Labelling of the integral proteins of sarcoplasmic-reticulum membranes

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(Received 20 February 1984/Accepted 11 April 1984)

Photoreactive lipids were introduced into rabbit and rat sarcoplasmic-reticulum membranes to label specifically integral proteins. Evidence was obtained that, in addition to the Ca^{2+} -dependent ATPase, the 160000- M_r glycoprotein, the 53000–56000- M_r components and polypeptides of M_r 30000, 20000 and 6000 are integral components of sarcoplasmic-reticulum membranes.

Sarcoplasmic reticulum (SR) is a highly specialized membrane system that regulates the cytoplasmic Ca^{2+} concentration of muscle cells (Tada *et al.*, 1978). Its polypeptide composition is still not clearly established; it appears to vary with the source (Heilmann *et al.*, 1977; Salviati *et al.*, 1981b), the different purification procedures (Meissner *et al.*, 1973; Michalak *et al.*, 1980) and with the resolving power of the gel electrophoresis used (Campbell *et al.*, 1980). Of the several polypeptides clearly identified in highly resolving SDS/polyacrylamide-gel electrophoresis (see Fig. 2 below), a ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase (Ikemoto, 1982), calsequestrin (MacLennan & Wong, 1971), a high-affinity Ca^{2+} -binding protein (M_{55} ; Meissner *et al.*, 1973) (Ostwald & MacLennan, 1974), an intrinsic glycoprotein (M_r 53000) (Campbell & MacLennan, 1981) and a proteolipid (Laggner & Graham, 1976) have been purified and studied with respect to structure, function and localization. Less-well characterized are a 56000- M_r protein (Campbell *et al.*, 1980), some 'acidic' proteins (M_r range 20000–32000) (MacLennan *et al.*, 1972) and a 160000- M_r glycoprotein (Campbell & MacLennan, 1983).

The topological arrangement of the components of SR membranes has been investigated with different approaches. These studies indicate that the Ca^{2+} -dependent ATPase and the 53000- M_r glycoprotein are transmembrane (Bercovici & Gitler, 1978; Michalak *et al.*, 1980). Calsequestrin and M_{55} have been located alternatively in the vesicle's interior, on the outside surface or within the membrane (Thorley-Lawson & Green, 1973;

Hidalgo & Ikemoto, 1977; Michalak *et al.*, 1980). The 160000- M_r glycoprotein and two polypeptides of approx. M_r 30000 have been suggested to be integral membrane components (Campbell *et al.*, 1980; Campbell & MacLennan, 1983); there is evidence that one of the two 30000- M_r proteins is a NADH-cytochrome b_5 reductase similar to that of liver microsomes (Salviati *et al.*, 1981a).

To gain more information on the arrangement of SR polypeptides with respect to the membrane, we have used in the present study two radioactive photoactivatable lipids. They bear at the fatty acid methyl terminus a nitroarylazido group; on illumination this is converted into a nitrene intermediate able to react with neighbouring molecules, thereby labelling them radioactively. Two chemically unrelated probes were used in order to avoid possible artefacts due to selective head-group-mediated interactions with certain membrane proteins. There are several lines of evidence indicating that these photoreactive lipid analogues label selectively the hydrophobic domain of membrane proteins (Bisson & Montecucco, 1984). In fact they have been shown to label only the hydrophobic tail of cytochrome b_5 (Bisson *et al.*, 1979), and none of the subunits of the hydrophilic F_1 domain of the mitochondrial and the *Escherichia coli* ATP synthetase were cross-linked by arylazidophospholipids (Montecucco *et al.*, 1983). Moreover, only intrinsic peptide fragments of the coliphage M13 coat protein were derivatized by an azidoglycolipid probe (Hu & Wisnieski, 1979). In the case of the cytochrome oxidase subunit II and subunit b of the ATP synthetase, the precise sites of cross-linking with azidophospholipids were determined, and sequence analysis showed that none of the hydrophilic segments of these proteins were labelled (Bisson *et al.*, 1982; Hoppe *et al.*, 1983b).

Abbreviations used: SDS, sodium dodecyl sulphate; SR, sarcoplasmic reticulum; PMSF, phenylmethanesulphonyl fluoride.

