Biochimica et Biophysica Acta 903 (1987) 374–380
Elsevier

BBA 73701

State of aggregation of the $(Ca^{2+} + Mg^{2+})$-ATPase studied using chemical cross-linking

R.M. Napier, J.M. East and A.G. Lee

Department of Biochemistry, University of Southampton, Southampton (U.K.)

(Received 17 February 1987)

Key words: Chemical cross-linking; ATPase, $(Ca^{2+} + Mg^{2+})$-; Sarcoplasmic reticulum; Spin label; Saturation-transfer ESR; ESR; (Rabbit muscle)

We have studied cross-linking of the $(Ca^{2+} + Mg^{2+})$-ATPase in sarcoplasmic reticulum and in reconstituted systems, using glutaraldehyde, cupric-1,10-phenanthroline and 3,3′-dithiobis(sulphosuccinimidylpropionate). All reagents produce extensive cross-linking, forming aggregates too large to enter polyacrylamide gels. Only traces of cross-linked dimeric ATPase species are formed. Saturation transfer electron spin resonance spectra of spin-labelled sarcoplasmic reticulum cross-linked with glutaraldehyde are also consistent with the formation of extensively cross-linked aggregates in the membrane. The results are interpreted in terms of dynamic clusters of ATPase molecules in the membrane, probably in the form of rows of ATPase molecules.

Introduction

As described in the previous paper [1], electron micrographs of negatively stained samples of sarcoplasmic reticulum show the presence of extended linear arrays of dimers of the $(Ca^{2+} + Mg^{2+})$-ATPase [2–4]. Studies of spin-labelled ATPase using saturation-transfer electron spin resonance (ST-ESR), however, show that the ATPase molecules have considerable rotational freedom of motion within the membrane [1]. These apparently disparate results can be reconciled if the lifetime of any particular ATPase molecule in a crystalline cluster of ATPase molecules is short (on the millisecond time scale), with ATPase molecules rapidly entering and leaving the clusters.

Another technique for investigating the state of aggregation of membrane proteins is chemical cross-linking [5], but unfortunately previous applications of this technique to the $(Ca^{2+} + Mg^{2+})$-ATPase have given contradictory results. Louis and Shooter [6] reported that cross-linking the ATPase with suberimidate led to the successive formation of dimers, trimers, tetramers, pentamers and hexamers. Murphy [7] reported that cross-linking with cupric phenanthroline formed tetramers with no dimers or trimers, from which he concluded that the ATPase was present in the sarcoplasmic reticulum membrane as a tetramer. In more recent studies, however, no specific oligomeric forms were found to predominate [6–11]. Here we have re-examined the patterns of cross-linking, in conjunction with saturation-transfer ESR studies.

Materials and Methods

Sarcoplasmic reticulum vesicles and $(Ca^{2+} + Mg^{2+})$-ATPase were prepared as described in the

Abbreviations: ATPase, $(Ca^{2+} + Mg^{2+})$-ATPase; ESR electron spin resonance; Mops, 4-morpholinepropanesulphonic acid; MSL, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide; SDS, sodium dodecyl sulphate; DTSP, 3,3′-dithiobis(sulphosuccinimidylpropionate).

Correspondence: A.G. Lee, Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, U.K.

previous paper [1]. ATPase was reconstituted into lipid vesicles at a molar ratio of lipid to protein of 3000 : 1 as described (Gould, G.W., McWhirter, J.M., East, J.M. and Lee, A.G., unpublished studies). Briefly, phospholipid was dispersed into buffer (0.25 M sucrose, 1 M KCl, 50 mM potassium phosphate, pH 8.0) and potassium cholate was added to give a cholate : lipid ratio (w/w) of 1 : 1, and the suspension sonicated to clarity in a bath sonicator (Branson). The purified ATPase (0.3–1.0 mg protein in 15–50 μl of buffer) was mixed with deoxycholate to give a deoxycholate to ATPase ratio of 0.6 : 1.0 (mg/mg). The mixture was vortexed for 5 s and then spun at $10000 \times g$ in a microfuge for 5 min to remove any unsolubilized aggregates. The lipid and protein samples were then mixed to give the required lipid to protein ratio. The detergent was removed by centrifugation through a Sephadex G-50 column (5 ml).

