Localization and Partial Characterization of the Oligomeric Disulfide-Linked Molecular Weight 95 000 Protein (Triadin) Which Binds the Ryanodine and Dihydropyridine Receptors in Skeletal Muscle Triadic Vesicles†

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ABSTRACT: A monoclonal antibody, GE 4.90, has been produced following immunization of mice with the 95-kDa protein (triadin) of terminal cisternae of rabbit fast skeletal muscle isolated in nondenaturing detergent. The antibody binds to a protein of M_r 95K in Western blots of microsomal vesicles electrophoresed in the presence of mercaptoethanol. The greatest intensity of the immunoblot reaction is to enriched terminal cisternae vesicles while little binding is seen to longitudinal reticulum and transverse tubules. The content of antigen in different microsomal subfractions has been estimated by immunoassay: terminal cisternae/triads contain 5.6 μg/mg of protein while heavy terminal cisternae contain 32 μg/mg. The molar content of triadin in vesicles is approximately the same as that of the ryanodine receptor. When Western blots of gels of terminal cisternae are run in nonreducing conditions, little protein of M_r 95K is visible. A number of bands, however, forming a ladder of higher molecular weight are discerned, indicating that the 95-kDa protein forms a disulfide-linked homopolymer. A biotinylated aromatic disulfide reagent (biotin–HPDP) labels the 95-kDa protein, the junctional foot protein, and the M_r 106K protein described by others as a Ca^{2+}-release channel (SG 106). This latter protein migrates in gel electrophoresis under nonreducing conditions at a molecular weight different from that of the 95-kDa protein. We did not detect any alteration of binding of the 95-kDa protein to the dihydropyridine receptor or junctional foot protein dependent on the state of oxidation of cysteine residues of either triadin or receptor protein used as the overlay probe.

Excitation–contraction coupling in skeletal muscle requires the transmission of the excitation signal from the transverse (T) tubule to the terminal cisternae (TC) of the sarcoplasmic reticulum (SR). The voltage sensor in the T-tubule which detects the depolarization and conveys the message in the first stage toward the SR has been identified as the dihydropyridine (DHP) receptor (Rios & Brum, 1987). The Ca^{2+}-release channel in the SR which responds to the message from the T-tubule has been identified as the high molecular weight junctional foot protein (JFP) (Kawamoto et al., 1986; Inui et al., 1987; Lai et al., 1987; Campbell et al., 1987). The mechanism through which the message is transmitted from the sensor to the release channel is still obscure. Two alternative hypotheses have found favor in recent research. One proposes that a water-soluble transmitter, inositol 1,4,5-trisphosphate, is released from the T-tubule and binds to the Ca^{2+}-release channel, causing activation of Ca^{2+} release. The merits and limitations of this hypothesis have been the subject of recent reviews (Caswell & Brandt, 1989; Hidalgo & Jaimovich, 1989).

A second hypothesis invokes direct communication between the DHP receptor and the JFP. The DHP receptor undergoes an alteration of shape following depolarization which is conformationally transmitted to the Ca^{2+}-release channel. Models suggesting direct coupling have appeared widely in recent publications (Caswell & Brandt, 1989; Takeshima et al., 1989; Hymel et al., 1988; Gill, 1989). This latter hypothesis, being molecular in nature, is harder to test than the hypothesis of transmitter–receptor interaction. The DHP receptor appears to be confined to the junctional T-tubules. In addition, the extensive protrusion of the JFP across the cytoplasmic space toward the T-tubule makes this protein an attractive candidate for effecting the physical coupling between the T-tubule and TC. A simple test of this hypothesis would be the observation that the DHP receptor binds to the JFP. Subsequent to the isolation of the DHP receptor and the JFP, no evidence has been presented that these two proteins do, in fact, bind to each other. The tools for carrying out the investigations exist, and it is known that the junction is stable after vesicle isolation. This failure to observe communication, therefore, suggests that it may not occur.

In a recent paper, we have proposed that the stable coupling between the T-tubule and TC is effected by binding of the α_1 subunit of the DHP receptor to an intrinsic junctional protein of the TC of M_r 95K. This 95-kDa protein, in its turn, binds to the JFP (Brandt et al., 1990; Kim et al., 1990b). We therefore propose that the triad junction forms a ternary complex between the voltage sensor, the 95-kDa protein, and the JFP. This can account for the apparent propinquity in vivo between the JFP and the DHP receptor and, in addition, for the fact that no report has appeared demonstrating that the DHP receptor and JFP bind to each other directly.

In order to characterize the 95-kDa protein more fully, we have prepared a monoclonal antibody against this protein. This paper describes the interaction of the monoclonal antibody with the 95-kDa protein. The antibody has been employed to assay quantitatively the distribution of protein among microsomal organelles in relation to the distribution of the JFP and DHP receptors. In the original purification of the 95-kDa protein, we found, surprisingly, that the protein migrated in molecular sieve chromatography as a very high molecular weight species despite its modest molecular weight in SDS Laemmli gels. In this paper, we will describe the oligomeric nature of the protein in its native state and discuss its relationship to other triadic proteins.

In earlier papers and more extensively in this paper, we demonstrate the localization and confinement of the 95-kDa protein to the triadic junction. We have also shown previously

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the ability of this protein to bind to the two junctional proteins, the JFP and the DHP receptors. In this paper, we propose the name "triadin" for the 95-kDa protein.

