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Arylisothiocyanate Modification of Sarcoplasmic Ca^{2+}-Stimulated ATPase

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Abstract

The related probes phenylisothiocyanate and *p*-sulfophenylisothiocyanate possess comparable reactivity with nucleophiles but are dissimilar in their solubility characteristics. The reagents are utilized to topologically characterize the sites of covalent interaction with the Ca^{2+}-stimulated ATPase of sarcoplasmic reticulum membranes. The hydrophobic probe phenylisothiocyanate binds covalently to the membrane-integrated protein. The extent of covalent interaction of this probe is reduced to a limited level of label incorporation by either preincubation with *p*-sulfophenylisothiocyanate or by exposing the labeled protein to alkaline reductive conditions. With respect to the chemical nature a dual interaction of phenylisothiocyanate is postulated. Phenylisothiocyanate modifies the Ca^{2+}-ATPase hydrophobically. In addition, aqueous-exposed nucleophiles (cysteine thiols) interact with both arylisothiocyanates. Inhibition of the Ca^{2+}-stimulated ATPase activity is effected by either probe.

Key Words: sacroplasmic Ca^{2+}-stimulated ATPase; phenylisothiocyanate; *p*-sulfophenylisothiocyanate; sarcoplasmic reticulum; membranes; hydrophobic.

Introduction

The growing interest in structural and functional aspects of membrane proteins requires selective labeling techniques applicable for the discernible domains of amphipathic protein molecules. An innumerable amount of reagents and various techniques are presently available for the modification of those parts of membrane constituents which are accessible from the aqueous medium [1–4]. In contrast, the investigation of the hydrophobic membrane domain has been explored to date by mainly nonspecifically

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interacting hydrophobic photolabels, i.e., arylazides and aryldiazirines [5–8]. Consequently, specific labeling procedures for the apolar membrane phase are urgently needed not only for topological but also for structural and functional investigations.

To elucidate both structural and functional aspects of membrane proteins, the use of group-specific hydrophobic protein reagents, i.e., arylisothiocyanates, has been proposed [9]. The selective interaction of these reagents is based on pH-controlled reactivity with proteinaceous nucleophiles. The chemically reactive form is the deprotonated state of the nucleophile [10]. Lysine ϵ-amino groups present in the apolar membrane phase are the most likely candidates for covalent hydrophobic modification by arylisothiocyanates. Amino groups exposed to the aqueous phase are not reactive with the reagents due to protonation at neutral pH. The possible modification of cysteine thiols in the aqueous phase by phenylisothiocyanate can be corrected for by pretreatment of the membranes with water-soluble hydrophilic *p*-sulfophenylisothiocyanate.

Utilizing this group-specific labeling technique, stoichiometric modification of bacteriorhodopsin [11], topologically discernible labeling of erythrocyte anion transport protein [12, 13] and covalent binding of phenylisothiocyanate to beef heart cytochrome *c* oxidase [14] have been reported. In this study the previously observed covalent interaction of phenylisothiocyanate with sarcoplasmic Ca^{2+}-ATPase [9] is further analyzed. The existence and minimum number of hydrophobically located nucleophilic groups in the sarcoplasmic reticulum Ca^{2+}-ATPase is investigated. Additionally, the Ca^{2+}-ATPase has been chosen as a representative of a functionally defined membrane-integrated protein whose structural features permit specific examination of the implication of abundantly present cysteine thiol functions [15] on the arylisothiocyanate modification.

Methods and Materials

Materials

Fragmented sarcoplasmic reticulum was prepared from rabbit white muscle [16]. Phenyl-[^{14}C]-isothiocyanate (12.5 Ci/mol) was obtained from Amersham, England. The Ca^{2+}-ionophore A 23187 was a gift from Eli Lilly Research Laboratory, Ind. USA. All chemicals were of the highest purity commercially available.

Methods

Phenylisothiocyanate Labeling of Sarcoplasmic Reticulum Membranes. Fragmented sarcoplasmic reticulum membranes were labeled at a protein concentration of 4 mg/ml with various amounts of phenyl-[^{14}C]-isothiocyanate (1 hr, 37°C in 10 mM sodium phosphate buffer, pH 7.3, stirred suspension). Upon incubation the membranes were sedimented (30 min, 100,000 *g*, 4°C) and washed twice with 10 mM sodium phosphate buffer, pH 7.3.