For cross-linking with glutaraldehyde, sarcoplasmic reticulum vesicles were suspended in buffer (50 mM Mops, 20 mM KCl, 5 mM $MgSO_4$, 50 μM Ca^{2+} (pH 6.8)) at 1.6 mg protein/ml. Glutaraldehyde was added at 23° C to give a final concentration of 20 mM, and at given times samples were removed and the reaction quenched with hydrazine (100 mM). For saturation-transfer ESR, samples were labelled at a 1 : 1 molar ratio of maleimide spin label (MSL) to ATPase following cross-linking with glutaraldehyde, as described in Ref. 1. For electrophoresis, samples were solubilized in 60 mM Tris-HCl (pH 6.8) containing 2% (w/v) sodium dodecyl sulphate (SDS), 1% β-mercaptoethanol, 0.002% bromophenol blue and 10% (v/v) glycerol, and incubated at 37° C for 30 min before electrophoresis.

Cross-linking with cupric-1,10-phenanthroline was performed in buffer (50 mM Tris-HCl (pH 8.0) or 50 mM Mops, 100 mM KCl (pH 7.0)) at a protein concentration of 4 mg/ml. Cupric-1,10-phenanthroline was added from a stock solution of cupric sulphate (100 mM) and 1,10-phenanthroline (15 mM) to give a final concentration of 0.2 mM cupric sulphate and 0.6 mM phenanthroline. The reaction was terminated by addition of *N*-ethylmaleimide (final concentration 50 mM) and EDTA (final concentration 10 mM).

For cross-linking with 3,3′-dithiobis(sulphosuccinimidylpropionate) (DTSP, Pierce), sarcoplasmic reticulum was resuspended in buffer (10 mM Tris-HCl, 100 mM NaCl, 5 mM $MgSO_4$, 0.1 mM Ca^{2+} (pH 8.0)) at 4 mg protein/ml. DTSP was added to a final concentration of 0.1 mM from a stock solution (100 mM) in dimethylsulphoxide. After incubation for various times at 22° C, the reaction was terminated by dilution into the SDS solubilization medium containing 50 mM glycine but no β-mercaptoethanol.

Cross-linked and control samples were solubilized in the sample buffer given above. SDS-polyacrylamide gels were run using the method of Laemmli and Faure [12]. Running gels (pH 8.8) contained 7% (w/v) acrylamide and 0.1% SDS and stacking gels (pH 6.8) 4% acrylamide and 0.1% SDS. Approx. 16 μg protein were applied per track and samples were electrophoresed at a constant current of 40 mA for 3 h. Gels were stained with Coomassie blue, and, after destaining, were scanned on a Joyce Loebel gel scanner. Relative peak areas were determined by cutting and weighing.

Saturation-transfer ESR spectra were recorded as described in Ref. 1.

Results

Homogenisation of muscle gives a heterogeneous preparation of vesicles derived from the sarcoplasmic reticulum which can be separated by sucrose gradient centrifugation into light, intermediate and heavy sarcoplasmic reticulum [13]. Fig. 1 shows densitograms of electrophoretically separated components of native sarcoplasmic reticulum vesicles obtained by our preparative procedure. Three major bands were observed at apparent molecular weights of 97000, 62000 and 54000 corresponding to ($Ca^{2+} + Mg^{2+}$)-ATPase, calsequestrin and the M55 glycoprotein, respectively [14]. The relative protein composition estimated from gels stained with Coomassie blue was 65% ($Ca^{2+} + Mg^{2+}$)-ATPase, 25% calsequestrin and approx. 8% M55 glycoprotein, consistent with our sarcoplasmic reticulum preparation being predominantly a mixture of intermediate and heavy vesicles.

