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Covalent labeling of the cytoplasmic or luminal domains of the sarcoplasmic reticulum Ca^{2+}-ATPase with fluorescent azido dyes

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Sarcoplasmic reticulum (SR) vesicles were incubated with azido derivatives of Cascade blue (ACB), Lucifer yellow (ALY), 2,7-naphthalene-disulfonic acid (ANDS), and fluorescein (AF) for 0.1–24 h at 2°C. All four dyes gave intense reaction with the cytoplasmic domain of the Ca^{2+}-ATPase on photoactivation after brief incubation. The penetration of the dyes into the luminal space of the SR was determined after centrifugation through Sephadex microcolumns to remove the external dye, followed by photolabeling and gel electrophoresis of the photolabeled proteins. The reaction of ACB and ANDS with the Ca^{2+}-ATPase and with calsequestrin increased progressively during incubation up to 24 h indicating their slow accumulation in the luminal space, while ALY and AF did not show significant penetration into the vesicles. The distribution of the covalently attached ACB in the Ca^{2+}-ATPase was tested by tryptic proteolysis after labeling exclusively from the outside (OS), from the inside (IS) or from both sides (BS). In all cases intense ACB fluorescence was seen in the A fragment with inhibition of ATPase activity. In the OS preparations the A_1, while in IS the A_2 fragment was more intensely labeled. There was no significant incorporation of ACB into the region of B fragment identified by FITC fluorescence. The crystallization of the Ca^{2+}-ATPase by EGTA + decavanadate was completely inhibited in the BS samples after labeling either in the Ca_2E_1 or E_2V conformation. There was no inhibition of crystallization in the OS preparations. In the IS preparations labeled in the Ca_2E_1 state the crystallization was impaired, while in the E_2V state there was only slight disorganization of the crystals. The total amount of ACB photoincorporated into SR proteins after incubation for 24 h was 1.75 nmol/mg protein; 2/3 of this labeling occurred from the outside and 1/3 from the inside. Similar level of labeling was obtained in media that stabilize the E_1 or the E_2 conformation of the Ca^{2+}-ATPase.

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Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, the Ca^{2+},Mg^{2+}-activated ATPase of sarcoplasmic reticulum (EC 3.6.1.38); FITC, fluorescein 5'-isothiocyanate; ALY, *N*-(((*p*-azidobenzoyl)amino)ethyl)-4-amino-3,6-disulfo-1,8-naphthalimide, dipotassium salt (Lucifer yellow AB); ACB, Cascade blue aminoethyl *p*-azidobenzamide trisodium salt; AF, azidofluorescein; ANDS, 3-azido-2,7-naphthalenedisulfonic acid disodium salt; SDS, sodium dodecylsulfate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DOC, deoxycholic acid; $C_{12}E_8$, octaethyleneglycol dodecyl ether; OS, outside; IS, inside; BS, both sides.

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Introduction

Hypothetical models of the structure of Ca^{2+}-ATPase based on hydropathy plots [2,3], site specific mutagenesis [4,5], the reaction of impermeant sulfhydryl reagents [6] and on immunological studies [7–9] predict that the Ca^{2+}-ATPase contains 10 transmembrane helices and that both the N-terminal and the C-terminal sequences are located on the cytoplasmic side of the membrane. Structures containing 8 transmembrane sequences have also been considered [9–12], but appear less likely [8,9].

All models assume that about 2/3 of the mass of the Ca^{2+}-ATPase is exposed on the cytoplasmic side of the membrane, in agreement with electron microscope reconstruction of the structure of Ca^{2+}-ATPase from

two dimensional Ca^{2+}-ATPase crystals [13–21], and from data obtained by neutron diffraction analysis of oriented multilayers of sarcoplasmic reticulum [22–26].

By contrast, very little is known about the disposition of the transmembrane and luminal segments of the molecule. The model proposed by MacLennan and his colleagues [3,9] predicts that residues 79–87, 284–294, 784–789, 859–896 and 951–960 form luminal loops between transmembrane helices M_1-M_2, M_3-M_4, M_5-M_6, M_7-M_8 and M_9-M_{10}, respectively. This would imply that of the approx. 1000 amino acid residues of the Ca^{2+}-ATPase only approx. 72 would be exposed in the luminal space of the sarcoplasmic reticulum. About half of these residues (859–896) are assigned to a single loop between the M_7 and M_8 helices. The luminal localization of this loop is supported by the observation that the monoclonal antibody A20 with epitopes in the 870–890 region [9] and a polyclonal antibody produced against the synthetic peptide TEDHPHFEGLDC, representing the 877–888 region [8], react with the Ca^{2+}-ATPase only after the membrane was permeabilized or solubilized with detergents. In view of its proximity to the putative Ca^{2+} binding region [4] the 859–896 sequence may contribute to the low affinity Ca^{2+} binding on the luminal side of the membrane. There is no direct information about the proposed loops connecting the M_1-M_2, M_3-M_4, M_5-M_6 and the M_9-M_{10} helices.

The purpose of our studies was to develop techniques for side-specific covalent labeling of sarcoplasmic reticulum proteins for the identification of those regions of the Ca^{2+}-ATPase that are exposed on the luminal side of the membrane. In this initial study the usefulness of the photoactivatable azido-derivatives of Cascade blue [27], naphthalenedisulfonate [28–33], Lucifer yellow [34] and fluorescein [35,36] was explored. The lack of specificity that characterizes the reaction of nitrenes with various protein side-chain groups increases the probability that even short loops will react. All these probes are relatively hydrophilic and their reactions are expected to be directed primarily against protein groups exposed on the surface. The sensitivity of detection presently is in the microgram protein range, but can be further enhanced by the use of radioactive reagents and antibodies directed against the covalently attached labels.

A preliminary report was presented [1].

Experimental procedures

Materials

Fluorescein 5′-isothiocyanate (FITC), *N*-(((p-azidobenzoyl)amino)-ethyl)-4-amino-3,6-disulfo-1,8-naphthalimide dipotassium salt (Lucifer yellow AB) (ALY), Cascade blue aminoethyl *p*-azidobenzamide trisodium salt (ACB) and rabbit anti-Cascade blue antibody were supplied by Molecular Probes, Inc., Eugene, OR 97402; 3-azido-2,7-naphthalenedisulfonic acid disodium salt (ANDS) was from Serva, Westbury, NY 11590; fluorescein amine (isomer I), iso-amil nitrite and glycylglycine from Aldrich Chemical Co., Milwaukee WI 53233; dimethylformamide (DMF), sodium azide, sodium vanadate (meta), ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific Co., Rochester, NY 14624; ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Tris, trypsin (bovine pancreas), trypsin inhibitor (soybean), imidazole, disodium-ATP, phospho*enol*pyruvate, NADH, pyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), albumin (bovine serum), sodium deoxycholate, anti-rabbit IgG conjugated with horseradish peroxidase, bisacrylamide, ammonium persulfate, Tris-maleate, thimerosol, 4-chloro-1-naphthol, antifoam A, Tween 20, Mops, Sephadex G-50 were obtained from Sigma Chemical Co., St. Louis, MO 63178; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) from Eastman Organic Chemicals, Rochester, NY, 14650; Ca^{2+}-ionophore A23187 from Calbiochem-Behring Diagnostics, La Jolla, CA 92112; acrylamide, nitrocellulose sheets, Coomassie brilliant blue R-250, β-mercaptoethanol were from Bio-Rad, Inc., Richmond, CA 94804; sodium dodecylsulfate (SDS) from Polysciences, Inc., Washington, PA, 18975; uranium diacetate oxide was supplied by Alfa Products, Thiakol Ventman Division, Danvers, MA 01923, U.S.A. All other chemicals were of analytical grade.

