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Inactivation of the Na,K-ATPase by modification of Lys-501 with 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS)

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The sodium pump or Na,K-ATPase, maintains the Na^+ and K^+ gradients across eukaryotic cell membranes at the expense of ATP. Incubation of purified canine renal Na,K-ATPase with 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) inhibited the ATPase activity. Both the labeling of the protein and the loss of ATPase activity were prevented by co-incubation with ADP (acting as an ATP analog) or KCl. Only the α-subunit was labeled by SITS. The α-subunit from the inhibited enzyme was extensively digested with trypsin, and SITS-labeled peptides were purified by reverse-phase HPLC and sequenced. The amino acid sequence determined, His-Leu-Leu-Val-Met-X-Gly-Ala-Pro-Glu, indicated that SITS modifies Lys-501 (X) on the α-subunit of Na,K-ATPase.

Na,K-ATPase; Enzyme inactivation; Active transport; Ion pump; 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS)

1. INTRODUCTION

Stilbenes, like SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) and the related compound H_2DIDS (dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate), have been shown to inhibit the Na,K-ATPase found in the basolateral membrane of jejunum [1] and rat brain microsomes [2]. We have reported [3–6] that H_2DIDS irreversibly inhibited the pNPPase and Na,K-ATPase activities of canine renal enzyme. Inactivation occurs after formation of an inhibitory reversible complex. The reversible inhibition was not affected by Na pump ligands, whereas the irreversible inactivation was prevented by either ATP or K. The inactivation by H_2DIDS could have been produced by intramolecular cross-linking, as has been observed with Band 3 [7]. To avoid this potential complication, we have utilized SITS, which cannot form cross-links as it has only one isothiocyanate group. We show here that SITS inactivates the Na,K-ATPase by covalent reaction with Lys-501, which has been shown to be the site of modification of other amino-reactive reagents whose effects are prevented by the simultaneous presence of ATP.

2. EXPERIMENTAL

2.1. *Materials*

TPCK-treated trypsin was from Worthington. Dithiothreitol (DTT), SDS, urea and the Bio-Sil TSK size exclusion columns were from Bio-Rad Laboratories; iodoacetamide (IAM) and SITS were from Sigma Chemical Co. All buffer salts and HPLC grade acetonitrile were obtained from Fisher Scientific. The 300 Å pore size C18 (218-TP-5415) and C4 (214-TP-5415) reverse-phase columns were from the Separations Group (Vydac). A LDC-Milton Roy HPLC system was used as described previously [12]. Peptides were sequenced on a Porton 2090E gas phase sequenator. The amino acid analyses and protein sequences were determined by the Protein Core Facility of the Department of Pharmacology and Cell Biophysics of the University of Cincinnati.

The Na,K-ATPase was purified from dog kidneys and assayed as described previously [6]. Na,K-ATPase used in these studies had a specific activity of about 20 μmol of P_i liberated/mg/min.

2.2. *Inactivation of the Na,K-ATPase by SITS*

The enzyme (1 μg) was incubated at 37°C in a medium (30 μl) containing (in mM): Tris-HCl 50 (pH 9.2); EDTA 1; with the concentrations of H_2DIDS and SITS indicated in Fig. 1. After 60 min, 20 μl of a mixture containing 0.75 mg/ml bovine serum albumin and 125 mM 2-mercaptoethanol was added. The total volume was diluted to 600 μl with the assay mixture and the Na,K-ATPase activity determined. These conditions are sufficient to eliminate all non-reacted SITS, to stop further reaction, and to prevent the reversible inhibition during enzyme assay.

