Lys515-Lys492 Cross-Linking by DIDS Interferes with Substrate Utilization by the Sarcoplasmic Reticulum ATPase

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ABSTRACT Sarcoplasmic reticulum (SR) Ca^{2+} ATPase was derivatized with 4,4′-diisothiocyanatostilbene-2,2′-sulfonic acid (DIDS), and complete enzyme inactivation was produced with a molecular stoichiometry of one DIDS per ATPase. It was determined by peptide analysis and sequencing that Lys492 and Lys515 were the ATPase residues derivatized by DIDS. Lack of electrophoretic resolution of the two peptide fragments that result from a single tryptic cut at Arg505 demonstrated that the two derivatized residues were cross-linked. Cross-linking of Lys492 and Lys515 by DIDS interfered with ATPase utilization of both ATP and *p*-nitrophenyl phosphate substrates, whereas derivatization of only Lys515 with fluorescein isothiocyanate interfered with ATPase utilization of ATP but not of *p*-nitrophenyl phosphate. Cross-linking with DIDS implies a distance of approximately 13 Å between Lys492 and Lys515, which corresponds to the length of ATP bound in an extended configuration. Therefore, within the groove of the nucleotide binding domain, the ATP substrate is positioned with the adenosine moiety near Lys515 and its terminal phosphate near Lys492.

INTRODUCTION

Derivatization of a single lysine residue (Lys515) with fluorescein isothiocyanate (FITC) produces inactivation of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (Pick and Bassilian, 1981; Pick and Karlish, 1982; Mitchison et al., 1982; Andersen et al., 1982). The derivatization reaction, as well as the related enzyme inactivation, can be partially prevented if ATP is added to the derivatization mixture (Clore et al., 1982). Even though the derivatized enzyme loses the ability to utilize ATP, catalytic activity is still obtained if the pseudosubstrate *p*-nitrophenylphosphate (*p*NPP) is used instead of ATP. The protective effect of ATP during the derivatization reaction and the lack of ATP utilization by the derivatized enzyme suggest partial overlap of ATP and FITC binding domains within the nucleotide binding site and reciprocal hindrance of the two ligands.

With the experiments to be described here we have cross-linked two lysine residues by the use of 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), as already done with band 3 of the anion exchanger (Jennings and Passow, 1979; Okubo et al., 1994) and with the Na^+,K^+ ATPase (Pedemonte and Kaplan, 1988; Gatto et al., 1997). We have then determined which lysine residues were in fact cross-linked, characterized the enzyme inhibition produced by cross-linking two lysine residues with DIDS (as compared with derivatization of only Lys515 by FITC), and considered the implications of these experiments with regard to the ATPase structure at the catalytic site.

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MATERIALS AND METHODS

SR vesicles were prepared from rabbit leg skeletal muscle as described by Eletr and Inesi (1972). Protein concentration was determined by the method of Lowry et al. (1951) or by measurements of light absorption at 280 nm after solubilization in 1% SDS and 0.1 N NaOH (standardized with bovine serum albumin). Most chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). DIDS was purchased from Molecular Probes (Eugene, OR).

Derivatization of SR ATPase with DIDS

Derivatization of the enzyme with DIDS was performed at room temperature by incubating SR vesicles (1 mg protein/ml) with different concentrations of DIDS in a medium containing 10 mM Tris, pH 9.2, 0.1 M KCl, 0.3 M sucrose, and 0.1 mM EGTA in the absence or in the presence of Ca^{2+} or ATP. At serial times, 5 μl of the mixture was taken for measurements of ATPase activity. Alternatively, the reaction was stopped by 20-fold dilution with 10 mM MOPS, pH 7.0, and 10% sucrose. The labeled protein was then pelleted at 100,000 × *g* for 45 min and resuspended in the same dilution buffer.

Determination of DIDS derivatization stoichiometry

Derivatized SR vesicles were sedimented by centrifugation and resuspended in 10 mM MOPS, pH 7.0, and 10% sucrose. The resuspended vesicles were then solubilized in 1% SDS and 0.1 N NaOH. The protein concentration was determined by light absorption measurements at 280 nm (standardized with bovine serum albumin), and the DIDS concentration was determined by light absorption measurements at 342 nm (standardized with DIDS). The ATPase catalytic site stoichiometry is 5 nmol/mg protein in our preparation.