Methods

Preparation of sarcoplasmic reticulum

Skeletal muscle sarcoplasmic reticulum vesicles were prepared from rabbit hind leg and back muscles as described by Nakamura et al. [37] and stored until use at −70 °C in a medium of 0.3 M sucrose, 10 mM Tris-maleate (pH 7.0) at a protein concentration of 30-40 mg/ml. Before starting the experiments the vesicles were washed with 9 volumes of a medium containing 0.1 M KCl, 20 mM Tris-Cl, 5 mM $MgCl_2$, (pH 7.4). This medium (standard medium) was used in all photolabeling experiments. In several experiments the sarcoplasmic reticulum preparations were further purified by deoxycholate extraction [38] or by extraction of microsomes at alkaline pH (pH 8.0) in the presence of 1 mM EDTA [39]. The final pellet obtained after extraction was resuspended in the standard medium; supernatants were dialyzed at 2 °C against 10 mM KCl, 2 mM Tris-HCl, 0.5 mM $MgCl_2$ (pH 7.4) overnight, lyophilized, and resuspended in one-tenth of the original volume with water.

Preparation of azidofluorescein

The azidofluorescein was synthesized as described by Rotman and Heldman [36]. All steps of this preparation were performed under red light in cold room. 200 mg fluorescein amine (isomer I) were dissolved in 10 ml of dimethylformamide (DMF) and diluted with 10 ml distilled H_2O. The solution was acidified by the addition of 2 ml concentrated sulfuric acid, followed by 400 μl of amyl nitrite. After stirring for 30 min at 2 °C, sodium azide (400 mg in 8 ml of water) was added. The mixture was stirred and cooled for a further 30 min and a saturated solution of sodium chloride was added to a final volume of 40 ml. The dark red precipitate was filtered on Buchner funnel, washed with an ice cold solution of 1% w/v NaCl, and dried under vacuum. The yield was 170 mg.

Preparation of 'monovanadate' and 'decavanadate' stock solutions

Stock solution of 50 mM monovanadate was prepared by boiling Na_3VO_3 at pH 10 in water for 15 min; the pH was adjusted to 7.4 by stepwise addition of HCl and boiled for 10 min to minimize the decavanadate content. Stock solutions containing primarily the decavanadate polyanion were prepared by adjusting the pH with HCl below pH 4 and storing the solution overnight or longer at 4 °C. The final pH was adjusted to neutral directly before the experiment was started [40,41].

The centrifuged column procedure

For selective photolabeling from the luminal side of the vesicles the dye was removed from the outside before photoactivation, using the centrifuged column procedure of Penefsky [42]. The microcolumns of Sephadex G-50 were prepared in 1 ml plastic tuberculin syringes fitted with a porous polyethylene disk as described by Penefsky [42]. The columns were precentrifuged before sample application in a swinging bucket rotor of the IEC DPR-600 centrifuge at $100 \times g$ at the tip of the syringe for 2 min at room temperature. Samples of 125 μl were applied to the top of the column in the dark and centrifugation was immediately repeated under exactly the same conditions as in precentrifugation, except that a clean centrifuge tube was used to collect the column effluent. The volume of effluent collected was 105–115 μl and the recovery of SR proteins was 70–80%. Protein concentration was measured according to Lowry et al. [43].

Photochemical surface labeling of sarcoplasmic reticulum vesicles

The native SR vesicles (30 mg protein/ml) were incubated with 20 mM azido-dye in dark (unless otherwise stated), either in the E_1 medium (0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (pH 7.4)) or in the E_2V medium (0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM vanadate (pH 7.4)) at 0 °C for 0–24 h. Before column centrifugation or illumination, the samples were diluted 10-fold in the same medium.

For illumination the samples were transferred into a quartz cuvette with 1 mm lightpath and 430 μl volume (Markson Science Inc., Del Mar, CA 92014, U.S.A.). The light source for photoactivation of azido-dyes was a high-pressure mercury/xenon lamp (USH-5086A) from Ushio, Inc., Japan, except in the crystallization studies, where a high-pressure xenon lamp (XBO 450W/Z) from Osram, Germany, was used. The activating light was filtered by a UV filter (Corning 7-60) through a 10 cm water heat filter. The UV filter has < 1% transmittance below 320 nm and above 380 nm. The irradiation time was routinely 5 min at a distance of 60 cm at room temperature. After photolysis the excess label was removed by washing the vesicles in the standard medium.

Labeling of Ca^{2+}-ATPase with fluorescein 5'-isothiocyanate

Sarcoplasmic reticulum vesicles (2 mg protein/ml) suspended in 0.3 M sucrose, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EGTA, were reacted with 5 nmol FITC/mg SR protein at 25 °C for 30 min in the dark [44]. After labeling the samples were diluted 10-fold with 20 mM K-Mops (pH 7) and centrifuged at 2 °C at $49000 \times g$ for 40 min to remove the unreacted dye; the sedimented vesicles were resuspended in 0.1 M KCl, 10 mM imidazole (pH 7.4) and 5 mM $MgCl_2$.

Partial tryptic proteolysis of sarcoplasmic reticulum

Tryptic digestion of sarcoplasmic reticulum proteins was carried out essentially as described by Dux and Martonosi [45]. Sarcoplasmic reticulum vesicles containing 2–3 mg protein per ml were digested with trypsin (0.05 mg/ml) usually in a medium of 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$ and 0.5 mM EGTA (pH 7.4) at 25 °C for times ranging from 0.5 to 15 min. The digestion was stopped by the addition of soybean trypsin inhibitor (0.2 mg/ml). For zero time samples trypsin and trypsin inhibitors were added together. Departures from these digestion conditions are described in the legends of the relevant figures.

SDS-polyacrylamide gel electrophoresis

Samples taken for electrophoresis were diluted with equal volume of sample buffer containing 10% SDS, 20 mM Tris-HCl (pH 8.0), 2% β-mercaptoethanol, 20% glycerol and 0.2% Bromophenol blue. After incubation for 5 min at 100 °C, aliquots containing 50–300 μg protein were applied for gel electrophoresis. Electrophoresis was performed essentially according to Laemmli [46], using 6–18% polyacrylamide gradient gels. Phosphorylase *b* (94 kDa), bovine serum albumin

(67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) were used as molecular weight markers (Pharmacia, Piscataway, NJ 08854, U.S.A.). Fluorescent protein bands were visualized by irradiating the gel with UV light from an MR4 UV lamp from Gates, G.W. and Co., Franklin Square, Long Island, NY. Permanent records were obtained by photographing the fluorescence on Kodak Plus-X pan 36 mm film, ASA 125 through a Promaster Spectrum 7 yellow filter to remove the reflected UV light. Exposure times were 1 to 5 min at f 4. The photographic prints of the fluorescent gels were analyzed with a Shimadzu dual-wavelength flying-spot scanner CS-9000 (Shimadzu Corporation, Kyoto, Japan). After photography, the gels were stained with Coomassie blue and photographed.

To get better separation of the A and B tryptic fragments of the Ca^{2+}ATPase the SDS-polyacrylamide gel electrophoresis was in some cases carried out in the modified Weber-Osborn system according to Thorley-Lawson and Green [47].

Electrophoretic transfer and immunoblot analysis of ACB labeled sarcoplasmic reticulum proteins and their tryptic fragments using anti-Cascade blue antibody

After SDS-polyacrylamide gel electrophoresis the proteins were transferred to nitrocellulose sheets as described earlier [48]. For localization of ACB labeled proteins, the nitrocellulose sheets were immunostained with 1 : 1000 and 1 : 4000 dilution of rabbit anti-Cascade blue antibody (Molecular Probes, Inc., Eugene, OR) and 1 : 1000 dilution of a peroxidase conjugated anti-rabbit secondary antibody, as described by Molnar et al. (1990) [48].

ATPase activities

ATPase activities were measured essentially as described by Varga et al. [49] using a coupled enzyme assay at 25 °C in a medium of 0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.45 mM $CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 0.42 mM phospho*enol*pyruvate, 0.42 mM NADH, 7.5 IU/ml pyruvate kinase, and 1 μM A23187 at pH 7.4. Final sarcoplasmic reticulum protein concentration in the assay was 5 μg/ml.