Materials and Methods

Isolation of Organelles. Organelles employed in this study were prepared as described previously (Kim et al., 1990a). Briefly, the sacrospinalis muscle of rabbit was homogenized in a Waring blender, and microsomes were prepared by sequential differential centrifugation. Sucrose density gradient fractionation of the microsomes separates a light fraction (longitudinal reticulum) from a heavy fraction (TC/triads). The TC/triads were passed through a French press at 6000 psi. The fragmented triads were then separated into three bands on a continuous sucrose density gradient. The lightest band is mainly of T-tubular origin. The intermediate band, described as light TC, contains a mixture of light SR vesicles and strong triad vesicles. The heavy band is almost completely pure TC vesicles.

Protein Isolation. The 95-kDa protein was isolated, essentially, as described previously by dissolution in 1% Lubrol PX followed by sequential hydroxyapatite chromatography and elution with 250 mM phosphate buffer followed by chromatography through sequential copper chelate and S500 Sephacryl columns (Kim et al., 1990b). The output close to the void volume of the molecular sieve column was passed onto a small hydroxyapatite column and eluted with 0.25 M phosphate buffer.

JFP was isolated as described previously (Brandt et al., 1990). TC/triads were dissolved in 1.4% CHAPS medium in the presence of protease inhibitors and passed onto a hydroxyapatite column. The protein was eluted in a medium which contained 0.18 M potassium phosphate. The eluate was incubated with 0.5 mg of sulfo-NHS-biotin (Pierce) for 20 min at 0 °C. The reaction was stopped by addition of Tris-HCl, pH 7.4 (20 mM final concentration). After incubation for 20 min, the sample was diluted 2 times with 0.5% CHAPS/0.05% egg phosphatidylcholine and passed onto a heparin–agarose column (Sigma). JFP was eluted with 0.6 M NaCl, 0.5% CHAPS, 0.05% phosphatidylcholine, 20 mM Tris-HCl, pH 7.4, and the protease inhibitors leupeptin, pepstatin, and phenylmethanesulfonyl fluoride. DHP receptor was isolated and labeled as described previously (Kim et al., 1990b).

Antibody Preparation. Four mice were each injected with 10–20 μg of purified triadin initially with Freund's complete adjuvant and subsequently with Freund's incomplete adjuvant at biweekly intervals. A few days before extraction of the spleen and fusion of the lymphocytes, the mice were boosted with the isolated protein injected into the peritoneal cavity and subcutaneously in the neck. The fusion and subsequent culturing of the hybridomas followed conventional protocols. Purified triadin (50 μL) in 0.5 M NaCl, 0.1% polyoxyethylene(9) lauryl ether ($C_{12}E_9$), and 0.01% egg phosphatidylcholine was deposited onto microtiter plates and then incubated with supernatants from the hybridoma cultures followed by antibody assay using the ELISA technique. The substrate for visualization of horseradish peroxidase was either 4-aminoantipyrine or 2,2′-azinobis(3-ethylbenzothiazoline-sulfonate) (ABTS). Positive cultures from this test were further grown and then tested individually in a Western blot of the purified triadin. The Western blots were developed by employing biotinylated secondary antibody and streptavidin peroxidase. The substrate for color development was 4-chloronaphthol. A strong color development at a molecular weight of approximately 95K was considered indicative of a positive culture. Sometimes accessory bands were seen at higher molecular weights, the origin of which will be described in this paper. The single monoclonal antibody described here was cloned 3 times at a density of one or less cells per well; the clone was then grown in large culture flasks. Some of the Western blot assays performed in this study employed the supernatant of these cultures directly. In others, the antibody was grown by injection of hybridoma culture into the peritoneal cavity of mice. The ascites fluid was harvested and centrifuged on a clinical centrifuge to remove cells and stored frozen. The antibody was purified from the ascites on a Bakerbond ABx (J. T. Baker) ion-exchange column following the protocol described by the manufacturer. The purified antibody was identified by using SDS gel electrophoresis. The light and heavy chains were the dominant protein, but smaller quantities of impurities were observed in higher molecular weight regions of the gel. The estimated purity is 80–90%. The antibody was concentrated on an Amicon ultrafiltration apparatus and stored at −20 °C in the presence of 35% glycerol.

The monoclonal antibody against the JFP was prepared by growing the hybridoma clones of Kawamoto et al. (1986) in Balb/c male mice. The antibody purification from ascites fluid was as described for the mAb α triadin above. Coupling to CNBr-activated Sepharose (Sigma) was carried out as described previously (Kawamoto et al., 1986).

Immunoassays. Vesicle fractions, 20 μg in 200 μL of 200 mM NaCl/20 mM Tris-HCl, pH 7.4 (TBS), were laid on the first well of a row on a microtiter plate and serially diluted 1:1 with TBS. The dilution series (100-μL final volume) was normally allowed to adsorb overnight at 4 °C. The wells were washed with TBS containing 0.1% Tween 20 and then incubated in TBS with 10 μg/mL mAb for 2 h at 22 °C. The wells were subsequently treated with biotinylated anti-mouse Ig antibody and streptavidin horseradish peroxidase (Amersham) followed by color development with ABTS and H_2O_2.

