# Synthesis of a Radioactive Azido Derivative of Thapsigargin and Photolabeling of the Sarcoplasmic Reticulum ATPase†

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*Received January 17, 1997; Revised Manuscript Received July 22, 1997*⊗

ABSTRACT: A thapsigargin C8-derivative (ZTG) was synthesized by acylating debutanoylthapsigargin with 4-azido[*carboxyl*-$^{14}$C]benzoic acid. ZTG retains the inhibitory activity of thapsigargin (TG) with respect to the $Ca^{2+}$ ATPase of sarcoplasmic reticulum (SR). Covalent ATPase labeling was obtained by photoactivation of the ZTG azido moiety under conditions optimized to reduce nonspecific association of ZTG with SR vesicles and to approximate a matching ZTG:ATPase stoichiometry. Specific photolabeling of the $Ca^{2+}$ ATPase with ZTG was obtained with 30% efficiency and was competitively inhibited by TG. Analysis of the labeled protein and its proteolytic fragments demonstrates that the ZTG label is associated covalently with the membrane-bound portion of tryptic subfragment A1, which spans the sequence between Leu253 and Arg324 and includes segments of S3 and S4 in the stalk, the M3 and M4 transmembrane helices, and the intervening lumenal loop. This finding is in agreement with previous spectroscopic observations and mutational analysis.

Thapsigargin (TG), a plant-derived sesquiterpene lactone (Christensen et al., 1982, 1988), is a highly specific inhibitor of endo- and sarcoplasmic reticulum (SR) $Ca^{2+}$ transport ATPases (Thastrup et al., 1987a,b; Sagara & Inesi, 1991; Lytton et al., 1991; Kijima et al., 1991; Campbell et al., 1991). Due to a very high binding affinity, inhibition can be obtained with amounts of TG approximately equivalent to the ATPase stoichiometry, consistent with binding of one inhibitor molecule per molecule of enzyme (Sagara & Inesi, 1991). Furthermore, mutational and spectroscopic studies suggest that TG binds near or within the membrane region of the SR ATPase (Sumbilla et al., 1993; Norregaard et al., 1994; Hua et al., 1995). The TG binding topology, however, was never demonstrated directly. Therefore, with the experiments to be described here, we have sought to obtain direct evidence of binding topology.

A specific feature of the TG molecule (Christensen et al., 1993) is that the ester bond at C8 can be cleaved selectively by reacting thapsigargin with triethylamine. The product, debutanoyl-thapsigargin (DBTG), can be used as a reactive species for acylation. For instance, we have recently reacted DBTG with *N*-dansylglycine to obtain a fluorescent derivative which retains the inhibitory properties of thapsigargin and can be used for fluorescence energy transfer within the SR ATPase (Hua et al., 1995). We report here the synthesis of a radioactive azido derivative by acylation of DBTG with 4-azido[*carboxyl*-$^{14}$C]benzoic acid and describe its use for photolabeling and determination of binding topology within the ATPase protein.

## MATERIALS AND METHODS

Chemical reagents were obtained from Sigma unless specified otherwise. *p*-Amino[*carboxyl*-$^{14}$C]benzoic acid (20 mCi; 0.4 millimol) was purchased from Amersham, non-radioactive 4-azidobenzoic acid from Molecular Probes, and TG from LC Laboratories. Sarcoplasmic reticulum vesicles were prepared from rabbit leg muscles as described by Eletr and Inesi (1972). Protein concentration was measured by the method of Lowry et al. (1951).

### *Synthesis of Radioactive 8-(4-Azidobenzoyl)thapsigargin (ZTG)*

*1. Preparation of Radioactive 4-Azidobenzoic acid.* An aliquot (54.8 mg = 0.4 millimole) of $^{14}$C-carboxyl labeled 4-aminobenzoic acid was suspended in 1.1 mL of concentrated hydrochloric acid while it was stirred on ice. Sodium nitrite (59.4 mg = 0.86 mmol) dissolved in 1.0 mL of cold water was added to the suspension to generate a diazonium ion. The mixture was kept on ice and was stirred until the material was completely dissolved. Sodium azide (78.0 mg = 1.2 mmol) dissolved in 0.4 mL of cold water was then added dropwise over half an hour while stirring, and the mixture was kept stirring on ice for another hour. The white precipitate formed over this period of time was collected by vacuum filtration on a Pyrex funnel with fritted disc, washed thoroughly with water, and dried under vacuum. The yield was approximately 70%.

Analysis of the radioactive 4-azidobenzoic acid product yielded a single spot on TLC, with an $R_f$ value identical to that of non-radioactive azidobenzoic acid standard purchased from Molecular Probes. Furthermore, product and standard coeluted within the same peak when subjected to HPLC, using a linear gradient of 0.5% acetic acid in water and 0.5% acetic acid in methanol for elution (monitored at 254 nm wavelength).

*2. Selective Hydrolysis of Thapsigargin (TG).* TG (2.0 mg = 3.1 $\mu$mol) was dissolved in 0.2 mL of methanol and 10 $\mu$L of triethylamine, and incubated with stirring at room temperature for about 5 h in a small capped tube. The reaction was followed by HPLC analysis of serial samples, using a linear gradient of 0.5% acetic acid in water and 0.5% acetic acid in methanol for elution (monitored at 230 nm).

† This work was supported by NIH Grant PO1HL-27867.
⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1997.