Determination of fluorescence intensity

Fluorescence intensity was measured using Varian SF-330 spectrofluorometer. Excitation and emission wavelengths were 415 nm and 530 nm for ALY, 400 nm and 418 nm for ACB, 495 nm and 525 nm for AF, respectively.

Crystallization of Ca^{2+}-ATPase and electron microscopy

The formation of two-dimensional Ca^{2+}-ATPase crystals were induced in native sarcoplasmic reticulum membranes by 5 mM decanavadate in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 0.5 mM EGTA. Protein concentration: 1 mg/ml. The crystal formation at 2 °C can be seen within a few hours and becomes quite extensive after 24 h [50,51]. After 24 h aliquots were taken for electron microscopy; the samples were deposited on a carbon-coated parlodion film,

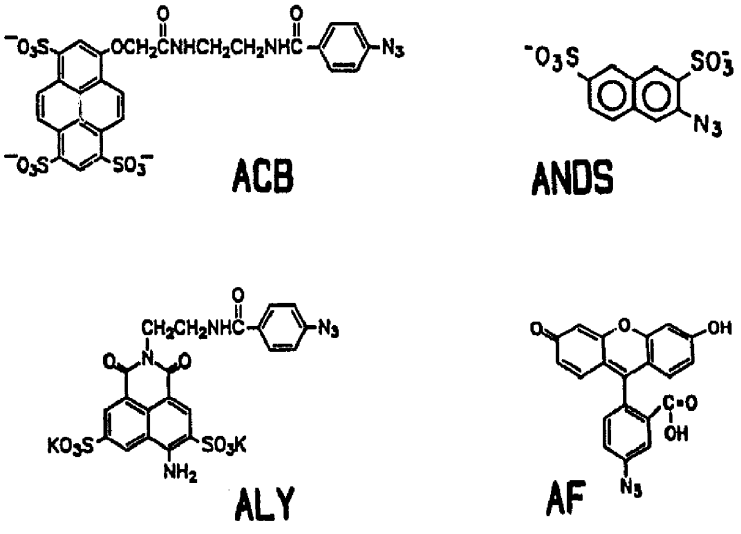


Fig. 1. Chemical formulas of hydrophilic photoactivated fluorescent probes. ACB, Cascade blue aminoethyl-*p*-azidobenzamide trisodium salt; ANDS, 3-azido-2,7-naphthalenedisulfonic acid sodium salt; ALY, *N*(((*p*-azidobenzoyl)amino)ethyl)-4-amino-3,6-disulfo-1,8-naphthalimide dipotassium salt (Lucifer yellow AB); AF, azidofluorescein.

and negatively stained with 1% uranyl acetate, pH 4.3 at 4°C. The samples were viewed in a Siemens Elmiskop I microscope at 60 kV.

Results and Discussion

Brief outline of the experimental approach

The purpose of these studies was to develop conditions for the selective covalent labeling of the cytoplasmic and luminal domains of sarcoplasmic reticulum proteins in the native membrane. Four water soluble fluorescent dyes with photoactivatable azido groups were chosen for these studies; the dyes slowly penetrate into the interior of the vesicles and after illumination establish covalent bonds with a variety of side-chain groups on the proteins. These four dyes are: Cascade blue aminoethyl-*p*-azidobenzamide (ACB), azido Lucifer yellow or *N*(((*p*-azidobenzoyl)amino)-ethyl)-4-aminodisulfo-1,8-naphthylamide dipotassium salt (ALY), 3-azido-2,7-naphthalenedisulfonic acid sodium salt (ANDS) and azidofluorescein (AF). Their formulas are given in Fig. 1.

In view of the slow penetration of the dyes across the membrane, it is expected that after brief incubation with sarcoplasmic reticulum vesicles the labeling will be confined to the outward facing cytoplasmic domains of the membrane proteins.

After full equilibration with the vesicle interior the dye can be removed from the outside medium by rapid centrifugation through a gel column [42] and protein side chains exposed on the luminal surface can be selectively labeled by the dye trapped in the vesicle lumen. Since the interior volume of sarcoplasmic reticulum vesicles is only approx. 5 μl/mg protein [39], significant labeling from the vesicle interior requires high (10–30 mM) dye concentration. The labeled proteins and their partial proteolysis products are separated by SDS-polyacrylamide gel electrophoresis and the extent of labeling determined by measuring the fluorescence of the covalently bound dye. The distribution of the bound label among the proteolytic frag-

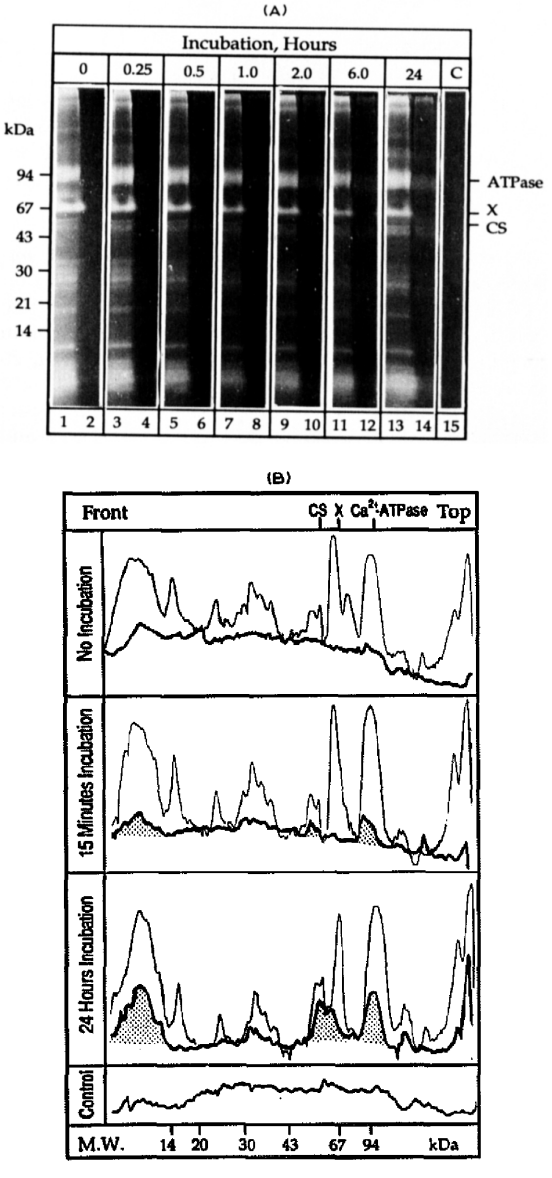


Fig. 2. The penetration of ACB into the sarcoplasmic reticulum vesicles. (A) Sarcoplasmic reticulum vesicles (20 mg protein/ml) were suspended in a medium of 0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (pH 7.4), and 10 mM ACB and incubated at 0°C for times ranging from 0 to 24 h. Aliquots taken at the indicated times were diluted 10-fold in the same medium but without ACB and used for photolabeling. One set of samples were illuminated directly for 5 min to label the sarcoplasmic reticulum proteins from both sides of the membranes (odd numbered samples). The other set of samples was subjected first to column centrifugation that removed the dye from the outside, but retained the dye on the inside of the sarcoplasmic reticulum; illumination of these samples for 5 min is expected to produce labelling only from the luminal side of the membrane (even numbered samples). (B) The densitograms made from the photographic print of the fluorescent gel. The thin lines represent the samples photolabeled on both sides without column centrifugation after 0, 15 min and 24 h incubation. The shadowed regions represent densitogram of the column centrifuged samples after 0, 15 min, 24 h incubation. The sample labeled C in Fig. 2A and the same control sample in Fig. 2B was incubated for 24 h, and column centrifuged as above; after incubation for a further 24 h to permit the release of trapped dye from the vesicles, there was a second column centrifugation followed by illumination for 5 min. The absence of labeling indicates that essentially all ACB was released from the vesicles in 24 h.

ments of the Ca^{2+}-ATPase should provide a useful test of the predicted transmembrane topology of the enzyme [2,5,7,8].