Gel Electrophoresis and Western Blot Overlay. Gel electrophoresis normally employed conventional Laemmli SDS gels. Western blotting was carried out by transfer to nitrocellulose filters for 1.5 h at 12 V in Laemmli running buffer containing 20% methanol. Blue prestained standards from Sigma or Bio-Rad were employed to calibrate the molecular weights and to delineate the lanes in the blot. After transfer, the blot was blocked in TBS with 3% bovine serum albumin (BSA) either overnight at 4 °C or 3 h at room temperature. The subsequent immunoblots or protein overlays were carried out as described previously (Kawamoto et al., 1986; Brandt et al., 1990). In Western blot assays employing purified antibody, 10 μg/mL in TBS was routinely used.

An extract of sea urchin sperm flagella enriched in dynein was a gift from Dr. M. Pratt, Department of Cell Biology and Anatomy, Univeristy of Miami. Myofibrils freshly prepared from rabbit psoas muscle (Solaro et al., 1971) were employed as the source of nebulin and titin.

Biotin–HPDP Labeling. PDP–biotin hydrazide [*N*-biotinyl-*N*′-[3′-(2′-pyridyldithio)propionyl]hydrazide] was synthesized from *N*-succinimidyl-PDP and biotin hydrazide in DMSO as described by Zaidi et al. (1989). In later experiments, the biotin–HPDP conjugate *N*-[6-(biotinamido)-hexyl]-3′-(2′-pyridyldithio)propionamide was purchased from Pierce and dissolved in DMSO. All labeling reactions were carried out on 2 mg of TC/triads per milliliter in 100 mM NaCl, 1 mM $MgCl_2$, and 20 mM MES (pH 5.5, pH 6.5), MOPS (pH 7.0), or HEPES (pH 7.5). The biotin–HPDP concentration was 100 μM, giving a final DMSO concentration of <1%. Reactions were terminated by centrifuging the

Table I: Antibody and Ligand Binding to Vesicle Subfractions

vesicle	activity: mAb triadin relative to heavy TC	activity: mAb JFP relative to mAb triadin	μg of triadin/ mg of protein	[^{3}H]PN 200–110 (1 nM) (pmol/mg)	[^{3}H]ryanodine (3.6 nM) (pmol/mg)
LR	0.03	0.018	0.2	1.7	0.61
TC/triads	0.17	0.42	5.6	11.6	3.82
T-tubule	0.04	0.05	0.6	18.4	0.50
light TC	0.16	0.31	5.6	15.9	3.32
heavy TC	1.0	1.4	32.0	8.1	8.80

membranes through a 20% sucrose cushion using either a Beckman ultracentrifuge at 100000*g* for 1 h or a Beckman Airfuge at 20 psi for 15 min. Pellets were suspended in 100 μL of nonreducing sample buffer for electrophoresis.

Ligand Binding. DHP binding was determined in 20 mM Tris-HCl/1 mM $CaCl_2$, pH 7.4, using [^{3}H]PN 200–110 as ligand. Ryanodine binding was assayed in 600 mM choline Cl, 10 mM HEPES/Tris, pH 7.4, 2.5 mM ATP, and 1 mM $CaCl_2$ as described previously. Filtration was carried out with Whatman GF/F membranes (Kim et al., 1990a).

Results

Monoclonal antibody GE 4.90 was prepared from mouse lymphocytes in animals which had been inoculated with purified triadin dissolved in a nondenaturing detergent (Lubrol PX and $C_{12}E_9$). This antibody was identified as anti-triadin by Western blot immunoabsorption employing the purified protein. Figure 1 shows a Western blot of proteins from microsomal vesicles using the antibody. The immunoblot shows a very faint band at approximately 95 kDa in lane 1 indicating that very little antigen is present in the longitudinal reticulum fraction. A single band at approximately 95 kDa protein is observed in the TC/triad immediately underlying the Ca^{2+}-ATPase (lane 2). The presence of a smear of activity above this band may be attributed to uneven running of the band in the gel in part owing to the presence in the preparation of high concentrations of Ca^{2+}-ATPase of almost identical molecular weight. Following French press treatment to disrupt the triads and fractionation of the vesicles on a sucrose density gradient, the T-tubular band shows very low antigen activity (lane 3) while a higher activity is observed in the light TC (lane 4). The heavy TC fractions show the strongest antigenic response (lane 5). Comparison with the Coomassie-stained gel (Figure 1B) shows that the higher antigenic activity is found in vesicles with high JFP content indicated by the band immediately below the stacking gel. This distribution conforms very closely to that which was observed for triadin distribution on the basis of its ability to bind to the DHP receptor and the JFP. It is also an antigenic distribution which is in accord with the confinement of the triadin to the junctional TC.

