POSSIBLE SITES OF ION FLOW IN THE SARCOPLASMIC RETICULUM MEMBRANE

David H. MacLennan, Amira Klip[1], Reinhart Reithmeier, Marek Michalak[2] and Kevin P. Campbell[1].

Banting and Best Department of Medical Research
C. H. Best Institute, University of Toronto
Toronto, Ontario, Canada

I. INTRODUCTION

In this paper, dedicated to Dr. Efraim Packer on his 65th birthday, we will summarize some of our approaches and recent observations on potential sites of ion flow across the sarcoplasmic reticulum membrane.

II. ACTIVE SITES WITHIN THE Ca^{2+} + Mg^{2+} ATPase MOLECULE

Dr. Efraim Packer carried out one of his most remarkable experiments in reconstitution when he incorporated the purified Ca^{2+} + Mg^{2+} dependent ATPase of sarcoplasmic reticulum into phospholipid vesicles containing Ca^{2+} precipitable

---

[1]Post-doctoral fellow of the Medical Research Council of Canada. [2]Post-doctoral fellow of the Muscular Dystrophy Association of Canada.

This research was supported by Grant MT-3399 from the Medical Research Council of Canada and by a grant from the Muscular Dystrophy Association of Canada.

C. P. Lee, G. Schatz, L. Ernster (eds.), Membrane Bioenergetics
Based on the International Workshop held at Cranbrook Schools, Bloomfield Hills, Michigan. July 5-7, 1979, in honor of Efraim Racker ISBN 0-201-03999-0

anions and demonstrated that the enzyme could catalyse ATP-dependent Ca^{2+} transport (1). Warren et al (2) confirmed this observation using a single, defined phospholipid and concluded that the ATPase protein must carry, within its polypeptide chain, sites or ATP hydrolysis and Ca^{2+} lonoplioric activity.

We became interested in the possibility that these two activities might be difined biochemically when we found that the ATPase protein, when incorporated into a biomolecular lipid layer, dramatically increased the conductivity of the bilayer to a series of divalent metal ions (3) At the same time that these experiments were being carried out we (4) and others (5-7) observed that the 100,000 dalton ATPase was cleaved in two discrete points when trypsin was applied to the surface of intact sarcoplasmic reticulum vesicles. We subsequently isolated the 55,000 and 45,000 dalton fragments resulting from the first cleavage and the 30,000 and 20,000 dalton fragments resulting from further cleavage of the 55,000 dalton fragment. The site of ATP hydrolysis could be assayed by way of incorporation of ^{32}P, from γ-^{32} P-ATP, into the peptide chain. The radioactive label found in the 100,000 dalton enzyme was also found in 55,000 and 30,000 dalton fragments (4,6). The ionophoric site, assayed by its ability to increase divalent metal ion conductance in a bimolecular lipid layer, was found in the 55,000 dalton fragment and,after the second cleavage, it was found in the 20,000 rather than the 30,000 dalton fragment (8). The two sites are, therefore, distinctly segregated in the peptide backbone and they can be separated and studied independently of each other.

The first cleavage of the ATPase into two large fragments did not affect either ATPase or overall calcium transport activity (4,9). At this stage it was possible to

dissolve the fragmented enzyme in SDS solution and to reconstitute Ca^{2+} transport activity after removal of SDS on an anion exchange column (10). The second, slower cleavage of the 55,000 dalton fragment into subfragments was correlated with the loss of calcium transport activity. This cleavage may, therefore, constitute a physical and functional uncoupling between ATP hydrolytic and calcium transport functions. This interpretation is supported by the finding that the action of trypsin in the second cleavage is limited to a single Arg-Ala bond and that no peptide material is cleaved out of the molecule (see table 1).

In more recent experiments carried out over the past two years we have tried to obtain more information about the chemistry of the Ca^{2+} ionophoric site. This has been an arduous task because of the difficulty of working with a very hydrophobic protein. The interpretation of our results has been greatly aided, however, by the remarkable achievements of Drs. Geoffrey Allen and Michael Green who have succeeded in sequencing nearly 3/4 of the ATPase molecule (11).