Derivatization of SR ATPase with FITC

FITC labeling was performed under the same condition as DIDS labeling, except for the use of 40 μM FITC instead of DIDS.

Measurements of enzyme activity

ATPase activity was assayed by adding 5 μl of the derivatization mixture to 1.0 ml of a medium containing 20 mM MOPS, pH 6.8, 80 mM KCl, 3

mM $MgCl_2$, 200 μM $CaCl_2$, 200 μM EGTA, 3 μM A23187 (Ca^{2+} ionophore), 5 mM NaN_3, and 3 mM ATP. Hydrolytic cleavage of P_i was measured by the method of Lanzetta et al. (1979). Alternatively, 70 μl of derivatization mixture were added to a medium identical to that used for measurements of ATP hydrolysis, except for the use of 3 mM *p*NPP instead of ATP. In this case, hydrolytic cleavage of *p*-nitrophenol was monitored spectrophotometrically at 400 nm wavelength.

Ca^{2+} binding in the absence of ATP

One milligram of control or DIDS-derivatized SR vesicles was incubated for 10 min at 25°C in 1.0 ml of a medium containing 20 mM MOPS, pH 6.8, 80 mM KCl, 3 mM $MgCl_2$, 50 mM EGTA, and 34 μM [^{45}Ca]$CaCl_2$ (i.e., 1.78 μM free Ca^{2+}). The sample was then passed through a chromatography column and eluted with equilibration medium. Fractional elution samples were collected for determination of radioactivity and protein concentration as described by Inesi et al. (1980).

Formation of phosphorylated enzyme intermediate

A 100-μg aliquot of control or DIDS-derivatized SR vesicles were incubated for 10 s at 2–3°C, in 100 μl of a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 10 mM $MgCl_2$, 100 μM $CaCl_2$, and 100 μM [γ-^{32}P]ATP. The mixture was quenched with 1.0 ml of 1.0 M Perchloric acid and 4.0 mM P_i, kept in ice for 10 min, and pelleted by centrifugation at 5,000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 0.1 M Perchloric acid, and 0.4 M P_i and then twice with ice-cold water. Finally, the denatured protein was solubilized and analyzed by polyacrylamide gel electrophoresis (PAGE) (Weber and Osborn, 1969) at pH 6.3. The gels were stained, dried, and utilized for autoradiography.

Mild trypsin digestion of SR vesicles

Mild trypsin digestion (for characterization of large tryptic fragments) was carried out with a trypsin:SR protein ratio of 0.005 (w:w). The reaction mixture contained 1.5 mg of SR protein/ml, 0.0075 mg of trypsin/ml, 20 mM MOPS, pH 7.0, 80 mM KCl, and 5 mM $MgCl_2$. The mixture was incubated for 4 min at room temperature. The digestion was stopped by the addition of 0.23 mg of soybean trypsin inhibitor. The tryptic fragments were separated by SDS-PAGE according to Weber and Osborn (1969).

Extensive trypsin digestion of DIDS-labeled SR vesicles

DIDS-labeled SR vesicles (4 mg protein/ml) were incubated with trypsin (0.4 mg/ml) for 10 min at 37°C in a medium containing 50 mM Tris/HCl, pH 8.1, and 0.25 M sucrose. Digestion was stopped by addition of 4 mg of trypsin inhibitor in 300 μl of 50 mM Tris buffer, pH 8.1. The mixture was centrifuged at 110,000 × *g* for 1 h, and the supernatant was taken for separation of peptide fragments by high-pressure liquid chromatography (HPLC).

Separation and purification of peptides

The trypsin digests were subjected to HPLC separation, using a linear gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 90% acetonitrile. Elution was monitored at 215 and 342 nm for peptide material and DIDS label, respectively. The elution fractions exhibiting absorption at both 215 and 342 nm were collected and subjected to further HPLC purification using a linear gradient of 10 mM NH_4Ac, pH 6.5, and 10 mM NH_4Ac in 90% acetonitrile. Elution was again monitored at 215 and 342 nm.

Peptide sequencing

HPLC-purified peptide was sequenced using a Hewlett Packard protein sequencer model G1005A equipped with an online HP 1090 series II/L system to analyze the phenylthiohydantoin derivatives. The phenylthiohydantoin derivatives of labeled residues were not recovered during sequencing but were identified indirectly by the appearance of a gap in the sequence. Residues within sequenced peptides were identified by comparison with the complete cDNA-derived amino acid sequence of the Ca^{2+} ATPase from rabbit skeletal muscle SR (MacLennan et al., 1985).

