Biochimica et Biophysica Acta, 468 (1977) 114—126

BBA 77733

CHEMICAL MODIFICATION OF SARCOPLASMIC RETICULUM MEMBRANES *

TED CHYN and ANTHONY MARTONOSI **

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Mo. 63104 (U.S.A.)

(Received November 19th, 1976)
(Revised manuscript received February 28th, 1977)

Summary

The usefulness of chemical cross-linking and ^{125}I-labeling techniques in the analysis of protein-protein interactions and membrane polarity was evaluated on sarcoplasmic reticulum membranes. Treatment of fragmented sarcoplasmic reticulum vesicles with glutaraldehyde, dimethylsuberimidate, or copper-phenanthroline leads to the formation of high molecular weight aggregates of the Ca^{2+} transport ATPase; intermediate polymers of functionally and structurally interesting sizes accumulated only occasionally and in amounts of questionable significance. Coupling of membrane proteins with tolylene 2,4-diisocyanate-albumin inhibited the ATPase activity and caused the appearance of high molecular weight aggregates and a band of about 160 000 dalton which corresponds to the ATPase-albumin complex.

Even after the 100 000 dalton band of the Ca^{2+} transport ATPase was severely diminished by cross-linking with copper-phenanthroline or toluene diisocyanate-albumin, the Ca^{2+} binding proteins of sarcoplasmic reticulum remained unreacted. A consistent finding was the presence of dimers of the Ca^{2+} transport ATPase in aged preparations of sarcoplasmic reticulum which were converted upon reduction with β-mercaptoethanol into 100 000 dalton units.

Microsomes were labeled with ^{125}I in the presence of lactoperoxidase, glucose oxidase, and glucose and the radioactivity of the various protein components was measured after sodium dodecyl sulfate-polyacrylamide gel electro-

* Preliminary reports of parts of this work were presented at the Nato Advanced Studies Institute on Biomembranes-Lipids, Proteins, and Receptors held in Espinho, Portugal in September, 1974 [1] and at the First International Symposium on Calcium Transport in Contraction and Secretion held in Bressanone, Italy on May, 1975 [2].

** To whom all correspondence should be addressed.

Abbreviations: TDIC, toluene-2,4-diisocyanate; SDS, sodium dodecyl sulfate. The terms C_1 and C_2 proteins refer to calsequestrin and the high affinity calcium binding protein, respectively.

phoresis. The specific activity of calsequestrin was many times greater than that of the Ca^{2+} transport ATPase suggesting that it is exposed on the oustide surface of the membrane. The binding site of calsequestrin on the outside surface may be sterically hindered from access by bulky reagents (tolylene diisocyanate-albumin, ferritin-labeled anti-calsequestrin antibodies, proteolytic enzymes, etc.), as calsequestrin becomes highly reactive with these agents only after its release from the membrane.

Introduction

The functional and structural implications of protein-protein interactions in biological membranes are widely recognized primarily on the basis of extensive work on the electron transport chain of mitochondria [3], the structure of the F_1 complex [4], the acetylcholine receptor [5], the purple membrane [6], and the phosphoenolpyruvate phosphotransferase system of bacteria [7].

Information is only beginning to emerge on the role of protein-protein interactions in active ion transport systems involved in $Na^+ + K^+$ [8] and Ca^{2+} [2] translocation.

The Ca^{2+} transport system of sarcoplasmic reticulum is ideally suited for the analysis of the functional significance of association-dissociation equilibria between membrane proteins in view of its relatively simple protein composition [9]. In fact, stable oligomers of the Ca^{2+} transport ATPase were commonly observed already in the earliest gel electrophoresis studies of sarcoplasmic reticulum [9,10] although their functional significance at that time was not recognized. Since then similar observations were made using dimethylsuberimidate [11,12], copper-phenanthroline [2,13], and *N,N'-p*-phenylenedimaleimide [14] as cross-linking agents, with somewhat divergent results.

The purpose of this report is to evaluate the value of chemical cross-linking techniques in the light of recent electron microscopic [15] and fluorescence energy transfer observations [16] which indicate the existence of ATPase oligomers in the membrane. The studies were extended to the localization of the Ca^{2+} binding proteins in the membrane with the aid of chemical cross-linking agents and enzyme-catalyzed iodination.

Experimental procedures

Fragmented sarcoplasmic reticulum was prepared from predominantly white skeletal muscles of rabbit [15] and chicken muscle [17] as described earlier.

The ATPase activity was assayed in a solution of 0.1 M KCl/10 mM imidazole (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), and 0.45 mM $CaCl_2$ at 25°C in a total volume of 2.1 ml. Protein concentration was usually 0.05—0.1 mg/ml. Incubation was stopped after 2—15 min with 0.5 ml 10% trichloroacetic acid, and aliquots were used for the assay of inorganic phosphate by the method of Fiske and SubbaRow [18]. Protein concentration was determined according to Lowry et al. [19]. ^{45}Ca transport was measured by the Millipore technique (as described earlier [20]). The labeling of the membranes with ^{125}I was performed

in the presence of glucose oxidase and lactoperoxidase essentially according to Hubbard and Cohn [21,22]. Disulfide bond formation was catalyzed by copper-phenanthroline [23].