A more quantitative evaluation of the distribution of antigen among microsomal vesicles was afforded by performing ELISA assays on vesicles deposited on microtiter plates. The vesicles were deposited at 10 μg per well in the first lane and in 2-fold serial dilutions through the subsequent 11 lanes. After development of the color reaction, the OD was plotted as a function of vesicle dilution to obtain parallel curves for the various vesicles. The dilution required to achieve a similar degree of color development was employed to indicate the relative antigen concentration in each fraction. The data of Table I are the average of two experiments in which all the assays were performed on a single preparation, but other analyses of parts of these assays gave very similar results. In Table I, the antigen concentration is arbitrarily set at 1 for the heavy TC vesicles. A comparison with the other vesicle

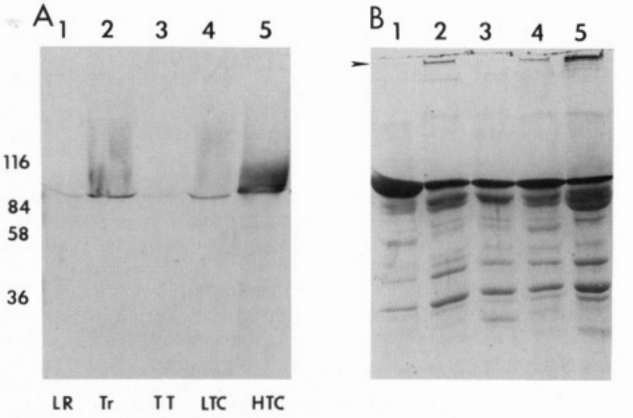

FIGURE 1: Western blot of anti-triadin antibody on microsomal subfractions from skeletal muscle. Vesicles (20 μg in each lane) were layered on a 6% acrylamide gel. Blue-stained standards (Sigma) separate (A) the immunoblot with GE 4.90 from (B) the Coomassie-stained gel. Lane 1, longitudinal reticulum (LR); lane 2, TC/triads (Tr); lane 3, T-tubules (TT); lane 4, light TC (LTC); lane 5, heavy TC (HTC). The JFP in (B) is indicated by the arrowhead.

subfractions shows the highest concentration of triadin in the heavy TC while lower concentrations are observed in the TC/triads and in the light TC fraction. The content of triadin in longitudinal reticulum and T-tubules is very low. A comparison with the distribution of DHP receptor and JFP, obtained through the binding assays of the ligands PN 200-110 and ryanodine, shows that the distribution of triadin corresponds more closely to that of ryanodine. This is in conformity with the view that triadin is confined to the junctional portion of the TC. We have carried out a comparison of the activity of triadin with that of JFP by performing simultaneous assays using a mAb directed against the JFP (column 3) as well as the anti-triadin mAb (column 2). If each antibody binds to a single site on each antigen, if the affinity of the antibody for the antigen is high, and if the antibody is present in excess, then the development of the ELISA reaction should allow a quantitative comparison of the JFP to triadin. We have added the antibody in excess in each case. Neither antigen is released from the antibody in salt solutions containing 1 M KCl, indicating that the affinity for the antigen is high in each case. We do not know that there is a single epitopic site per molecule although this is normally the case for a monoclonal antibody. The data of Table I show that the concentration of vesicles required to achieve an identical degree of color development in the assay is quite comparable for the two antibodies. The two proteins are therefore present in very comparable molecular ratios.

The ELISA assay has been quantitated by comparing the activity in the vesicles with that of the purified protein. We have not succeeded in obtaining the purified triadin in a concentrated form and have therefore run SDS gels and compared by densitometry of the 95-kDa band with that of standard albumin concentrations run on the same gel in order to estimate most accurately the protein concentration of

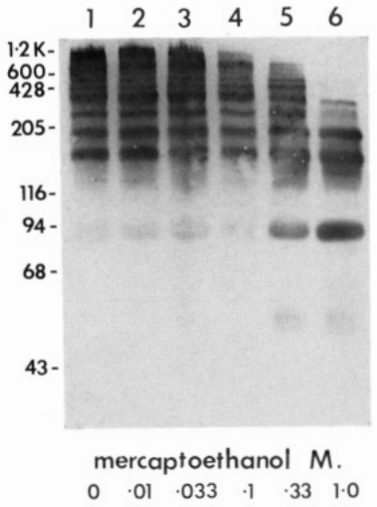

FIGURE 2: Western blot of a nonreducing SDS gel of TC/triads which were preincubated with varying concentrations of mercaptoethanol. TC/triads (100 μL of 1 mg/mL) in 250 mM sucrose/2 mM histidine were incubated with the indicated concentration of mercaptoethanol. Iodoacetamide, 100 mM, was added, and after 20-min incubation, the vesicles were centrifuged through 20% sucrose in a Beckman Airfuge. The pellet was resuspended in sample buffer, and 25 μg/lane was electrophoresed on a 4–10% acrylamide gradient gel. Standards run on the same gel included titin (1.2×10^6 Da) and nebulin (600 kDa) from a preparation of myofibrils, dynein (428 kDa) from sea urchin sperm flagella, and high molecular weight standards (Sigma).

