Functional Significance of Quaternary Organization of the Sarcoplasmic Reticulum Ca^{2+}-ATPase

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There is both structural and functional evidence for protein-protein interaction between Ca^{2+}-ATPase polypeptide chains in the sarcoplasmic reticulum membrane. Studies on detergent solubilized ATPase indicate that the monomeric form is capable of performing a normal cycle of ATP hydrolysis, but some of the modulatory effects of the substrates disappear after detergent solubilization. However, the necessity of protein-protein interactions for the Ca^{2+} transport function remains unclarified. A new approach is described, employing ATPase reconstituted with a large excess of phospholipid, which may help to resolve this question.

There is increasing evidence that many intrinsic membrane proteins are associated in oligomeric form in the membrane. The possible functional implications of selfassociation have recently been discussed: for transport proteins oligomerization provides on apportunity to form a hydrophilic channel within the area delimited by protein-protein contacts [1–3], but whether such a common channel is an essential feature of transport mechanisms has not been resolved. It is the purpose of the present communication to review recent evidence bearing on the role of oligomerization for the Ca^{2+} transport function of the sarcoplasmic reticulum Ca^{2+}-ATPase.

Studies on detergent solubilized ATPase

There are now several reports showing that under appropriate conditions it is possible to maintain full ATP hydrolysis of the Ca^{2+}-ATPase, solubilized in monomeric form by deoxycholate [4] or the non-ionic detergent $C_{12}E_8$ [5, 6]. The solubilized protein is easily inactivated by complexation of Ca^{2+} with EGTA, presumably as the consequence of conversion to the E^* conformation under these conditions [6]. In contrast to membraneous ATPase Watanabe *et al.* [7] suggest that the enzyme only is activated by one Ca^{2+} in the monomeric state as indicated by a Hill coefficient of 1. Verjovski-Almeida and Silva [8] reported retention of positive cooperativity when solubilization was performed in the presence of a high concentration of Ca^{2+}. This was thought to result in oligomerization of the ATPase. However, in our experiments the monomeric protein exhibits positive cooperativity with respect to Ca^{2+} as indicated both by enzyme activity [6] and fluorescence [9] measurements. Probably the conditions used for solubilization are critical for maintaining the enzyme in a native like state [3]. It should be noted that at the protein concentration used for the enzymatic assay the protein is predominantly in monomeric form (> 90%) so that the effect of oligomeric ATPase on the activity curve is expected to be negligible. However, it has been pointed out that there is a possibility that the aggregational state of the enzyme might change during activation, so as to promote e.g. formation of a dimer [2]. If this is the case the aggregational state of the ATPase before activation is clearly irrelevant. During ordinary measurement of activity by enzymatic spectrophotometry the ATPase is present at high dilution (3–15 µg/ml), and enzyme activity is proportional to the protein concentration, in contrast to what would have been expected if ATP hydrolysis were dependent on reversible selfassociation of the ATPase. The protein does have a tendency for aggregation at high protein concentrations as indicated both by sedimentation velocity [6] and sedimentation equilibrium [5] analysis. The gel electrophoretic results

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Abbreviations: $C_{12}E_8$, octaethyleneglycol *n*-dodecyl monoether; DCCD, dicyclohexylcarbodiimide; Tes, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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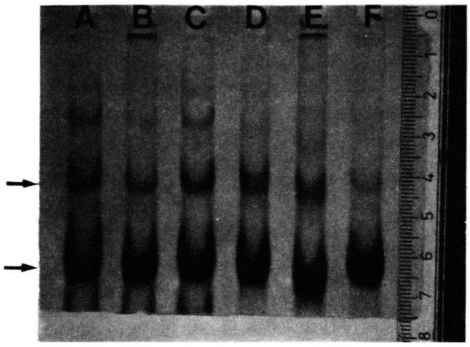