Materials and methods

SR of leg muscles was prepared from rabbit in the presence of $5\mu\text{M}$ -PMSF as described by Robinson *et al.* (1972) and from rat by the method of Boland *et al.* (1981). ATPase activity was measured with a NADH-coupled system (Warren *et al.*, 1974). 1-Myristoyl-2-[12-amino-*N*-(3-nitro-1-azidophenyl)]dodecanoyl-*sn*-glycero-3-phospho-[^{14}C]choline (174 Ci/mol) and 12-amino-*N*-(2-nitro-4-azidophenyl)dodecanoyl[6- ^3H]glucosamine (azidoglycolipid) (38 Ci/mmol) were prepared as described by Bisson & Montecucco (1981) and Bramhall *et al.* (1979). All operations involving photoactive probes were carried out under a red safety light.

The labelling of SR membranes was performed as follows. The phospholipid (1.1 nmol) or the glycolipid (0.024 nmol) was dried at the bottom of a tube with a nitrogen flux and then under vacuum for 2 h. To the phospholipid probe, 250 μl of de-aerated 50 mM-Tris/HCl (pH 7.4)/0.1 mM-EDTA was added and sonicated under nitrogen for 10 min. Rabbit or rat SR (200 μg of protein) was incubated with this mixture for 6 h at 0°C . This leads to a 50–70% incorporation. To the azidoglycolipid, 2 μl of dimethyl sulphoxide were added, followed by 250 μl of de-aerated Tris buffer, and then the SR membranes (200 μg of protein) were incubated in this mixture for 1 h at 37°C . In some experiments, MgATP^{2-} (4 mM) and CaCl_2 (2 mM) were present.

Samples were illuminated with a 100 W long-wave u.v. lamp with the protection of a glass filter at 0°C for 15 min; no decrease of ATPase activity was observed after u.v. irradiation. The samples were layered on to 10% (w/v) sucrose/50 mM-Tris/HCl (pH 7.4)/0.1 mM-EDTA and centrifuged for 3 h at 230 000 g in a Sorvall AH 650 rotor. The pellets were resuspended in electrophoresis sample buffer. SDS/polyacrylamide-gel electrophoresis was performed in a 20 cm-long linear 12–20% (w/v)-polyacrylamide gradient or on a 7.5% (w/v)-polyacrylamide gel as previously described (Gutweniger *et al.*, 1983). M_r standards were as described by Gutweniger *et al.* (1981). Fluorography was performed by the method of Bonner & Laskey (1974).

Results and discussion

Fig. 1 shows the Coomassie Blue staining (a,d) and labelling patterns (b,c) with azidoglycolipid of rat and rabbit SR membranes after electrophoresis on a 7.5% (w/v)-polyacrylamide gel. As expected, the highest amount of radioactivity is associated with the Ca^{2+} -ATPase, the major constituent of SR proteins. Also labelled are the 160 000- M_r glycoprotein and the 53 000–56 000- M_r polypep-

Fig. 1. SDS/polyacrylamide-gel electrophoresis and autoradiographic labelling profiles of rat and rabbit SR membranes on 7.5% gels

(a,d) Coomassie Blue staining pattern of rat (a) and rabbit (d) SR vesicles (150 μg of protein). (b,c) Fluorographic labelling profile of rat (b) and rabbit (c) SR vesicles with azidoglycolipid. Electrophoresis was carried out as described by Laemmli (1970). Abbreviations used: CS, calsequestrin; M_{55} , M_{55} protein; M_{53} , 53 000- M_r protein.

tides, which cannot be easily resolved from each other. Radioactivity is also associated with some polypeptides running near the gel front, where unbound lipids are found. Fig. 2 shows the polypeptide pattern for rat (a) and rabbit (e) SR membranes after electrophoresis on a linear polyacrylamide gradient system able to resolve proteins in the low- M_r region. It is apparent that many more polypeptides are present. This is unlikely to be due to proteolysis, because proteinase inhibitors were used during membrane preparation and gel electrophoresis; moreover, this is in agreement with the findings of other workers (Michalak *et al.*, 1980; Campbell *et al.*, 1980).