S0006-2960(97)00105-0 CCC: $14.00 

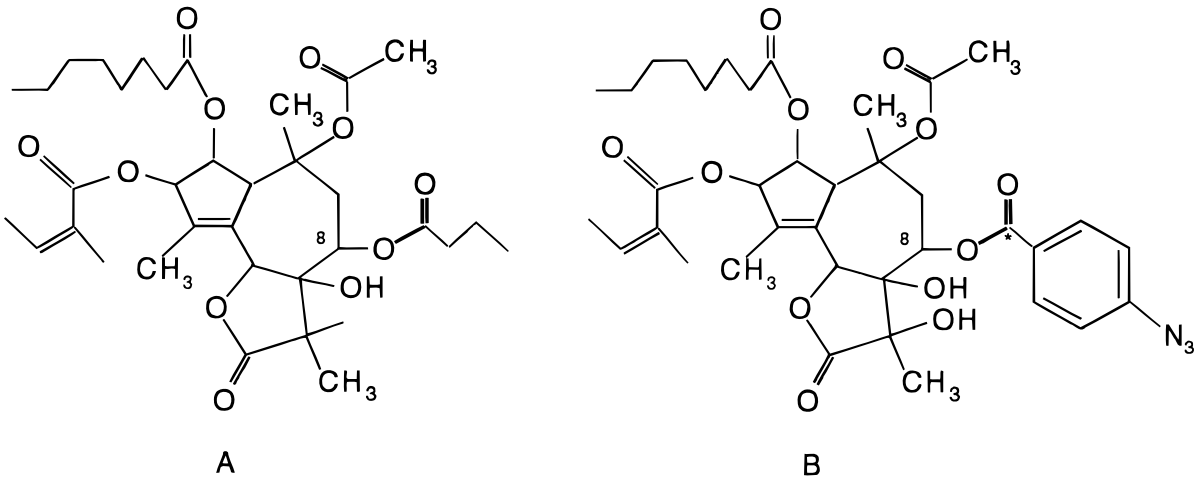


FIGURE 1: Structures of native thapsigargin (TG, structure A) and 8(4-azidobenzoyl)thapsigargin (ZTG, structure B).

Under these conditions, the retention times for TG and debutanoylthapsigargin (DBTG) were 23.3 and 17.4 min, respectively. When the TG peak was almost gone and the DBTG peak had developed to a maximum, the product was collected by evaporation of solvent by speedvac. The yield of product was approximately 90%.

*3. Acylation of DBTG*. A 1.6 mg amount of DBTG was dissolved in 50 $\mu$L of methylene dichloride, to which 0.75 mg of dimethylaminopyridine and 1.5 mg of radioactive 4-azidobenzoic acid were added. Following 5 min of stirring on ice, 1.38 mg of dicyclohexylcarbodiimide dissolved in 10 $\mu$L of $CH_2Cl_2$ was added. After 4 h of incubation with stirring on ice, the reaction was incubated at 4 °C overnight. The dicyclohexylurea precipitate was filtered, and the solvent was evaporated under vacuum. The residue was dissolved in ethylene dichloride, and the product was purified by HPLC using the same elution medium as before, monitoring elution at 230 nm (for the TG moiety) and 254 nm (for the benzoyl moiety). The retention time for 8(4-azidobenzoyl)thapsigargin (ZTG) was 39.15 min. A 0.78 mg amount of radioactive ZTG was obtained under these conditions, corresponding to a yield of approximately 41% relative to the DBTG used for the acylation reaction.

*Measurements of ZTG Association with SR Vesicles*

ZTG association with SR vesicles was measured by first equilibrating 2.0 mg of SR protein with 1.0 mL of 20.0 mM MOPS, pH 7.0, 80.0 mM KCl, 5.0 mM $MgCl_2$, and various concentrations of ZTG. After 5 min of incubation, this mixture was loaded on a column containing Sephadex G25-150 (particle size: 50−150 $\mu$m) (1.5 × 40.0 cm) pre-equilibrated with 8 column volumes of the same medium but without ZTG. Elution was monitored for protein at 280 nm, and samples were collected for determination of radioactivity.

*Photolabeling*

SR vesicles (1 mg/mL) in 20 mM MOPS, pH 7.0, 80.0 mM KCl, 5.0 mM $MgCl_2$, 10 $\mu$M ZTG, and 20% (v:v) glycerol, were placed in quartz cuvettes, chilled in ice, and illuminated for 1 min with UV light from a UV hand-held mineral light lamp (UVSL-25; 4W/115V). Alternatively, the chromatography (as explained above) elution fractions (1.0 mL each) containing SR protein and bound ZTG were collected and, after addition of 0.2 mL glycerol, used for illumination. The illuminated vesicles were pelleted by centrifugation at 100000*g* for 45 min and resuspended in 10 mM MOPS buffer, pH 7.0, and 10% sucrose.

*ATPase Activity*

Hydrolytic cleavage of ATP by the SR ATPase was measured with a coupled enzymatic assay (Horgon et al., 1972), and monitored at 340 nm in an Aminco DW2 recording spectrophotometer. The composition of the reaction mixture is given in the legend to Figure 5.

*Tryptic Digestion and Peptide Characterization*

Mild trypsin digestion (for characterization of large fragments of the ATPase) was carried out with a trypsin:SR protein ratio of 0.005 (w:w). The reaction mixture contained 2.0 mg of SR protein/mL, 0.01 mg of trypsin/mL, and 20.0 mM MOPS, pH 7.0. The reaction was incubated at 25 °C for 1−20 min and was stopped by the addition of 0.3 mg of soybean trypsin inhibitor. The tryptic fragments were resolved by SDS−PAGE according to Weber and Osborn (1969).