Partial Purification of Phenylisothiocyanate-Labeled Ca^{2+}-ATPase, Chloroform–Methanol Extraction, Reduction, and Carboxymethylation. Labeled membranes were solubilized in 2% SDS, 5 mM dithiothreitol, and 10 mM sodium phosphate buffer, pH 7.3, during 15 min at 37°C. Following Sepharose 4B chromatography in 25 mM sodium phosphate buffer, pH 7.3, containing 0.2% SDS and 0.1% (v/v) mercaptoethanol, the first of three separated peaks (absorbance 280 nm, radioactivity) was concentrated by vacuum dialysis (Ultra thimbles UH 100/25, Selectron). The dialysis buffer contained 0.2% SDS and 25 mM sodium phosphate buffer, pH 7.3. As judged by SDS gel electrophoresis, 95% of the total protein was present as Ca^{2+}-ATPase.

For the determination of lipid-associated, noncovalently bound radioactivity present in the protein fraction, aliquots were diluted with water to a final concentration of 0.02% SDS and extracted by chloroform–methanol according to Renkonen [17]. To distinguish between irreversibly (lysine ϵ-amino) and reversibly (cysteine thiol) bound label, the fraction containing purified Ca^{2+}-ATPase was repeatedly reduced and carboxymethylated with dithiothreitol (1 mM) and iodoacetic acid (3 mM) in 6 M guanidine · HCl, 0.5 M Tris, and 2 mM EDTA at pH 8.0 as described by Allen and Green [18]. The samples were then exhaustively dialyzed, the final dialysis solution containing 0.2% SDS and 25 mM sodium phosphate buffer, pH 7.3.

Phenyl-[^{14}C]-Isothiocyanate Incorporation into Ca^{2+}-ATPase as Analyzed by Gel Electrophoresis. Labeled membranes were solubilized in 1% SDS and 10 mM sodium phosphate buffer, pH 7.3, and the protein was separated by SDS gel electrophoresis. The relative amount of electrophoretically separated Ca^{2+}-ATPase protein was measured by triangulation of the area in the densitometric scan of Coomassie blue stained gels (absorbance 560 nm). The Ca^{2+}-ATPase band was then cut out and dissolved by incubation with H_2O_2/ammonia 99:1 (v/v). The ratio of radioactivity per peak area (cpm/cm^2) served as a relative measure for label incorporation into the Ca^{2+}-ATPase protein.