The pattern and ratio between the ATPase and the 30 000- M_r proteins is characteristic of a mixed muscle preparation (Wiehrer & Pette, 1983).

Fig. 2. SDS/polyacrylamide-gel electrophoresis and autoradiographic labelling profiles of rat and rabbit SR membranes on 12–20% acrylamide gels

(a,e) Coomassie Blue staining pattern of rat (a) and rabbit (e) SR proteins (150 μ g of protein). (b,c) Fluorographic profile of rat (b) and rabbit (c) SR proteins with azidoglycolipid in the presence of MgATP^{2-} (4mM) and CaCl_2 (2mM). (d) Labelling pattern of rabbit SR in the absence of MgATP^{2-} . Photographic reproduction is unable to show that the label at the 20000- M_r position of lane (c) is distributed over three components and in lane (b) over two components.

Several differences in mobility and staining intensity between the polypeptide components of rabbit and rat SR preparations can be seen. In particular, rat calsequestrin has a slightly lower mobility (cf. Figs. 1a and 1d), and the 30000- M_r protein has a higher staining intensity than its rabbit counterparts.

The pattern of labelling with the azidoglycolipid (Figs. 2b and 2c) again shows that the Ca^{2+} -

ATPase and the 53000–56000- M_r components are labelled. From this experiment it is also evident that polypeptides of apparent M_r 30000 and 20000 are labelled. This is in agreement with the finding that one of the 30000- M_r polypeptides is a NADH-cytochrome b_5 reductase, whose integral nature in microsomes is well documented (Salviati *et al.*, 1981a). More radioactivity is associated with the rat than with the rabbit 30000- M_r component. The 20000- M_r polypeptide is resolved in three polypeptides in the rabbit and in two in the rat preparation; all these proteins are labelled. The rabbit proteolipid is also labelled. In the case of rat SR, a polypeptide that could correspond to the rabbit proteolipid (M_r ~7000) is slightly modified by azidoglycolipid. These patterns were all obtained in the presence of MgATP^{2-} . In its absence the rabbit proteolipid is more labelled (see Fig. 2d), whereas the rest of the pattern is unaltered, suggesting that it undergoes a conformational rearrangement in the membrane. Very similar results were obtained with the chemically unrelated phospholipid probe. Hence the differential labelling of the protein bands does not appear to be due to any specific interaction with the probes.

As with any labelling technique this approach is unable to give any information about the unlabelled polypeptides, which can be either external to the membrane or inside the membrane but shielded by the labelled components or poorly reactive towards the nitroarylnitrene.

The different extents of labelling cannot be strictly correlated with the amount of protein surface exposed to lipids, since it has been shown that the nitrene intermediate, although very reactive, does show differing reactivities towards the different amino acid lateral residues (Bisson & Montecucco, 1984).

In a previous study of hydrophobic photolabelling of SR membranes, Bercovici & Gitler (1978) used 5-[^{125}I]iodonaphthyl 1-azide. They found labelling of the Ca^{2+} -ATPase and a very low labelling, if any, of two proteins of M_r 20000 and 10000. The lack of reaction of this azide with some polypeptides found labelled in the present study is most probably related to the low reactivity of the naphthyl nitrene with respect to the nitrophenylnitrene (Bisson & Montecucco, 1984; Hoppe *et al.*, 1983a).

The present study strengthens the notion that the Ca^{2+} -ATPase, the 53000- M_r glycoprotein and the proteolipid are intrinsic components of the SR membranes and shows that they expose part of their surface to lipids. Moreover, it provides strong evidence that a glycoprotein of M_r 160000, the 53000- M_r components and polypeptides of M_r about 30000, 20000 and 6000 are integral membrane proteins and that they interact with lipids.

When the primary sequence of the hydrophobic domain of these integral proteins is available, it will be possible with this method to identify those polypeptide segments forming the lipid/protein boundary of the hydrophobic domain; this is particularly interesting in such proteins as the Ca^{2+} -ATPase, whose polypeptide chain has been demonstrated to traverse the lipid bilayer several-fold (Allen, 1983).