For cross-linking of membrane proteins with TDIC-albumin, bovine serum albumin was coupled with TDIC as described by Likhite and Sehon [24]. Conditions for the reactions of the membranes with dimethyl-suberimidate and glutaraldehyde are given in the figure legends.

SDS-polyacrylamide gel electrophoresis was carried out as described earlier [10]. In some instances gels of lower cross-linkage were used as described by Murphy [13].

The content of SH groups in the membranes was measured by the Ellman technique [25] in a medium of 0.025 M imidazole, pH 7.0, 5 mM EDTA, 0.25 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.5 mg microsomal protein/ml at 25° C essentially as described earlier [10].

Antibodies against the Ca^{2+} transport ATPase and the C proteins (Ca^{2+} binding protein and calsequestrin) were prepared as described earlier [26,27]. The antibodies were labeled with ferritin according to Tillack et al. [28].

Materials

Glucose oxidase (Type II from *Aspergillus niger*) and 1,10-phenanthroline monohydrate were obtained from Sigma Chemical Co., St. Louis, Mo. Milk lactoperoxidase was supplied by Calbiochem., San Diego, Calif. Dimethylsuberimidate was the product of Pierce Chemical Co., Rockford, Ill.; glutaraldehyde was obtained from Fisher Scientific, Fair Lawn, N.J., and $Na^{125}I$ from Industrial Nuclear Corp., St. Louis, Mo.

Results

Effects of glutaraldehyde and dimethylsuberimidate upon the protein composition and enzymatic activity of sarcoplasmic reticulum

The typical protein composition of rabbit sarcoplasmic reticulum is shown in Fig. 1 (A). The major band represents the Ca^{2+} transport ATPase which usually forms a small amount of dimers and traces of other oligomers which appear near the top of the gel. The amount of oligomers varies from preparation to preparation and it usually constitutes a significant fraction only in aged microsome preparations. After reduction of the vesicles with 1.6 M β-mercaptoethanol the oligomers dissociate into 100 000 dalton units (not shown) indicating that they are stabilized by intermolecular disulfide bonds.

Other minor components are the C_1 and C_2 proteins of 45 000—55 000 molecular weight which were first observed in our laboratory [29] and were subsequently named calsequestrin and calcium binding protein, respectively [30]. Trace amounts of minor protein components of relatively high mobility are observed in the area of the gel near the phospholipids.

The relationship between electrophoretic mobility and the molecular weight is shown in Fig. 2. For calibration in the range of 100 000—400 000 daltons, rabbit muscle myosin and purified Ca^{2+} transport ATPase were used which upon aging formed distinct bands of dimers, trimers, and tetramers. It is noted

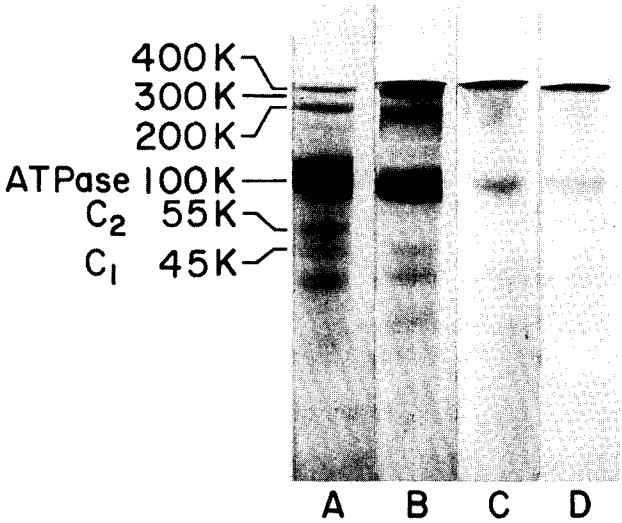

Fig. 1. Cross-linking of sarcoplasmic reticulum proteins with glutaraldehyde. Rabbit skeletal microsomes (3.5 mg/ml) were cross-linked with increasing concentrations of glutaraldehyde at room temperature for 60 min in 1 ml phosphate buffer (0.05 M). Concentration of glutaraldehyde: A, none; B, 0.1 mM; C, 0.5 mM; D, 1 mM. Reaction was stopped with 0.01 M NH_4HCO_3. Polyacrylamide gel (5%) was prepared as described in Materials and Methods. About 200 μg microsomal proteins were used for each gel. Gel electrophoresis for 12 h in room temperature.

that the relationship between mobility and the log of molecular weight is non-linear above 100 000 daltons under the conditions used in most experiments except those in Fig. 4.

Treatment of rabbit skeletal muscle microsomes with 0.1—1 mM gluteraldehyde (Fig. 1) or with 1—10 mM dimethylsuberimidate (not shown) causes progressive disappearance of the bands corresponding to the Ca^{2+} transport ATPase, and the C_1 and C_2 proteins with the accumulation of high molecular weight aggregates that are retained on the top of the gel. Similar observations were made on microsomes isolated from the superficial pectoralis muscle of chicken. ATPase oligomers of intermediate size (dimers, trimers, tetramers, etc.) accumulated only to a slight extent at moderate concentrations of cross-linking agents, in agreement with Louis and Shooter [11]. At higher concentrations of cross-linking agents the concentration of oligomers initially present in sarcoplasmic reticulum membranes actually decreased during the cross-linking process, accompanied by marked inhibition of the ATPase activity, and Ca^{2+} transport [12,31].

