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Examination of the molecular mechanism of SH reagent-induced inhibition of the intestinal brush-border membrane Na^+/phosphate cotransporter

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Abstract

SH residues on the rabbit intestinal brush-border membrane Na^+/phosphate cotransporter were examined using a variety of SH specific reagents, proteolytic digestion and HPLC separation of SH-labeled cotransporter, and partial reaction assays. Of the seven SH-containing peptide fragments on the non-denatured non-reduced cotransporter six peptides were labeled: five SH-containing peptides were labeled with acrylodan or IAF (iodoacetamidofluorescein) and three peptides were labeled with IAEDANS. One SH-containing peptide was labeled with IAEDANS or fluorescein maleimide only. Selective SH labeling conditions employing acrylodan and IAEDANS were used to identify the environments of these SH-containing peptides in the native cotransporter. The nature of SH reagent-induced inhibition of Na^+-dependent phosphate uptake was examined using substrate-induced conformational changes, and substrate-induced changes in IAEDANS and acrylodan fluorescence of the SH-labeled Na^+/phosphate cotransporter. The results indicate that five of the SH-labeled peptides sense the Na^+-induced conformational change, three peptides sense the Na^+ + difluorophosphate-induced conformational change, and one peptide senses only the Na^+ + monofluorophosphate-induced conformational change. Five of the SH-labeled peptides are passive participants in the substrate-induced conformational changes with only SH 51 involved in cotransporter function. Alkylation of SH 51 resulted in a cotransporter conformation which differed from the substrate-mediated conformations and was characterized by increased monofluorophosphate sensitivity.

Keywords: Sodium ion/phosphate cotransporter; Phosphate transport; Inhibition; Sulfhydryl specific reagent; Molecular mechanism; Brush-border membrane; (Intestine)

1. Introduction

Sulfhydryl residues are thought to be involved in substrate transport in a variety of membrane transport proteins including the intestinal [1–3] and renal [4,5] brush-border membrane Na^+/phosphate cotransporters. The role of these residues in substrate transport is poorly understood. Chemical modification studies examining the role of SH residues in membrane transport have been limited by multiple SH residues per transport protein, the non-competitive nature of the sulfhydryl reagent-induced inhibition, transport protein specific and non-specific SH reagent effects, the absence of partial reaction assays to define SH involvement in the transport cycle, and the absence of corroborating effects in site-directed mutagenesis studies. Considering the significant role of SH residues in carrier-mediated substrate transport across biological membranes and protein structure [6–8] understanding the mechanism of SH chemical modification-induced inhibition of membrane transport proteins is an important area of research.

An initial step in understanding the molecular mechanism of SH reagent chemical modification and inhibition of substrate transport is the development of methods of identifying the number of labeled residues, defining the environments of the residues in the native protein and examining the effect of SH residues on the transport protein using partial reaction assays. We have developed partial reaction assays which identified four conformations of the Na^+/phosphate cotransporter which are consistent with these conformations being involved in substrate transport [9]. Using these conformational assays the macromolecular mechanism of SH reagent-induced inhibition of Na^+/phosphate cotransport was suggested to result from a

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distinct but altered Na^+ conformation [9]. These results contrast with similar studies of the Na^+/glucose cotransporter which suggested an altered substrate-free conformation [10,11].

We have extended the examination of the role of SH residues in substrate transport using the environment-sensitive SH reagents, acrylodan and IAEDANS, to examine the SH-containing peptides of the intestinal brush-border membrane Na^+/phosphate cotransporter. We have examined the effect of SH reagents on substrate-induced conformational changes, and characterized the direct effects of substrates on the SH reagent modified cotransporter. Our results suggest that inhibitory SH reagents alter the Na^+/phosphate cotransporter Na^+ conformation without inhibiting the Na^+-induced conformational change. This modified Na^+ conformation resulted in a modified Na^+ + phosphate conformation which had a reduced V_{max}. The results are consistent with all but one of the SH-containing peptides being affected by the Na^+-induced conformational change as passive participants (fluorescence affected, but SH-labeling having no effect on Na^+ or Na^+ + phosphate conformation). Modification of the SH 51 peptide altered the Na^+ and Na^+ + phosphate conformation directly. The results also suggest that with respect to SH reagent modified proteins and perhaps other non-competitive inhibitor-modified proteins, structure/conformation relationships might be a more appropriate paradigm than structure/function relationships.