The side-specific labeling of other sarcoplasmic reticulum proteins should provide information about the sidedness of their distribution in the sarcoplasmic reticulum.

The rate of penetration of azide-Cascade blue (ACB) into sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were incubated with 10 mM ACB at 0 °C in the dark for times ranging between 0 and 24 h (Figs. 2A and 2B). The penetration of ACB into the interior space of the vesicles was determined on aliquots taken at various times by comparing the extent of photolabeling after illumination of the total reaction mixture, that results in labeling from both sides of the membrane (odd numbered samples), with the intensity of photolabeling after column centrifugation, that permits labeling only from the luminal side of the membrane (even numbered samples).

The samples collected after short incubation with ACB show intense labeling in several proteins from the outside (Fig. 2A, lane 1), but essentially no reaction from the luminal side of the membrane (Fig. 2A, lane 2). The most intensely labeled proteins are the Ca^{2+}-ATPase (≈ 110 kDa) and an unidentified X protein (≈ 67–80 kDa) that is not identical with serum albumin (see below). The mobility of the X protein varied in different experiments due perhaps to an effect of Ca^{2+} on the net charge and structure of the X protein.

During continued incubation the extent of labeling from the cytoplasmic side changed only moderately (Figs. 2A and 2B), but beginning at 0.25 h there was a progressive increase in the reaction of ACB with the Ca^{2+}-ATPase, the calsequestrin [52,53] and with a group of low molecular weight components (≈ 10 kDa) from the luminal side of the membrane (Fig. 2A, lanes 4, 6, 8, 10, 12 and 14). After 24 h of incubation the ACB incorporation from the luminal side of the membrane accounted for approx. 1/5 of the total labeling in the Ca^{2+}-ATPase, nearly 3/4 of the labeling in calsequestrin and more than 1/2 of the labeling in the low molecular weight components (Fig. 2B). Essentially all ACB that penetrated into sarcoplasmic reticulum vesicles during 24 h of incubation in the dark was released during the subsequent 24 h of incubation in a dye-free medium (Fig. 2A, sample C and Fig. 2B, control sample).

There are significant differences in the specificity of ACB, ALY and AF for the different protein components of the sarcoplasmic reticulum. All three dyes reacted strongly with the cytoplasmic domain of the Ca^{2+}-ATPase (Fig. 3A) and with a 53–55 kDa doublet. However, the X protein was labeled only by ACB. The

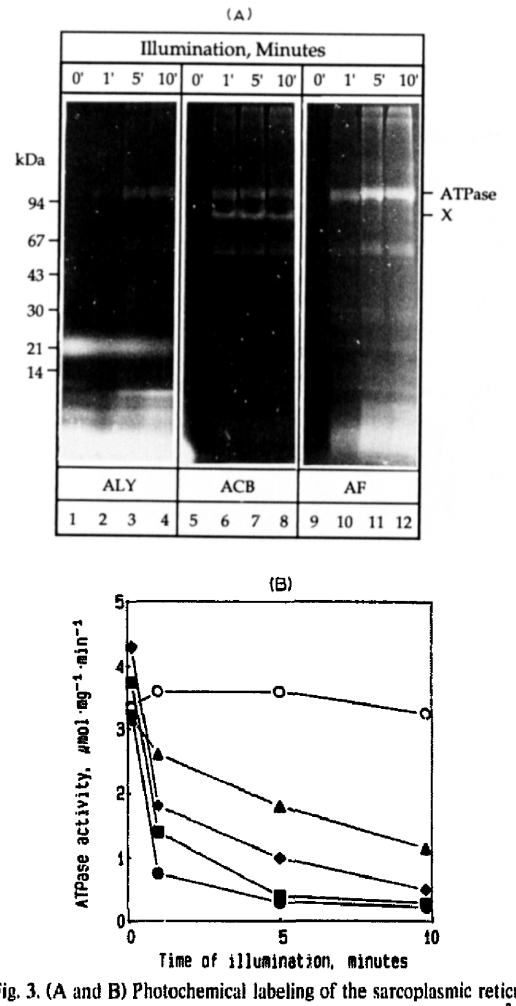


Fig. 3. (A and B) Photochemical labeling of the sarcoplasmic reticulum vesicles by ALY, ACB and AF and their effect on the Ca^{2+}-stimulated ATPase activity. Sarcoplasmic reticulum vesicles (2 mg protein/ml) were suspended at 2 °C in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, containing 1 mM ALY (1–4, ▲), 1 mM ACB (5–8, ■), 1 mM AF (9–12, ●) or 1 mM ANDS (13–15) and illuminated by ultraviolet light for times ranging from 0 to 10 min. Aliquots of 100 μg protein were taken at the indicated times for SDS-polyacrylamide gel electrophoresis (A) and aliquots of 5 μg for ATPase assay (B). Control samples (○) were illuminated in the same medium, but without the dyes.

ACB-X emitted strong greenish fluorescence, while the fluorescence emission of the Ca^{2+}-ATPase and other ACB labeled proteins was blue. The labeling of the 21 kDa component by ALY is anomalous, as its intensity appears to decline with longer illumination.

The photolabeling of the cytoplasmic surface of sarcoplasmic reticulum by ALY, ACB and AF is accompanied by inhibition of ATPase activity (Fig. 3B); the inhibition is in rough proportion with the extent of labeling by the three dyes (Fig. 3A).

ANDS penetrates in the sarcoplasmic reticulum at rates similar to that of ACB and can also be used for covalent photolabeling of the Ca^{2+}-ATPase both from the cytoplasmic and from the luminal surface of the membrane, but the sites of labeling within the Ca^{2+}-ATPase have not been analyzed in detail.

Analysis of the sidedness of labeling by ACB using deoxycholate (DOC) extraction

Sarcoplasmic reticulum vesicles were labeled with ACB without preincubation (outside), after 20 h of incubation (both sides), and after 20 h of incubation followed by column centrifugation (inside) (Fig. 4).

In agreement with the data in Fig. 2, ACB labels primarily the Ca^{2+}-ATPase and the X protein from the outside, the Ca^{2+}-ATPase and the calsequestrin from the inside, and all three proteins from both sides, in addition to several minor components (Fig. 4 , top panels). Extraction of the labeled vesicles with low concentration of deoxycholate removes much of the fluorescence associated with calsequestrin, as shown by the appearance of the fluorescent calsequestrin band in the DOC extract of the vesicles labeled either from inside or from both sides (Fig. 4, bottom panels); there is a corresponding decrease in the fluorescence at the 60 kDa region in the deoxycholate extracted membranes (Fig. 4, middle panels). These observations are consistent with the known localization of calsequestrin in the luminal space of the sarcoplasmic reticulum cisternae [52]. The ACB label associated with the