Effect of copper-phenanthroline on the protein composition and enzymatic activity of sarcoplasmic reticulum

Copper-phenanthroline catalyzes the formation of intermolecular disulfide bonds and was used extensively to study proximity relationships between proteins in erythrocyte membranes [23].

In sarcoplasmic reticulum membranes exposed to copper and phenanthroline for 2—60 min at 25°C, the 100 000 dalton band which corresponds to the Ca^{2+} transport ATPase diminished with time without major change in the amount of

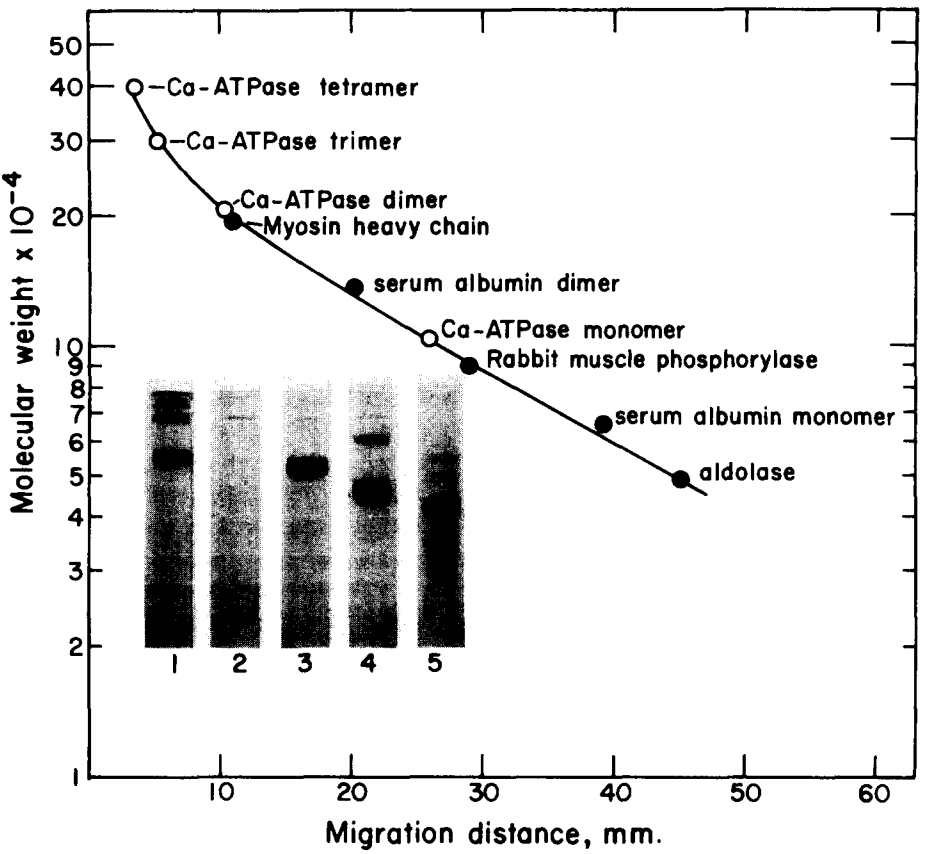

Fig. 2. Correlation between electrophoretic mobility and molecular weight. Conditions for electrophoresis were as described earlier [10]. For details see text. Insert: 1, purified Ca^{2+} transport ATPase from rabbit sarcoplasmic reticulum; 2, myosin; 3, rabbit muscle phosphorylase; 4, serum albumin; 5, aldolase.

C_1 and C_2 proteins (Fig. 3). Essentially all the cross-linked protein appeared in the form of high molecular weight aggregates which were retained on the surface of the gel.

There is no evidence for the accumulation of the sizeable amounts of intermediate polymers of 200 000—400 000 daltons mass. In this respect the copper-phenanthroline system shares the all or none character of reaction with glutaraldehyde and dimethylsuberimidate. With increasing cross-linking the ATPase activity is inhibited with a decrease in the titratable SH group content of the membrane. Essentially no activity remains when the ATPase monomers are completely converted into high molecular weight aggregates.

The absence of sizeable accumulation of intermediate polymers of 400 000 daltons mass during cross-linking with copper-phenanthroline is in conflict with the observations of Murphy [13]. In an attempt to resolve this difference, the experiments were repeated (Fig. 4) under the cross-linking and electrophoresis conditions used by Murphy [13] with essentially the same results as shown in Fig. 3.