On incubation with 20 mM glutaraldehyde, the absorbance of the main 97 kDa band decreases as

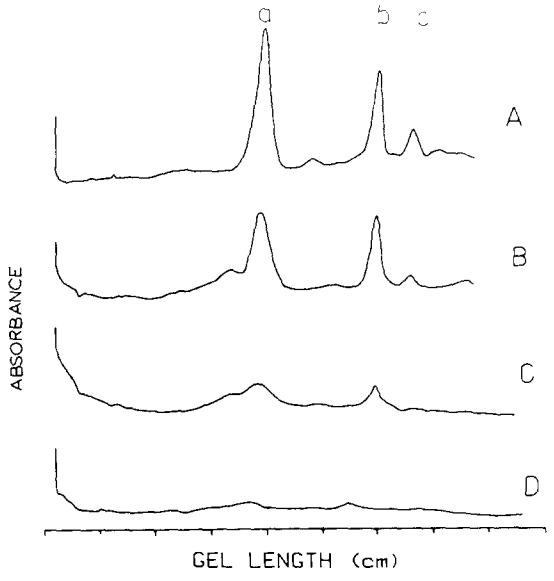

Fig. 1. Densitograms of native and cross-linked sarcoplasmic reticulum. Sarcoplasmic reticulum was cross-linked with 20 mM glutaraldehyde and SDS-polyacrylamide gels were carried out as described in Material and Methods. (A) Native sarcoplasmic reticulum and (B–D) sacroplasmic reticulum after cross-linking with glutaraldehyde for: (B) 0.5 min; (C) 3 min; (D) 17 min.

a function of the time of reaction (Figs. 1 and 2). On cross-linking, a band is observed at the top of the running gel, attributable to small aggregates of the ATPase, which first increases in intensity with increasing reaction time but then decreases in intensity for longer reaction times. The decrease in total protein staining with time indicates the formation of protein aggregates too large to enter the stacking gel. No indication was found for the formation of a distinct dimeric species. As shown in Fig. 1, on cross-linking with glutaraldehyde the band attributable to the $(Ca^{2+} + Mg^{2+})$-ATPase became distinctly asymmetric due to the appearance of a shoulder at 112 kDa. The 112 kDa band was attributable to ATPase since, after blotting onto nitrocellulose paper, it reacted with monoclonal antibody 1/2H7 raised to the purified ATPase (Colyer, J., Lee, A.G. and East, J.M., unpublished observations). McIntosh and Ross [15] have suggested that the new band is due to altered hydrodynamic properties of the modified ATPase in SDS gels.

Fig. 3 shows saturation-transfer ESR spectra for sarcoplasmic reticulum labelled with the maleimide spin label MSL after cross-linking with glutaraldehyde. With increasing time of cross-lin-

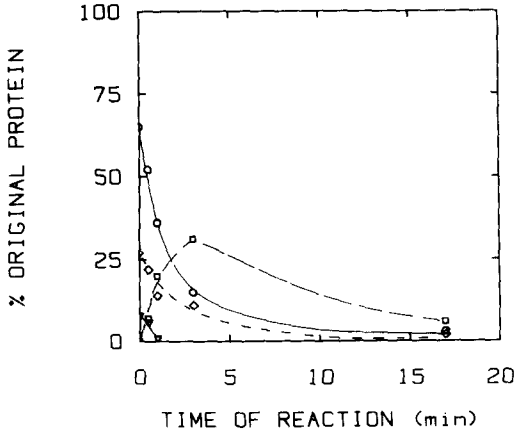

Fig. 2. Time-course of cross-linking determined from polyacrylamide gels stained with Coomassie blue. Reaction conditions as in legend to Fig. 1. Band intensities are expressed relative to total protein in native sarcoplasmic reticulum. ○, monomeric ATPase; □, high molecular weight, cross-linked species of ATPase; ◇, calsequestrin; ▽, M55-glycoprotein.