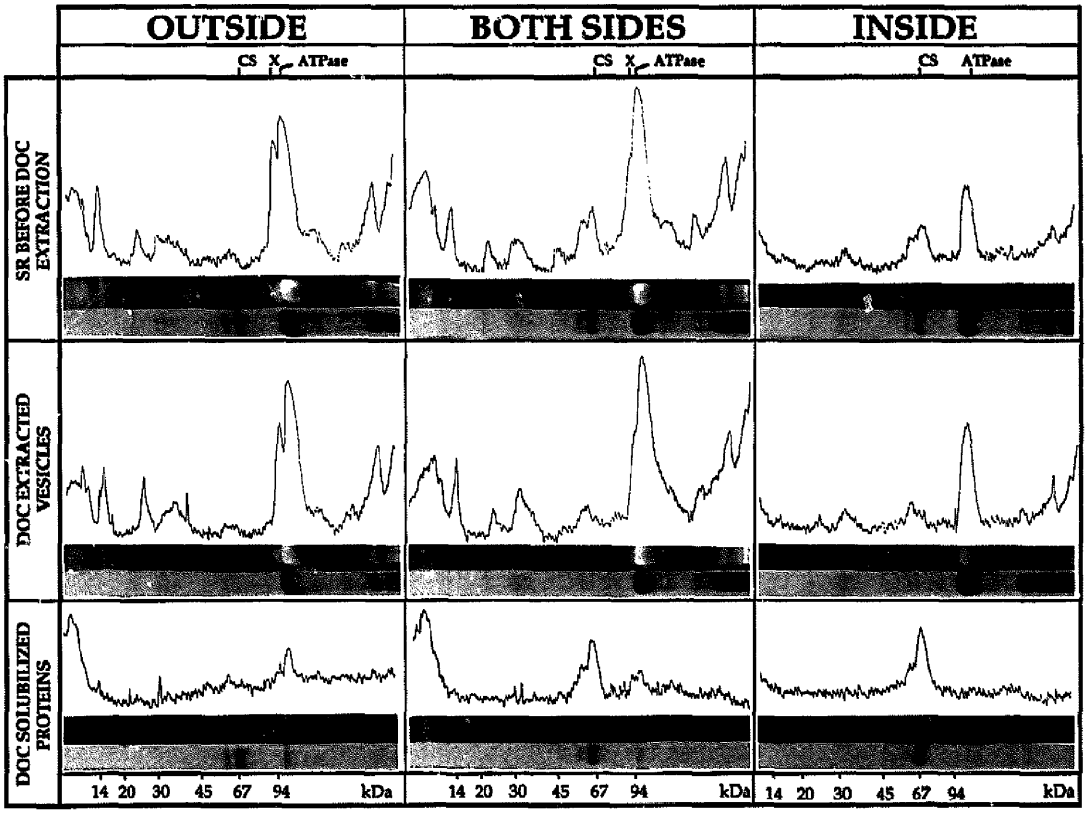


Fig. 4. Labeling of sarcoplasmic reticulum vesicles by ACB from the outside, from both sides, and only from the inside. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were suspended at 2 °C in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 20 mM ACB. Aliquots were diluted ten-fold at the beginning (outside) and at the end (both sides) of a 20-h incubation at 0 °C in the dark and illuminated for 5 min, as described under Methods. For analysis of the selective labeling from the inside the samples taken after 20 h incubation were column centrifuged and immediately illuminated for 5 min, as described above (top row). Aliquots of the labeled vesicles were extracted with deoxycholate as described by Meissner et al. [38] to remove extrinsic proteins such as calsequestrin; the DOC extracted vesicles (middle row) and the dialyzed and lyophilized extracts (bottom row) were applied for electrophoresis as described under Methods. The figure shows the densitograms made from the photographic prints of ACB labeled fluorescent bands. The inserts show the corresponding gel slices photographed in UV light (above) or stained with Coomassie blue (bottom).

Ca^{2+}-ATPase and with the X protein was quantitatively retained in the DOC extracted membranes (Fig. 4, middle panels).

The effect of Ca^{2+} and vanadate on the labeling of sarcoplasmic reticulum vesicles by ACB

The extent of labeling of sarcoplasmic reticulum by ACB was measured in media containing 0.5 mM Ca^{2+} where the Ca^{2+}-ATPase is in the Ca_2E_1 state, and in the presence of 0.5 mM EGTA and 1 mM monovanadate that stabilize the Ca^{2+}-ATPase in the E_2V state (Table I). The proportion of the label attached from the inside of the vesicles is significantly greater in the E_2V as compared with the Ca_2E_1 state, resulting in lower OS/IS ratios after short or long incubation with ACB (Table I). These observations are consistent with greater exposure of the Ca^{2+}-ATPase on the luminal surface of the membrane in the E_2V state. Deeper immersion of the Ca^{2+}-ATPase into the membrane in the presence of vanadate was suggested earlier based on freeze-etch electron microscopy of vanadate-induced Ca^{2+}-ATPase crystals [54].

After 32 h of preincubation the total amount of ACB covalently bound to the sarcoplasmic reticulum was estimated to be approx. 17.4 nmol/mg in the E_1 and 16.8 nmol/mg in the E_2 state (Table I).

These estimates are based on a comparison with the fluorescence intensity of standard ACB solutions measured in the absence of sarcoplasmic reticulum. As the reaction of ACB with the sarcoplasmic reticulum measured at 0.1–1.0 μM dye concentration did not cause significant change in the fluorescence intensity, the quantum yield of the fluorescence of the free and covalently bound dye is expected to be similar. Photolysis produced about 2-fold increase in the fluorescence intensity of ACB either in the absence or in the presence of sarcoplasmic reticulum.

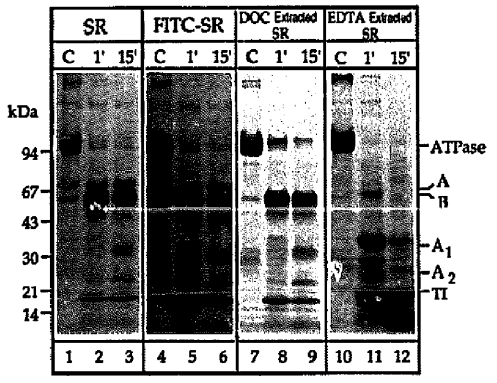


Fig. 5. Comparison of tryptic cleavage patterns of native, FITC labeled, DOC-extracted and EDTA-extracted SR vesicles. Sarcoplasmic reticulum vesicles (2 mg protein/ml) were digested by trypsin (0.05 mg/ml) as described under Methods. Aliquots were taken before the addition of trypsin (lanes 1, 4, 7, 10) and after 1 min (lanes 2, 5, 8, 11) and 15 min (lanes 3, 6, 9, 12) of reaction. Aliquots of 150 μg were applied for SDS-polyacrylamide gel electrophoresis as described under Methods. Lanes 1–3, native sarcoplasmic reticulum. Lanes 4–6, sarcoplasmic reticulum labeled with FITC according to Papp et al. [44]. Lanes 7–9, sarcoplasmic reticulum extracted with deoxycholate according to Meissner et al. [38]. Lanes 10–12, sarcoplasmic reticulum extracted with 1 mM EDTA at pH 8.0 [39].

Distribution of covalently bound ACB among the tryptic cleavage fragments of the Ca^{2+}-ATPase

During tryptic hydrolysis of sarcoplasmic reticulum the Ca^{2+}-ATPase is first cleaved at the T_1 cleavage site (Arg-505–Ala-506) into two major fragments [2,55], with apparent molecular weights of 57 kDa (A frag-

TABLE I

Fluorescence intensities of side specifically photolabeled sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were labeled from the outside (OS), from the inside (IS) or from both sides with ACB as described under Methods and legend to Fig. 4. The incubation medium was 0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.4), and either 0.5 mM $CaCl_2$ to stabilize the E_1 conformation or 0.5 mM EGTA and 1 mM monovanadate to stabilize the E_2 conformation. The samples were diluted to 20 μg protein/ml and the fluorescence was determined using a Varian SR-330 spectrofluorometer. Excitation and emission wavelengths were 400 nm and 418 nm, respectively. The fluorescence of OS and IS labeled samples are expressed as % of total fluorescence intensity measured in samples labeled from both sides. The ratio of the fluorescence intensity of samples labeled only on the outside vs. samples labeled only on the inside is given as OS/IS. The numbers in parentheses give the amount of covalently attached ACB in nmoles per mg protein, calculated by comparison of the fluorescence intensity with standard ACB solutions under the same conditions as the measurements with sarcoplasmic reticulum vesicles. The calculations are based on the assumption that the quantum yield of fluorescence is not affected by the reaction of ACB with the sarcoplasmic reticulum

Incubation (h)	E_1				E_2			
	Total fluorescence intensity	OS (%)	IS (%)	OS/IS	Total fluorescence intensity	OS (%)	IS (%)	OS/IS
0	134.4	98.6	0.14	704.2	138.0	94.7	5.3	17.8
32	217.6 (17.4)	69.4 (11.9)	30.58 (5.27)	2.3	212.8 (16.8)	61.5 (10.4)	38.5 (6.5)	1.6