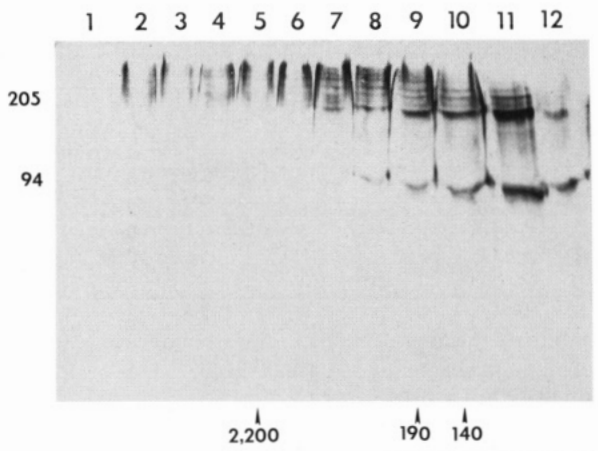

FIGURE 3: Rate zonal centrifugation of triadin in TC/triads after dissolution. TC/triads (5 mg/mL) were suspended in 900 μL of 1 M NaCl, 1% Lubrol PX, 0.1% egg phosphatidylcholine, 20 mM Tris-HCl, pH 7.4, 10 μM leupeptin, and 10 μM pepstatin. After centrifugation in a Beckman Airfuge, the supernatant was loaded on a 5–30% linear sucrose density gradient containing the medium above in a Beckman SW 50.1 centrifuge. Centrifugation was performed for 16 h at 25 000 rpm, and 0.4-mL samples were withdrawn. Samples are numbered from the bottom of the tube to the top. Sample (100 μL) was layered separately on two 5% acrylamide gels and electrophoresed under nonreducing conditions. One gel was employed for Coomassie staining and the other for Western blot using the mAb. Samples from the gradient were also laid on a microliter plate and assayed by serial dilution using the anti-JFP mAb. The standards for the rate zonal run were glyceraldehyde-3-phosphate dehydrogenase (140 kDa) and phosphorylase *b* (190 kDa) detected from the Coomassie gel and JFP (2.2×10^6 Da) obtained from the ELISA assay.

triadin. The medium in which triadin is isolated contains high salt concentrations and detergent which may inhibit the deposition of protein on the microtiter plates. We have added to each well of the triadin assay a small amount of Biobeads SM to absorb the detergent. This considerably improves the ELISA assay so that color development is maintained even at high detergent levels. The data show that the heavy TC contains approximately 32 μg of triadin/mg of protein. The content in TC/triads and LTC is lower while T-tubules contain practically no triadin. If we assume a molecular weight of the triadin of 95K, then the molar content in heavy TC is 340 pmol/mg while that in TC/triads is 59 pmol/mg. In the same experiment, we have determined the B_{max} of ryanodine in TC/triads as 7.8 pmol/mg by Scatchard plot analysis of binding data. If, as has been claimed (Lai et al., 1988), there is one ryanodine binding site per JFP tetramer, then the JFP molar content is 31 pmol/mg. The relative content of triadin and JFP estimated from the absolute contents is therefore quite similar.

Disulfide Linkage of Triadin. Figure 2 shows a Western blot of an SDS gel of TC/triads in which varying concentrations of mercaptoethanol were incubated with the intact vesicles and subsequently removed by centrifugation through a 20% sucrose cushion. The gel was run in the absence of disulfide reducing agents. Lane 1 shows the Western blot of triadin in the absence of mercaptoethanol. The Western blot overlay pattern is markedly distinct from that observed when high concentrations (2.5%, ~0.35 M) of mercaptoethanol are present in the dissolution medium (Figure 1). A ladder of bands is seen of varying molecular weights from 170K to approximately 10^6. This gradient gel was calibrated with high molecular weight markers including dynein (428K), nebulin (600K), and titin (1200K) and molecular weight estimates obtained for the immunoreactive bands. The bands migrate in the gel with apparent molecular weights of 94K, 155K, 200K, 260K, 330K, 420K, 510K, 600K, and 680K which represents somewhat less than multimers of 95 kDa. However, it is likely that the presence of disulfides prevents the full opening of the polypeptide chain to random coils in the denaturing medium. Several proteins with disulfide linkages are observed to show anomalously low Stokes radii in nonreducing conditions (Fish et al., 1970). The interpretation of the data is that the protein forms homopolymers of varying length linked by disulfide bonds. The effect of mercaptoethanol on the intact vesicles is considerably less pronounced than when the reagent is present in the denaturing condition of the usual SDS-containing sample buffer. At a concentration of 100 mM, the highest molecular weight polymers start to disappear, and even at the highest concentration employed here of 1 M, polymers up to hexamers are still detectable although the monomer is now the major constituent. A small amount of lower molecular weight protein of M_r 60K probably represents proteolytic product. Thus, 1 M mercaptoethanol (~8%) is only partially effective in reducing the protein in the intact organelle whereas it is fully effective in reducing the protein after denaturation. We have assayed the depolymerization of triadin employing a range of reducing agents. Glutathione, 4-mercaptopyridine, 2-mercaptopyridine, and dithiothreitol are without effect at 100 mM concentration. Dithiothreitol at 1 M concentration causes partial depolymerization. Opening access of the reducing agents to the interior of the organelle by adding Triton X-100 does not affect the potency of the thiol reagent. The absence of reduction by glutathione, the physiological reducing agent in the cytoplasm, suggests that the formation of intermolecular disulfide bonds is a physiological condition of this protein.

