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Labeling of High Affinity ATP Binding Sites on the 53,000- and 160,000-dalton Glycoproteins of the Sarcoplasmic Reticulum with the Photoaffinity Probe 8-N_3-[α-^{32}P]ATP*

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8-Azido-[α-^{32}P]ATP (8-N_3-ATP) was used as a photoaffinity label for ATP binding sites in the sarcoplasmic reticulum membrane. The radioactive 8-N_3-ATP was specifically incorporated into proteins of 53,000, 105,000, and 160,000 daltons when intact sarcoplasmic reticulum vesicles were incubated with 0.14-1.6 μM 8-N_3-ATP. The presence of 100-500 μM ATP during the incubation inhibited the binding of 8-N_3-ATP, while cAMP and AMP did not affect binding. Analysis of various membrane fractions during purification of the sarcoplasmic reticulum from muscle homogenates showed concomitant purification of the 53,000-, 105,000-, and 160,000-dalton proteins that bound 8-N_3-ATP. The 8-N_3-ATP-labeled proteins had identical mobilities to the 53,000-dalton glycoprotein, the 105,000-dalton (Ca^{2+} + Mg^{2+})-ATPase and the 160,000-dalton glycoprotein, respectively. 8-N_3-ATP labeling of deoxycholate extracts of sarcoplasmic reticulum resulted in the specific labeling of two proteins of 40,000 and 53,000 daltons, while calsequestrin (63,000 daltons) was not labeled. The 53,000- and 160,000-dalton labeled proteins bound to Con A Sepharose columns and were eluted by α-methyl-D-mannoside. Endo-β-*N*-acetylglucosaminidase H digestion of 8-N_3-ATP-labeled proteins reduced the 53,000- and 160,000-dalton 8-N_3-ATP-labeled proteins to 49,000 and 155,000 daltons, respectively. These observations show that the major intrinsic glycoproteins of the sarcoplasmic reticulum (Campbell, K. P., and MacLennan, D. H. (1981) *J. Biol. Chem.* 256, 4626-4632) are the 53,000- and 160,000-dalton that bind 8-N_3-[α-^{32}P]ATP and, therefore, these glycoproteins contain ATP binding sites.

Antiserum to the 53,000-dalton glycoprotein specifically immunoprecipitated the 53,000- and 160,000-dalton 8-N_3-ATP-labeled proteins from extracts of sarcoplasmic reticulum. When cardiac sarcoplasmic reticulum was labeled with 8-N_3-ATP, label was incorporated into proteins of 53,000 and 130,000 daltons. These proteins were precipitated with antiserum against the skeletal muscle, 53,000-dalton glycoprotein and the molecular weights of these proteins were reduced to 49,000 and 125,000, respectively, after incubation with Endo-β-*N*-acetylglucosaminidase H. Thus the 8-N_3-ATP binding proteins were identified as the 53,000- and 130,000-dalton, intrinsic glycoproteins of cardiac sarcoplasmic reticulum.

The mechanism of Ca^{2+} transport by the sarcoplasmic reticulum of rabbit skeletal muscle has been extensively studied (1-3) but regulation of Ca^{2+} transport in skeletal muscle sarcoplasmic reticulum by phosphorylation has only recently been explored (4, 5). The sarcoplasmic reticulum membrane from rabbit skeletal muscle has been shown to contain endogenous protein kinase activity (4-10) and Varsanyi and Heilmeyer (9) have recently reported that the (Ca^{2+} + Mg^{2+})-ATPase can be phosphorylated by a membrane-bound kinase. We have found a calmodulin plus Ca^{2+}-dependent protein phosphorylation system in sarcoplasmic reticulum which phosphorylates a 60,000-dalton substrate (10). Although the substrates of protein kinase are readily identified, the identities of the membrane-bound protein kinases in the sarcoplasmic reticulum membrane are not known.