The first experiments were to align the tryptic fragments. The N-terminal sequence of the 100,000, 55,000 and 20,000 dalton fragments could not be obtained (12). This was because each of these fragments began with a blocked N-terminal amino acid, Ac-Met (13). N-terminal sequences were obtained on the 30,000 and 45,000 dalton fragments and, accordingly; the fragments could be aligned as Ac-N-20,000-30,000-45,000-COOH.

The second set of experiments was to subfragment the 20,000 dalton fragment using CNBr. There are 4 methionines in the fragment (8) but since one Met is N-terminal, CNBr treatment should yield 4 peptides. We were, indeed, able to identify four CNBr fragments of molecular weights about

TABLE 1: Relationship between amino acid sequences in tryptic and CNBr fragments and sequences 1 and 3 of G. Allen (11). Residues identified by sequence analysis, ——→; residues identified by carboxypeptidase digestion, ←——.

13,000, 7,500, 4,500 and < 1,000. We have obtained amino terminal sequences on the two larger fragments (Table 1). The 13,000 dalton fragment had an amino terminal sequence identical to that beginning with the second residue, of the intact ATPase. Therefore it could be aligned as beginning with the second amino acid, Glu, and running some 120 amino acids into the chain. The sequence of the first 19 amino acids in this fragment is listed in Table 1.

The 7,500 dalton fragment did not contain any homoserine lactone, the product of the CNBr reaction with methionine, and its C-terminal amino acid was Arg, indicating that it was released from the C-terminal end of the 20,000 dalton tryptic fragment. Several pieces of evidence have allowed us to assign this fragment to part of sequence number 3 obtained by Allen (11). Our N-terminal sequence of the 30,000 dalton tryptic fragment was found to be identical with the 6 C-terminal residues in Allen's sequence 3 and these 6 residues were preceded by an Arg residue. These observations established that sequence 3 covered the point of tryptic cleavage between the 20,000 and 30,000 dalton fragments. Some 73 residues proximal to the Arg residue was a Met-Gly bond that would be susceptible to CNBr cleavage. Allen's amino acid sequence distal to the. Met-Gly bond was found to be identical to our N-terminal amino acid sequence for the 7,500 dalton fragment (Table 1) and the overall composition of the 7,500 dalton fragment and the sequence intervening between Gly and Arg were found to be essentially identical. Therefore, the 7,500 dalton CNBr fragment is C-terminal in the 20,000 dalton tryptic fragments and its primary sequence is known.

A Tyr residue is found just proximal to the 7,500 dalton fragment in Allen's primary sequence 3. The 4,500 dalton fragment contains a Tyr group but the 1,000 dalton

fragment does not. Therefore, the 4,500 dalton fragment must lie proximal to the 7,500 dalton fragment with the 1,000 dalton fragment intervening between the 13,000 and 4,500 dalton fragments. The order of alignment of the fragments is, therefore, 13,000-1,000-4,500-7,500.

Studies carried out on file ionophoric properties of the fragments are in progress. The 7,500 dalton fragment does not have any selective capacity for conduction of divalent metals through a lipid bilayer. The 13,000 dalton fragment, however, has divalent metal ionophore activity and is several fold selective for cations over anions. The selectivity sequence among the divalent cation is, however, different from chat of the 100,000, 55,000 and 20,000 dalton components. Its order of selectivity is Mn>Ca>Ba>Sr>Mg (T. Herrmann and A. E. Shamoo, personal communication) whereas the selectivity sequence observed for the larger peptides was Ba>Ca>Sr>Mg>Mn. The ionophoric properties of the 4,500 and 1,000 dalton fragments have not yet been measured. Therefore, we do not know whether additional activity is present in these fragments. If the only Ionophoric activity observed is that in the 13,000 dalton fragment-, we must assume that its selectivity has been somewhat modified at this stage of purification.