2.3. *Preparation of SITS-labeled enzyme*

The enzyme (3 mg) was incubated at 37°C in a medium (50 ml) containing (mM): Tris-HCl 50 (pH 9.2); EDTA 1; and SITS 0.012. The medium also contained 0.5 mM PMSF and 1 μg/ml aprotinin. Samples containing either KCl (0.1 mM), or ADP (3 mM), or no inhibitor, were prepared in parallel. The concentration of SITS was calculated from its absorbance spectrum using a molar extinction coefficient of 40 $mM^{-1} \cdot cm^{-1}$ at 336 nm. The spectrum of the SITS solution was determined before each use to determine reagent concentration and integrity. After treatment, an aliquot of each sample was used to determine the remaining Na,K-ATPase activity. The samples were centrifuged 430,000 × g for 10 min, the supernatants discarded, and the pellets were resuspended with 1 ml of 0.5 M EDTA-Tris, pH 6.9. Samples were centrifuged at 430,000 × g for 20 min and the pellets were resuspended with 1 ml of 100 mM EDTA-Tris, 0.5% 2-mercaptoetha-

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nol, pH 7, containing 1 M KCl. After 1 h at room temperature, the samples were washed by centrifugation and resuspension with water.

2.4. *Proteolysis of the SITS-labeled protein, purification and sequence analysis*

The pelleted Na,K-ATPase samples (inactivated and protected) were homogenized with 150 μl water and solubilized with 200 μl of 20% SDS. The solubilized protein was alkylated with 100 mM iodoacetamide (IAM) in 200 mM Tris-HCl, pH 8.3, at 37°C for 15 min. The α-subunit was isolated by HPLC using a size-exclusion column (Bio-Sil TSK250) equilibrated with 50 mM NaH_2PO_4, pH 5.77, containing 0.1% SDS and 0.01% NaN_3 [8]. The absorbance of the eluent was monitored at 280 nm and the α-subunit was collected. The solution was concentrated to 100 μl using Centricon-30 devices, treated with 20 mM DTT for 30 min at 60°C, and realkylated as before. The samples were then reinjected onto the HPLC column to remove excess reagents. Aliquots of the repurified α-subunit were withdrawn for amino acid analysis. The purified α-subunit was concentrated in a Centricon-30 to 250 μl, and the protein was precipitated by the addition of 4 vols. of −20°C acetone followed by incubation in dry-ice and ethanol for 1 h. The precipitated protein was pelleted by centrifugation and the supernatant removed. 100 μl of 8 M urea was added and the pellet dislodged from the side of the tube and then sonicated for 10 min. Then, 80 μl of 500 mM NH_4HCO_3, pH 8.3, 200 μl of H_2O, and 10 μl of 2 mg/ml of trypsin in 1 mM HCl were added, and the sample was incubated at 37°C for 2 h with occasional mixing. The ratio of trypsin to α-subunit was approximately 1:50. A second 10 μl aliquot of trypsin was added to the sample, and the digestion was continued overnight. The peptide digest was injected onto a HPLC C18 column and the peptides were separated using a linear gradient of 10–40% CH_3CN in water containing 0.1% TFA over 30 min. The peptides were monitored at 333 nm, which was the wavelength of maximum absorption for SITS in 0.1% TFA/H_2O. The SITS-labeled peptides were repurified on a C4 column using a linear gradient of 15–20% CH_3CN containing 0.1% TFA over 30 min.