The major proteins of the sarcoplasmic reticulum of rabbit skeletal muscle sarcoplasmic reticulum are: a (Ca^{2+} + Mg^{2+})-ATPase (M_r 105,000), calsequestrin (M_r 63,000), and two intrinsic glycoproteins (M_r 53,000 and 160,000) (11-13). The (Ca^{2+} + Mg^{2+})-ATPase is an intrinsic membrane protein which, alone, is responsible for active transport of calcium across the sarcoplasmic reticulum membrane (14). Calsequestrin is a very acidic protein which binds some 1,000 nmol of Ca^{2+}/mg of protein. It is probably involved in calcium storage within the lumen of the sarcoplasmic reticulum (12). The intrinsic, 53,000-dalton glycoprotein is a transmembrane protein, largely exposed on the cytoplasmic surface of the sarcoplasmic reticulum (13). Its function is not known. Calsequestrin, the 53,000-dalton glycoprotein, and a larger glycoprotein (160,000 daltons in skeletal; 130,000 daltons in cardiac) have been found to be invariant constituents of both skeletal and cardiac sarcoplasmic reticulum membranes (15).

8-N_3-ATP[1] has been used as a photoaffinity label for the catalytic subunit of the cAMP-dependent protein kinase (16). It competes with ATP for the high affinity ATP binding site and it is accepted as a substrate in the phosphotransferase reaction. It has also been used to label ATP binding sites in bovine heart mitochondrial ATPase (17) and in bacterial ATPase (18). In this paper, we describe experiments using 8-N_3-ATP to photoaffinity label high affinity ATP binding sites in the sarcoplasmic reticulum membrane in an attempt to identify possible membrane-bound protein kinases.

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[1] The abbreviations used are: 8-N_3-ATP, 8-azido-adenosine 5'-triphosphate; SDS, sodium dodecyl sulfate; ConA, concanavalin A; Endo H, Endo-β-*N*-acetylglucosaminidase H; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid.

EXPERIMENTAL PROCEDURES

Materials—SDS, acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad Laboratories. Concanavalin A Sepharose 4B and Protein A Sepharose 4B were obtained from Pharmacia. Endo H (*Streptomyces plicatus*) was a product of Health Research Inc. 8-N_3-[α-^{32}P]ATP was obtained from ICN at a specific activity of 16 Ci/mmol.

Preparation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of MacLennan (11) as modified by Campbell and MacLennan (13). Crude sarcoplasmic reticulum vesicles were membranes obtained in the initial 44,000 × *g* centrifugation step (11). Light membranes were obtained as previously described (13). Cardiac sarcoplasmic reticulum vesicles were prepared according to Jones *et al.* (19).

Photoaffinity Labeling with 8-N_3-[α-^{32}P]ATP—Photoaffinity incorporation of 8-N_3-[α-^{32}P]ATP into sarcoplasmic reticulum proteins was carried out in 100 μl of solution containing 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EGTA, and 100 μg of sarcoplasmic reticulum in the presence or absence of 0.25 mM $CaCl_2$ and 0.14–1.6 μM 8-N_3-[α-^{32}P]ATP (16 Ci/mmol). Preincubation was carried out for 1–5 min in the dark at 4 °C and the samples were then irradiated for 10 min with a Mineralight UVS-11 hand lamp at a distance of 8 cm. Controls were run in the presence of 100–500 μM ATP. Photoaffinity labeling was also carried out on preparations of light vesicles separated from sarcoplasmic reticulum vesicles, on crude muscle microsomes, and on deoxycholate extracts of sarcoplasmic reticulum vesicles. Endo H treatment of the labeled proteins was carried out as described in Ref. 10 following UV irradiation. Immunoprecipitation of labeled proteins was carried out using antiserum to the 53,000-dalton glycoprotein and Protein A Sepharose 4B beads as previously described (10). The analysis of labeled sarcoplasmic reticulum proteins by SDS-polyacrylamide slab gel electrophoresis was performed using the discontinuous buffer system of Laemmli (20) in 1.5-mm thick slab gels of 7.5% acrylamide and in gradient gels of 5–15% acrylamide. Autoradiography of dried slab gels was performed using Kodak X-Omat film and a Dupont Cronex Lightning Plus enhancing screen.

Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Incubation of sarcoplasmic reticulum vesicles with μM 8-N_3-[α-^{32}P]ATP under conditions where (Ca^{2+} + Mg^{2+})-ATPase activity was activated (11) resulted in very low levels of incorporation of 8-N_3-ATP into a protein of 95,000 daltons (not shown). This incorporation was not specific since 500 μM ATP did not compete with 8-N_3-ATP. 8-N_3-ATP can be used as a substrate by various ATPases (17, 18) and the resulting compound is 8-N_3-ADP. Therefore, specific labeling probably did not occur because of hydrolysis of 8-N_3-ATP during the 5-min incubation period prior to UV irradiation. We were not able to evaluate this possibility because the 8-N_3-ATP was labeled with ^{32}P in the α-position. Our results, in which 8-N_3-ATP was probably hydrolyzed to 8-N_3-ADP, suggest that the photoaffinity probe 8-N_3-ADP at μM concentrations does not specifically label any sarcoplasmic reticulum proteins.

Specific labeling with 8-N_3-ATP was detected following incubation of sarcoplasmic reticulum vesicles with 1.6 μM 8-N_3-[α-^{32}P]ATP in the buffer system used to measure Ca^{2+}-calmodulin-dependent kinase activity (10). In this buffer system, there was very low ATPase activity. Fig. 1 shows that 8-N_3-ATP was bound to five proteins of 35,000, 53,000, 95,000, 105,000, and 160,000 daltons. Labeling of the 35,000- and 95,000-dalton proteins was nonspecific since it could not be removed with excess ATP. Specific labeling, which could be competed off with a 300-fold excess of ATP, was observed in the 53,000-, 105,000-, and 160,000-dalton proteins (Fig. 1). The presence of 500 μM cAMP or 500 μM AMP had no effect on the labeling (not shown). Binding quantitation is presented in Table I. In the absence of Ca^{2+}, 0.35 pmol of 8-N_3-ATP were bound to the 53,000-dalton protein/mg of sarcoplasmic reticulum protein. The addition of Ca^{2+} caused a 25% increase in the labeling in the 53,000-dalton protein and a 400% increase in the labeling of the 105,000-dalton protein (Fig. 1 and Table I). The 8-N_3-ATP-labeled proteins migrated with identical mobility to the (Ca^{2+} + Mg^{2+})-ATPase (M_r 105,000), intrinsic glycoprotein (M_r 53,000), and the high molecular weight intrinsic glycoprotein (M_r 160,000). The binding of 8-N_3-ATP to the (Ca^{2+} + Mg^{2+})-ATPase was expected since it is known that the (Ca^{2+} + Mg^{2+})-ATPase binds ATP (1–3). In our study, we concentrated on the binding of 8-N_3-ATP to the 53,000- and 160,000-dalton proteins.

In a previous study of 8-N_3-cAMP labeling of the sarcoplasmic reticulum (13), we observed specific labeling of a 53,000-dalton protein. Further analysis revealed that this cAMP labeled protein was not a sarcoplasmic reticulum pro-