These studies show that an ionophoric activity can be defined and traced to a particular part of the ATPase molecule. The physiological role of this ionophoric activity has yet to be proven although some experiments (9,14) suggest that it is involved in overall Ca^{2+} transport and that its function can be uncoupled from ATP hydrolysis. In any consideration of whether or not the site is physiologically relevant, it must be kept in mind that the ionophore does not display the same selectivity as is observed for overall ion transport. The sarcoplasmic reticulum only transports

Ca^{2+} and Sr^{2+} and these are the two ions which activate ATP hydrolysis. Ba^{2+}, Mg^{2+} and Mn^{2+}, which are conducted by the ionophore, have no capacity to stimulate ATP hydrolysis and are not transported by the sarcoplasmic reticulum. Therefore, if the ionophore is involved in physiological ion transport, access to it must be stringently controlled by the site of ATP hydrolysis. Studies of the physical and functional, relationship between these two sites will undoubtedly provide new insights into the mechanism of ion transport.

III. RECOGNITION OF A GLYCOPROTEIN IN THE SARCOPLASMIC RETICULUM.

In our initial studies of the sarcoplasmic reticulum protein composition we routinely used SDS-polyacrylamide gels prepared by the method of Weber and Osborn (15). We found major protein bands at 100,000, 55,000, 44,000, 30,000 and 6,000 daltons. Subsequently we were successful in purifying the 100,000 dalton ATPase, the 55,000 dalton high affinity calcium binding protein, the 44,000 dalton calsequestrin and a proteolipid with a mobility corresponding to 6,000 daltons (16). Recently we reexamined the contribution of the high affinity calcium binding protein to the 55,000 dalton band using the Laemmli slab gel system (17) and a two-dimensional slab gel system utilizing a Weber-Osborn separation in one dimension and a Laemmli system in the second dimension. We found some four protein bands in the 55,000 dalton region, the most prominent of which was a diffusely staining protein of about 53,000 daltons (18). As we were also interested in the question of whether proteins of the sarcoplasmic reticulum were glycosylated, we treated the gels with ^{125}I-concanavalin A (19). While

calsequestrin and two high molecular weight proteins bound a small amount of concanavalin A, the 53,000 dalton protein was by far the most heavily labelled of the proteins separated in the slab gel. This staining capacity has permitted us to follow the protein during fractionation.

Fig 1 shows that a large proportion of the protein is extracted from the sarcoplasmic reticulum by low concentrations of deoxycholate in the presence of KCl; conditions which release calsequestrin but do not dissolve the ATPase. The glycoprotein remains soluble during the process of depletion of deoxycholate and it can be fractionated on DEAE cellulose with a KCl gradient. A part: of the protein remains with the ATPase but is fractionated away during the process of ammonium acetate purification of the ATPase, being precipitated with the first ammonium acetate addition whereupon it remains insoluble. The purified ATPase is essentially free of the glycoprotein.

When sarcoplasmic reticulum vesicles were highly purified and separated into several fractions by density gradient centrifugation following the methods of Meissner (20) or of Lau et al (21) the glycoprotein was found in light, intermediate and heavy vesicles. When the heavy vesicles were treated in a French pressure cell to free transverse tubular elements, the glycoprotein remained with heavy sarcoplasmic reticulum, its content being sharply reduced in the transverse tubular fraction. We have not as yet attempted to quantitate the protein to determine if there is a molar stoichiometry between the glycoprotein and the ATPase in each fraction but a first approximation from the amount of Coomassie Blue bound indicates that a rather constant stoichiometry does exist between these two proteins.