Varying the concentration of phenanthroline and copper over a wide range

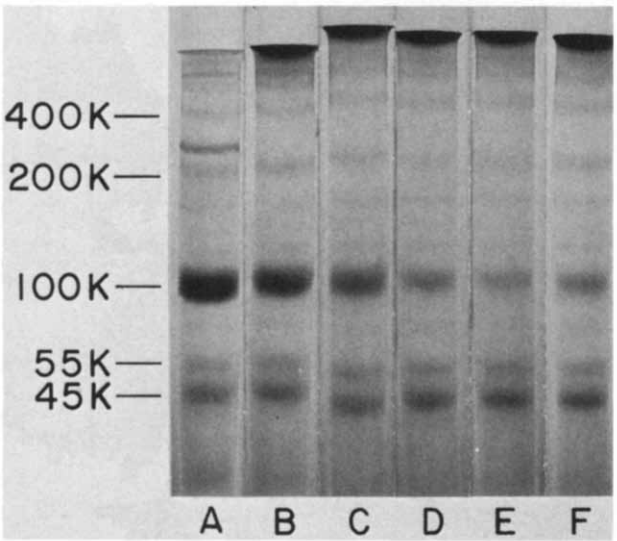

Fig. 3. The reaction of microsomal proteins with copper-phenanthroline. Rabbit skeletal microsomes (2 mg/ml) were cross-linked with 10^{-4} M of 1,10-phenanthroline and $CuSO_4$ for various times in an incubation solution containing 50 mM Tris · HCl, pH 8.4. Reactions were stopped by addition of 10^{-3} M EDTA. Polyacrylamide gels (5%) were prepared as described under Materials and Methods. Electrophoresis for 20 h in room temperature. Incubation times: A, 0 min; B, 2 min; C, 5 min; D, 10 min; E, 20 min; F, 60 min.

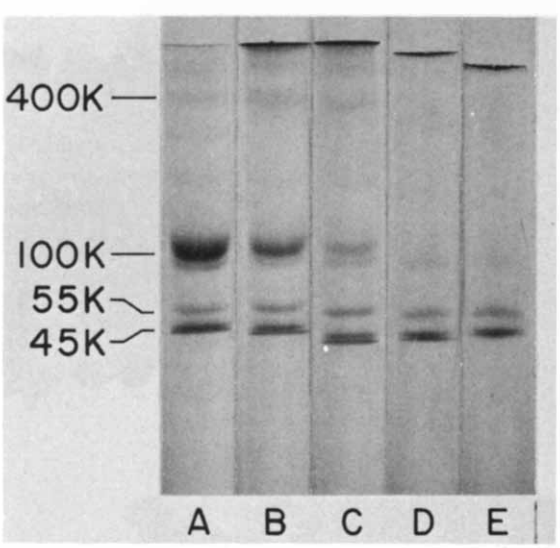

Fig. 4. Cross-linking of sarcoplasmic reticulum proteins with copper-phenanthroline. Rabbit skeletal microsomes (2 mg/ml) were cross-linked with 10^{-4} M $CuSO_4$ and $3 \cdot 10^{-4}$ M, 1,10-phenanthroline in 1 ml incubation solution containing 1 mM $CaCl_2$ and 0.14 M morpholinopropanol sulfonic acid adjusted to pH 7.2 with triethanolamine essentially as described by Murphy [13]. Reaction was stopped with 10 mM EDTA, 16 mM *N*-ethylmaleimide and 10 mg/ml SDS. Polyacrylamide gels (5.1%) were prepared according to Murphy [13]. Incubation times: A, 0 min; B, 1 min; C, 5 min; D, 20 min; E, 60 min.

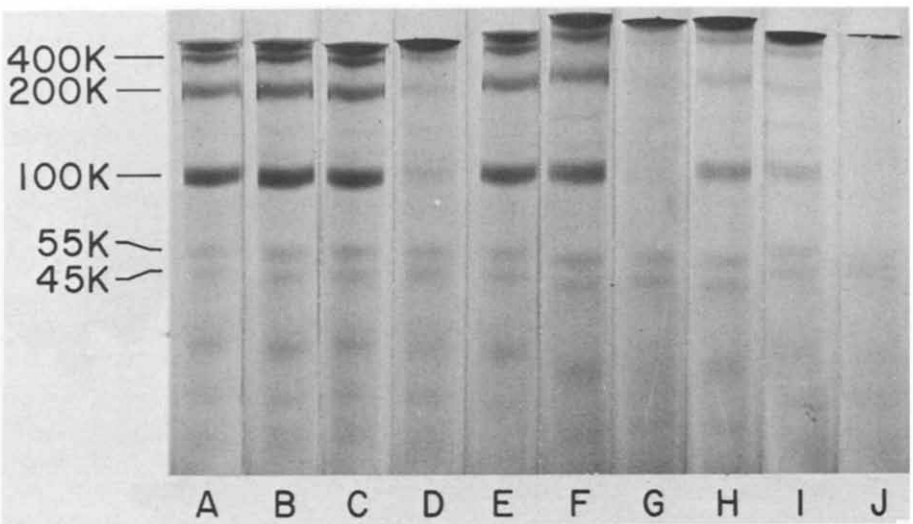

Fig. 5. Effect of $CuSO_4$ and 1,10-*O*-phenanthroline concentration on the cross-linking of microsomal proteins. Microsomes (2 mg/ml) in 0.05 M Tris · HCl buffer, pH 8.4, were reduced by 1 mM dithiothreitol at 4°C for 4 h. Free dithiothreitol was removed by repeated centrifugation. Microsomes (2 mg/ml) were cross-linked in an incubation mixture containing 0.05 M Tris · HCl, pH 8.4, with various concentrations of $CuSO_4$ and 1,10-*O*-phenanthroline as indicated in the figure. Reactions were stopped after 1 h by addition of 0.02 mM EDTA (pH 7.4). Polyacrylamide gel (5%) was prepared as described in Materials and Methods. $CuSO_4$ concentrations: A, 0; B, E, H, 10^{-6} M; C, F, I, 10^{-5} M; D, G, J, 10^{-4} M. *O*-Phenanthroline concentrations: A, O, B, C, D, 10^{-5} M; E, F, G, 10^{-4} M; H, I, J, 10^{-3} M.

