSSION: CuPA has been shown to catalyze the air oxidation of sulfhydryls to disulfides (2). Its ability to effect cross-linking of membrane-bound proteins has also been demonstrated (7,8). Given the bond distance between atoms in a disulfide (<2Å), formation of intermolecular cross-links requires close juxtapositioning of participating reactants. In the absence of any indication that CAL and CBP undergo significant intermolecular cross-linking, their existing as units of stable or transient oligomers might be ruled out, although the possible lack of accessible or properly oriented sulfhydryls may be responsible.

The cross-linking of the ATPase protein in the present work yields an oligomer with an apparent mol wt of 4×10^5, a value which corresponds most closely to that of a tetramer. The absence of dimers and trimers on the gels would seem to eliminate random polymerization due to thermal collisons of unassociated monomers, for such a mechanism would produce more dimers than trimers, more trimers than tetramers, etc. More plausible is a specific association of the ATPase protein in the form of a tetramer. Generation of band b and larger polymers (which do not enter the gels) is presumably the result of linking of the initially formed tetramers.

Partial disruption of SR with deoxycholate or Triton X-100, a procedure which does not inactive the ATPase, also does not prevent oligomer formation by CuPA. In fact cross-linking is somewhat faster, probably because the sulfhydryls become more accessible. Disperson in the "strong" detergent SDS, on the other hand, prevents intermolecular cross-linking. Intramolecular cross-linking probably does occur; the more diffuse ATPase band on the gel may be attributable to mixtures of different Stokes radii.

In studies to be reported later, we find that CuPA treatment of another SR, prepared from chichen breast muscle, gives results very similar to those obtained for rabbit SR; in addition, other cross-linking reagents will be shown to reveal the existence of a tetrameric ATPase. Evidence of oligomers using two other techniques has recently been reported. Using electron microscopy, Malan et al. (9) proposed a trimeric arrangement based on differences in densities of the outer projections seen by deep etching and of the particles seen on the concave faces after freeze fracture. Le Maire and Tanford (10), employing ultracentrifugation in the presence of the detergent Tween 80, report that "the smallest fully active particles contain 3 to 4 ATPase polypeptide chains. Taken together, these observations would seem to argue strongly that the ATPase exists within the SR membrane as an oligomer consisting of probably 4 ATPase units.

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