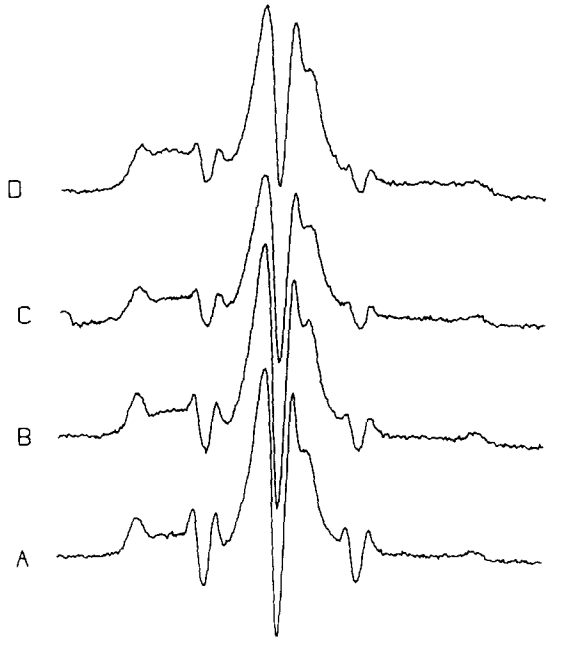

Fig. 3. Saturation-transfer ESR spectra at 4° C of sarcoplasmic reticulum (A) and after cross-linking with glutaraldehyde under the conditions in legend to Fig. 1, for: (B) 0.5 min; (C) 3 min and (D) 17 min.

TABLE I

THE EFFECT OF TIME OF CROSSLINKING OF SARCOPLASMIC RETICULUM WITH 20 mM GLUTARALDEHYDE ON SATURATION-TRANSFER ESR PARAMETERS AND DERIVED EFFECTIVE ROTATIONAL CORRELATION TIMES AT 4°C

Reaction time (min)	Spectral parameter L''/L	Effective correlation time (μs)
0	0.61	40
0.5	0.63	43
1.0	0.74	70
3.0	0.76	75
17.0	0.94	180

king, the spectra change as expected for a decrease in rotational motion for the ATPase (Table I): cross linking for longer times than 17 min had no further effect.

Reaction of sarcoplasmic reticulum with cupric-1,10-phenanthroline for up to 1 h at 22°C also led to the disappearance of the band due to the ATPase, with the formation of aggregates too large to enter the gel. Only traces of dimeric species were observed (data not shown).

Reaction with DTSP also led to the disappearance of the band due to monomeric ATPase, with relatively little effect on the band due to calsequestrin. As shown in Fig. 4, the intensity of the monomer band remaining after reaction with DTSP at 22°C for 30 min decreased with increasing concentration of DTSP. No clear band attributable to dimeric species appeared in the gels. Reaction with 0.05 mM DTSP was studied as a function of time, and after 60 min the intensity of the band due to monomeric ATPase had decreased to 30% of its original intensity, but again at no time point were any significant amounts of dimeric species observed.

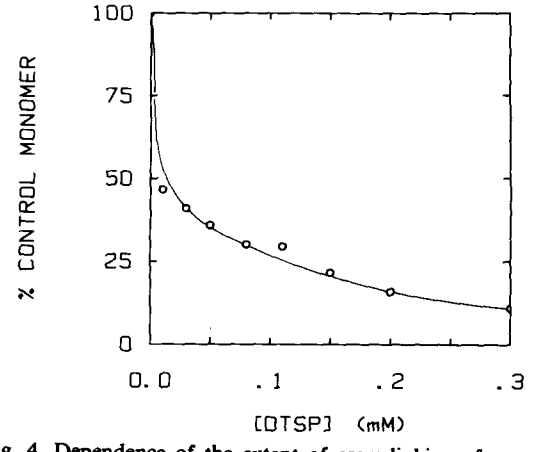

Fig. 4. Dependence of the extent of cross-linking of sarcoplasmic reticulum on the concentration of DTSP, determined as the decrease in density of the ATPase band. Reactions and SDS-polyacrylamide gels of the resulting samples were carried out as described in Materials and Methods.