The glycoprotein is heavily labelled with a cycloheptaamylose-fluorescamine-complex (18) which cannot

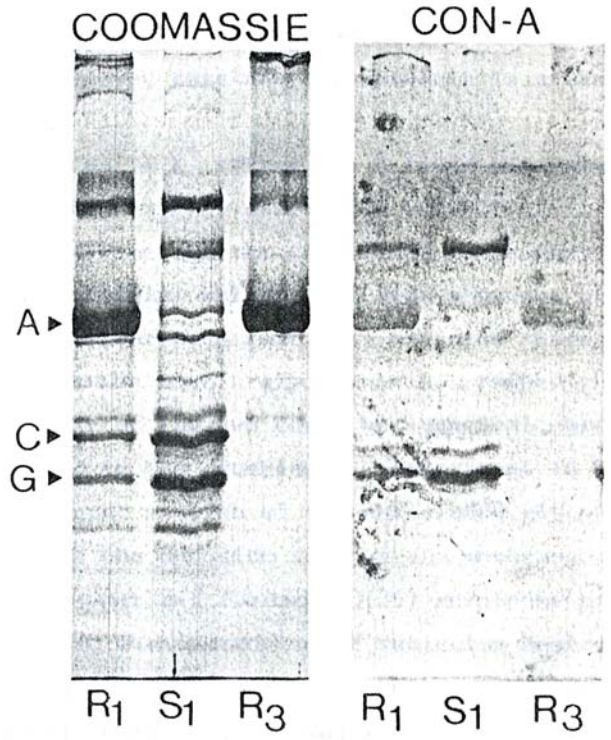

Fig 1 Gel electrophoretic patterns of: sarcoplasmic reticulum (R_1); extract containing the glycoprotein (S_1); purified ATPase (R_3): Left, Coomassie Blue stain; right, ^{125}I concanavalin A stain: A, ATPase; C, calsequestrin; G, glycoprotein.

penetrate the interior of the sarcoplasmic reticulum and does not label calsequestrin or the high affinity calcium binding protein (22). Thus the protein must be exposed to the exterior of the vesicles. We have not yet proven that the protein is embedded in the lipid bilayer but it is not removed by sucrose or salt washing or by extraction with EGTA. Therefore, we think that it interacts hydrophobically with the lipid bilayer. The sugar moieties on glycoproteins of rough microsomes are found in luminal spaces (23). Since the sarcoplasmic reticulum has the same orientation as rough microsomes, it is probable that the sugars on the glycoprotein

are also located in the lumen of the sarcoplasmic reticulum and, therefore, that the glycoprotein is a transmembrane protein.

The $Na^+ + K^+$ ATPase has a lower molecular weight glycoprotein tightly associated with it (24). Since the $Na^+ + K^+$ ATPase and the Ca^{2+} ATPase share many properties such as molecular weight, and even sequence homology (25) it is not surprising that they may also share the property of association with a glycoprotein. Indeed the molecular arrangement of the two could be identical, with ATP being hydrolysed in the cytoplasmic compartment in both cases and the sugar portion of the associated glycoprotein being located on the opposite side of the membrane.

Meissner and Fleischer (26) and Repke et al (27) reported that when sarcoplasmic reticulum membranes were dissolved in deoxycholate and reconstituted under defined dialysis conditions, two major protein hands were observed in the reformed membranes, the ATPase and the 55,000 dalton band. We showed that tills band was not the high affinity calcium binding protein but was the 53,000 dalton glycoprotein (18).

When Dr. Packer reconstituted Ca^{2+} transport using the purified ATPase (1) he found that the preparation did not transport anions but was dependent on the presence of anions in the interior of the vesicles. On the other hand, Meissner and Fleischer (26) reconstituted low lipid vesicles from either whole sarcoplasmic reticulum or partially purified ATPase that retained the ability to transport anions. The possibility arose that the lipid composition or content of the reconstituted vesicles might determine anion penetration. More recently, however, Chiesi et al (28) and Peterson et al (29) have reconstituted transporting vesicles, rich in phospholipid, from Triton X-100 extracts. These

systems do transport anions and in both cases the starting material was the whole sarcoplasmic reticulum. These observations raise the possibility that there is a protein required for anion transport in the sarcoplasmic reticulum which can he purified away from the ATPase but which will reassociate with membranes containing the ATPase under the right conditions. A possible candidate for this protein is the glycoprotein.