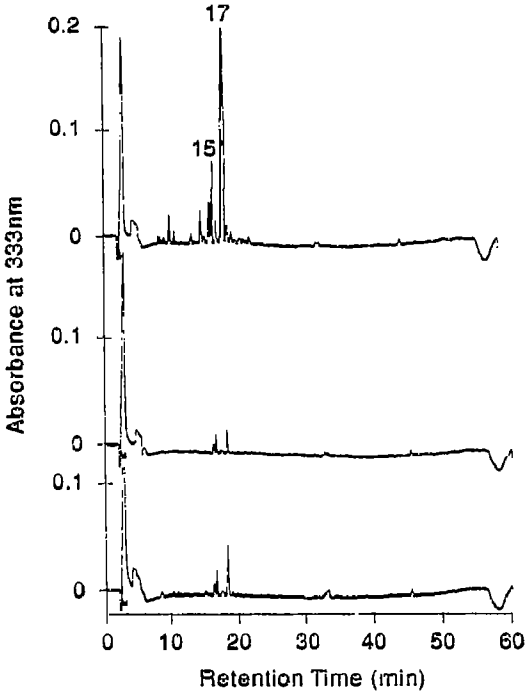

Fig. 2. Peptide mapping of the SITS-labeled α-subunit. After trypsin treatment of 3 mg of SITS-labeled Na,K-ATPase, the fragments produced were separated by HPLC as indicated in section 2. The chromatograms are of the tryptic fragments from the inactivated control- (top), the K-protected- (middle), and the ADP-protected (bottom) Na,K-ATPase samples obtained by C18 HPLC. The peaks labeled 15 and 17 were repurified and sequenced (see Table 1).

3. RESULTS

Both SITS and H_2DIDS irreversibly inhibited the Na,K-ATPase (Fig. 1). The enzyme was incubated with different concentrations of either SITS or H_2DIDS, and assayed after 30 min. As previously described for H_2DIDS [5], the inactivation by SITS was protectable by either ADP or K. The remaining Na,K-ATPase activity was 100% and 88% with K (0.1 mM) and ADP (3 mM), respectively, when the protein was incubated for 1 h with 12 μM SITS, as compared with the activity remaining in the treated control. After labeling, the Na,K-ATPase subunits were separated by SDS-PAGE. As previously observed with H_2DIDS [5], SITS appears to label only the α-subunit (data not shown).

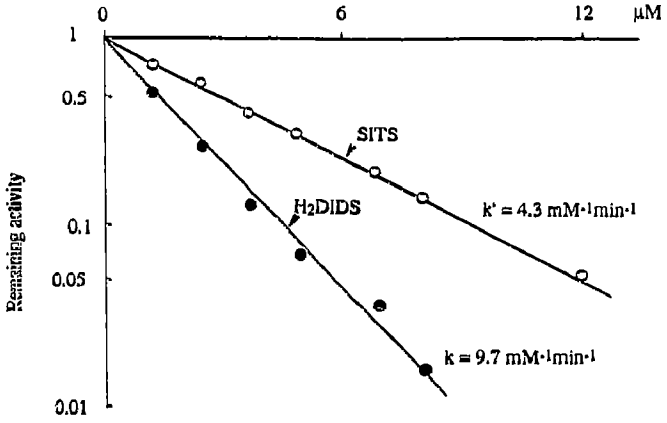

Fig. 1. Inactivation of the Na,K-ATPase by H_2DIDS and SITS. The reaction was performed as described in section 2. The apparent first order reaction constants were calculated using the formula $\log v_i/v_o = k_i I \cdot t/2.3\ k_i$ where v_i and v_o are the reaction velocities in the absence and presence of the inhibitor, I, indicates the concentration of inhibitor, and t the time of treatment. The formula was calculated assuming the following reaction:

$$E + I \underset{k_i}{\rightleftharpoons} (EI) \xrightarrow[k_1]{} E_i \quad \text{and} \quad k' = k_1 I/K_i,$$

as previously described [18].

ADP-protected-, K-protected- and SITS-inactivated samples of the Na,K-ATPase were solubilized with SDS and processed as described above (see section 2). Fig. 2 shows chromatograms of the SITS-labeled proteolytic fragments of the inactivated enzyme, and those corresponding to the K- and ADP-protected samples. The apparent absorbance in the void volume (approximate retention time of 3 min) is due to refractive index effects caused by urea. Most of the SITS bound to the protein was contained in several peaks which were observed in much smaller amounts in the K- or ADP-protected samples. The two major peaks of SITS-labeled peptides (peaks 15 and 17 in Fig. 2) were further purified on a

C4 column using a linear gradient of 15–20% CH_3CN containing 0.1% TFA over 30 min, and sequenced. Table I shows the amino acids detected in each cycle and the corresponding recovery. The sequence determined corresponds to the sequence that starts at His-496 in the α-subunit of the sheep kidney Na,K-ATPase [9] ((the dog kidney α-subunit sequence has not been published). The residue at position 501 corresponding to a Lys in the cDNA sequence of lamb kidney Na,K-ATPase was not detected, indicating that this is the amino acid modified by SITS. The reason for the different elution positions in the C18 column chromatography of peptides 15 and 17 is not clear, since they yielded the same amino acid sequence and the same site of labeling.