Extensive trypsin digestion for removal of the extramembranous ATPase region from labeled vesicles was carried out in a mixture containing 1.0 mg of SR protein (whole labeled vesicles)/mL, 0.25 mg of trypsin/mL (trypsin:SR protein ratio = 0.25, w:w), 50 mM Tris/HCl, pH 8.1, 0.25 M sucrose. After 10 min of incubation at 37 °C, the reaction was stopped by the addition of 2.5 mg of soybean trypsin inhibitor. The peptide fragments still associated with the membrane vesicles were separated from the soluble peptide fragments by centrifugation at 110000*g* for 1 h (Shin et al., 1994). The sediment was then solubilized and subjected to tricine SDS−PAGE as described by Shin et al. (1994).

Electrophoresis-purified peptides were sequenced using a Hewlett Packard protein sequencer model G1005A equipped with an online HP 1090 series II/L system to analyze the PTH derivatives. The PTH derivatives of labeled residues were not recovered during sequencing but were identified by a gap or reduced yield in the sequence. Sequenced ATPase segments were identified by comparison with the complete cDNA-derived amino acid sequence from rabbit skeletal muscle (MacLennan et al., 1985).

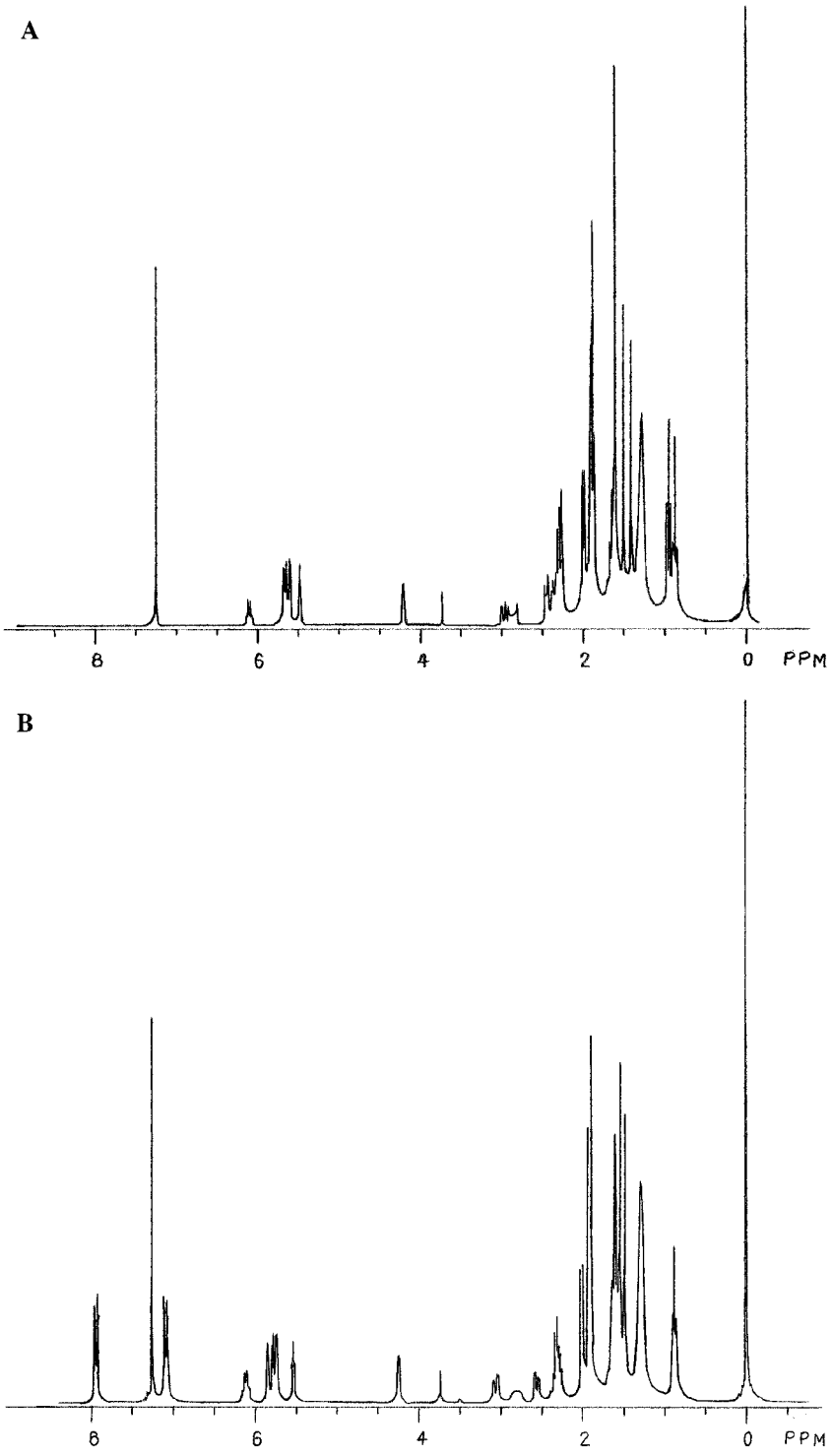


FIGURE 2: Proton NMR spectra of TG (A) and ZTG(B). The butanoyl protons of TG are visible at 0.9 ppm for $CH_3$, 1.6 ppm for $CH_2$, and 2.3 ppm for another $CH_2$ (A). The benzene protons of ZTG are visible at 7.1 and 7.9 ppm (B).