In the isolation of triadin, we observed that the protein eluted from molecular sieve columns apparently as a high molecular weight species. Intermolecular disulfide links could explain this observation. We have investigated the degree of association of the protein in nondenaturing conditions by rate zonal centrifugation of Lubrol-dissolved TC/triads. Figure 3 shows Western blot immunoreaction of a nonreducing SDS gel from fractions obtained from a density gradient after rate zonal centrifugation. Under the conditions of centrifugation, the

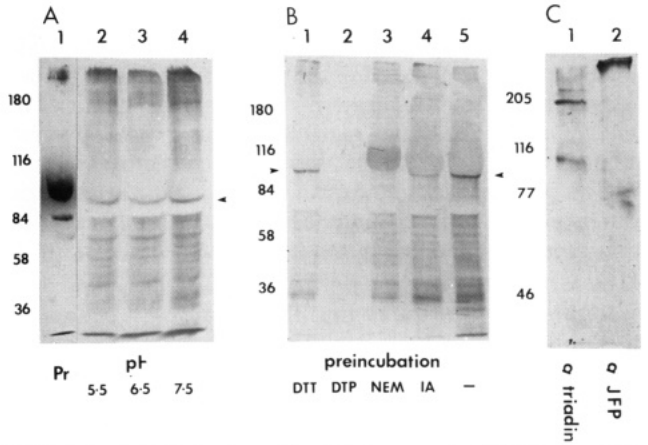

FIGURE 4: Labeling of reactive thiol groups in TC/triads. (A) TC/triads (1 mg/mL) were pretreated with 5 mM iodoacetamide at pH 7.5, centrifuged, and resuspended in reaction buffer containing 0.1 mM biotin–HPDP at the pH indicated. After 5 min at 20 °C, the reaction was stopped by centrifugation through 20% sucrose, and the pellets were resuspended in nonreducing sample buffer. Lanes were loaded with 50 μg of protein each. Lane 1 is the Coomassie-stained gel. Lanes 2–4 are developed for biotin–HPDP. The arrowhead indicates the probable SG 106 protein. (B) TC/triads (2 mg/mL) in reaction buffer were pretreated with 10 mM dithiothreitol (DTT), 0.1 mM 2,2-dithiopyridine (DTP), 10 mM *N*-ethylmaleimide (NEM), or 10 mM iodoacetamide (IA) for 10 min and then centrifuged through 20% sucrose. The pellets were resuspended in 100 mM NaCl, 20 mM MOPS, pH 7.0, 1 mM $MgCl_2$, and 100 μM biotin–HPDP, incubated for 5 min, and centrifuged through 20% sucrose. Pellets were resuspended in nonreducing solubilizing buffer, and 50 μg of protein was loaded in each lane. The arrowheads indicate the SG 106 protein. (C) Antibodies mAb GE 4.90 and mAb anti-JFP were coupled to CNBr-activated Sepharose. TC/triad membranes prelabeled with biotin–HPDP as described for (B) were washed once with 0.1 M NaCl/20 mM MOPS, pH 7.0, and then dissolved with 1 M NaCl, 20 mM HEPES, pH 7.6, and 1% Lubrol PX at 1 mg/mL protein. Equal portions of the extract were incubated with 100 μL of the antibody–Sepharose gel for 16 h at 4 °C, and the gel was recovered and washed by centrifugation with 1 M NaCl, 1% Lubrol PX, and 20 mM MOPS, pH 7.0. The gel was then suspended with equal volumes of nonreducing sample buffer and 100 μL applied directly to a slab gel.

monomer migrates only a short distance during centrifugation. The figure shows that the higher molecular weight polymers migrate further and that the distance of migration is dependent upon the size of the polymer. The diffusion rate has been calibrated with proteins of known molecular weight. The extent of migration of the monomer is that expected of a protein of approximately 100000 molecular weight, indicating that in its native state the protein is dissociated. Similarly within the limits of accuracy of determination of the distance of diffusion, the higher molecular weight species run according to the number of units in the polymer. The data show, therefore, that the protein is not held as a polymer by noncovalent forces.