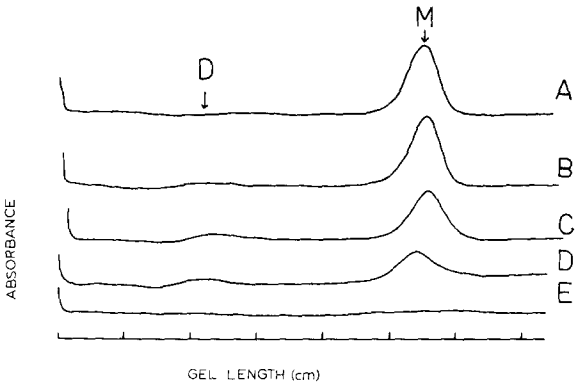

Fig. 5. Densitograms of reconstituted ATPase cross-linked with DTSP. The ATPase was reconstituted with egg yolk phosphatidylcholine at a molar ratio of lipid to protein of 3000:1 (A) and cross-linked with 0.01 mM DTSP for 1 min (B) or 10 min (C) or with 0.1 mM DTSP for 1 min (D) or 10 min (E). Marked are the expected positions of monomeric and dimeric species of the ATPase.

Reaction of the purified ATPase (at a lipid:protein molar ratio of 30:1) with DTSP gave results very similar to those observed with sarcoplasmic reticulum. The effect of DTSP on the ATPase reconstituted into bilayers of egg yolk phosphatidylcholine at a molar ratio of lipid to protein of 3000:1 is shown in Fig. 5. Reaction again gave aggregates too large to enter the running gel, but intensity attributable to dimer was observed under some conditions, although again the proportion of such dimeric species was very small (Fig. 5). Similar results were obtained with cupric-1,10-phenanthroline, with a more distinct band attributable to dimeric ATPase being formed than with native sarcoplasmic reticulum, but the major effect being the formation of large aggregates (data not shown).

Discussion

As described in the previous paper [1], there is much evidence that the ATPase is present in the

sarcoplasmic reticulum membrane and in reconstituted systems in dimeric form and that, at least under some conditions, these dimers can associate to give extended arrays [2–4,20]. Chemical cross-linking has been used extensively to probe the state of aggregation of membrane proteins, with variable degrees of success [5,18]. The major problem in such studies is to distinguish between cross-linking of pre-existing aggregates and cross-linking that follows from diffusional collisions between initially separated molecules within the membrane. Although it is very difficult to distinguish rigorously between these two possibilities, studies with red blood cell membranes have suggested that cross-linked products, in fact, derive very largely from long-term protein associations [18]. Further, Dellweg and Sumper [19] found that cross-linking of bacteriorhodopsin in the purple membrane gave predominantly trimeric species, consistent with the trimeric structure of bacteriorhodopsin in the membrane. In contrast, Brett and Findlay [17] found that cross-linking rhodopsin in the retinal rod membrane under a variety of conditions gave decreasing amounts of dimeric, trimeric and tetrameric species (with most of the rhodopsin being present as the monomer), consistent with a monomeric structure for rhodopsin in the membrane, with cross-linking following from collisions between molecules.

The studies of cross-linking of the $(Ca^{2+} + Mg^{2+})$-ATPase in the sarcoplasmic reticulum membrane reported here fail to find evidence for clear dimeric or tetrameric species, with cross-linking predominantly producing aggregates too large to enter the gels, in agreement with previous studies [8–11,22,23]. As shown in Fig. 2, on cross-linking with glutaraldehyde, there is a gradual loss of monomeric ATPase with increasing time of cross-linking, with formation of large aggregates. The loss of monomeric ATPase follows pseudo first order kinetics, with a half-time of 1.4 min. First order kinetics could follow either from cross-linking of a pre-existing aggregate of protein molecules or from an initial rate limiting covalent reaction followed by a very rapid collision of monomers [5,16], but the formation of large aggregates of ATPase under a variety of conditions of cross-linking with little dimer formation is difficult to understand except in terms of pre-existing clusters of ATPase molecules.