## RESULTS

### *Chemical Characterization of the ZTG Product*

The structures of native TG and of the ZTG derivative are shown in Figure 1, and the proton NMR spectra of TG and ZTG product in Figure 2. It is clear from the spectra that the butanoyl moiety protons ($CH_3$ triplet at 0.9 ppm; $CH_2$ broad peak at 1.6 ppm; $CH_2$ multiple peaks at 2.3 ppm) of TG are replaced by azidobenzoyl protons (7.1, 7.9 ppm) in ZTG. Furthermore, it is shown in Figure 3 that the HPLC elution properties of the azidobenzoyl derivative are changed by exposure to UV light, demonstrating that the analog is photolabile, which is as expected of an azido derivative.

### *Inhibitory Properties of the Azidobenzoyl TG Derivative*

The concentration dependence of the inhibitory effect of ZTG on the SR ATPase is shown in Figure 4. ZTG has a somewhat lower affinity than TG for the ATPase ($K_I = 50$ nM, as compared to 10 nM for TG). It is noteworthy, however, that the affinity for the enzyme is actually increased by the addition of the azidobenzoyl moiety to DBTG, which is the intermediate obtained by cleavage of the butanoyl

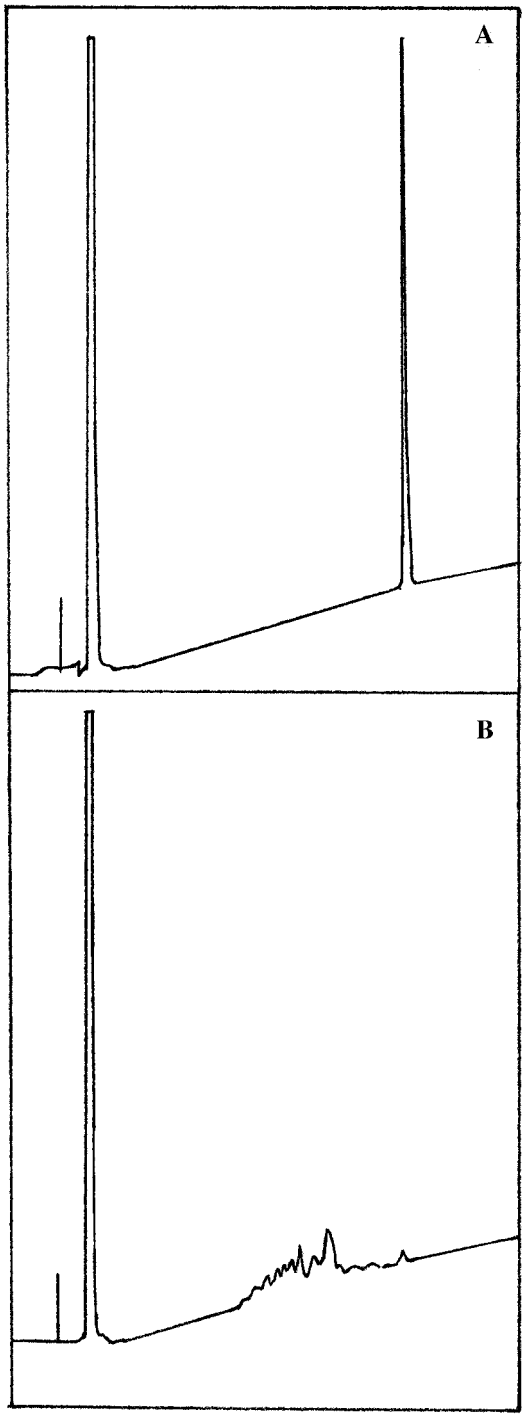


FIGURE 3: HPLC elution of ZTG before and after UV exposure. Note the disappearance of the ZTG peak due to photolysis.

moiety from TG. Therefore, the azidobenzoyl moiety is a rather satisfactory replacement of the native butanoyl moiety, with regard to the steric requirement for specific binding to the enzyme. Replacement of the butanoyl moiety with the fluorescent dansyl group (Hua et al., 1995) is less satisfactory (Figure 4).

The relatively high affinity of ZTG for the ATPase indicates that the derivative can be used for binding and photolabeling studies.

*ZTG Association with SR Vesicles*

Previous kinetic studies (Sagara & Inesi, 1991) indicate that the TG inhibitory effect is related to stoichiometric

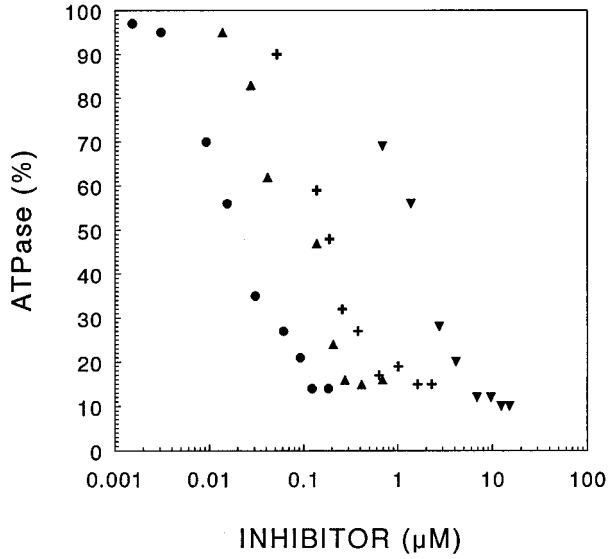


FIGURE 4: Inhibition of SR ATPase by TG, ZTG, DBTG, and DTG (dansylglycine TG). ATPase activity was measured in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 20 $\mu$M $CaCl_2$, 2 mM A23187 ionophore, 10.9 $\mu$g of SR protein/mL, 0.1 M phosphoenolpyruvate, 5000 units of pyruvate kinase/mL, 5000 units of lactic dehydrogenase/mL, 15 mM nicotinamide adenine dinucleotide, reduced form, and 0.2 mM ATP. TG = ●; ZTG = ▲; DBTG = +; DTG = ▼.