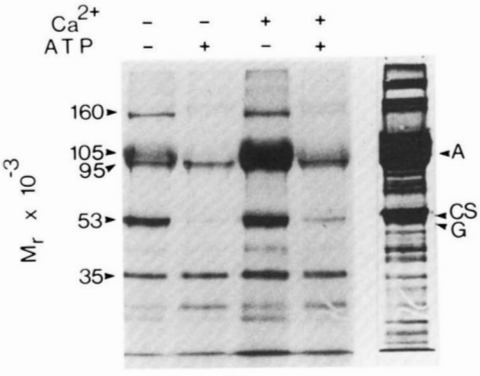

FIG. 1. **8-N_3-[α-^{32}P]ATP incorporation into sarcoplasmic reticulum.** Sarcoplasmic reticulum vesicles (1 mg/ml) were pre-incubated at 4 °C for 5 min in 100 μl of a solution of 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EGTA with 1.6 μM 8-N_3-[α-^{32}P]ATP in the presence (+) or absence (−) of 0.25 mM Ca^{2+} or 500 μM ATP. Following irradiation for 10 min with UV light, the labeled vesicles were analyzed by SDS-gel electrophoresis in 7.5% acrylamide and autoradiography. *A*, ATPase; *CS*, calsequestrin; *G*, 53,000-dalton glycoprotein. The 8-N_3-ATP was incorporated into proteins of 35,000, 53,000, 105,000, and 160,000 daltons. ATP inhibited the 8-N_3-ATP labeling of the 53,000-, 105,000-, and 160,000-dalton proteins. No labeling was observed when the sarcoplasmic reticulum vesicles were first solubilized with SDS, or under conditions of high ATPase activity.

TABLE I

Photoaffinity labeling of sarcoplasmic reticulum vesicles with 8-N_3-ATP

Photoaffinity incorporation of 8-N_3-[α-^{32}P]ATP into 100 μg of sarcoplasmic reticulum protein was carried out in 100 μl of a solution of 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EGTA, in the presence or absence of 0.25 mM $CaCl_2$ and 1.6 μM 8-N_3-ATP (16 Ci/mmol). Preincubations were carried out for 1–5 min in the dark at 4 °C and the samples were then irradiated for 10 min with a Mineralite UVS-11 hand lamp at a distance of 8 cm. The labeled vesicles were analyzed by SDS-polyacrylamide gel electrophoresis, the resulting slab gels were subjected to autoradiography and the radioactive bands were cut out and analyzed by liquid scintillation counting. Specific incorporation was calculated as the difference in 8-N_3-ATP labeling in the presence and absence of cold excess ATP and is given in pmol of 8-N_3-ATP incorporated per mg of sarcoplasmic reticulum protein.

Sarcoplasmic reticulum protein	8-N_3-ATP incorporation	
	$-Ca^{2+}$	$+Ca^{2+}$
molecular weight	*pmol/mg*	
53,000	0.35	0.43
105,000	0.20	0.81
160,000	0.13	0.12

tein but was probably localized in lighter membranes such as the transverse tubular or sarcolemmal membranes. Therefore, we tested whether the 8-N_3-ATP-labeled 53,000-, 105,000-, and 160,000-dalton proteins were sarcoplasmic reticulum proteins. Fig. 2 shows the results of labeling various fractions obtained during the purification of sarcoplasmic reticulum vesicles with 0.14 μM 8-N_3-ATP. Specific incorporation of 8-N_3-[α-^{32}P]ATP into the 53,000-, 105,000-, and 160,000-dalton proteins was increased in the sarcoplasmic reticulum (Fig. 2*C*) and in the KCl-washed sarcoplasmic reticulum vesicles (Fig. 2*D*). The 40,000-dalton labeled band was found both in the sarcoplasmic reticulum and in the lighter membranes. It was probably the catalytic subunit of the cAMP-dependent protein kinase which has been shown to bind 8-N_3-ATP (16). Nonspecific labeling of 35,000- and 95,000-dalton proteins was enriched in fractions that were relatively deficient in sarcoplasmic reticulum membranes (Fig. 2, *A* and *B*).

In order to test whether the 53,000-dalton glycoprotein was labeled by 8-N_3-ATP, we extracted sarcoplasmic reticulum vesicles with deoxycholate to solubilize calsequestrin and the 53,000-dalton glycoprotein and labeled the detergent extract. Fig. 3 (*lane 1*) shows that the extract-contained 8-N_3-ATP-labeled proteins of 53,000 and 40,000 daltons. The labeling of these proteins in the extract had the same specificity as that seen in the intact vesicles. Calsequestrin (M_r 63,000) was not labeled in either intact vesicles or in detergent extracts of sarcoplasmic reticulum vesicles.