Fig. 1. Effect of ligands on the cross-linking properties of detergent solubilized ATPase. Sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle according to de Meis and Hasselbach [22]. The membranes were solubilized with $C_{12}E_8$ at a protein and detergent concentration of 5.5 mg/ml and 10 mg/ml, respectively, in a solution containing 10 mM Tes (pH 7.5) and 0.1 M KCl. The sample was centrifuged on an Airfuge for 15 min. Aliquots (50 µl) of the sample were added to media (200 µl) containing 10 mM Tes, 0.1 M KCl and 1 mg $C_{12}E_8$/ml with various additions as detailed below. A. 0.1 mM Ca^{2+}, 0.5 mM glutaraldehyde; B. 0.1 mM Ca^{2+}, 0.1 mM Cu^{2+}, 0.3 mM phenantroline; C. 1 mM EGTA, 0.5 mM glutaraldehyde; D. 1 mM EGTA, 5 mM ATP, 5 mM Mg^{2+}, 1 mM phosphoenolpyruvate and pyruvate kinase (60 I.U./ml), 0.5 mM glutaraldehyde; E. 0.1 mM Ca^{2+}, 5 mM ATP, 5 mM Mg^{2+}, 1 mM phosphoenolpyruvate, pyruvate kinase (60 I.U./ml), 0.1 mM Cu^{2+}, 0.3 mM phenantroline; F. 0.1 mM Ca^{2+}, 5 mM ATP, 5 mM Mg^{2+}, 1 mM phosphoenolpyruvate, pyruvate kinase (60 I.U./ml), 0.5 mM glutaraldehyde. Cross-linking was terminated after 2 min by addition of NH_4HCO_3 to glutaraldehyde containing samples and EDTA to the samples containing Cu^{2+}-phenantroline. Dodecylsulfate (50 µl of 18% (w/v) solution), with or without mercaptoethanol (in the Cu^{2+}-phenantroline samples), was added, and the samples were boiled for 5 min before being subjected to dodecylsulfate gel electrophoresis [23]. The arrows point at monomeric and dimeric ATPase.

of Fig. 1 show the effect of Cu^{2+}-phenantroline and glutaraldehyde as cross-linking agents of $C_{12}E_8$ solubilized ATPase at a rather high protein concentration (1 mg/ml). The presence of aggregated ATPase is suggested by the formation of distinct dimer bands in addition to the major band of monomeric ATPase. Interestingly these bands are observed in the same or less amounts in the presence of activating substrates. Thus this experiment provides no support for the view that oligomerization is favored under these conditions. It may be noted that under the present experimental conditions Cu^{2+}-phenantroline is the more efficient cross-linker of membraneous ATPase. With this agent there is a band of highly aggregated ATPase which does not enter the gel and which presumably is derived from non-solubilized ATPase which was incompletely removed from the preparation during the centrifugation prior to the cross-linking. The fact that there is no appreciable difference between the intensity of the dimer and trimer bands formed in the presence of Cu^{2+}-phenantroline and glutaraldehyde and that no intervening forms such as tetramers and pentamers are observed with Cu^{2+}-phenantroline suggests that cross-linking mainly occurred in preformed oligomers rather than by a collisional process during the conditions of the assay.

Studies on membraneous ATPase

It has been reported that modification of less than one amino acid residue per polypeptide chain of ATPase by FITC [10] or DCCD [11] suffices to produce complete inactivation of ATP hydrolysis and Ca^{2+} transport. Furthermore, fluorescamine has been found to affect Ca^{2+} transport and phosphorylation with inorganic phosphate to a higher extent at low modification levels than ATP hydrolysis [12]. These observations have been offered as evidence of coupling between ATPase polypeptide chains in the membraneous state, equivalent to half of the sites behaviour proposed for many soluble enzymes. During a study of the FITC treated preparations we found that remaining enzyme activity was unchanged after solubilization with $C_{12}E_8$. This observation does not readily lend support to the view that modification of one chain affects the activity of a neighboring chain of ATPase. Furthermore, the extent of modification required to block enzyme activity (7 nmol/mg ATPase) was found to be equivalent to the ATP binding capacity of the unmodified preparation. In agreement with Pick and Bassilian [13] we found that after modification the enzyme is active with acetylphosphate as a substrate and still exhibits a Ca^{2+} dependent equilibrium between the two principal conformations E and E^* [14]. Fig. 2 shows a reciprocal plot of relative Ca^{2+}-ATPase activity (V/V_{max}) versus MgATP concentration for unmodified and a partially modified preparation. In the latter case V_{max} was reduced by 48%. It is seen that the curves are identical, indicating the same reduction of the ATPase activity of the modification preparation at all ATP concentrations. The downward deviation reflects modulatory effects of ATP at concentrations higher than those which produce maximal phosphorylation of the protein [6,