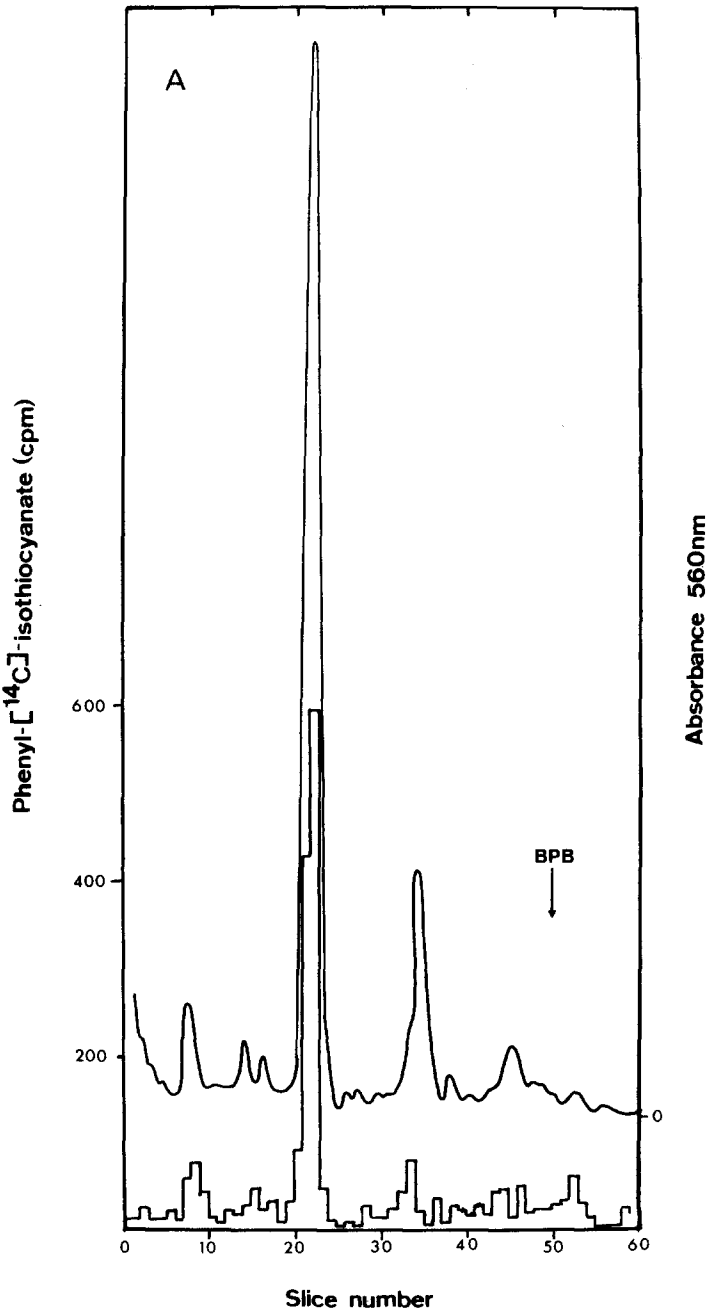

Fig. 1. Gel electrophoretic pattern of phenyl-[^{14}C]-isothiocyanate-labeled sarcoplasmic reticulum membranes and 95% purified Ca^{2+}-ATPase. Sarcoplasmic reticulum membranes were labeled with 2.4 mM phenyl-[^{14}C]-isothiocyanate by incubation at 37°C for 1 h in 10 mM sodium phosphate buffer, pH 7.3. The repeatedly sedimented labeled membranes (A) and the isolated Ca^{2+}-ATPase (B) were analyzed by 5.8% acrylamide SDS-gel electrophoresis in Tris-acetate, pH 7.4. The densitometric trace of the Coomassie-blue-stained gels is depicted above the incorporated radioactivity. BPB: bromophenolblue.

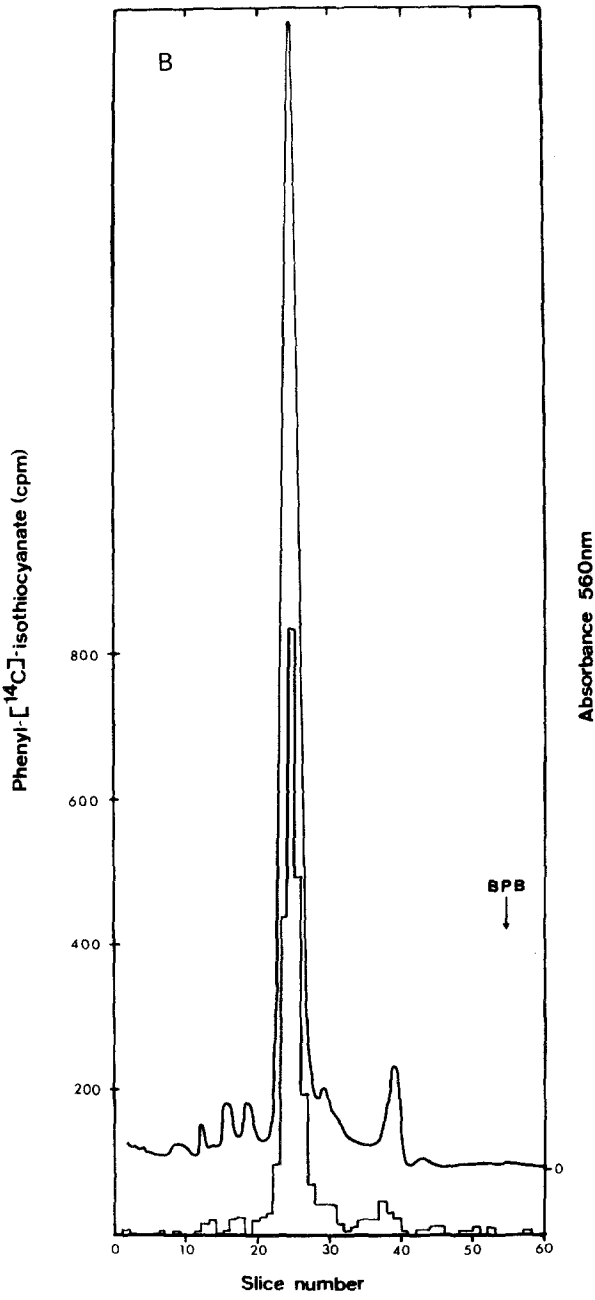

Fig. 1. Continued.