RESULTS

ATPase derivatization and catalytic inactivation

It is shown in Fig. 1 *A* that inactivation of Ca^{2+} ATPase is produced after a 60-min incubation of SR vesicles with

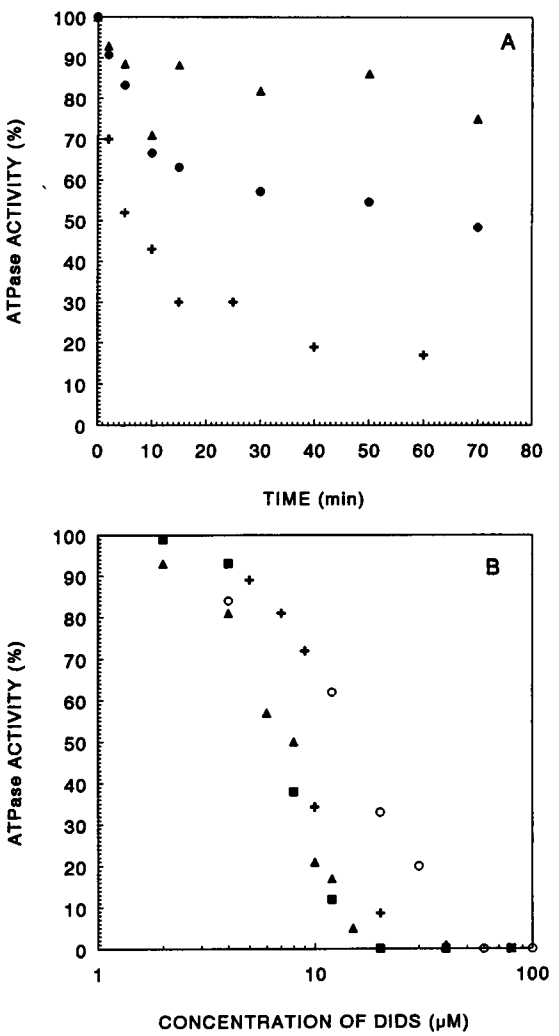

FIGURE 1 Inactivation of SR ATPase by DIDS. (*A*) Time dependence. SR vesicles were incubated with DIDS (▲, 4 μM; ●, 7 μM; +, 12 μM), and samples were taken at various times for ATPase measurements as explained in Materials and Methods. (*B*) DIDS concentration dependence: ATPase activity remaining after a 60-min incubation of SR vesicles with various concentrations of DIDS in the absence (▲) or in the presence of 4 mM ATP (○), 2 μM Ca^{2+} (□), or 1 mM Ca^{2+} (+).

DIDS. The DIDS concentration dependence of the inhibitory effect is given in Fig. 1 *B*, which also shows that some protection is observed if 4 mM ATP is present in the incubation mixture. No protection is obtained with 2 μM or 1 mM Ca^{2+}. A plot of residual ATPase activity as a function of the DIDS label stoichiometry indicates that inactivation is produced by reaction of one DIDS molecule per ATPase (Fig. 2).

In addition to ATP as substrate, the SR ATPase can utilize *p*NPP as a pseudosubstrate in a Ca^{2+}-dependent manner, coupled to Ca^{2+} transport (Inesi, 1971; Lacapere and Garin, 1994). Interestingly, we now find that ATPase derivatization with DIDS produces enzyme inactivation not only when ATP is used as a substrate but also when *p*NPP is used (Fig. 3). On the other hand, SR ATPase derivatization with FITC produces ATPase inhibition of ATP utilization but not of *p*NPP utilization (Fig. 3).