The effect of cross-linking on the rotational mobility of the ATPase was studied using saturation-transfer ESR spectroscopy (Fig. 3, Table I). As shown in the previous paper [1], the ratio of spectral line heights L''/L can be used to characterise the rotational motion, and the change ofh L''/L with time of cross-linking shown in Fig. 3 is consistent with a gradual reduction in rotation rate, in agreement with the results of Thomas and Hidalgo [21]. The spectra shown in Fig. 3 could represent a fairly homogeneous population of cross-linked species, or could represent a mixture of monomeric and totally immobilized, large aggregates. If the population of molecular species is homogeneous then the increase in L''/L value from 0.61 for native sarcoplasmic reticulum to 0.94 for sarcoplasmic reticulum cross-linked with glutaraldehyde for 17 min would correspond to a change in effective rotational correlation time from 40 μs to 180 μs (Table I). As shown in the previous paper [1] these effective rotational correlation times can be converted into approximate particle radii, indicating that after cross-linking, the ATPase would be present in an aggregate of approximate radius 120 Å, corresponding to a 16-mer: such a result would be consisitent with the results of gel electrophoresis shown in Fig. 1.

The most interesting of the studies presented here is that of cross linking in reconstituted systems containing the ATPase at a molar ratio of lipid to protein of 3000 : 1 (Fig. 5). Although more cross-linked dimeric species are formed in this system than in sarcoplasmic reticulum, the production of dimeric species is still very small compared to that of large aggregates. It might have been expected that in these systems, where the lipid to protein ratio is very high, the ATPase would have been present as a monomeric species, as found, for example, under some conditions in detergent solution. Cross-linking would then have been expected to give a pattern of decreasing amounts of dimer, trimer etc., as found for rhodopsin [17]: this was not observed. Alternatively, it might have been expected that the ATPase would have been present in the reconstituted membranes as isolated dimers, as also found under the appropriate conditions in detergent solution. Cross-linking would then have been expected

to give extensive dimeric species, analogous to the formation of trimers on cross-linking of bacteriorhodopsin [19]: again, this was not observed. The results therefore suggest that even when diluted into excess lipid, the ATPase has a tendency to form clusters containing a large number of ATPase molecules, rather than forming isolated monomers or dimers. This conclusion is in agreement with preliminary studies of the fluorescence polarisation of the ATPase labelled with fluorescein isothiocyanate, which suggest the presence of ordered structures (on the fluorescence timescale) even at high dilution in lipid (Munkonge, F. and Lee, A.G., unpublished observations).

The results of this paper can be combined with the saturation-transfer ESR results presented in Ref. 1 to produce a coherent model for the dynamic state of the ATPase molecule in the sarcoplasmic reticulum membrane and in reconstituted systems. The cross-linking studies reported here are most easily interpreted assuming that the ATPase is present in the membrane in large clusters or aggregates. The saturation-transfer ESR spectra, however, are consistent with rapid (on a millisecond time scale) rotational motion for the ATPase in the plane of the membrane, suggesting that the ATPase is present as a monomeric or dimeric species. Together, the experiments therefore suggest a dynamic structure for the membrane, in which the ATPase molecules tend to form clusters (probably as linear arrays of monomers or dimers) but with the lifetime for any particular ATPase molecule within the cluster being short.

Neither the cross-linking experiments reported here, nor the saturation-transfer ESR experiments [1] provide information as to whether the predominant structure for the ATPase within the clusters is monomeric or dimeric. Radiation inactivation experiments [5], however, suggest that the primary structure is dimeric, and the simplest possible aggregate of proteins in the membrane is a dimeric structure with a dyad axis running through the membrane [24] as observed for negatively stained sarcoplasmic reticulum [2–4]. There is also much evidence for the formation of dimeric ATPase species in detergent solution [32,33].

A number of observations suggest that a structure consisting of rows of molecules may be a recurring theme in membrane structure. Thus it has been suggested that both the $(Na^+ + K^+)$-ATPase [25] and the acetylcholine receptor [26] are present in membranes as rows of dimeric species. Further, it has been suggested that cholesterol in membranes occurs as alternating rows of lipid molecules and lipid-cholesterol complexes [27,28]: in this case also, it was suggested that the existence of the alternating rows was transient. More generally, it has been shown that the mixing of phospholipid molecules in the liquid crystalline phase is non-ideal, with a tendency for like molecules to be nearest neighbours [29,30], and it is possible that transient formation of linear arrays of like molecules occurs in this case also. The formation of such super lattices is common in ferromagnets [31], and comes about when the attraction between unlike molecules is greater than the attraction between like molecules.