Competitive Binding. Sarcoplasmic reticulum membranes (4 mg protein/ml) were preincubated with specified concentrations of *p*-sulfophenylisothiocyanate (30 min, 37°C in 10 mM sodium phosphate buffer, pH 7.3, stirred suspension). After the preincubation, phenyl-[^{14}C]-isothiocyanate (2 mM) was added and the membranes were incubated for a further hour at 37°C. The modified membranes were then sedimented (30 min, 100,000 *g*, 4°C), washed twice with 10 mM sodium phosphate buffer, pH 7.3, and analyzed by SDS gel electrophoresis.

Ca^{2+}-ATPase Activity. Ca^{2+}-stimulated ATPase activity was determined according to McLennan [19] in presence of 100 mM KCl, 0.05 mM $CaCl_2$ (or 0.5 mM EGTA), 5 mM ATP, 5 mM $MgCl_2$, and 100 mM sodium maleate, pH 7.3. The resulting basal (EGTA) ATPase activity was subtracted from the total activity to give the Ca^{2+}-dependent rate. The ATPase assay was performed with 0.1 mg protein in the presence of Ca^{2+}-ionophore A 23187 (1 μg per assay) to ensure equilibration of Ca^{2+}. The incubation at 37°C was stopped after 4 min with 10% trichloroacetic acid and aliquots were used for the determination of inorganic phosphate by the method of Fiske and Subbarow [20]. For inhibition studies membranes (4 mg protein/ml) were preincubated with various concentrations of arylisothiocyanate in 100 mM KCl and 100 mM sodium maleate buffer, pH 7.3.

Analytical Procedures. Protein was determined in presence of 0.1% SDS according to Lowry *et al.* [21]. Radioactivity was measured as described by Fox [22]. For SDS acrylamide gel electrophoresis the method of Fairbanks *et al.* [23] was followed, using 5.8% acrylamide gels in Tris-acetate buffer, pH 7.4. Protein bands were visualized by Coomassie blue staining. For determination of phenyl-[^{14}C]-isothiocyanate incorporation, destained gels were cut into 1-mm slices. The slices were then dissolved by incubation overnight at 80°C with H_2O_2 containing 0.01 volume 25% ammonia. The resulting solution was measured for radioactivity.

Results

Covalent Binding of Phenylisothiocyanate to Ca^{2+}-ATPase

As demonstrated by SDS gel electrophoresis, the hydrophobic label phenyl-[^{14}C]-isothiocyanate binds covalently to the Ca^{2+}-ATPase, which is the major protein present in sarcoplasmic reticulum membranes (Fig. 1A). Upon solubilization of the labeled membranes in SDS/dithiothreitol, followed by Sepharose 4B chromatography and dialysis, the probe remains protein associated (Fig. 1B). By this purification step the Ca^{2+}-ATPase