Ca^{2+} binding and phosphoenzyme formation

The catalytic cycle of the SR ATPase begins with binding of 2 mol of Ca^{2+} per mol of ATPase and proceeds to form a phosphorylated enzyme intermediate by transfer of the ATP terminal phosphate to an ATPase aspartyl residue. We then performed experiments to clarify whether ATPase inactivation by DIDS involves enzyme activation by Ca^{2+} or interferes specifically with formation of the phosphoenzyme intermediate. Our measurements of Ca^{2+} binding in the absence of ATP yielded levels of 7.7 ± 0.7 nmol of Ca^{2+} per mg of SR protein, in agreement with previous data obtained with SR vesicles by other methods (Inesi et al., 1980). We then found levels of 6.7 ± 0.6 nmol of Ca^{2+} per mg of protein after derivatization with DIDS and 6.4 ± 0.1 nmol of Ca^{2+} per mg of protein when we used SR vesicles incubated with the derivatization medium in the absence of DIDS. These results indicate that a slight inhibition of Ca^{2+} binding is produced by exposure of the SR vesicles to the alkaline derivatization medium, but no effect on Ca^{2+} binding is produced by DIDS per se.

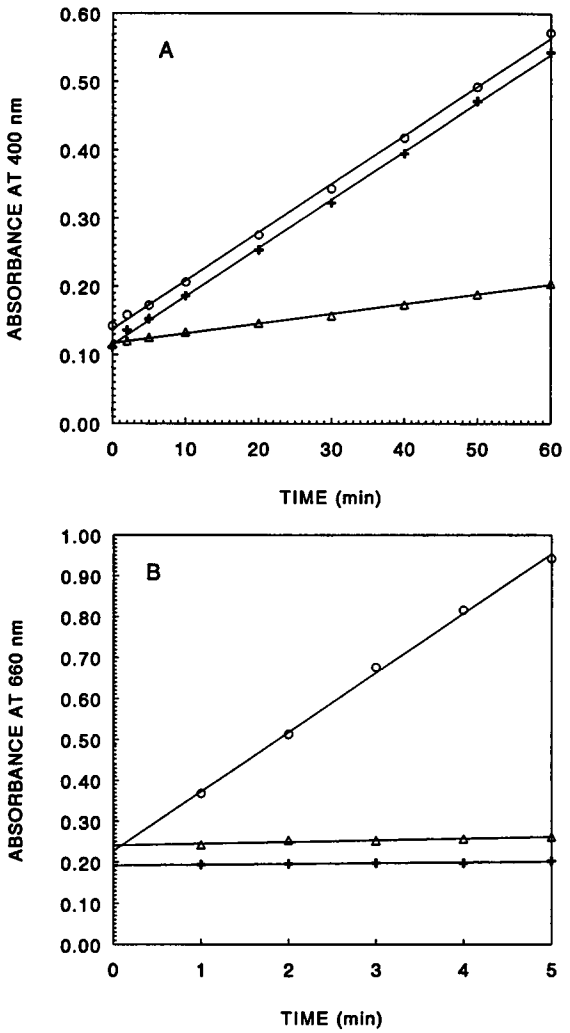

FIGURE 3 Utilization of pNPP (*A*) or ATP (*B*) by the SR ATPase. ○, control vesicles; △ and +, vesicles derivatized with 20 μM DIDS (△) or 40 μM FITC (+). After 60 (DIDS) or 10 (FITC) min of incubation, pNPP (*A*) and ATPase (*B*) activities were determined as explained in Materials and Methods.

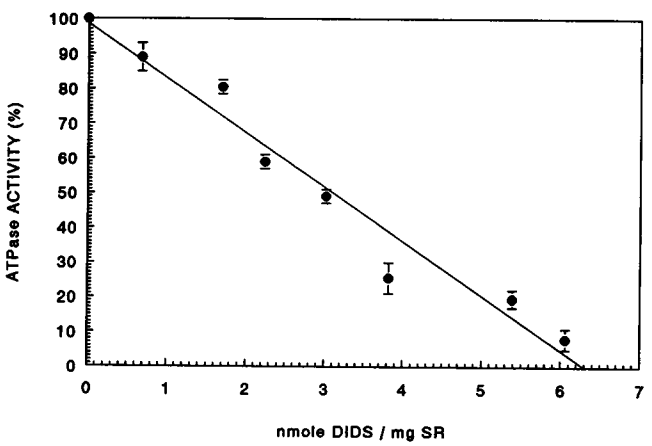

FIGURE 2 Inactivation of SR ATPase as a function of DIDS label stoichiometry. SR vesicles were incubated with DIDS in the presence of increasing concentrations (0, 2, 4, 6, 8, 10, 12, and 15 μM) and the stoichiometry of labeling determined as explained in Materials and Methods.