We investigated the role of sulfhydryl groups in the triadin molecule by employing biotin–HPDP following the method described by Zaidi et al. (1989) in their studies of the low molecular weight Ca^{2+}-release channel SG 106. Figure 4 shows the labeling of protein from TC/triad vesicles which have been incubated with the biotinylated disulfide, separated by SDS gel electrophoresis, and developed by streptavidin–peroxidase reaction. We carried out the incubation at three different pHs (Figure 4A, lanes 2–4) since the thiol–disulfide exchange reaction occurs through the mercaptide anion and the reaction is faster in more alkaline pHs (Torchinsky, 1981). The figure shows an extensive pattern of labeling of SR proteins. A small strongly labeled band (arrowhead) immediately below the Ca^{2+}-ATPase (seen in the Coomassie-stained gel, lane 1) may represent the protein described by Zaidi et al. (1989). These authors have reported that under the conditions of incubation employed in this experiment at all pHs tested, the SG 106 protein is the only one labeled. In our hands, in several experiments we have been unsuccessful in obtaining the degree of specificity which they report. We did not detect any significant difference between the labeling at different pHs. It is possible that, under all pHs of reaction described here, the rate of reaction is sufficiently fast to give rise to complete reaction with accessible and reactive SH groups. Figure 4B shows the influence of pretreatment with a variety of sulfhydryl reagents on the pattern of labeling. Pretreatment with dithiothreitol, (DTT, lane 1) did not influence the labeling although administration of DTT after biotin–HPDP caused loss of labeling. Prior oxidation of the vesicles with dithiopyridine (DTP, lane 2) caused complete loss of labeling, as is to be expected since this reagent should react with the same thiol groups as the biotinylated reagent. *N*-Ethylmaleimide (NEM, lane 3) preincubation obliterated labeling of the protein immediately below the Ca^{2+}-ATPase (arrowhead). Some other proteins of lower molecular weight also lost label, but the majority of proteins were unaffected. Treatment with iodoacetamide (IA, lane 4) gave rise to a similar response except that the labeling of the protein below the Ca^{2+} pump was only partially reduced. The solubilization, electrophoresis, and Western blot were carried out in the absence of disulfide reducing agent, and we would, therefore, expect that triadin would appear in the gel in the polymeric forms as in Figure 2. It is, however, possible that some monomer is present in the preparation since in some preparations we have observed monomeric protein (e.g., Figure 3). If the monomer contains reactive thiol while the disulfide-linked polymers lack a reactive sulfhydryl group, the monomer alone may be labeled by the biotinylated disulfide. In Figure 4C, vesicles were prelabeled with biotin–HPDP, then dissolved in detergent in the presence of 1 M KCl, and incubated with either immobilized anti-triadin antibody (lane 1) or immobilized anti-JFP antibody (lane 2). After the column was washed, the bound proteins were released with nonreducing gel sample buffer and separated by gel electrophoresis. The Western blot development of the biotinylated disulfide shows the presence of bands of M_r 100K and 200K with smaller amounts of higher polymers from the anti-triadin column (lane 1), indicating that the reagent labeled non only the monomer but also higher polymers. Therefore, the intense band below the Ca^{2+}-ATPase, in Figure 4A,B, is not triadin. In addition, the anti-JFP column (panel C, lane 2) shows that labeled material at the molecular weight of JFP is bound to the anti-JFP column, indicating that the JFP can be labeled by biotin–HPDP.

Triadin was originally identified as a participant in the triad junction on the basis of its binding to the DHP receptor and the JFP using protein overlay assay. The original overlay procedures were carried out on proteins which had been electrophoresed in reducing conditions. It is possible, however, that subsequent oxidation during transfer and binding could have occurred. In Figure 5, we have investigated the binding of JFP to reduced and nonreduced triadin. Gel electrophoresis has been carried out either in the presence (A) or in the absence of reducing agent (B). In addition, the blot has been preincubated either in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of mercaptoethanol (as indicated by "Blot" under the gel lanes) immediately prior to incubation with JFP. The reducing agent was not included in the protein overlay incubation medium in order to avoid reduction of disulfides of JFP or the DHP receptor since they contain SH

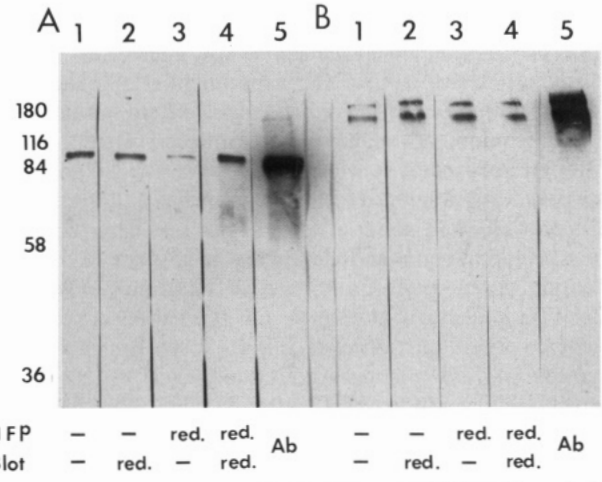

FIGURE 5: Triadin was purified by dissolution of TC/triads (1 mg/mL) in 1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1% Lubrol PX, 0.1% phosphatidylcholine, and protease inhibitors followed by 1-h incubation with a column of GE 4.90 coupled to an Actidisc (FM Corp). The disk was washed with 1 M NaCl, 0.1% Lubrol PX, 0.0% phosphatidylcholine, 20 mM Tris-HCl, pH 7.4, and protease inhibitors and eluted with the same medium containing 2 M NaSCN and 30 mM sodium phosphate, pH 7.4, instead of NaCl. The eluate was passed onto a small hydroxyapatite column and eluted with 0.5 M sodium phosphate, pH 7.4, 0.1% Lubrol PX, 0.01% phosphatidylcholine, and protease inhibitors. Equal aliquots were electrophoresed on 5% slab gels under reducing (A) or nonreducing (B) conditions. After being blocked with 3% BSA in TBS, alternate strips were incubated with 5% mercaptoethanol in 20 mM potassium gluconate/20 mM MOPS, pH 7.2, buffer for 1 h at 20 °C. All strips were then washed until all detectable odor was removed. JFP was isolated and labeled as described under Materials and Methods by hydroxyapatite chromatography and labeled with 0.5 mg of NHS–biotin (Pierce). Half of the preparation was then incubated for 20 min at 20 °C with 100 mM mercaptoethanol. Both preparations were then purified by heparin–Sepharose chromatography. Blotted strips were overlayed with biotinylated-JFP as indicated in a medium containing 20 mM potassium gluconate, 20 mM Tris/MOPS, pH 7.2, and 0.5% CHAPS. N_2 gas was passed into each tube which was then sealed and incubated overnight at 4 °C. The lanes for immunoblot were incubated with mAb anti-triadin followed by biotinylated sheep anti-mouse Ig. All strips were then developed with streptavidin–peroxidase and 4-chloronaphthol.