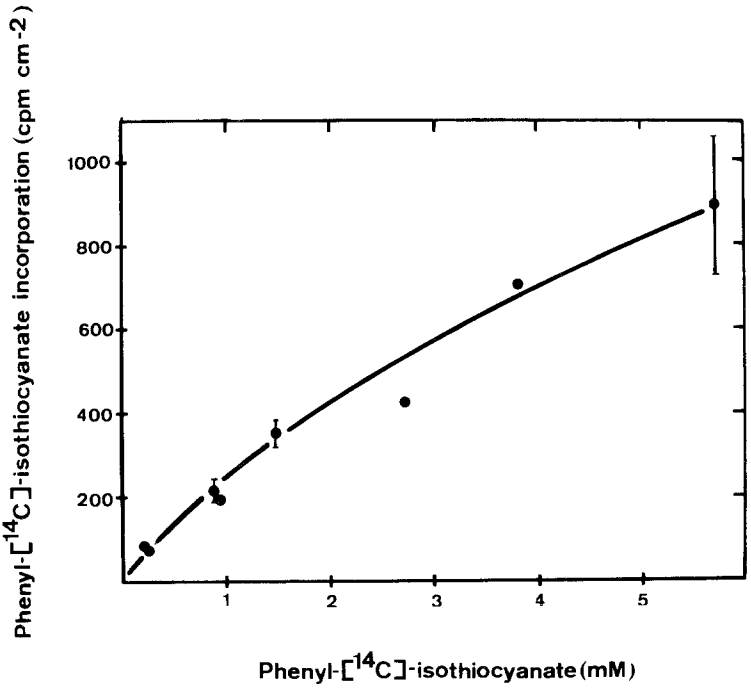

Fig. 2. Phenylisothiocyanate incorporation into sarcoplasmic Ca^{2+}-ATPase. Sarcoplasmic reticulum membranes were labeled with phenyl-[^{14}C]-isothiocyanate by incubation for 1 h at 37°C in 10 mM sodium phosphate buffer, pH 7.3. The labeled membranes were repeatedly sedimented and the proteins separated by SDS gel electrophoresis. Phenyl-[^{14}C]-isothiocyanate incorporation is defined as the radioactivity recovered in the acrylamide gel, corresponding to the separated Ca^{2+}-ATPase protein band. Protein content was quantified by triangulation of the area in the densitometric scan (absorbance 560 nm) of Coomassie-blue-stained gels. Duplicate values of two independent series are shown.

protein, comprising 80–85% of the total protein in sarcoplasmic reticulum membranes, is enriched to about 95% of total protein as determined by densitometric quantification of Coomassie-blue-stained gels.

Phenylisothiocyanate binding to the membrane protein is further demonstrated in the electrophoretically separated Ca^{2+}-ATPase protein. As shown in Fig. 2 increasing protein modification occurs with increasing label concentration. Label incorporation does not saturate in the concentration range used for membrane modification. Concentration-dependent label incorporation into the purified ATPase is further documented in Table I. The incorporation data shown in column B refer to the binding ratios determined

in ATPase preparations where lipid-associated noncovalently bound radioactive material was extracted by chloroform–methanol.

Competitive Binding by p-Sulfophenylisothiocyanate and the Effect of the Alkaline Reductive Treatment

To investigate the possible interaction of phenylisothiocyanate with water-exposed reactive nucleophiles (cysteine thiols), sarcoplasmic reticulum membranes were preincubated with the water-soluble probe *p*-sulfophenylisothiocyanate. Pretreatment of the membranes with 2 mM *p*-sulfophenylisothiocyanate followed by 2 mM phenyl-[^{14}C]–isothiocyanate (Fig. 3) reduces the phenyl-[^{14}C]-isothiocyanate incorporation by about 25% as compared to the control sample (without *p*-sulfophenylisothiocyanate present in the preincubation, Fig. 2). Furthermore, preincubation of the membranes with up to 4 mM *p*-sulfophenylisothiocyanate results in a constant phenyl-[^{14}C]-isothiocyanate binding to the membrane protein (Fig. 3). It is concluded from these experiments that phenylisothiocyanate and *p*-sulfophenylisothiocyanate competitively interact with those nucleophiles accessible from the aqueous medium. *p*-Sulfophenylisothiocyanate, however, does not interfere with the hydrophobically located phenylisothiocyanate binding sites. These sites are accessible solely by the hydrophobic probe.

To obtain information on the chemical nature of the covalent interaction of arylisothiocyanates with sarcoplasmic Ca^{2+}-ATPase, the purified protein fraction was subjected to reduction and carboxymethylation, a procedure which includes treatment of the protein with denaturing conditions (6 M

Table I. Phenylisothiocyanate Labeling of Sarcoplasmic Reticulum Ca^{2+}-ATPase[a]

(A) Initial concentration of phenyl-[^{14}C]-isothiocyanate in the incubation medium (mM)	Phenylisothiocyanate incorporation (mol reagent/mol protein)	
	(B) Protein-bound phenyl-[^{14}C]-isothiocyanate after chloroform–methanol treatment	(C) Protein-bound phenyl-[^{14}C]-isothiocyanate after reduction and carboxymethylation
0.4	1.5	0.6
2.4	5.2	3.0
4.4	6.6	3.3

[a]Sarcoplasmic reticulum membranes were modified with various phenyl-[^{14}C]-isothiocyanate concentrations. Labeled Ca^{2+}-ATPase was then solubilized and purified to 95% by Sepharose 4B chromatography. Lipid-associated noncovalently bound phenyl-[^{14}C]-isothiocyanate present in the ATPase fraction was extracted by chloroform–methanol. The reagent/protein binding ratio was calculated assuming a molecular weight of 100,000 dalton for the Ca^{2+}-ATPase. The reductive treatment at pH 8.0 (reduction and carboxymethylation) was performed as described in Methods.