(Fig. 5) did not lead to an increase in the concentration of intermediate polymers. Exposure of the cross-linked membrane to 1.6 M β-mercaptoethanol, which reduces the intramolecular disulfide bonds, caused complete dissociation of the high molecular weight aggregates into ATPase monomers of 100 000 daltons.

Cross-linking of sarcoplasmic reticulum proteins with toluene, 2,4-diisocyanate

Reaction of sarcoplasmic reticulum membranes in the absence of complement with antibodies directed against the Ca^{2+} transport ATPase leaves the ATPase activity and Ca^{2+} transport unaffected [26,27]. These observations ruled out rotary carrier mechanism as a plausible scheme to explain Ca^{2+} translocation [26,27,32].

In an attempt to achieve immobilization of the Ca^{2+} transport enzyme by the covalent attachment of a bulky group, we reacted sarcoplasmic reticulum with toluene diisocyanate-albumin (Fig. 6). Reaction with increasing concentrations of toluene diisocyanate-albumin progressively decreased the amount of 100 000 dalton protein with only slight changes in the protein content of C_1 and C_2 bands. A faint new band of about 160 000 daltons mass appeared (arrow) which presumably represents the ATPase-albumin complex. Most of the ATPase that underwent cross-linking appeared among the high molecular weight aggregates retained on the top of the gel. The formation of large aggregates with toluene diiisocyanate-albumin is presumably due to the presence of several reactive groups on each toluene diisocyanate-albumin molecule which allows formation of multiple cross-links.

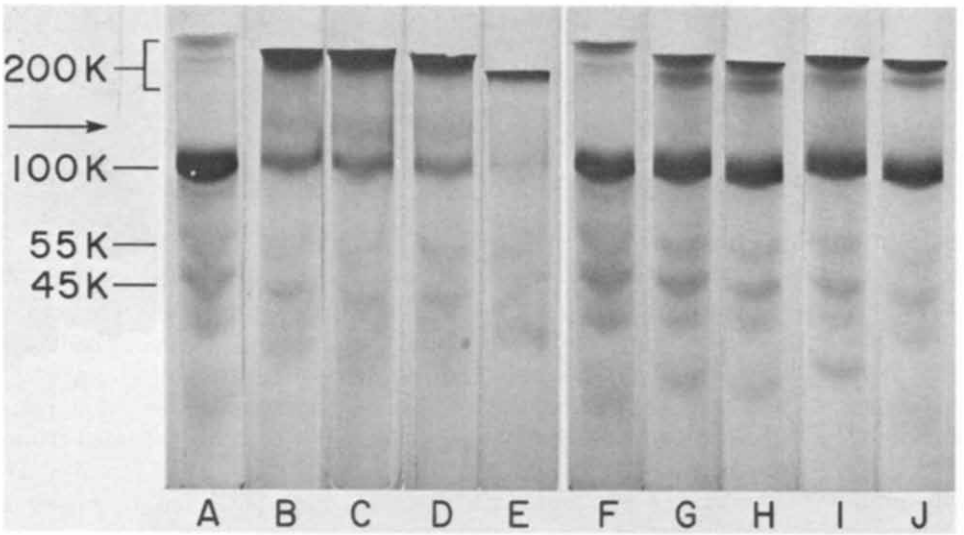

Fig. 6. Cross-linking of microsomal proteins by bovine serum albumin-toluene 2,4-diisocyanate (TDIC-albumin) conjugate. TDIC-albumin conjugate was prepared by reacting 0.2 ml of TDIC with 100 mg albumin in a volume of 10 ml sodium phosphate buffer (0.1 M, pH 8.0) at 0°C for 25 min. Free TDIC was removed by centrifugation at 6000 rev./min for 10 min. The supernatant containing TDIC-albumin was further reacted for 1 h at 0°C. Microsomes (1 mg/ml) were cross-linked with TDIC-albumin by addition of conjugate in increasing amounts. The reaction was stopped with 0.08 M NH_4Cl (pH 8.0) and the unreacted conjugate was removed by repeated centrifugation. Polyacrylamide gel electrophoresis was carried out as described in Materials and Methods. TDIC-albumin microsomal protein weight ratios were as follows: A, 0; B, 1; C, 3; D, 6; E, 9. To samples F—J only TDIC was added at the same starting concentrations as in A—E. About 200 μg of microsomal protein were used for each gel.

In the absence of albumin but otherwise under identical conditions toluene diisocyanate causes the appearance of a faint band of oligomers with close to 200 000 daltons mass accompanied by a slight decrease in the amount of 100 000 dalton protein (Fig. 6).