4. DISCUSSION

The sequence of the peptide fragments labeled by SITS indicated that the binding of the probe was to Lys-501 on the α-subunit of Na,K-ATPase. The two major peptides sequenced account for most of the SITS labeling (see Fig. 2, top panel). A repeat of the entire labeling/purification/sequencing scheme gave identical results (data not shown). There were other minor peaks labeled, and their labeling was prevented by K or ADP, but they were not sequenced. The SITS labeling was specific and it was nearly abolished by the presence of ADP (an ATP analog) or K, both of which are ligands of the enzyme. The fact that these two ligands affected the inactivation suggests that the region around the labeled amino acid (Lys-501) may be involved in or is modified by conformational changes associated with the binding of these ligands. It is not yet clear why SITS apparently covalently modifies Lys-501, while preliminary data suggests that H_2DIDS is attached to a lysine residue closer to the carboxyl-terminus [10].

Lys-501 has been shown to be labeled by FITC (fluorescein isothiocyanate) which also inactivates the Na,K-ATPase [11,12] and also by NIPI (*N*-(2-nitro-4-isothiocyanophenyl)-imidazole) [13,14]. SITS, FITC, and NIPI are all aryl isothiocyanates. The different extents to which K ions protect against NIPI and SITS inactivation probably has a kinetic basis and depends upon the relative reactivities of Lys-501 with these reagents. The fact that labeling and inactivation of the Na,K-ATPase by these probes can be prevented by ATP supports the hypothesis that they bind to the nucleotide binding site. However, recent experimental evidence has cast doubt on the idea that Lys-501 is an essential part of the ATP site. For example, antibodies which specifically bind to the segment that includes Lys-501 failed to inhibit Na,K-ATPase activity [15]. Site-directed mutagenesis of the corresponding FITC-reactive Lys to Glu in the sarcoplasmic reticulum Ca^{2+}-ATPase resulted in only a 5% decrease in Ca^{2+} transport [16], and more recent analysis of Ca^{2+} pump mutants suggests that this lysine residue is not essential for ATP binding [17]. These data indicate that Lys-501 and its amino group may not be essential for activity, but that the binding of chemical probes to Lys-501 may inhibit the enzyme activity by interfering with neighboring amino acids, by filling a space necessary for the binding/hydrolysis of ATP or disturbing conformational changes associated with the enzymatic cycle of the protein.

Table I

Sequence data from the SITS-labeled peptides isolated from the α-subunit

Cycle	Peak 17		Peak 15	
	Amino acid (residue no.)	pmol	Amino acid (residue no.)	pmol
1	His (496)	14	His (496)	7
2	Leu (497)	36	Leu (497)	8
3	Leu (498)	38	Leu (498)	7
4	Val (499)	42	Val (499)	9
5	Met (500)	28	Met (500)	6
6	X		X	
7	Gly (502)	16	Gly (502)	4
8	Ala (503)	21	Ala (503)	4
9	Pro (504)	10	Pro (504)	3
10	Glu (505)	10	Glu (505)	2
11	Arg		Arg	

Amino acids in cycles 6 and 11 were not identified, and they correspond to Lys and Arg, respectively, in the published sequence of the sheep kidney Na,K-ATPase α-subunit [9]. Peptide C-terminal Arg residues are very often not detected in protein sequencing. No other amino acids were present in several sequencer cycles after 11 on either sample, consistent with the presumed trypsin cleavage at the undetected Arg residue.

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