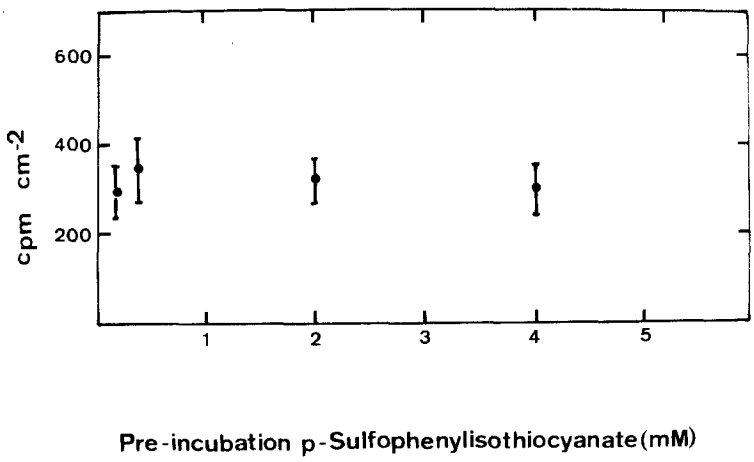

Fig. 3. Effect of preincubation with *p*-sulfophenylisothiocyanate on phenylisothiocyanate binding to Ca^{2+}-ATPase. Sarcoplasmic reticulum membranes were preincubated with indicated amounts of *p*-sulfophenylisothiocyanate (30 min, 37°C), followed by a phenyl-[^{14}C]-isothiocyanate (2 mM, 60 min, 37°C) incubation. Radioactive label incorporation (cpm/cm^2) was determined as described in Fig. 2.

guanidine · HCl), at basic pH (pH 8.0) in a reducing medium (1 mM, 10 mM dithiothreitol) for 50 min at 42°C. Such treatment effects considerable dissociation of bound phenylisothiocyanate (Table I, column C). Complete removal of the probe could, however, not be achieved. When sarcoplasmic reticulum membranes were modified with 4.4 mM phenyl-[^{14}C]-isothiocyanate, 3.3 mol reagent remained associated with the protein and could not be extracted by either chloroform–methanol nor detached by the alkaline reductive treatment under denaturating conditions.

Effect of Arylisothiocyanates on the Ca^{2+}-Stimulated ATPase Activity

Both reagents, the water-soluble *p*-sulfophenylisothiocyanate and the hydrophobic phenylisothiocyanate, inhibit Ca^{2+}-stimulated ATPase activity to the same extent. As shown in Fig. 4, the time-dependent inhibition is comparable for both reagents. Interestingly, in all the experiments performed, the basal ATPase (measured in the presence of EGTA instead of Ca^{2+}) was not affected by either of the probes at any inhibitor concentration (data not shown). As reported earlier [24, 25] the spin-label probe 2,2,6,6 tetramethyl-4-isothiocyanate piperidine nitroxide does not affect the Ca^{2+}-stimulated ATPase activity. These results are explicable by the remarkably lower reactivity of alkylisothiocyanates as compared to arylisothiocyanates toward nucleophiles [10]. Ca^{2+}-ATPase inhibition, however, was observed by

Chyn and Martonosi [26] using the bifunctional probe toluene-2,4,-diisocyanate as a chemical modifier on fragmented sarcoplasmic reticulum.

Discussion

Chemical modification of functionally defined membrane proteins (erythrocyte anion transport protein [12, 13]; bacteriorhodopsin [11]) by the hydrophobic probe phenylisothiocyanate has been previously reported. For both membrane proteins it was found that the most likely candidate for hydrophobic modification is the lysine ϵ-amino group, located in a hydrophobic environment. If, however, the membrane protein possesses cysteine thiol functions exposed to the aqueous medium, which are present in the deprotonated form, thiolate modification by arylisothiocyanates would be expected.