We then checked the formation of phosphoenzyme intermediate after addition of ATP in the presence of Ca^{2+} and found that this reaction was totally abolished by derivatization with DIDS (Fig. 4). Therefore, derivatization with DIDS interferes with substrate binding and/or utilization rather then enzyme activation by Ca^{2+}.

Identification of labeled residues

Prolonged incubation of SR vesicles with trypsin produces complete digestion of the globular (i.e., extramembranous) ATPase region and solubilization of its peptide fragments (Inesi and Scales, 1974). We collected and purified by HPLC the peptide fragments obtained by digestion of ATPase derivatized with DIDS. The elution peak containing

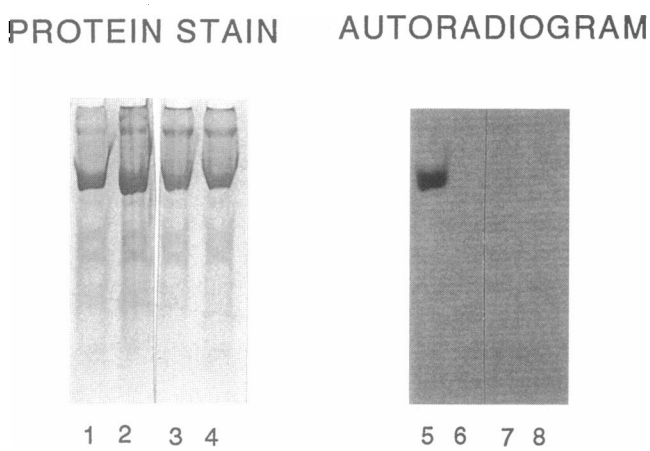

FIGURE 4 Formation of phosphoenzyme. Autoradiographic detection of phosphoenzyme was obtained by incubation with radioactive ATP in the presence of Ca^{2+} (see Materials and Methods). Lanes 1 and 5, control vesicles; lanes 2 and 6, background radioactivity determined by acid quenching before addition of ATP; lanes 3 and 7, SR vesicles derivatized with 20 μM DIDS; lanes 4 and 8, background radioactivity for the DIDS derivatized vesicles.

the labeled peptide fragment, as revealed by light absorption at 215 and 342 nm, was then submitted to sequence analysis. The results of this analysis (Table 1) are consistent with the presence of two ATPase peptide segments in the sample, one spanning from Met512 to Arg524, and the other from Asp490 to Met494. Lys492 and Lys515 are missing from the sequences obtained from the first and the second fragment, respectively, indicating that these two lysines are the residues derivatized by DIDS. Identical residues (Lys492 and Lys515) were found to be labeled whether ATPase derivatization with DIDS was carried out in the absence (0.1 mM EGTA) or in the presence of 20 μM free Ca^{2+}. It is of interest that we did not find evidence of Lys684 derivatization, as produced by adenosine triphosphatepyridoxal in the presence of Ca^{2+} (Yamamoto et al, 1988).

To find out whether Lys492 and Lys515 were cross-linked by DIDS, we performed electrophoretic analysis of the large peptide fragments obtained by mild trypsin digestion of the ATPase (Migala and al., 1973; Inesi and Scales, 1974; Stewart and MacLennan, 1974). It is shown in Fig. 5 that such a digestion produces two complementary fragments (A and B) of the ATPase, which can be easily separated by PAGE. On the other hand, digestion of ATPase derivatized with DIDS did not yield the same electrophoretic pattern, as the entire protein behaved as a single unit, and the two fragments did not separate upon electrophoresis. As the pertinent trypsin site (Arg505) resides between Lys492 and Lys515, it is apparent that cross-linking of these two lysines prevents separation of the two fragments resulting from mild trypsin digestion. It is noteworthy that stoichiometric ATPase derivatization with DIDS prevents completely the separation of the two tryptic fragments (Fig. 5). This suggests that cross-linking of Lys492 and Lys515 involves homogeneously the entire ATPase population in the reaction mixture. Furthermore, identical results were obtained when ATPase derivatization with DIDS was carried out in the absence (0.1 mM EGTA) or in the presence of 20 μM Ca^{2+}, confirming that cross-linking of Lys492 and Lys515 was obtained in either case, as indicated by sequence analysis (see above).