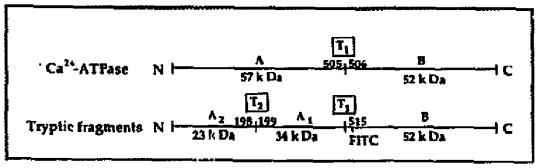


Fig. 6. The location of the tryptic cleavage sites (T_1 and T_2) in the Ca^{2+}-ATPase, and the identification of the tryptic cleavage fragments (A, B, A_1, A_2). For details, see text.

ment) and 52 kDa (B fragment) (Fig. 5, lane 2). Further digestion of the A fragment at the T_2 site (Arg-198–Ala-199) produces the 34 kDa A_1 and the 23 kDa A_2 subfragments (Fig. 5, lane 3). The B fragment is digested more slowly giving rise to a family of bands ranging in size between 30 and 10 kDa (not shown). Prior labeling of the Ca^{2+}-ATPase with fluorescein 5'-isothiocyanate at lysine-515 [2,3] permits clear identification of the FITC-labeled B fragment on the basis

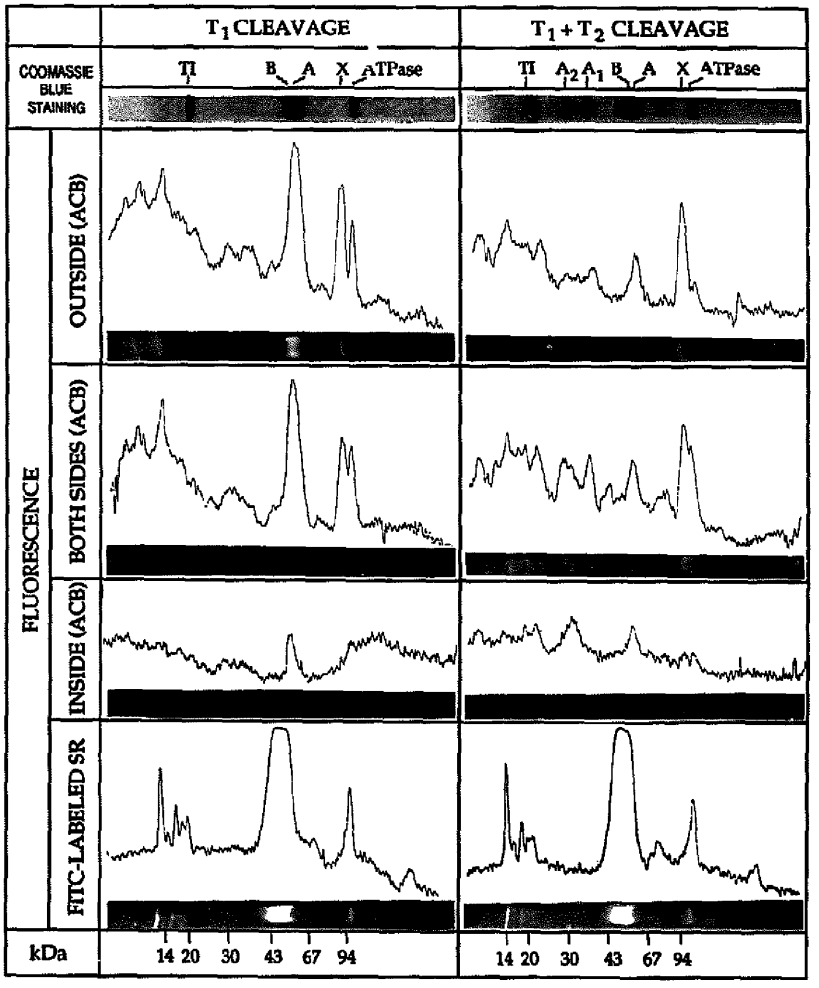


Fig. 7. The distribution of the covalently attached ACB in the tryptic digested Ca^{2+}-ATPase after labeling exclusively from the outside, from the inside or from both sides. Native sarcoplasmic reticulum vesicles (30 mg protein/ml) were incubated with 20 mM ACB for 20 h at 0 °C in the dark in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ medium in the presence of either 0.5 mM $CaCl_2$ ($T_1 + T_2$ cleavage, second column), or 0.5 mM EGTA and 1 mM monovanadate (T_1, cleavage, first column). In the presence of 1 mM vanadate the T_2 cleavage site is blocked [45]. The side-specific labeling was performed as described in Fig. 4. After photolabeling the Ca^{2+}-ATPase was purified by DOC extraction [38] and digested with trypsin for 1 min, as described under Methods. The positions of the Ca^{2+}-ATPase, and its principal tryptic fragments, the band of the trypsin inhibitor and the intensely labeled band of the X fragment are marked on the Coomassie blue stained gel slices (top of figure). The middle part of the figure shows the densitometric scans of the tryptic fragments of Ca^{2+}-ATPase after labeling from the outside, both sides or from the inside. To determine the location of the B fragment, Ca^{2+}-ATPase labeled with FITC in lysine-515 was digested under identical conditions (bottom of figure). The fluorescent gel patterns are shown under the corresponding densitograms.

of its fluorescence, but otherwise has no influence on the pattern of tryptic cleavage (Fig. 5, lanes 4–6). These observations are summarized in Fig. 6, based on the work of several laboratories [55].

The removal of calsequestrin and other extrinsic proteins either by deoxycholate extraction (Fig. 5, lanes 7–9, Ref. 38) or by extraction with EDTA (Fig. 5, lanes 10–12, Ref. 39) simplifies the protein composition and causes an increase in the steady state rate of ATP hydrolysis in the absence of Ca^{2+} ionophores due to increase in the Ca^{2+} permeability of the membrane. The pattern of tryptic cleavage remains essentially unchanged after DOC extraction (Fig. 5, lanes 7–9), consistent with retention of the native structure of the Ca^{2+}-ATPase. After extraction with EDTA the rate of tryptic hydrolysis is greatly accelerated (Fig. 5, lanes 10–12); there is no significant accumulation of the B fragment, and instead of the A_1 and A_2 subfragments, two new fragments with apparent molecular weights of ≈ 40 kDa and ≈ 20 kDa are seen. This may imply that the site of the T_2 cleavage shifted after EDTA extraction to a peptide bond nearer to the N-terminus of the Ca^{2+}-ATPase, resulting in the formation of an A'_1 fragment that is larger than the A_1 and an A'_2 that is smaller than the A_2 fragment normally observed during proteolysis of native sarcoplasmic reticulum vesicles. The effect of EDTA extraction on the digestion of Ca^{2+}-ATPase by trypsin was also tested on FITC-labeled sarcoplasmic reticulum preparations, with essentially identical results to those obtained on unlabeled preparations. Under these conditions the cleavage of the B fragment can be followed on electrophoresis by the fluorescence of the covalently attached FITC. Already after 1 min digestion the B fragment was completely decomposed into fluorescent subfragments of about 30–10 kDa, due to removal of mass from the C-terminal end of the B fragment.

The underlying change in the structure of the Ca^{2+}-ATPase may account for the irreversible increase in the Ca^{2+} permeability of the membrane and for the inhibition of Ca^{2+} accumulation after EDTA extraction that cannot be fully reversed by readdition of Ca^{2+}, Mg^{2+}, and the extracted extrinsic proteins of sarcoplasmic reticulum [39,56].

The distribution of ACB fluorescence among the tryptic fragments of the Ca^{2+}-ATPase was analyzed after labeling from the outside, from both sides or only from the inside, either in a Ca^{2+}-free vanadate containing medium (T_1 cleavage) or in the presence of 0.5 mM Ca^{2+} (T_1 and T_2 cleavage) or in the presence of 0.5 mM Ca^{2+} (T_1 and T_2 cleavage) (Fig. 7). To simplify the protein composition and to enrich the Ca^{2+}-ATPase content the extrinsic proteins of sarcoplasmic reticulum were removed by extraction with low concentration of deoxycholate after photolabeling but before the tryptic digestion.