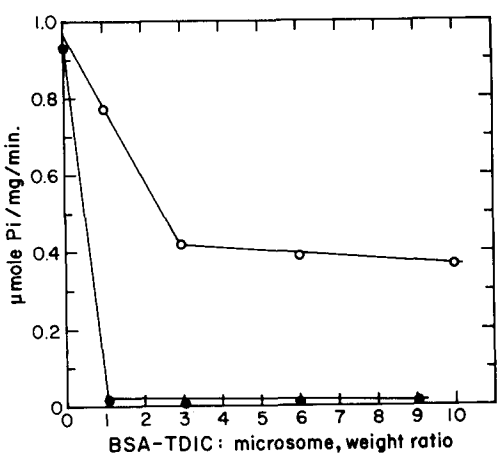

Fig. 7. Effect of TDIC-albumin conjugate and TDIC on the ATPase activity. Microsomal proteins were reacted with TDIC-albumin conjugate or TDIC as in Fig. 6. ATPase activities were measured as described under Experimental Procedures. ●, TDIC-albumin; ○, TDIC.

The ATPase activity of sarcoplasmic reticulum treated with TDIC-albumin at a 1 : 1 weight ratio is nearly completely inhibited (Fig. 7) although some of the ATPase is still present in the monomer form. Even toluene diisocyanate alone markedly inhibits ATPase activity (Fig. 7) although the decrease in the amount of ATPase monomer is less than 10%. This is probably due to intramolecular cross-links or active site modification.

Iodination of microsomal proteins

Reaction of muscle microsomes with copper-phenanthroline and with toluene diisocyanate left the C_1 and C_2 proteins largely unaffected. As the two acidic proteins were also resistant to treatment of microsomes with trypsin [33] and did not react readily with ferritin-labeled antibodies directed against them [1], it was suggested that they are located on the inside surface of sarcoplasmic reticulum membrane [34]. However, the same proteins were readily released from the vesicles by exposure to 1 mM EGTA at pH 8.0 [29] and brief reaction of native membranes with ^{125}I [35] or with fluorescamine [36] led to massive labeling of the C_1 and C_2 proteins, suggesting outside localization.

TABLE I

EFFECT OF EGTA, Mg-ATP AND Ca^{2+} ON THE ^{125}I LABELING OF SARCOPLASMIC RETICULUM PROTEINS

Microsomes (5 mg/ml) were iodinated in 0.1 M Tris · HCl buffer, pH 8.0, containing 50 μg/ml glucose oxidase, 50 μg/ml of lactoperoxidase and 5 mM glucose at a 25°C temperature for 5—30 min. Reaction was terminated by addition of $2 \cdot 10^{-4}$ M $Na_2S_2O_3$. Microsomes were collected by centrifugation and dialyzed against 0.1 M Tris · HCl buffer, pH 8.0, containing $2 \cdot 10^{-4}$ M $Na_2S_2O_3$. Aliquots were taken for radioactivity measurements. Electrophoresis was carried out as described under Experimental Procedures. Gels were sliced and the bands of ATPase, C proteins, and phospholipids were cut out and counted. Specific radioactivities were calculated in terms of protein concentration as described by Martonosi and Halpin [10].

Medium composition	Band	Protein-bound radioactivity after incubation for					
		5 min (cpm)	15 min (cpm)	30 min (cpm)	5 min (cpm/mg)	15 min (cpm/mg)	30 min (cpm/mg)
Control	Top	7 051	9 213	14 037	94 167	120 324	173 605
	ATPase	13 864	18 072	23 648	40 678	58 979	92 764
	C_2	6 243	7 387	10 360	109 334	123 682	170 456
	C_1	6 015	7 280	12 365	146 939	201 837	263 999
	Phospho-lipid	4 011	4 113	5 212	100 396	114 636	193 834
5 mM EGTA	Top	6 922	14 831	20 533	120 632	180 003	350 366
	ATPase	18 633	22 732	28 415	42 678	55 976	92 734
	C_2	7 223	10 937	16 051	113 634	153 223	286 963
	C_1	8 481	11 655	17 054	183 736	270 116	406 374
	Phospho-lipid	4 122	5 363	6 214	113 674	149 391	256 913
5 mM MgATP	Top	4 000	11 244	12 678	87 630	176 354	223 467
0.5 mM $CaCl_2$	ATPase	10 116	13 015	17 398	39 843	46 738	69 780
	C_2	2 414	4 432	6 099	73 865	99 738	163 261
	C_1	2 635	4 659	9 533	89 367	170 638	213 764
	Phospho-	2.311	4 286	4 617	63 178	156 045	223 764

In an attempt to resolve this conflict the conditions of ^{125}I labeling were further investigated using native sarcoplasmic reticulum membranes, purified Ca^{2+}-transport ATPase vesicles and isolated C proteins.

After labeling of rabbit skeletal muscle microsomes with ^{125}I the distribution of radioactivity among the various protein fractions was analyzed by SDS-polyacrylamide gel electrophoresis. Although the C_1 and C_2 proteins represent only 3—5% of the protein content of sarcoplasmic reticulum membranes, the level of ^{125}I radioactivity incorporated into them calculated per mg protein far exceeded the radioactivity of the Ca^{2+} transport ATPase, which constitutes 70—80% of the membrane proteins (Table I). The specific activity of C_1 protein is high already after 5 min incubation which is consistent with external labeling. Significant incorporation was also observed in the phospholipid fraction which is presumed to contain the proteolipid. These observations are in essential agreement with those of Thorley-Lawson and Green [35].