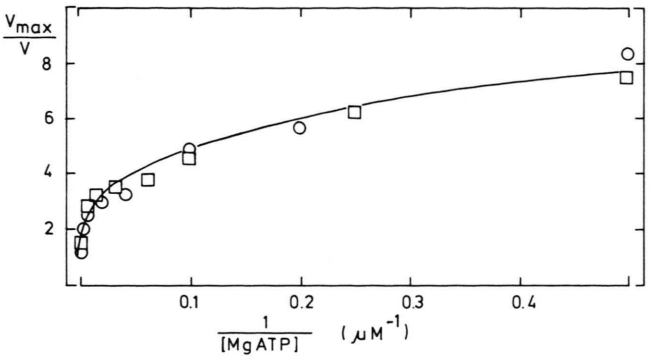

Fig. 2. Effect of MgATP on Ca^{2+}-ATPase activity of partially fluorescein labeled (○) and unlabeled (□) Ca^{2+}-ATPase. Purified ATPase was labeled with 3.5 nmol FITC/mg protein and the resultant preparation had a V_{max} which was 52% of the V_{max} of unlabeled ATPase. Enzyme activity measurements were performed by enzymatic spectrophotometry [6]. The detailed procedure is published elsewhere [9].

15]. This result suggests that also the modulatory effects of ATP are independent of protein-protein contacts, in contrast to earlier proposals [6, 16]. A possible mechanism is that ATP accelerates the dephosphorylation rate or the E^2 to E conversion of monomeric ATPase after the dephosphorylation step [14].

There are other studies which suggest that protein-protein interactions do affect the transport properties of the enzyme. Fassold *et al.* [17] reported that the ratio of Ca^{2+} transported: GTP hydrolyzed may vary between 2 and 6 during the initial phase, depending on the Ca^{2+} and GTP concentration. It was suggested that protein-protein contacts may lead to induction of Ca^{2+} transport by neighboring molecules of non-phosphorylated ATPase. Among other possibilities considered to account for high transport ratios of Ca^{2+} were translocation of Ca^{2+} bound at low affinity sites. Ikemoto *et al.* [18] found that after addition of a large excess of EGTA to the Ca^{2+}-equilibrated enzyme only half of the bound Ca^{2+} was released quickly. The Ca^{2+} which remained bound could be translocated by the further addition of ATP shortly after addition of the EGTA, confirming previous observations by Sumida *et al.* [19]. The non-equivalence of the ATPase peptide chains was suggested to arise from protein-protein interactions. In agreement with this view the slow phase of Ca^{2+} release disappeared after solubilization of the membranes with $C_{12}E_8$. In a following paper [20] it was reported that also a rapid and slow phase in the Ca^{2+} translocation could be discerned under special conditions (high concentrations of choline chloride and a low temperature). The slow phase was considered to be controlled by the release of Ca^{2+} to the intravesicular space from a neighboring chain that initially bound Ca^{2+} tenaciously. The picture which emerges from these results is that one of the two sets of subunits is one step ahead of the other in carrying out the sequential steps of the translocation process (phosphorylation, translocation of Ca^{2+}, and release of translocated Ca^{2+} to the intravesicular space).

The question arises whether the coupling of ATPase chains described by Ikemoto and collaborators is an obligatory feature of the Ca^{2+} translocation process. This could be the case if Ca^{2+} translocation occurs by a common channel formed by the hydrophilic interface of a dimer of ATPase. Such a mechanism would imply half-of-the-sites behaviour. Ordinarily the ATPase is only partially phosphorylated by ATP [4], and Watanabe *et al.* [7] report that high affinity Ca^{2+} binding is also limited to a ratio of 2:1 per phosphorylated ATPase peptide chain. However, the level of phosphorylation of monomeric ATPase, solubilized by $C_{12}E_8$, is identical to that of membraneous enzyme [9], and accordingly other factors than protein-protein contacts may be responsible for the incomplete phosphorylation with ATP.

Studies on reconstituted ATPase

The specificity of protein-protein interactions in the native membrane should be amenable to study by the use of ATPase reconstituted with a large excess of lipid. However, previous studies have shown a tendency for ATPase to aggregate in protein rich areas [21]. This study was done with deoxycholate as solubilizing agent. By a procedure using both $C_{12}E_8$ (for solubilization of the protein) and cholate (for solubilization of exogenous lipid) we have succeeded in dispersing a large fraction of the ATPase. The ratio of phospholipid to protein