Acknowledgment

We thank the Wellcome Trust for financial support, J.M.E. is a Wellcome Trust Lecturer.

References

1 Napier, R.M., East, J.M. and Lee, A.G. (1987) Biochim. Biophys. Acta, 903, 365–373
2 Taylor, K., Dux, L. and Martonosi, A. (1986) J. Mol. Biol. 187, 417–427
3 Buhle, E.L., Knox, B.E., Serpersu, E. and Aebi, U. (1983) J. Ultrastruct. Res. 85, 186–203
4 Castellani, L., Hardwicke, P.M.D. and Vibert, P. (1985) J. Mol. Biol. 185, 579–594
5 Ji, T.H. (1979) Biochim. Biophys. Acta 39–69
6 Louis, C. and Shooter, E.M. (1972) Arch. Biochem. Biophys. 153, 641–655
7 Murphy, A.J. (1976) Biochem. Biophys. Res. Commun. 70, 160–166
8 Chyn, T. and Martonosi, A. (1977) Biochim. Biophys. Acta 468, 114–126
9 Baskin, R.J. and Hanna, S. (1979) Biochim. Biophys. Acta 576, 61–70
10 Hebdon, G.M., Cunningham, L.W. and Green, N.M. (1979) Biochem. J. 179, 135–139
11 Kurobe, Y., Nelson, R.W. and Ikemoto, N. (1983) J. Biol. Chem. 258, 4381–4389
12 Laemmli, U.K. and Faure, M. (1973) J. Mol. Biol. 80, 575–599
13 Meissner, G. (1975) Biochim. Biophys. Acta 389, 51–68
14 Campbell, K.P. (1986) in Sarcoplasmic Reticulum in Muscle Physiology (Entman, M.L. and Van Winkle, W.B., eds), Vol. 1, pp. 65–99, CRC Press, Boca Raton

15 McIntosh, D.B. and Ross, D.C. (1985) Biochemistry 24, 1244–1251
16 Middaugh, C.R., Vanin, E.F. and Ji, T.H. (1983) Mol. Cell. Biochem. 50, 115–141
17 Brett, M. and Findlay, J.B.C. (1979) Biochem. J. 177, 215–223
18 Freedman, R.B. (1979) Trends Biochem. Sci. 4, 193–197
19 Dellweg, H.G. and Sumper, M. (1978) FEBS Lett. 90, 123–126
20 Hymel, L., Maurer, A., Berenski, C., Jung, C.Y. and Fleischer, S. (1984) J. Biol. Chem. 259, 4890–4895
21 Thomas, D.D. and Hidalgo, C. (1978) Proc. Natl. Acad. Sci. USA 75, 5488–5492
22 Louis, C.F. and Holroyd, J.A. (1978) Biochim. Biophys. Acta 535, 222–232
23 Bailin, G. (1980) Biochim. Biophys. Acta 624, 511–521
24 Klingenberg, M. (1981) Nature 290, 449–454
25 Hebert, H., Jorgensen, P.L., Skriver, E. and Maunsbach, A.B. (1982) Biochim. Biophys. Acta 689, 571–574
26 Heuser, J.E. and Salpeter, S.R. (1979) J. Cell Biol. 82, 150–173
27 Rogers, J., Lee, A.G. and Wilton, D.C. (1979) Biochim. Biophys. Acta 552, 23–37
28 Presti, F.T., Pace, R.J. and Chan, S.I. (1982) Biochemistry 21, 3831–3835
29 Lee, A.G. (1978) Biochim. Biophys. Acta 507, 433–444
30 Lee, A.G. (1983) in Membrane Fluidity in Biology (Aloi, R.C., ed.), Vol. 3, Academic Press, New York
31 White, R.M. and Geballe, T.H. (1979) Long Range Order in Solids, Academic Press, New York
32 Silva, J.L. and Verjovski-Almeida, S. (1985) J. Biol. Chem. 260, 4764–4769
33 Andersen, J.P., Vilsen, B., Nielsen, H. and Moller, J.V. (1986) Biochemistry 25, 6439–6447