It should be pointed out that Tran et al. (1994) generated a trypsin-resistant, 30-kDa fragment of the $Na+,K^+$ ATPase by photolabeling the ATP binding site with 2-azido-adenosine triphosphate, with no apparent cross-link-

TABLE 1 Yield of amino acids upon sequence analysis of a DIDS-labeled ATPase fragment collected by HPLC

Cycle number	Amino acid residue	Yield (pmol)
1	Met	337
	Asp	75
2	Phe	317
	Arg	67
3	Val	294
	X	
4	X	
	Ser	41
5	Gly	240
	Met	47
6	Ala	294
7	Pro	201
8	Glu	220
9	Gly	205
10	Val	169
11	Ile	112
12	Asp	202
13	Arg	84

The two sequences correspond to the amino terminals starting at Met 512 and Asp 490. This pattern is attributed to the presence of two small peptide fragments cross-linked by DIDS.

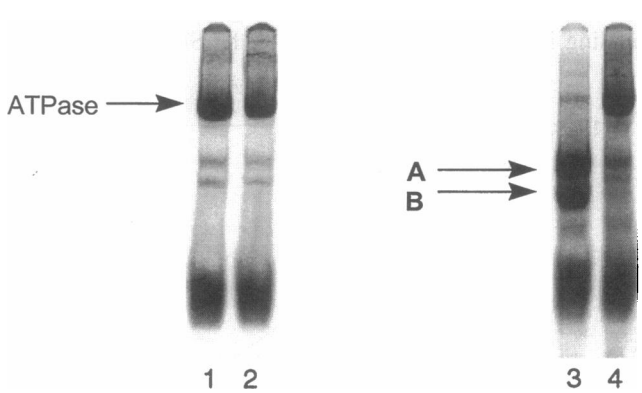

FIGURE 5 Electrophoresis of ATPase fragments obtained by mild trypsin digestion. The protein electrophoretic pattern of control and DIDS (20 μM) derivatized vesicles is shown in lanes 1 and 2, respectively. The same samples are shown after mild trypsin digestion in lanes 3 and 4. The arrow points to the undivided ATPase. Note that DIDS derivatization prevents separation of the two tryptic fragments in lane 4 if Lys492 and Lys515 are cross-linked. On the contrary, the two fragments would separate if cross-linking was between Lys515 and Lys684, due to the location of the first tryptic site at Arg505 (see diagram in Fig. 6). See Materials and Methods for experimental details.

ing. Therefore, to rule out that in our experiments the lack of trypsin fragment separation was in fact due to interference with trypsin digestion by the DIDS label, we carried out sequence analysis of the ATPase derivatized with DIDS and digested with trypsin. We obtained a prominent yield of the amino-terminal sequence of the B fragment (starting with Ala506), demonstrating that the two fragments were in fact produced by trypsin digestion but could not be separated as a consequence of cross-linking by DIDS. Even in these experiments, we obtained negligible yield of Lys515, consistent with its derivatization by DIDS.

DISCUSSION

The cytosolic head of the SR ATPase includes a region where significant analogies to other adenyl kinases suggest the presence of a binding domain for the nucleotide enzyme substrate (Brandl et al., 1986). Consistent with these predictions, bound nucleotide was demonstrated within a groove of this region, by analysis of electron diffraction data (Toyoshima et al., 1993; Yonekura et al., 1997). Models based on sequence and consensus analysis (Green, 1989; Green and Stokes, 1992) postulate that this region consists mainly of alternating α-helices and β-strands, with Lys492 and Lys515 placed near two β-strands (Fig. 6) that precede and follow the first trypsin site, respectively.

Lys515 is known to be reactive to FITC (Mitchison et al., 1982). The bound FITC interferes with enzyme utilization of ATP, but not of *p*NPP, consistent with overlap of the nucleotide adenosine moiety with the FITC label at the binding site. On the other hand, Lys492 reacts with adenosine triphosphopyridoxal (Yamamoto et al., 1989), and pyr-