The 53,000- and 160,000-dalton, intrinsic glycoproteins have been shown to bind to ConA Sepharose when the deoxycholate extract is passed through this lectin affinity column (13). When the 8-N_3-ATP-labeled extract was applied to a ConA Sepharose column, the 8-N_3-ATP-labeled, 53,000- and 160,000-dalton proteins were bound by ConA Sepharose. The effluent from the ConA affinity column contained the 40,000-dalton 8-N_3-ATP-labeled protein but very little of the 53,000- or 160,000-dalton proteins (Fig. 3, *lane 2*). The photoaffinity labeled 53,000- and 160,000-dalton protein could be eluted from the column with α-methyl-D-mannoside (Fig. 3, *lane 3*) under the same conditions required for elution of glycoproteins. Binding of the 8-N_3-ATP-labeled 53,000- and 160,000-dalton proteins to ConA Sepharose was inhibited by the presence of α-methyl-D-mannoside in the extract. Incubation of 8-N_3-ATP with the effluent from the ConA column, which

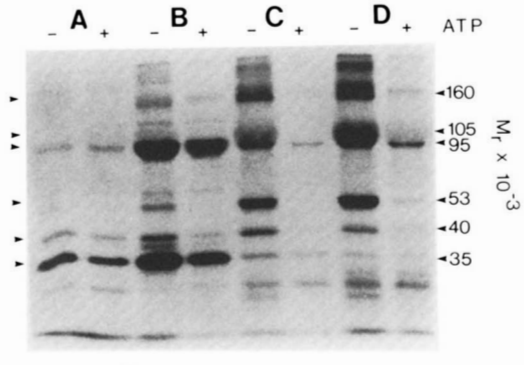

FIG. 2. **8-N_3-[α-^{32}P]ATP labeling of various fractions in the preparation of sarcoplasmic reticulum vesicles.** Membranes were pre-incubated at 4 °C as described in Fig. 1 in the absence of Ca^{2+} with 0.14 μM 8-N_3-[α-^{32}P]ATP, irradiated with UV light, and analyzed by gel electrophoresis in 7.5% acrylamide and autoradiography. *A*, crude muscle microsomes (44,000 g); *B*, light membranes; *C*, sarcoplasmic reticulum vesicles; *D*, KCl-washed sarcoplasmic reticulum vesicles. The labeled 53,000-, 105,000-, and 160,000-dalton proteins were enriched in the sarcoplasmic reticulum and KCl-washed sarcoplasmic reticulum. The labeled 40,000-dalton protein was found in the light membranes and in the sarcoplasmic reticulum.

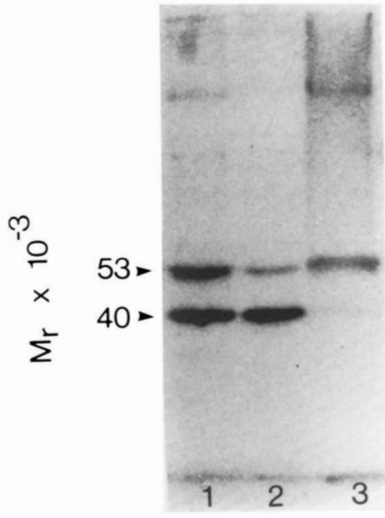

FIG. 3. **ConA affinity chromatography of the 8-N_3-[α-^{32}P] ATP labeled deoxycholate extract.** The deoxycholate extract of sarcoplasmic reticulum was labeled with 0.14 μM 8-N_3-[α-^{32}P]ATP as described in Fig. 1 in the absence of Ca^{2+}. Following UV irradiation, the labeled extract was analyzed by gel electrophoresis in 7.5% acrylamide (*lane 1*) or passed through a ConA Sepharose 4B column (*lane 2*) and analyzed by gel electrophoresis in 7.5% acrylamide. The ConA Sepharose column was then eluted with α-methyl-D-mannoside and analyzed by gel electrophoresis (*lane 3*). The 40,000-dalton labeled protein was not bound by the lectin affinity column while the 53,000-dalton labeled protein was retained by the lectin affinity column and eluted with α-methyl-D-mannoside.