References

- Allen, G. (1983) in *Structure and Function of Membrane Proteins* (Palmieri, F. & Quagliariello, E., eds.), Elsevier Publishing Co., Amsterdam, in the press
- Bercovici, T. & Gitler, C. (1978) *Biochemistry* **17**, 1484–1489
- Bisson, R. & Montecucco, C. (1981) *Biochem. J.* **193**, 757–763
- Bisson, R. & Montecucco, C. (1984) in *Techniques for the Analysis of Membrane Proteins* (Cherry, R. & Ragan, C. I., eds.), Chapman and Hall, London, in the press
- Bisson, R., Montecucco, C. & Capaldi, R. A. (1979) *FEBS Lett.* **106**, 317–320
- Bisson, R., Steffens, G. C. M. & Buse, G. (1982) *J. Biol. Chem.* **257**, 6716–6720
- Boland, R. L., Ritz, E. & Hasselbach, W. (1981) *Biochim. Biophys. Acta* **647**, 227–231
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Bramhall, J. S., Shiflett, M. A. & Wisnieski, B. J. (1979) *Biochem. J.* **177**, 765–768
- Campbell, K. P. & MacLennan, D. H. (1981) *J. Biol. Chem.* **256**, 4626–4632
- Campbell, K. P. & MacLennan, D. H. (1983) *J. Biol. Chem.* **258**, 1391–1394
- Campbell, K. P., Franzini-Armstrong, C. & Shamoo, A. E. (1980) *Biochim. Biophys. Acta* **602**, 97–116
- Gutweniger, H. E., Bisson, R. & Montecucco, C. (1981) *J. Biol. Chem.* **256**, 11132–11136
- Gutweniger, H. E., Grassi, C. & Bisson, R. (1983) *Biochem. Biophys. Res. Commun.* **116**, 272–283
- Heilmann, C., Brdiczka, D., Nickel, E. & Pette, D. (1977) *Eur. J. Biochem.* **81**, 211–222
- Hidalgo, C. & Ikemoto, N. (1977) *J. Biol. Chem.* **252**, 8446–8454
- Hoppe, J., Friedl, P. & Jorgensen, B. B. (1983a) *FEBS Lett.* **160**, 239–242
- Hoppe, J., Montecucco, C. & Friedl, P. (1983b) *J. Biol. Chem.* **258**, 2882–2885
- Hu, V. W. & Wisnieski, B. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 5460–5464
- Ikemoto, N. (1982) *Annu. Rev. Physiol.* **44**, 297–317
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laggner, P. & Graham, D. E. (1976) *Biochim. Biophys. Acta* **433**, 311–317
- MacLennan, D. H. & Wong, P. T. S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1231–1235
- MacLennan, D. H., Yip, C. C., Iles, G. H. & Seeman, P. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 469–477
- Meissner, G., Conner, G. E. & Fleischer, S. (1973) *Biochim. Biophys. Acta* **298**, 246–269
- Michalak, M., Campbell, K. P. & MacLennan, D. H. (1980) *J. Biol. Chem.* **255**, 1317–1326
- Montecucco, C., Dabbeni-Sala, F., Friedl, P. & Galante, Y. M. (1983) *Eur. J. Biochem.* **132**, 189–194
- Ostwald, T. J. & MacLennan, D. H. (1974) *J. Biol. Chem.* **249**, 974–979
- Robinson, J. D., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1972) *Biochemistry* **11**, 2903–2909
- Salviati, G., Salvatori, S., Betto, R. & Margreth, A. (1981a) *Biochem. J.* **197**, 515–518
- Salviati, G., Volpe, P., Salvatori, S., Betto, R., Damiani, E. & Margreth, A. (1981b) *Biochem. J.* **202**, 289–301
- Tada, M., Yamamoto, T. & Tonomura, Y. (1978) *Physiol. Rev.* **58**, 1–79
- Thorley-Lawson, D. A. & Green, N. M. (1973) *Eur. J. Biochem.* **40**, 403–413
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622–626
- Wiehrer, W. & Pette, D. (1983) *FEBS Lett.* **158**, 317–320