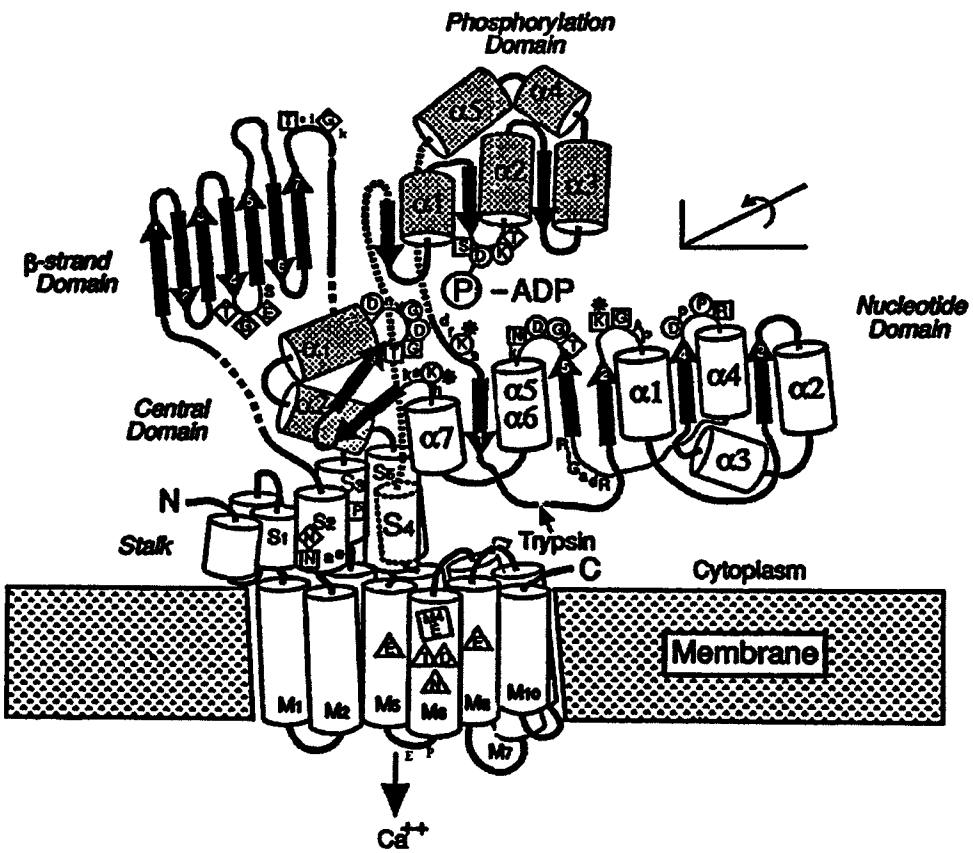

FIGURE 6 Topology of cross-linked lysine residues within a structural model of the SR ATPase. This is a somewhat simpler version of the model proposed by Green and Stokes (1992), based on sequence alignment and consensus of secondary structure predictions (Green, 1989). The large extramembranous region includes the nucleotide binding domain, with a prevalence of alternating α-helices and β-strands. The trypsin cleavage site affected by mild digestion is indicated by an arrow. The reactive lysines are denoted by asterisks. Lys515, distal to β-strand 2 and proximal to GAP, reacts with FITC and DIDS. Lys492, proximal to β-strand 1, is cross-linked to Lys515 by DIDS. Both Lys492 and Lys684 (distal to α-7) react with adenosine triphosphopyridoxal in the absence of Ca^{2+} and only with Lys684 in the presence of Ca^{2+} (Yamamoto at al., 1988, 1989). The phosphorylation domain (including Asp351, which undergoes phosphorylation) is separated by a cleft (or groove) from the nucleotide binding domain. Its position and distance, relative to the nucleotide binding domain, can change by rotation over the central domain hinge, about the axis indicated by the arrow. The effects of site-directed mutations are denoted by circles (total ATPase and transport inhibition), squares (reduced transport), diamonds (no transport, normal phosphorylation), and triangles (apparent interference with Ca^{2+} binding). According to Andersen (1995), mutations of Lys515 do not affect significantly the rates of transport and their ATP concentration dependence, whereas mutations of Lys492 (as well as of Arg489 or Phe487) reduce significantly the affinity of the enzyme for ATP. Capital letters refer to residues that were mutated without functional consequences. Lowercase letters refer to nonmutated residues as sequence references.

idoxal 5′-phosphate (Yamagata et al., 1993), suggesting proximity of this residue to the γ-phosphate of ATP.