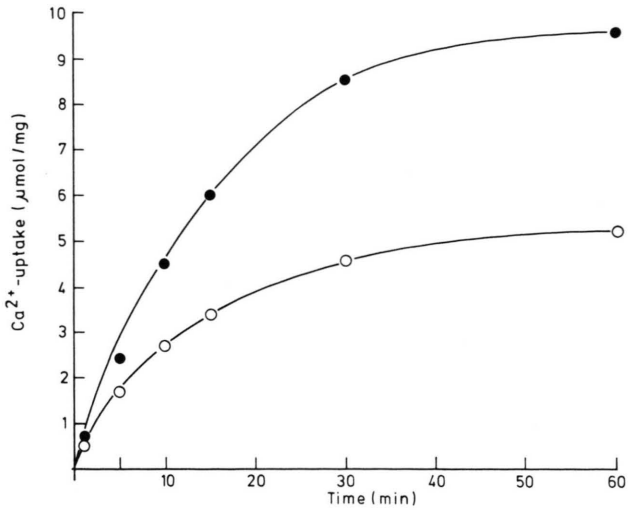

Fig. 3. Ca^{2+} transport of Ca^{2+}-ATPase reconstituted with an excess of phospholipid. Sarcoplasmic reticulum vesicles were solubilized with $C_{12}E_8$ and added in an amount corresponding to about 0.3 mg Ca^{2+}-ATPase to 30 mg egg lecithin, solubilized by cholate. The sample was reconstituted by dialysis by a procedure similar to that described by Skriver *et al.* [24]. After reconstitution the sample was divided in two parts: one was layered over a 7% (w/v) sucrose and 0.1 M phosphate (pH 7.1) and centrifuged for 15 h at $2-3 \times 10^5 \times g$ to sediment protein rich vesicles from the preparation. (○) Ca^{2+} transport of the sample after reconstitution; (●) Ca^{2+} transport after reconstitution and sucrose density centrifugation. The detailed procedure will be described elsewhere.

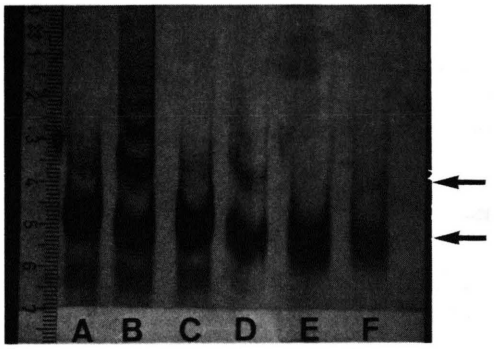

Fig. 4. Cross-linking with Cu^{2+}-phenantroline of native sarcoplasmic reticulum, $C_{12}E_8$-solubilized ATPase, and reconstituted ATPase. A. Native SR. B. Native SR after treatment with Cu^{2+}-phenantroline. C. $C_{12}E_8$ solubilized ATPase. D. $C_{12}E_8$ solubilized ATPase after treatment with Cu^{2+}-phenantroline. E. Reconstituted ATPase. F. Reconstituted ATPase after treatment with Cu^{2+}-phenantroline. The arrows point at monomeric and dimeric ATPase.

varied from 75–150:1. Freeze-fracture replica of the resulting preparation revealed a population of liposomes of fairly uniform size with few and scattered intramembranal particles. Occasionally, protein rich vesicles were found; these could be removed from the preparation by density centrifugation into a medium containing 8% (w/v) sucrose. Fig. 3 shows Ca^{2+} uptake in the presence of phosphate as a Ca^{2+} precipitating agent. It is seen that the highest levels of Ca^{2+} uptake are obtained after removal of protein rich vesicles by density centrifugation. This consistent with Ca^{2+} transport into the larger intravesicular volume per weight unit protein that must be presumed to exist for the protein rich vesicles. The ratio of Ca^{2+} transported to ATP hydrolyzed is around 1:1, which is as high a coupling ratio as has been previously reported for reconstituted ATPase.

Fig. 4 shows a comparison of the effect of cupric phenantroline as a disulfide linking catalyst of ATPase in sarcoplasmic reticulum, $C_{12}E_8$ solubilized ATPase, and reconstituted ATPase. In the native sarcoplasmic reticulum distinct bands of dimers, trimers, tetramers and pentamers are observed, whereas there is only one distinct dimer band in the case of $C_{12}E_8$-solubilized ATPase. Almost all of the reconstituted ATPase remains in monomeric form after the exposure to Cu^{2+}-phenantroline; only a faint band corresponding to a dimer can be seen. This is consistent with the proposal that the major part of the ATPase is present as a monomer in the reconstituted membranes. Taking into account the good transport properties of the preparation this results in the interesting possibility that a monomer of ATPase is capable of transporting Ca^{2+} across a phospholipid membrane. However, it should be stressed that many more experiments are needed to state that conclusion with confidence. But the approach outlined in this section may ultimately prove useful in deciding on the role of the aggregational state of the ATPase for Ca^{2+} transport.

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