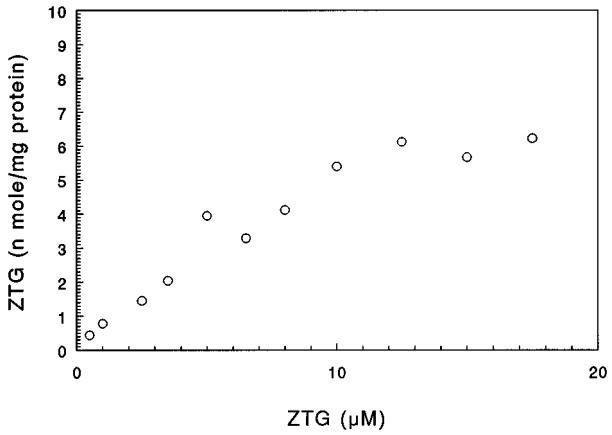


FIGURE 5: ZTG association with SR vesicles. Incubation mixture: 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1.0 mg of SR protein/mL, and ZTG as indicated. Following a 5 min incubation, the mixture was loaded in a Sephadex G-25-150 column (1.5 × 40.0 cm) and eluted with 20 mM MOPS, pH 7.0, 80 mM KCl, and 5 mM $MgCl_2$. The elution fractions containing proteins were pooled and centrifuged at 100000*g* for 45 min. The sediment was then collected for measurement of radioactivity and protein concentration. The SR vesicles used in these experiments yield maximal levels of 5 nmol of phosphorylated intermediate/mg of protein upon utilization of ATP.

interaction of TG with the SR ATPase. Taking advantage of the radioactive analog synthesized for the present studies, we assessed the association of ZTG with the vesicles separated by centrifugation from the equilibration medium (in the absence of photolabeling). Binding increased linearly as the ZTG concentration increased, even above the level yielding total functional inhibition. These results suggest that ZTG (and most likely TG as well) partitions in the membrane phase of the SR vesicles, in excess of specific ATPase site saturation.

In the light of the results described above, we assumed that total amount of ZTG bound to SR vesicles included a

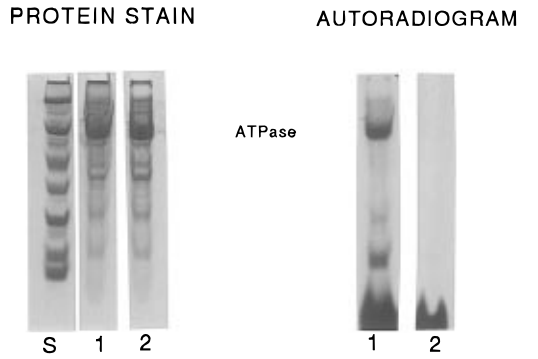


FIGURE 6: ZTG photolabeling of SR ATPase. A labeling mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1.0 mg of SR protein/mL, 10.0 $\mu$M ZTG, and 20% (v:v) glycerol was placed in a 1.0 cm ice-cooled quartz cuvette with an aluminum foil backing. This mixture was either illuminated with a hand-held mineral UV lamp (4 W, 115 V) for 1 min at 1−2 cm distance (lane 1) or not illuminated to obtain a control sample (lane 2). The samples were diluted 10-fold with a buffer containing 10 mM MOPS, pH 7.0, and 10% (w/v) sucrose and sedimented at 100000*g* for 45 min. The pellet was resuspended in the same buffer and subjected to SDS gel electrophoresis. The gels were stained for protein (left) and used for development of autoradiographs (right).

specific component and a nonspecific component, the latter exhibiting faster dissociation kinetics relative to the very slow dissociation from specific inhibitory sites (Sagara et al., 1992a). We sought to eliminate the nonspecific component by passing the binding equilibrium mixture through a chromatography column (see Materials and Methods) with an elution medium containing no ZTG. A discrete amount of radioactive ZTG eluted with the protein peak, with little or no radioactivity trailing this peak, due to adsorption of dissociated ZTG to the gel matrix. When we plotted the ZTG bound to the protein peak as a function of increasing ZTG concentrations in the initial binding medium, we obtained a saturable relation, with a maximal level of ZTG roughly matching the ATPase stoichiometry (Figure 5) which in our preparation of SR vesicles is approximately 5 nmol/mg of protein. We cannot exclude that, even under these conditions, the ZTG label may be distributed in specific and nonspecific components. Nevertheless, a matching ZTG:ATPase stoichiometry turned out to favor the specificity of photolabeling.

It should be pointed out that the concentration dependence of ZTG binding (Figure 5) reflects the ZTG concentration in the initial 1.0 mL reaction mixture, which was then diluted by the large elution volume of the chromatography column. For this reason, the $K_{1/2}$ in the binding experiment (Figure 5) is higher than the $K_I$ in the ATPase inhibition experiment (Figure 4).

*Photolabeling*

It is shown in Figure 6 that when photolabeled vesicles were solubilized with sodium dodecyl sulfate, and when the solubilized proteins were subjected to gel electrophoresis, the resulting ATPase band was radioactively labeled. No label is associated with the ATPase band of control samples which were incubated with radioactive ZTG without being subjected to illumination. A significant amount of label is also apparent on the 32 and 20 kDa bands which were identified by MacLennan et al. (1973) as acidic proteins. It is important to note that no radioactivity is associated with either the ATPase or the acidic proteins of control samples which were incubated with radioactive ZTG without being subjected to illumination. In total, 52% of the photoactivation dependent labeling is on the ATPase, 14% with the 32 kDa band, and 34% with the 20 kDa band. No ZTG label is associated with the 54 kDa band or with calsequestrin.