Our experiments were inspired by the observations of Gatto et al. (1997) on the Na^+,K^+ ATPase in which Lys501 was derivatized first by DIDS with loss of ATP binding, and cross-linking was formed with Lys480. We demonstrate here that it is possible to cross-link Lys515 and Lys492 with DIDS in the SR Ca^{2+} ATPase, indicating that Lys515 and Ly492 in the SR Ca^{2+} ATPase sequence correspond to Lys501 and Lys480 in the Na^+,K^+ ATPase. Cross-linking with DIDS demonstrates that the two reactive amino groups are separated by a distance of approximately 13 Å (the length of DIDS) in the folded ATPase structure. The Ca^{2+} ATPase sequence from Lys492 to Lys515,

492Lys Ser Met Ser Val Tyr Cys Ser
Pro Ala Lys Ala Ser Arg Ala Ala Val
Gly Asn Lys Met Phe Val Lys515

suggests that this structure folds with Lys492 to Ser499 in extended sheet conformation with a likely turn starting at Pro500 and extending for several residues followed by another turn probably involving Gly509. The rest of the chain would return in an extended sheet conformation from Asn510 to Lys515. This extended loop structure, as in the model of Fig. 6 (Green, 1989), could bring Lys492 and Lys515 to the ATP binding site surface at a distance of 13 Å from each other. Arg505, which is readily attacked by trypsin, would lie exposed in the loop region in such a mode.

The 13 Å distance between Lys492 and Lys515 is close to the length of the ATP molecule, which is approximately 13 Å in extended configuration, and is consistent with proximity of FITC to the adenine moiety and of Lys492 to the γ-phosphate of the bound nucleotide. Demonstration of the proximity of these residues to substrate molecular moieties may be helpful to sequence assignments within the ATP binding domain revealed by the electron image analysis of Yonekura et al. (1997). In the light of these structural considerations, it can be understood how cross-linking of Lys492 and Lys515 by DIDS interferes with substrate binding through the entire length of the ATP binding domain, including the adenosine moiety of ATP and the phosphate moiety of ATP or *p*NPP. This interference may be produced by the mere presence of the DIDS molecule and/or by a protein structural change produced by the cross-linking reaction. With regard to the possible role of derivatization in preventing participation of the derivatized residues in substrate complexation, it is known that mutations of Lys492 (as well as Arg489 and Phe487) decrease significantly the enzyme affinity for ATP, whereas mutations of Lys515 do not (Andersen, 1995).

It should be pointed out that Lys492 reacts with 5′-trinitrophenyl-8-azido-adenosine triphosphate (McIntosh et al., 1992) and 5′-trinitrophenyl-2-azido-adenosine triphosphate (Inesi et al., 1992), in addition to DIDS and adenosine triphosphopyridoxal. Furthermore, photolabeling of the Na+,K^+ ATPase Gly502 residue (the corresponding position of which would be close to Lys492 in the SR ATPase sequence) with 2-azido-adenosine triphosphate was obtained by Tran et al. (1994). These experiments are consistent with nucleotide binding in this region. They suggest, however, proximity of the adenosine moiety, rather than the γ-phosphate of ATP, to Lys492. It is noteworthy, in this regard, that the trinitrophenyl and azido moieties could cause displacement of the nucleotide analog with respect to the ATP substrate position within the binding site, and this may be one reason for the extremely slow rates of 5′-trinitrophenyl-adenosine triphosphate utilization by the SR ATPase (Watanabe and Inesi, 1982). Alternatively, the nucleotide site may allow alternate ligand positions, of which only the one stabilized by a specific derivatization reaction may be recognized in each type of experiment.

In the folded ATPase structure, Arg678 and neighboring residues are likely to be near Lys492 (see diagram in Fig. 6), as Arg678 and Lys492 can be cross-linked with glutaraldehyde (McIntosh, 1992). Furthermore, adenosine triphosphopyridoxal reacts with both Lys492 and Lys684 in the absence of Ca^{2+} (Yamamoto et al., 1989), whereas it reacts only with Lys684 in the presence of Ca^{2+} (Yamamoto et al., 1988). In our experiments we did not observe cross-linking of Lys515 with Lys684 by DIDS but only cross-linking of Lys492 and Lys515 either in the presence or in the absence of Ca^{2+}. It is possible that a primary reaction of DIDS with Lys515, or a local protein structural feature distinguishing DIDS from ATP, prevents DIDS from extending to Lys684 even in the presence of Ca^{2+}.

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