Sarcoplasmic Ca^{2+}-ATPase possesses cysteine residues which are reactive with the hydrophilic reagent 5,5'-dithiobis-(-2-nitrobenzoate) in the absence of detergent [15]. Additionally the active site bears catalytically important functions which can be blocked by the thiol reagent *N*-ethylmaleimide [27–29]. Monofunctional amino-group-directed reagents (formaldehyde, methylbutyrimidate) do not inhibit Ca^{++}-ATPase activity in sarcoplasmic reticulum [30, 31]. The present study demonstrates the availability of proteinaceous nucleophilic groups which are exposed to the aqueous phase and which are reactive with both *p*-sulfophenylisothiocyanate and phenylisothiocyanate. It is highly probable that the modified nucleophiles are the abundantly present cysteine groups which under labeling conditions (pH 7.3) are partially deprotonated and therefore reactive with the probes. This conclusion is further supported by the observed inhibition of the Ca^{2+}-stimulated ATPase activity by both labels (Fig. 4), implying that the catalytically important groups (thiol functions) are accessible to and therefore modified by arylisothiocyanates. Dissociation of covalently bound phenylisothiocyanate by the alkaline reductive treatment further implicates the involvement of cysteine residues in arylisothiocyanate binding. Both dissociation-decomposition reactions and exchange reactions in the presence of excess nucleophile have been described for S-esters of *N*-monosubstituted dithiocarbamic acids ($R_1NHCSSR_2$) [10].

In sarcoplasmic reticulum the interaction of arylisothiocyanates with proteinaceous nucleophiles is predominantly due to thiolate modification. In addition, however, there are nucleophiles present that are modified solely by hydrophobic phenylisothiocyanate but not by *p*-sulfophenylisothiocyanate

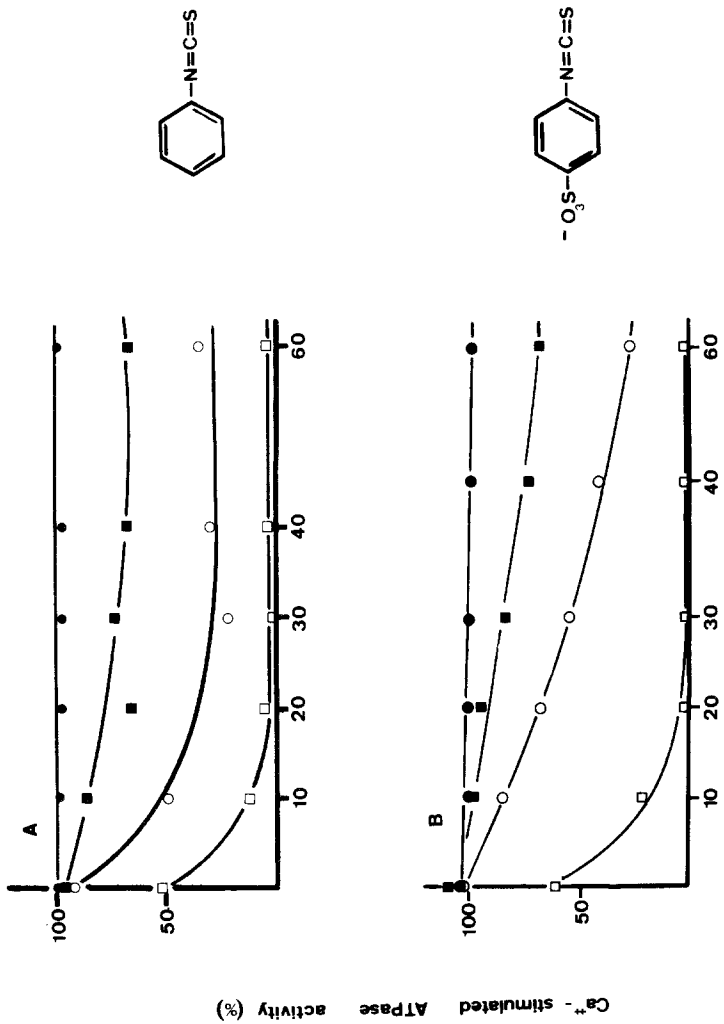

Fig. 4. Effect of arylisothiocyanates on the Ca^{2+}-stimulated ATPase activity. Inhibition experiments utilizing phenylisothiocyanate (A) and *p*-sulfophenylisothiocyanate (B) as inhibitors of Ca^{2+}-ATPase activity were performed in the presence of the Ca^{2+}-ionophore A 23178 as described in Methods. The arylisothiocyanate concentrations used were: none (●), 0.1 mM (■), 0.2 mM (○), 1 mM (□).

(Fig. 3). The binding ratio (reagent/protein), determined after the alkaline reductive treatment of the purified Ca^{2+}-ATPase, demonstrates the presence of at least three noncleavable, covalently modified phenylisothiocyanate binding sites. As these sites cannot be blocked by pretreatment of membranes with *p*-sulfophenylisothiocyanate, they are concluded to be lysine ϵ-amino groups located in the hydrophobic membrane phase.

Acknowledgments

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