It is also shown in Figure 6 that a significant amount of non-reacted (i.e., present even in the absence of photoactivation) ZTG is present at the end of the gels. In order to reduce nonspecific association of ZTG, we then performed our photolabeling experiments on SR vesicles passed through a Sephadex column. As explained above, the vesicles treated in this manner retain an amount of ZTG approximating the ATPase stoichiometry.

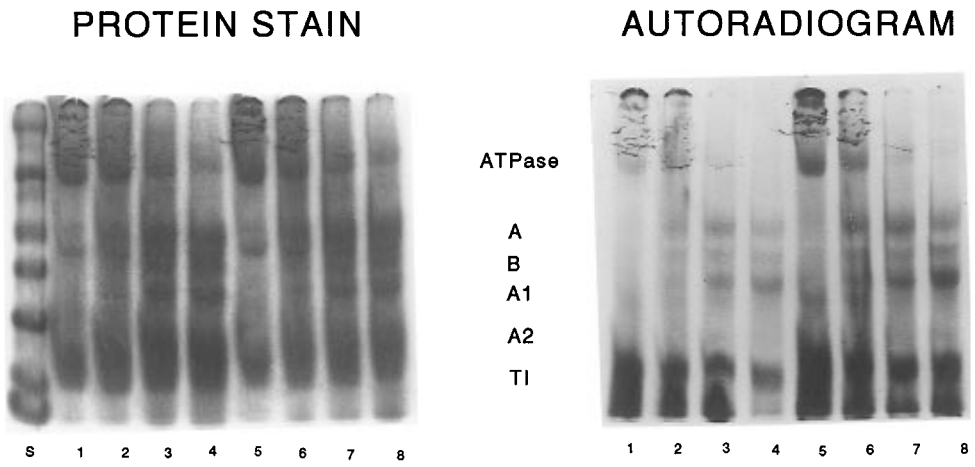


FIGURE 7: Electrophoretic separation of labeled ATPase and large tryptic fragments. A labeling mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 2.0 mg of SR protein/mL, and 10.0 $\mu$M ZTG (corresponding to 100% of available ATPase sites), with or without 100 $\mu$M TG, was loaded in a Sephadex G-25-150 column (1.5 × 40.0 cm) and eluted with 20 mM MOPS, pH 7, 80 mM KCl, and 5 mM $MgCl_2$. The elution fractions containing proteins were pooled, and 20% (v/v) glycerol was added. Aliquots were placed in a 1 cm quartz ice-cooled cuvette covered with aluminum in the back and illuminated with a hand-held mineral UV lamp (4 W, 115 V) for 1 min at a 1−2 cm distance. The mixture was diluted 10 times with a buffer containing 10 mM MOPS, pH 7.0, and 10% (w/v) sucrose. The vesicles were then sedimented by centrifugation and resuspended in the same buffer to a concentration of 10 mg of SR protein/mL. Samples were then prepared for "light trypsin digestion" (see Materials and Methods), which was allowed to proceed at 25 °C for 0 (lanes 1 and 5), 1 (lanes 2 and 6), 3 (lanes 3 and 7), or 20 (lanes 4 and 8) min. Samples were then collected for SDS gel electrophoresis, followed by protein staining (left) and autoradiography (right). Lanes 1−4 are derived from samples incubated in the presence of radioactive ZTG as well as non-radioactive TG; lanes 5−8 from samples incubated only with radioactive ZTG.