groups or disulfides which significantly alter the properties of the protein as detected by ligand binding experiments (Pessah et al., 1987; Brandt et al., 1985). The overlay data of this experiment show that JFP binds both to the triadin monomer obtained by reduction and to the higher molecular weight polymers associated with disulfide linkage. In addition, pretreatment of the JFP with mercaptoethanol (lanes 3 and 4) did not significantly affect the binding of JFP to the blot. Identical experiments have been carried out with the DHP receptor giving rise to similar results (not shown), suggesting that the binding of either of these proteins to triadin is not significantly diminished by reductions of the disulfides on either triadin or the overlaying receptor proteins. Lanes 5 in Figure 5 represent overlay with the mAb showing that the pattern of overlay with the JFP is identical with that of the antibody.

Discussion

The antibody GE 4.90 has been developed against triadin isolated in nondenaturing conditions. The initial evidence that the antibody was directed again triadin was obtained by Western blot overlay carried out in reducing SDS gels. Since there are several SR proteins with a similar molecular weight, this evidence did not fully resolve the correctness of the assignation. The finding that antigen forms disulfide-linked polymers of various sizes offers a diagnostic test of the presence of this protein. Triadin was originally identified by its ability to bind to the DHP receptor and JFP in protein overlay. Figure 5 shows that, in the absence of reducing agents, polymers may be detected in the gel overlays using JFP overlay. This corresponds exactly with the antigenic activity of triadin and demonstrates that the mAb is directed against triadin. We have detected no other microsomal proteins which bind to the antibody. Low quantities of a band at approximately 60 kDa are sometimes detected which we believe to be a proteolytic product of the main protein.

In our original detection of triadin using DHP and JFP overlay, we demonstrated that the protein was a terminal cisternae protein (Brandt et al., 1990; Kim et al, 1990b). In this paper, we extend these observations using the antibodies to quantitate the antigenic content in differing vesicle fractions. There is a close correlation between the distributions of the JFP as detected by ryanodine binding and the distribution of triadin demonstrated by using an antibody. Triadin is clearly absent except for a small degree of contamination from longitudinal reticulum and T-tubules. It is present in high concentration in the enriched TC fraction described by us as heavy TC. The data are consistent with triadin and JFP binding to each other in the membrane although each protein may also exist unassociated with the other.

We have employed two methods to quantitate triadin in vesicles. The direct assay of the antigenic content of isolated triadin and triadin in vesicles correlates well with the indirect method. In both cases, we find that triadin is present at approximately the same concentration as JFP. Therefore, although this protein is a minor one in SR, it is probably a major constituent of the triad junction. Its presence in the junction at a concentration comparable to the JFP suggests that its ability to bind to JFP could be a significant physiological factor in the organization and function of the latter protein.

The finding that triadin exists in isolated organelles in the form of a homopolymer of variable size explains some of the previous observations concerning this protein. We have found that chromatographic techniques such as hydroxyapatite chromatography and molecular sieve chromatography give a rather broad distribution of triadin. The explanation for this may lie in the large variety of size of the polymeric protein. In addition, the protein requires hypertonic salt concentration, moderately strong detergent treatment, and low protein concentration for dissolution. We have been unsuccessful in reducing the salt concentration or changing to a milder detergent such as CHAPS after dissolution since the protein precipitates in the column under these conditions. It is possible that the formation of higher polymers accounts for these observations since all subunits of a polymer need to dissolve for the polymer to be extracted from the membrane.

It is likely that the disulfide-linked polymer is the native form of the protein. The protein is not readily reduced by a variety of thiol reagents. Glutathione at 100 mM concentration does not reduce the protein. The physiological significance of the formation of this polymer is not clear; we have no evidence currently that it influences the reactivity of triadin. JFP and DHP receptor both overlay the disulfide-linked polymer and the reduced form of the protein. It is possible that triadin forms the matrix of the junctional portion of the TC in which the JFP is immersed and that the organization of the triad is influenced by the manner in which the subunits of the polymer interact with each other. A surprising feature of our observations is the wide range of molecular weights of the polymer. We have counted up to 12 different bands on SDS gels, but the number may be considerably more than this

since the larger units are difficult to distinguish. We do not know the shape of the polymer; it may exist as a linear chain, in a branched chain, or possibly a closed loop. It is possible that the polymers hook around each other and thereby form a more rigid matrix in the membrane.

It is possible that any monomer present in the native membrane represents protein with fully reduced cysteine residues while the polymer contains internal subunits with two intermolecular disulfide bonds and terminal subunits with one intermolecular bond. If this is the case, then there are two reactive SH groups which participate in the polymerization in each subunit molecule. It is possible, however, that all the cysteines which participate in polymerization are fully oxidized to form disulfides independent of the polymeric size since those disulfides which do not form intermolecular bonds may participate in intramolecular bonds or, at least in the higher polymers, a closed loop could be formed in which all cysteines are intermolecularly linked with disulfide bonds. In our experiments with biotin-HPDP, we were not able to distinguish a variability of active SH groups with polymer length. It is possible though that this reagent did not detect these thiols which participate in the polymerization reaction. The data indicate, however, that some SH groups are present in the reduced form and are readily reactive with this reagent.

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