contained no glycoprotein, resulted in no labeling at 53,000 or 160,000 daltons. Thus, the binding of the 8-N_3-ATP-labeled, 53,000- and 160,000-dalton protein to ConA Sepharose and their elution with α-methyl-D-mannoside indicate that the labeled proteins are glycoproteins.

For further evidence of identity between the labeled proteins and the 53,000- and 160,000-dalton glycoproteins, we have compared Endo H digestion of the two proteins. Endo H treatment of the sarcoplasmic reticulum or of the purified 53,000-dalton glycoprotein resulted in a reduction in the mass of the glycoprotein to 49,000 daltons (13). Endo H digestion of the 8-N_3-ATP-labeled sarcoplasmic reticulum showed that the 8-N_3-ATP-labeled 53,000- and 160,000-dalton proteins were also sensitive to Endo H. The mass of the 53,000-dalton 8-N_3-ATP-labeled protein was reduced to 49,000 daltons and the mass of the 160,000-dalton 8-N_3-ATP-labeled protein was reduced to 155,000 daltons upon Endo H digestion (Fig. 4).

We have also used immunoprecipitation to show that the 53,000- and 160,000-dalton 8-N_3-ATP-labeled proteins were intrinsic glycoproteins. The antiserum raised against the purified 53,000-dalton glycoprotein (10) cross-reacted with the 160,000-dalton glycoprotein and precipitated both proteins from extracts of sarcoplasmic reticulum or whole cells.[2] Sarcoplasmic reticulum was labeled with 8-N_3-ATP, the labeling reaction was stopped by the addition of the immunoprecipitation buffer which contained 1% Triton X-100, 1% Na deoxycholate, and 0.1% SDS, and immunoprecipitates were obtained within 2 h using antiserum and protein A Sepharose (10). Control antiserum did not precipitate any 8-N_3-ATP-labeled proteins. Antiserum to the 53,000-dalton glycoprotein immunoprecipitated both the 53,000- and 160,000-dalton 8-N_3-ATP-labeled protein (Fig. 5*A*). These labeled proteins

[2] Zubrzycka-Gaarn, E., Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. *J. Biol. Chem.* **258,** in press.

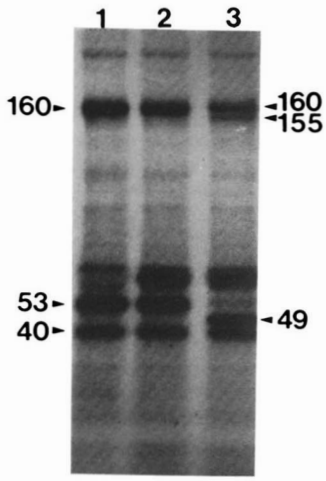

FIG. 4. **Effect of Endo H on 8-N_3-[α-^{32}P]ATP-labeled proteins.** A deoxycholate extract of sarcoplasmic reticulum was labeled as described in Fig. 1 in the absence of Ca^{2+} (*lane 1*) and then treated without (*lane 2*) or with (*lane 3*) Endo H as described in Ref. 10. The products were analyzed by gel electrophoresis in 5–15% acrylamide gradient gels and autoradiography. Endo H reduced the molecular weight of the labeled protein from 53,000 and 160,000 to 49,000 and 155,000, respectively. Some nonspecific labeling of calsequestrin (*lanes 2* and *3*) occurred during the 6-h incubation at 37 °C. This labeling was not observed when the labeling reaction was stopped by the addition of SDS sample buffer followed by boiling. Note that the molecular weight of the 40,000-dalton, 8-N_3-ATP-labeled protein was not sensitive to Endo H. *Numbers on the ordinate* represent $M_r \times 10^{-3}$.