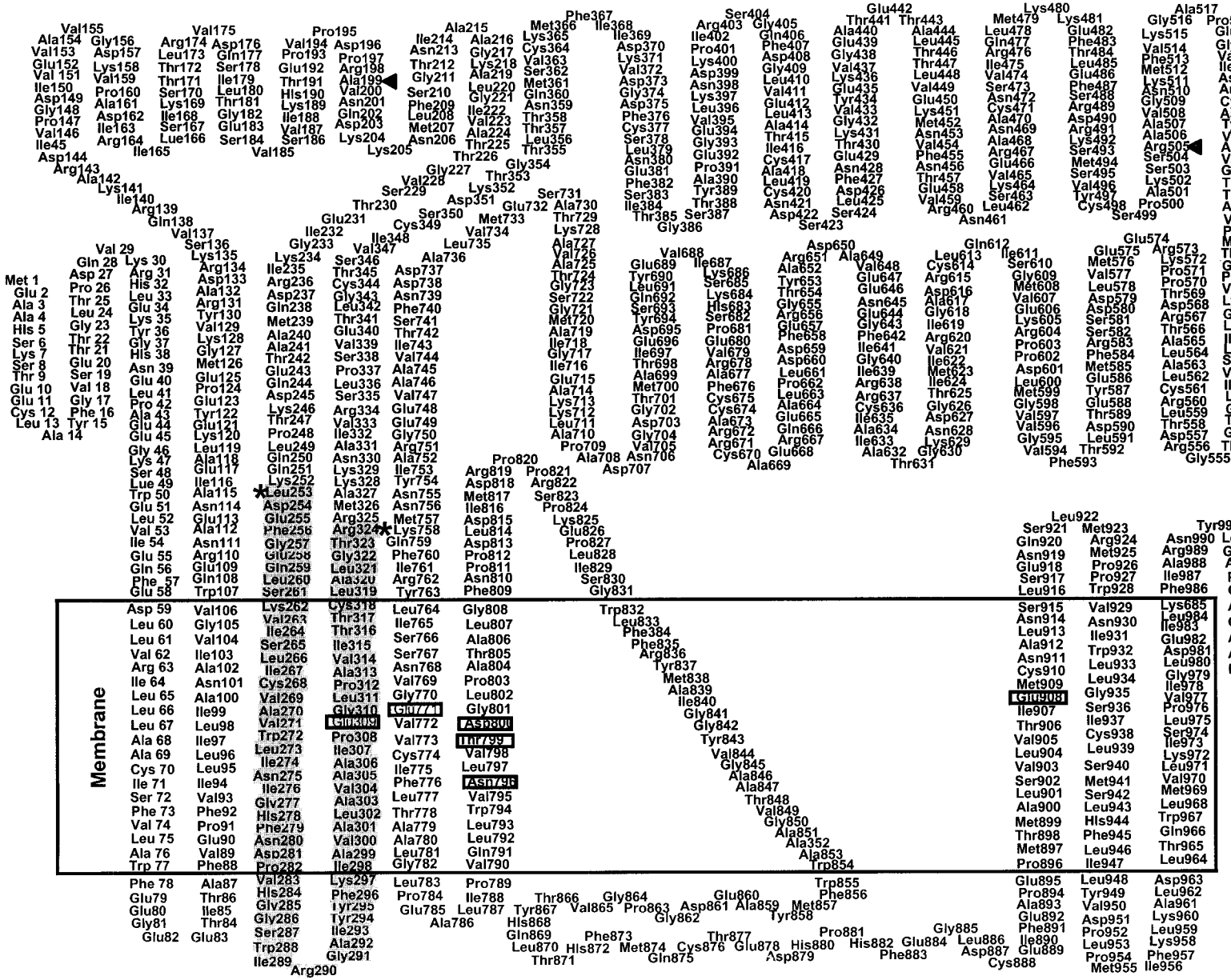


FIGURE 8: Rabbit skeletal muscle SR ATPase amino acid sequence and its proposed membrane distribution. The diagram is derived from MacLennan et al. (1985). Tryptic fragment A spans the sequence between Met1 and Arg505 (triangle) and subfragment A1 between Ala199 (triangle) and Arg505 (triangle). The asterisks correspond to the amino (Leu253) and carboxyl (Arg324) terminals of the labeled segment (shaded in the diagram) of subfragment A1. This labeled segment includes portions of S3 and S4 in the stalk, the M3 and M4 transmembrane helices, and the intervening lumenal loop. The residues (Glu309, Glu771, Asn796, Thr799, Asp800, and Glu908) involved in $Ca^{2+}$ binding (Clarke et al., 1987) are denoted by enclosures in boxes. Asp351 is the residue undergoing phosphorylation upon ATP utilization by the ATPase.

Addition of TG to the vesicles before radioactive ZTG largely prevents radioactive labeling of the ATPase (compare the ATPase in sample 1 to that of sample 5 in Figure 7), demonstrating that the TG and ZTG ligands compete for the same saturable and presumably specific ATPase sites. On the other hand, ZTG labeling of the acidic proteins is not prevented by TG (compare samples 1 and 5 of Figure 7), suggesting that this labeling component is not specific.

*Identification of Labeled Peptides*

We eluted the ATPase band from the gels for further characterization of the labeled protein. It is known that limited ATPase digestion with trypsin yields two main fragments (Migala et al., 1973; Stewart & MacLennan, 1974; Inesi & Scales, 1974) referred to as A and B. In turn, fragment A yields subfragment A1 and A2. The A1 fragment has a MW of approximately 35 000 and spans the ATPase sequence from Ala199 to Arg505. It is shown in Figure 7 that different levels of radioactive ZTG label are associated with fragments A and B and with subfragment A1. Densitometric evaluation of the peptide bands in Figure 7 indicates that 83% of the ATPase ZTG label is associated with tryptic fragment A, and 17% with tryptic fragment B. Upon further digestion the label of fragment A remains with subfragment A1.

With respect to the putative membrane topology derived from sequence analysis (MacLennan et al., 1985), subfragment A1 includes the M3 and M4 transmembrane helices, a short extramembranous loop between M3 and M4 facing the SR lumen, and two larger extramembranous segments facing the cytosol on the amino terminal side of the M3 membrane-spanning helix, and on the carboxyl terminal side of M4 (Figure 8). We then performed experiments to find out whether the covalent ZTG label was associated with the extramembranous or membrane bound segment of the labeled subfragment A1. For this purpose, we digested extensively photolabeled SR vesicles (i.e., without detergent solubilization and electrophoresis) with trypsin. Digestion under these conditions cleaves off the entire extramembranous region of the ATPase, leaving the membrane-bound region of the ATPase still associated with the vesicles (Inesi & Scales, 1974). Extramembranous and membrane bound peptides can then be separated by sedimentation of the residual vesicles in the centrifuge. It was demonstrated by Shin et al. (1994), and confirmed by our own sequence analysis, that in this procedure the A1 subfragment is cleaved above the S3−M3 and S4−M4 interfaces, leaving with the membrane-bound fraction the peptide segment between Leu253 and Arg324, which includes short segments of S3 and S4, the entire M3 and M4 transmembrane helices, and the intervening lumenal loop (Figure 8).