In vanadate containing systems where the tryptic cleavage was restricted to the T_1 cleavage site [45], most of the ACB incorporated from the outside was distributed in the A and B fragments with some residual fluorescence in the undigested Ca^{2+}-ATPase (Fig. 7, top left panel). After labeling from the inside most of the covalently bound ACB was localized within the A band that migrated slower than the B band identified by FITC labeling (Fig. 7, bottom left panel).

In Ca^{2+}-containing samples tryptic cleavage occurred at the T_1 and T_2 sites (Fig. 7, right panels) and under these conditions the covalently bound ACB was distributed among the A_1 and A_2 subfragments with some residual labeling in the A fragment.

These observations suggest that the exposed regions of the Ca^{2+}-ATPase both on the cytoplasmic and on the luminal surface of the sarcoplasmic reticulum are associated primarily with the N-terminal half of the Ca^{2+}-ATPase that forms the A fragment after tryptic cleavage. The regions corresponding to the B fragment contribute little to the labeling from the luminal side of the membrane.

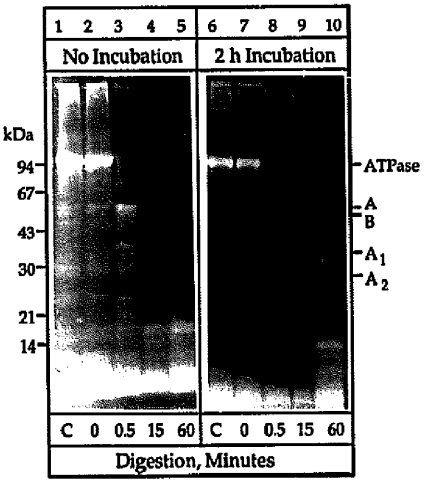


Fig. 8. The distribution of the covalently attached AF in the tryptic digested Ca^{2+}-ATPase. Sarcoplasmic reticulum (2 mg protein/ml) was suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM AF in the dark. Aliquots were taken before and after 2 h of incubation at 25 °C. The samples were illuminated for 5 min and the unreacted dye was removed by washing in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$. After 40 min centrifugation at 100000 × g at 2 °C the pellets were suspended in 0.1 M KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EGTA (pH 7.4), and the protein concentrations were adjusted to 2 mg/ml. Aliquots were taken before digestion with 0.05 mg/ml trypsin at 25 °C (lanes 1 and 6), and after 0 (2, 7), 0.5 (3, 8), 15 (4, 9) and 60 (5, 10) min of digestion. The proteolysis was terminated by the addition of 0.2 mg/ml trypsin inhibitor. Aliquots of 100 μg protein were applied for SDS gel electrophoresis, as described under Methods. The AF-labeled fragments were visualized by UV light and photographed before staining the gels for protein.

The X protein was labeled in both media only from the outside and it was not digested significantly by trypsin under conditions where the Ca^{2+}-ATPase was extensively fragmented.

The distribution of covalently attached azidofluorescein (AF) among the tryptic fragments of the Ca^{2+}-ATPase

In contrast to fluorescein 5'-isothiocyanate that labeled exclusively the B tryptic fragment of the Ca^{2+}-ATPase (Fig. 5 and Ref. 57), photolabeling with azidofluorescein gave rise to significant fluorescence in the A, A_1 and A_2 tryptic fragments of the Ca^{2+}-ATPase (Fig. 8, lanes 3 and 8). During further digestion for 15-60 minutes the movement of the label could be followed into lower molecular weight fragments (Fig. 8, lanes 4, 5, 9, 10). Most or all of the labeling occurred from the outside, since there was no significant difference between samples photolabeled before (Fig. 8, lanes 1–5) or after 2 h of incubation (Fig. 8, lanes 6–10) at 25 °C in the dark with azidofluorescein.

The effect of labeling with ACB on the crystallization of the Ca^{2+}-ATPase

Sarcoplasmic reticulum vesicles were incubated with 20 mM ACB for 0.1 or 48 h at 0 °C either in a medium containing 0.5 mM Ca^{2+} (Ca_2E_1 conformation, Table II, samples 1–8) or in a calcium-free medium containing 1 mM monovanadate (E_2V conformation, Table II, samples 9–16). From the control samples the dye was removed without exposure to light (NS). The experimental samples were illuminated either before or after column centrifugation for labeling from the outside (OS), from both sides (BS) or only from the inside (IS), respectively (Table II and Fig. 9).

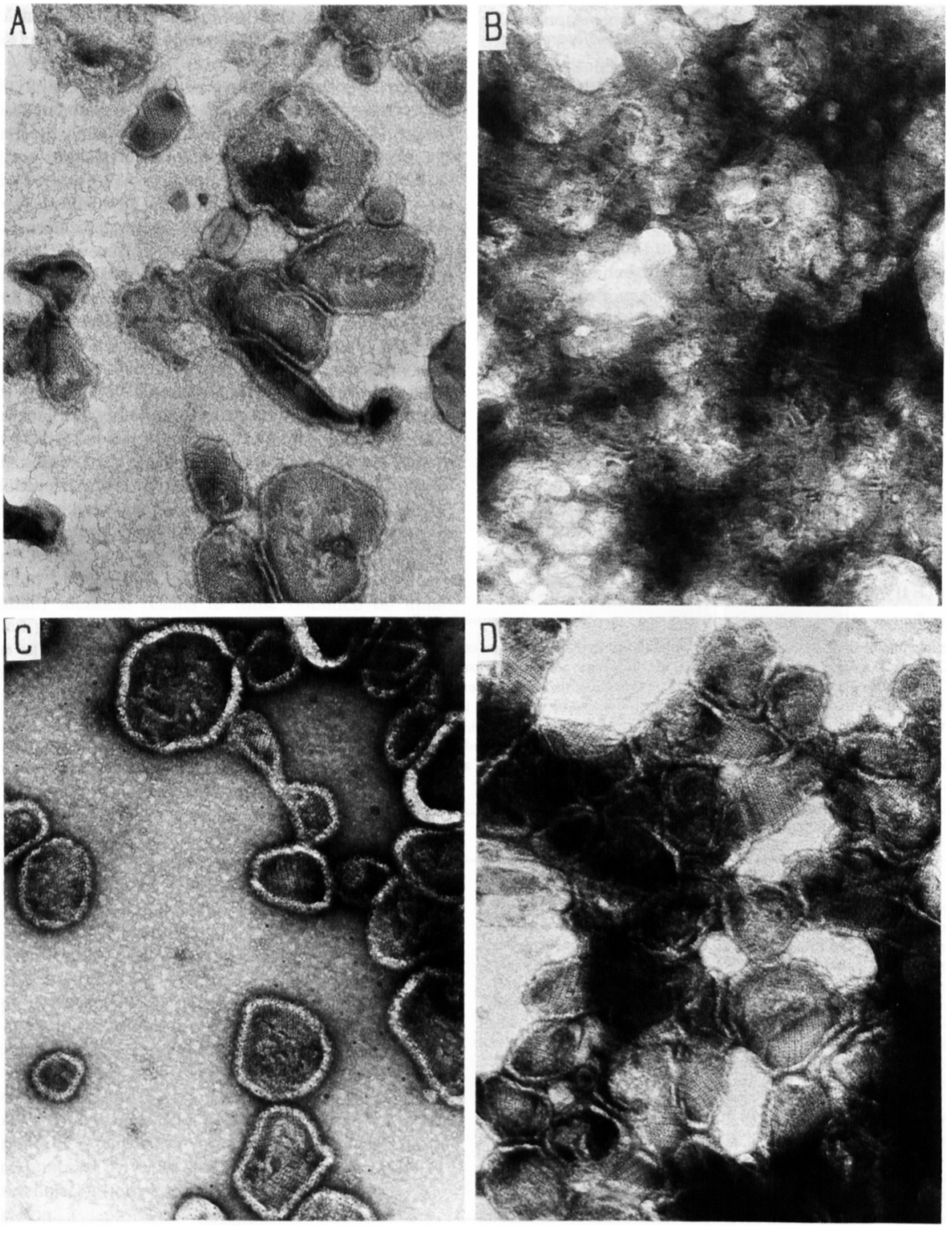
After exposure to the crystallization medium the control samples showed the normal development of Ca^{2+}-ATPase crystals, indicated by the high percentage of the vesicles that contained crystalline arrays (Table II, odd numbered samples; Figs. 9A and 9C). In the samples labeled with ACB from both sides (Table II, samples 6 and 14; Fig. 9B) the crystallization was inhibited and massive aggregation of the vesicles was observed. Since the total amount of ACB incorporated under these conditions is only approx. 17 nmol/mg protein (Table I) the inhibition of crystallization results from the binding of as little as 1–2 moles of ACB per mole of Ca^{2+}-ATPase. In the samples labeled only from the outside (Table II, samples 2 and 10) the crystallization was unimpaired. Curiously the labeling from the inside produced different effects depending on the conformation of the Ca^{2+}-ATPase during the labeling. In the Ca^{2+}-containing medium (Table II, sample 8) the labeling by ACB caused severe inhibition of crystallization, while in the vanadate-containing