The C_1 and C_2 proteins are released from the sarcoplasmic reticulum membrane by treatment with EGTA at pH 8.0 [29] and under these conditions their reactivity with ^{125}I increases (Table I).

The decreased incorporation of ^{125}I into all proteins in the presence of ATP, Mg^{2+} and Ca^{2+} (Table I) may be related to substrate-induced conformation change in the membrane that is reflected by changes in fluorescence [16], electron spin resonance [37], and SH group reactivity [38].

The reactivity of the Ca^{2+} transport ATPase and the C_1 and C_2 proteins in sarcoplasmic reticulum membranes was compared with the reactivity of the purified proteins (Table II).

The isolated C_1 and C_2 proteins showed far greater incorporation of ^{125}I than the same proteins on the sarcoplasmic reticulum membranes or in mixtures

TABLE II

IODINATION OF MICROSOMES, ATPase AND C_1 PROTEIN

Iodination of microsomes (5 mg/ml), purified ATPase (5 mg/ml) and C_1 protein (5 mg/ml) was carried out under the conditions described in Table I. The gels were stained, the visible bands were cut out and counted.

Preparations	^{125}I bound			
	No deoxycholate (cpm)	2% deoxycholate (cpm)	No deoxycholate (cpm/mg)	2% deoxycholate (cpm/mg)
Microsomes				
Top layer	713	583	31 726	15 310
ATPase	801	890	5 161	5 585
C_2	155	285	9 695	10 490
C_1	477	557	35 650	24 576
Phospholipid	260	175	26 260	10 472
Purified ATPase	1 126	1 157	11 871	11 930
Purified C_1 protein	110 081	182 997	2 228 542	1 170 981
ATPase + C_1 protein				
ATPase	1 594	1 169	33 233	22 026
C_1 protein	1 759	1 499	39 625	34 851

with the purified ATPase, where they may interact with the membranes. Although competition for ^{125}I by the Ca^{2+} transport ATPase and phospholipids makes a significant contribution to this difference, the observations suggest that the binding of C_1 and C_2 proteins to the membranes lowers their reactivity with ^{125}I.

Discussion

Based upon electron microscopy [2,15,16] and fluorescence energy transfer [16,39] observations, we proposed that the Ca^{2+} transport ATPase of sarcoplasmic reticulum tends to associate into oligomers and raised the possibility that an equilibrium between monomers and oligomers may have functional significance either in active Ca^{2+} uptake or in the passive Ca^{2+} release during excitation. These observations were supported by the presence of ATPase oligomers in sarcoplasmic reticulum membrane solubilized with sodium dodecyl sulfate (refs. 9 and 10, see also this report) or Triton-X-100 [40]. These oligomers are apparently stabilized by intermolecular disulfide bonds as they dissociate into monomers after reduction with high concentration of β-mercatoethanol. Therefore there is some doubt whether the presence of oligomers in such systems represents stabilization of a naturally occurring enzyme form or is simply an artifact.

Previous work using chemical cross-linking agents led to conflicting results. In the presence of dimethylsuberimidate a statistically expected mixture of monomers, dimers, trimers and tetramers was observed [11]; copper-phenanthroline was claimed to form primarily tetramers [13] and *N,N'-p*-phenylenedimaleimeide primarily hexamers [14]. The experiments reported here indicate that with glutaraldehyde, dimethylsuberimidate, copper-phenanthroline and toluene diisocyanate-albumin the dominant process is the formation of high molecular weight aggregates with molecular weights above 400 000. While intermediate polymers of 200 000—400 000 daltons were observed in aged microsomes and sometimes after treatment with copper-phenanthroline and TDIC, the amount of these was usually very small and hardly distinguishable from similar components in the starting material. In fact during conversion of ATPase into high molecular weight aggregates in the presence of copper-phenanthroline the amount of intermediate polymers usually decreased.

The observed tendency for formation of large aggregates without sizeable accumulation of intermediate polymers suggests a cooperative reaction, perhaps involving denaturation, and raises doubt that glutaraldehyde, dimethylsuberimidate, and copper-phenanthroline can be used to "stabilize" protein-protein interactions as they occur in the native sarcoplasmic reticulum membranes. The high concentration of Ca^{2+} transport ATPase in the membrane may facilitate the formation of random aggregates through collision between the particles during the cross-linking process.

An interesting finding is that copper-phenanthroline and TDIC-albumin cause extensive aggregation of the Ca^{2+} transport ATPase, without effect upon the C_1 and C_2 proteins. The low cysteine content of C_1 and C_2 proteins [30,34] explains in part their resistance to copper-phenanthroline. The lack of cross-linking with TDIC-albumin indicates their inaccessibility for reaction.

The reactivity of C proteins in solution was much greater than when bound to sarcoplasmic reticulum membranes or to reconstituted ATPase vesicles. Part of this difference may be explained by competition between various proteins for the ^{125}I, but the data indicate a genuine decline in ^{125}I incorporation into C proteins under conditions when they are bound. A similar difference in the sensitivity of calsequestrin to trypsin digestion was observed by Stewart and MacLennan [33].