It is shown in Table 1 that 6.63 nmol of ZTG/mg of protein is found in the total digest of the photolabeled SR vesicles, in conformity with the experiments shown in Figure 5. However, of this ZTG only 1.52 nmol can be considered specific label inasmuch as its covalent attachment to the ATPase is prevented by TG. Since our preparation of SR vesicles contains approximately 5 nmol ATPase per mg of protein, we conclude that 30% of the specific ATPase sites are photolabeled. Nearly all of the label is found in the membrane-bound fraction, and that includes the specific label. It is then apparent that the ZTG label resides within the ATPase segment between Leu253 and Arg324 (shaded in the diagram of Figure 8). Furthermore, on the basis of the experiments represented by Figures 6 and 7, we conclude that the remaning ZTG (5.11 out of 6.63 nmol) is partly non-reacted but still partitioned in the lipid phase, partly attached to SR proteins other than the ATPase, and partly attached to nonspecific sites of the membrane-bound ATPase region.

Table 1: Distribution of Radioactive ZTG Label in the Extramembranous and Membrane-Bound Regions of the SR ATPase[a]

| | ZTG (nanomoles) | | |
|---|---|---|---|
| sample | TG (−) | TG (+) | "specific" |
| total digest | 6.63 ± 0.08 | 4.91 ± 0.09 | 1.72 |
| supernatant | 1.04 ± 0.09 | 0.87 ± 0.04 | 0.17 |
| sediment | 5.45 ± 0.12 | 3.93 ± 0.08 | 1.52 |

[a] 1.0 mg of SR protein (labeled with ZTG in the absence or in the presence of 100 $\mu$M TG, as explained for Figure 7) was suspended in the buffer composed of 0.25 M sucrose, 50 mM Tris/HCl (pH 8.10), and digested with trypsin at 1:4 (w/w) ratio of enzyme to protein at 37 °C for 10 min. Digestion was stopped with addition of trypsin inhibitor and the mixture was centrifuged at 110000*g* for 1 h. Total mixture, supernatant, and resuspended pellet were then sampled for radioactivity. Each value was derived from eight samples which had been derived from two experiments. The difference between labeling in the absence and in the presence of TG is consider to be "specific" labeling. Note that 1 mg of SR protein contains approximately 5.0 nmol of ATPase and, therefore, that 1.52 nmol of "specific" labeling corresponds to 30% of the available ATPase sites.

## DISCUSSION

An intriguing feature of the SR ATPase inhibition by TG is that in addition to affecting the overall enzyme and transport activity, it interferes with specific partial reactions of the catalytic cycle, independent of each other. In fact, it can be shown that TG inhibits $Ca^{2+}$ binding by the ATPase in the absence of ATP, and ATPase phosphorylation by $P_i$ in the absence of $Ca^{2+}$ (Sagara et al., 1992a), under conditions permitting each of these two partial reactions to occur and reach equilibrium without going through the entire catalytic cycle. Since the $Ca^{2+}$ binding domain resides within the membrane region of the ATPase, and the phosphorylation (Asp351) domain resides within the extra membranous cytosolic region, it is apparent that the TG inhibitory effect is extended over an approximately 50 Å distance separating the two functional domains. A long-range effect of TG on the ATPase molecule is also manifested by its effect on the ATPase tendency to form ordered arrays (Sagara et al., 1992b) for electron diffraction studies (Stokes & Lacapere, 1994) and to acquire features attributed to an E2 conformation (de Meis & Vianna, 1979; Wictome et al., 1995).

The long-range effect of TG leaves open the question of its binding topology within the ATPase molecule. Spectroscopic and mutational experiments suggest that the TG binding domain resides within or near the membrane bound region of the ATPase. Firstly, the occurrence of energy transfer between a fluorescent TG derivative and ATPase tryptophanyl residues at the membrane interface, as well as the absence of energy transfer between the fluorescent TG derivative and a fluorescein isothiocyanate label of the extra membranous ATPase region, suggest that the fluorescent derivative resides within or near the SR membrane (Hua et al., 1995). Secondly, chimeric exchange of the larger extra membranous segment of the $Ca^{2+}$ ATPase with the corre-

sponding region of the $Na^+,K^+$ ATPase, yields an enzyme which is still sensitive to TG, suggesting that the extra membranous ATPase region (with the possible exception of the smaller extramembranous segment) is not involved in TG binding since the $Na^+,K^+$ ATPase is not sensitive to TG (Sumbilla et al., 1993).

As for a more precise localization of the TG binding site, it was reported that chimeric exchange of the M3 transmembrane segment of the $Ca^{2+}$ ATPase with the corresponding segment of the $Na^+,K^+$ ATPase eliminates TG sensitivity (Norregaard et al., 1994), suggesting that the M3 segment is either involved in binding TG or otherwise involved in transmitting the inhibitory effect of TG.

Synthesis of a radioactive azido derivative of TG has made it possible to label covalently the ATPase for analysis of the TG binding topology. Our photolabeling experiments demonstrate a specific ZTG label site within the peptide intervening between Leu253 and Arg324 (shaded in the diagram of Figure 8). This includes segments of S3 and S4 in the stalk, the M3 and M4 transmembrane helices, and the intervening lumenal loop. Such a topology of TG binding is in agreement with the spectroscopic and mutational studies listed above. It is then apparent that the inhibitory effect of TG on $Ca^{2+}$ binding is likely to be produced by perturbation of the neighboring transmembrane helical cluster (M4, M5, M6, and M8), which has been identified with the $Ca^{2+}$ binding domain (Clarke et al., 1989), and of the stalk segments which are involved in the functional linkage of $Ca^{2+}$ binding and phosphorylation domains (Zhang et al., 1995).

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BI970105N