TABLE II

Crystallization of Ca^{2+}-ATPase after labeling the sarcoplasmic reticulum with ACB

The labeling of the membrane was performed either in the E_1 medium containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 20 mM ACB or in the E_2 medium of the same composition except that it contained 0.5 mM EGTA and 1 mM monovanadate instead of $CaCl_2$. After incubation for 0.1 or 48 h at 0 °C some of the samples were subjected to column centrifugation and illumination (5 min at 25 °C) as indicated in the table. The unbound ACB was removed by washing as in Fig. 8, and the crystallization was induced by suspending the vesicles (1 mg protein/ml) in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, and 5 mM decavanadate by incubation for 24 h at 2 °C. The crystallization index was determined as described earlier [51]. The crystallization index denotes the percentage of the vesicular profiles that contain crystalline arrays in several fields totaling approx. 500 vesicles. The expected patterns of labeling are denoted as: NS, neither side; BS, both sides; OS, outside; IS, inside.

#	Medium	Incubation (h)	Column centrifugation	Illumination	Labeling pattern	Crystallization index
1	E_1	0.1	–	–	NS	89.9
2	E_1	0.1	–	+	OS	87.9
3	E_1	0.1	+	–	NS	90.8
4	E_1	0.1	+	+	NS	90.5
5	E_1	48.0	–	–	NS	81.5
6	E_1	48.0	–	+	BS	2.0
7	E_1	48.0	+	–	NS	83.8
8	E_1	48.0	+	+	IS	3.1
9	E_2	0.1	–	–	NS	90.8
10	E_2	0.1	–	+	OS	82.6
11	E_2	0.1	+	–	NS	90.3
12	E_2	0.1	+	+	NS	90.5
13	E_2	48.0	–	–	NS	89.7
14	E_2	48.0	–	+	BS	7.6
15	E_2	48.0	+	–	NS	91.5
16	E_2	48.0	+	+	IS	90.2


A
B
C
D

medium (Table II, sample 16) large number of somewhat disorganized crystals could still be observed (Fig. 9D). This difference cannot be attributed to Ca^{2+}-dependent proteolysis in sample 8 (Table II), since the corresponding control samples incubated under identical conditions (Table II, samples 5 and 7) showed good crystallization.

The reaction of ACB with the X protein

The ≈ 80 kDa X protein is intensely labeled by ACB but not by ALY or AF (Fig. 3). Its mobility was compared with that of bovine serum albumin in undigested and in trypsin-digested sarcoplasmic reticulum (Fig. 10). Although serum albumin, like the X protein, is labeled by ACB, and both proteins are relatively insensitive to trypsin digestion the mobility of X protein is significantly slower than that of serum albumin (Fig. 10). The possible identity of X protein with a component of the terminal cisternae/triad fraction is being explored.

Conclusions

A technique was developed using azido derivatives of fluorescent dyes for selective covalent photolabeling of the Ca^{2+}-ATPase and other sarcoplasmic reticulum proteins, either from the cytoplasmic or from the luminal side of the membrane. In the Ca^{2+}-ATPase labeled with azido-Cascade blue from the cytoplasmic surface the fluorescence was distributed between the A and B tryptic fragments, while in the enzyme labeled from the luminal side nearly all fluorescence was associated with the A fragment. This is unexpected, since in the predicted structure of the Ca^{2+}-ATPase [2,3] three of the five luminal loops are in the region of the B fragment. Among possible explanations of this behavior are:

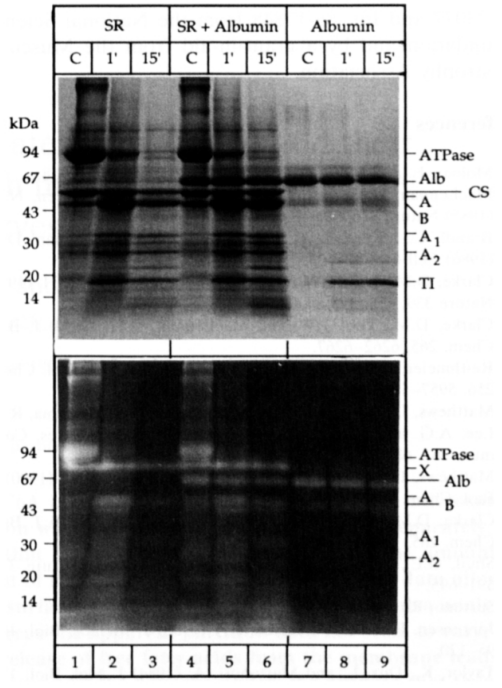


Fig. 10. Photolabelling of SR vesicles by ACB in the presence of bovine serum albumin. Sarcoplasmic reticulum vesicles (3 mg protein/ml) (lanes 1–6), and bovine serum albumin (0.6 mg/ml) (lanes 4–9) were suspended in 0.1 M KCl, 20 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM ACB and illuminated for 5 min. After illumination the samples were dialyzed at 2 °C overnight against 1200 volumes of buffer, followed by digestion with trypsin (0.05 mg/ml) in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$. Aliquots removed before digestion (1, 4, 7) and after 1 min (2, 5, 8), or 15 min (3, 6, 9) of digestion were processed for SDS polyacrylamide gel electrophoresis. The electropherograms were photographed in UV light (bottom half of figure) and stained with Coomassie blue (top half of figure).

Fig. 9. The effect of side specific photolabelling with ACB on the vanadate induced two-dimensional crystallization of Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were incubated at 0 °C for 48 h in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM monovanadate and 20 mM ACB. Aliquots were diluted 10-fold and the diluted samples were either processed without column treatment (A, B) or subjected to column centrifugation (C, D). The control samples (A, C) were kept in the dark, while portions of the other samples (B, D) were illuminated for 5 min at room temperature with a high pressure xenon lamp (XBO 450W/Z), Osram, Germany) as described under Methods. The unbound ACB was removed by washing the vesicles in 23 volumes of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA and 1 mM monovanadate. The suspensions were centrifuged at 100000× *g* for 40 min at 4 °C. The two-dimensional crystallization of Ca^{2+}-ATPase was induced by suspending the vesicles (1 mg protein/ml) in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM decavanadate and incubating for 24 h at 2 °C. Aliquots were taken for negative staining with 1% uranyl acetate and used for electron microscopy as described under Methods. Magnification: 90 720×.

(1) differences in the reactivity of the luminal loops with nitrenes;

(2) partitioning of the dye into the lipid phase or into high-affinity binding pockets within the protein, that would facilitate reactions at sites other than those on the luminal surface;

(3) insufficient exposure of some of the luminal loops prevents reaction with the photoactivated dye;

(4) the predictions about the transmembrane folding of the Ca^{2+}-ATPase may be incorrect.

An experimental examination of the various alternatives using radioactively labeled azido dyes is in progress.

Acknowledgements

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