The site of localization of C proteins in the membrane is disputed. Internal localization was suggested on the basis of extraction data [30], the inaccessibility of C proteins to digestion with proteolytic enzymes [33], and the lack of evidence indicating binding of anti-C antibodies to the membrane [26,41]. The rapid release of C proteins from the membrane by EGTA at pH 8.0 [29] and their great reactivity with ^{125}I and with fluorescamine [36] argue for external localization. A plausible interpretation of all available information is that the C proteins are located on the outside surface of the microsomes but their interaction with bulky reagents like anti-C antibodies [20,41], trypsin [33], and TDIC-albumin is prevented by the geometry of the binding site. The Ca^{2+} transport ATPase and phospholipids are likely to contain sufficient internal Ca^{2+} binding sites of low affinity to bind the accumulated calcium.

Acknowledgements

This work was supported by research grants from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM 18117); from the National Science Foundation (PCM-7600707); and from the Missouri Heart Association, Inc.

Part if this work was performed by Mr. T. Chyn, M.S., as a requirement for the Ph.D. degree, supported by a traineeship from St. Louis University.

References

1 Martonosi, A.N. (1975) in Biomembranes-Lipids, Proteins, and Receptors, Proceedings of A Nato Advanced Study Institute (Burton, R.M. and Packer, L., eds.), pp. 369—390, Biscience Publications Division, Webster Groves, Mo.
2 Martonosi, A. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, F., Drabikowski, W. and Margreth, A., eds.), pp. 313—327, North-Holland Publ. Co., Amsterdam
3 Hatefi, Y. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed), Vol. 4, pp. 3—41, Plenum Press, New York
4 Tzagoloff, A. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 4, pp. 103—124, Plenum Press, New York
5 Karlin, A. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 4, pp. 311—330, Plenum Press, New York
6 Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28—32
7 Kundig, W. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 3 pp. 31—55, Plenum Press, New York
8 Kyte, J. (1974) J. Biol. Chem. 249, 3652—3660
9 Martonosi, A. (1969) Biochem. Biophys. Res. Commun. 36, 1039—1044
10 Martonosi, A. and Halpin, R.A. (1971) Arch. Biochem. Biophys. 144, 66—77
11 Louis, C. and Shooter, E.M. (1972) Arch. Biochem. Biophys. 153, 641—655
12 Yuthawong, Y., Feldman, N. and Boyer, P.D. (1975) Biochim. Biophys. Acta 382, 116—124
13 Murphy, A.J. (1976) Biochem. Biophys. Res. Commun. 70, 160—166
14 Ikemoto, N., Garcia, A.M., O'Shea, P.A. and Gergely, J. (1975) J. Cell Biol. 67, No. 2, part 2, Abstr. 187c

15 Jilka, R.L., Martonosi, A. and Tillack, T.W. (1975) J. Biol. Chem. 250, 7511—7524
16 Martonosi, A.N., Nakamura, H., Jilka, R.L. and Vanderkooi, J.A. (1977) in the Biochemistry of Membrane Transport (Semiezza, G. and Carafoli, E., eds.), Springer, in the press
17 Boland, R., Martonosi, A. and Tillack, T.W. (1974) J. Biol. Chem. 249, 612—623
18 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648—658
21 Hubbard, A.L. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390—405
22 Hubbard, A.L. and Cohn, Z.A. (1975) J. Cell Biol. 64, 438—460
23 Steck, T.L. (1972) J. Mol. Biol. 66, 295—305
24 Likhite, V. and Sehon, A. (1967) in Methods in Immunology and Immunochemistry (Williams, C.A. Chase, M.W., eds.), Vol. I, pp. 150—167, Academic Press, New York
25 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
26 Martonosi, A. and Fortier, F. (1974) Biochem. Biophys. Res. Commun. 60, 382—389
27 Martonosi, A., Jilka, R. and Fortier, F. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., Klingenberg, M.E., Quagliariello, E. and Siliprandi, N., eds.), pp. 113—124, North-Holland Publ. Co., Amsterdam
28 Tillack, T.W., Scott, R.E. and Marchesi, V.T. (1972) J. Exp. Med. 135, 1209—1227
29 Duggan, P.F. and Martonosi, A. (1970) J. Gen. Physiol. 56, 147—167
30 MacLennan, D.H. and Wong, P.T.S. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1231—1235
31 Sommer, J.R. and Hasselbach, W. (1967) J. Cell Biol. 34, 902—905
32 Dutton, E., Rees, E.D. and Singer, S.J. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1532—1536
33 Stewart, P.S. and MacLennan, D.H. (1974) J. Biol. Chem. 249, 985—993
34 MacLennan, D.H. and Holland, P.C. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 3, pp. 221—259, Plenum Press, New York
35 Thorley-Lawson, D.A. and Green, N.M. (1973) Eur. J. Biochem. 40, 403—413
36 Hasselbach, W., Migala, A. and Agostini, B. (1976) Z. Naturforsch. 30c, 600—607
37 Nakamura, H., Hori, H. and Mitsui, T. (1972) J. Biochem. Tokyo 72, 635—646
38 Martonosi, A. (1976) FEBS Lett. 67, 153—155
39 Vanderkooi, J.M., Ierokomos, A., Nakamura, H. and Martonosi, A. (1977) Biochemistry 16, 1262—1267
40 LeMaire, M., Moller, J.V. and Tanford, C. (1976) Biochemistry 15, 2336—2342
41 MacLennan, D.H. (1975) Can. J. Biochem. 53, 251—261