We have begun the study of whether the glycoprotein is involved in the ion transport function. We have found that the purified ATPase (30) will transport Ca^{2+} at low rates when reconstituted under the conditions described by Meissner and Fleischer (26) and assayed in the presence of oxalate. If crude preparations of glycoprotein are added to the reconstitution mixture, the glycoprotein is selectively rebound to the reformed membranes and the Ca^{2+} transport rate is enhanced several fold. We do not know the mechanism of the stimulation, as yet, but we are testing the hypothesis that it is related to an enhanced anion transport function.

REFERENCES

1. Packer, E. (1972). J. Biol. Chem. 247:8198-8200.
2. Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975). Nature 255:684-687.
3. Shamoo, A.E. and MacLennan, D.H. (1974). Proc. Natl. Acad. Sci. U.S.A. 71:3522-3526.
4. Stewart, P.S. and MacLennan, D.H. (1974). J. Biol. Chem. 249:985-993.
5. Migala, A., Agostini, B. and Hasselbach, W. (1973). Z. Naturforsch. 28:178-182.
6. Thorley-Lawson, D.A. and Green, N.M. (1973). Eur. J. Biochem. 40:403-413.
7. Inesi, G. and Scales, D. (1974). Biochemistry 13: 3298-3306.
8. Shamoo, A.E., Ryan, T.E., Stewart, P.S. and MacLennan, D.H. (1976). J. Biol. Chem. 251:4147-4154.

9. Shamoo, A.E., Scott, T.L. and Ryan, T.E. (1977). J. Supramol. Struct. 6:345-353.
10. MacLennan, D.H., Khanna, K. and Stewart, P.S. (1976). J. Biol. Chem. 251:7271-7274.
11. Allen, G. (1977). FEBS llth Meeting, Copenhagen, Symposium A4, 45:159-168.
12. Klip, A. and MacLennan, D.H. (1978). In "Frontiers of Biological Energetics: from Electrons to Tissue (L. Dutton, J. Leigh and A. Scarpa, eds.) Academic Press, New York. pp. 1137-1147.
13. Tong, S.W. (1977). Biochem. Biophys. Res. Commun. 74:1242-1248.
14. Shamoo, A.E. and MacLennan, D.H (1975). J. Membrane Biol. 25:65-74.
15. Weber, K. and Osborn, M. (1969). J. Biol. Chem. 244: 4406-4412.
16. MacLennan, D.H. and Holland, P.C. (1975). Ann. Rev. Biophys. Bioengineer. 4:377-404.
17. Laemmli, U.K. (1970). Nature (Lond.) 227:680-685.
18. Michalak, M., Campbell, K.P. and MacLennan, D.H. (1979). J. Biol. Chem. (submitted for publication).
19. Rostas, J.A.P., Kelly, P.T. and Cotman, C.W. (1977). Anal. Biochem. 80:366-372.
20. Meissner, G. (1975). Biochim. Biophys. Acta. 389:51-68.
21. Lau, Y.H., Caswell, A.H. and Brunschwig, J.P. (1977). J. Biol. Chem. 252:5565-5574.
22. Hidalgo, C. and Ikemoto, N. (1977). J. Biol. Chem. 252:8446-8454.
23. Boulan, E.R., Sabatini, D.D., Pereyra, B.N. and Kreibich, G. (1978). J. Cell Biol. 78:894-909.
24. Kyte, J. (1972). J. Biol. Chem. 247:7642-7649.
25. Bastide, F., Meissner, G., Fleischer, S. and Post, R.L. (1973). J. Biol. Chem. 248:8385-8391.
26. Meissner, G. and Fleischer, S. (1974). J. Biol. Chem. 249:302-309.
27. Repke, D.I., Spivak, J.C. and Katz, A.M. (1976). J. Biol. Chem. 251:3169-3175.
28. Chiesi, M., Peterson, S.W. and Acuto, 0. (1978). Arch. Biochem. Biophys. 189:132-136.
29. Peterson, S.W., Hanna, S. and Deamer, D.W. (1978). Arch. Biochem. Biophys. 191:224-232.
30. MacLennan, D.H. (1970). J. Biol. Chem. 245:4508-4518.