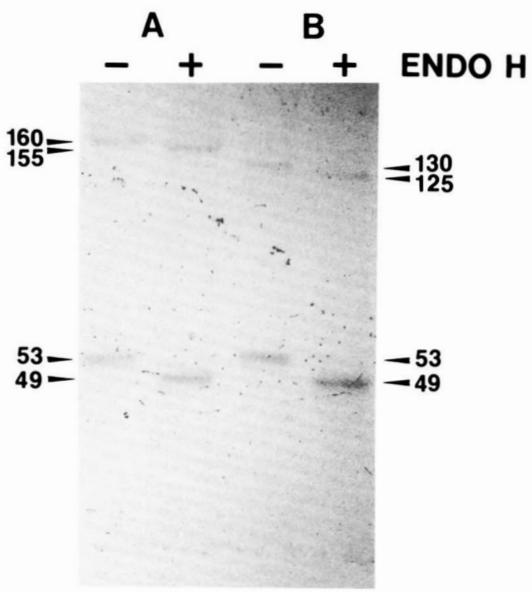

FIG. 5. **Immunoprecipitation of 8-N_3-ATP-labeled proteins with antiserum to the 53,000-dalton glycoprotein.** Deoxycholate extracts of skeletal (*A*) or cardiac (*B*) sarcoplasmic reticulum were labeled as described in Fig. 1 in the absence of Ca^{2+} and then immunoprecipitation was carried out using sheep anti-rabbit skeletal 53,000-dalton glycoprotein antiserum (according to Ref. 10). Following immunoprecipitation, the samples were treated with (+) or without (−) Endo H for 6 h at 37 °C (according to Ref. 10) and then analyzed by SDS-gel electrophoresis in 5–15% acrylamide gradient gels and autoradiography. The antiserum to the 53,000-dalton glycoprotein immunoprecipitated the 53,000- and 160,000-dalton 8-N_3-ATP-labeled glycoproteins from skeletal extracts and the 53,000- and 130,000-dalton 8-N_3-ATP-labeled glycoproteins from cardiac extracts. *Numbers on the ordinate* represent $M_r \times 10^{-3}$.

were shown to be glycoproteins by Endo H digestion of the immunoprecipitates. The molecular weights of both labeled proteins were sensitive to Endo H (Fig. 5*A*).

When cardiac sarcoplasmic reticulum was labeled with 8-N_3-ATP, radioactivity was incorporated into the 53,000 and 130,000 dalton proteins (not shown). We have previously shown that cardiac sarcoplasmic reticulum contains a 53,000-dalton glycoprotein which cross-reacts with antiserum to the skeletal glycoprotein and an Endo H-sensitive 130,000-dalton glycoprotein which is similar to the 160,000-dalton glycoprotein of skeletal muscle (15). Fig. 5*B* shows that the antiserum to the skeletal muscle 53,000-dalton glycoprotein immunoprecipitated both the 53,000- and the 130,000-dalton, 8-N_3-ATP-labeled glycoproteins from cardiac sarcoplasmic reticulum. Moreover, Fig. 5*B* shows that these immunoprecipitated proteins were glycoproteins since their molecular weights were reduced upon incubation with Endo H.

The data presented in this paper show that the 53,000- and 160,000-dalton glycoproteins of the sarcoplasmic reticulum bind 8-N_3-ATP and thus contain high affinity binding sites for ATP. The physiological roles of these ATP binding sites are not known but it is possible that the glycoproteins are membrane-bound protein kinases and that the 8-N_3-ATP is binding to the catalytic portion of these enzymes. Experiments are now in progress to determine the role of the ATP binding site on the glycoproteins and to evaluate whether there is protein kinase activity in the glycoproteins from skeletal or cardiac sarcoplasmic reticulum.

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