2. Materials and methods

2.1. Materials

Chromatofocusing chromatography supplies and Sepharose S-200 were purchased from Pharmacia, Piscataway, NJ. [^{32}P]Phosphate was purchased from NEN/Du Pont, Boston, MA. Fluorescent SH reagents were purchased from Molecular Probes, Eugene, OR. Proteolytic enzymes, PCMB, PCMBS, iodoacetamide, DTT, NEM, phosphatidylcholine, cholesterol, and buffers were purchased from Sigma, St. Louis, MO. Electrophoretic supplies were purchased from Bio-Rad, Richmond, CA. TFA was purchased from Pierce, St. Louis, MO. All organic solvents were purchased from Aldrich, Milwaukee, WI and were HPLC grade or better. All other chemicals were purchased from Fisher, Plano, TX and were reagent grade or better.

2.2. Methods

Intestinal brush-border membrane vesicle preparation

Rabbit intestinal brush-border membrane vesicles were prepared by Ca^{2+} precipitation (Ca^{2+}-BBMV) as previously described [12]. Ca^{2+}-BBMV protein was resuspended in 300 mM mannitol and 10 mM Hepes-Tris (pH 7.5), and stored at liquid N_2 temperatures until needed.

SDS-BBMV protein (SDS-treated Ca^{2+}-BBMV protein) was prepared as previously described [13,14]. SDS-BBMV protein was resuspended in 300 mM mannitol and 10 mM Hepes-Tris (pH 7.5), and stored at liquid N_2 temperatures until needed.

Ca^{2+}-BBMV protein and SDS-BBMV protein purification were assayed using the brush-border enzyme markers, alkaline phosphatase [15] and sucrase [16]. Compared to the intestinal homogenate activities, Ca^{2+}-BBMV protein was 24-fold to 32-fold enriched in alkaline phosphatase and sucrase. SDS-BBMV protein was 85-fold to 100-fold enriched in these brush-border membrane markers.

Na^+/phosphate cotransporter purification and proteoliposome reconstitution

The intestinal Na^+/phosphate cotransporter was purified as previously described [3]. Protein was resuspended in 150 mM KCl and 10 mM Hepes-Tris (pH 7.5) at a final concentration of 5 mg/ml to 10 mg/ml and stored at liquid N_2 temperatures until needed.

Na^+/phosphate cotransporter was reconstituted into phosphatidylcholine/cholesterol liposomes as previously described [3]. Cotransporter purification was determined using a rapid mixing/rapid quenching assay using Na^+-dependent [^{32}P]phosphate uptake into proteoliposomes [3,17]. Na^+ dependent uptake was determined measured as uptake in the presence of 100 mM cis Na^+ minus uptake in the presence of 100 mM cis TMA^+.

SH reagent conditions

50 μg of proteoliposome reconstituted protein were reacted with the indicated SH reagent (SH reagent concentration varied from 10 μM to 250 μM) in 100 mM TMACl, 50 mM KCl, and 25 mM Hepes-Tris (pH 7.5) (SH reaction media) for 2 min at 23°C. The reaction was terminated by addition of a 25-fold dilution of ice-cold 100 mM TMACl, 50 mM KCl, and 25 mM Hepes-Tris (pH 7.5). Protein was collected by centrifugation at 100 000 × g for 60 min. Pellets were resuspended in the appropriate media by 10 passes through a 0.23 gauge needle.

In some experiments proteoliposome reconstituted protein reacted with PCMB or PCMBS was treated with 50 mM DTT, and SH reaction media for 20 min at 4°C to reverse PCMB and PCMBS binding. The reaction was stopped and protein collected by centrifugation as described above.

Uniform fluorescent SH reagent modification of the intestinal brush-border membrane Na^+/phosphate cotransporter by acrylodan, IAEDANS, or IAF, was performed with the detergent solubilized cotransporter in 50 mM Tris-HCl (pH 7), and 0.1% NOG (n-octyl glucoside) using 15 μM fluorescent label in the dark for 15 min at 23°C. The reaction was terminated by addition of a 10-fold excess of 50 mM Tris-HCl (pH 7) and 0.1% NOG. The

Table 1
SH reagent labeling of papain fragments

Reagent	Fragment #					
	18	23	31	38	43	51
IAF	X	X	X	X		X
IAF + PCMBS	X	X	–	–		–
IAF + PCMB	X	–	X	X		–
Acrylodan	X	X	X	X		X
Acrylodan + PCMBS	X	X	–	–		–
Acrylodan + PCMB	X	–	X	X		–
IAEDANS		X		X	X	
IAEDANS + PCMBS		X		–	X	
IAEDANS + PCMB		–		X	X	
NEM + IAEDANS		–		–	–	

X indicates labeled with fluorescent derivative, no mark indicates did not react, and – indicates protected from labeling.

reactants were washed and concentrated by centrifugation (1500 × *g* for 30 min) through a 1 kDa cut-off filter using an Amicon micropartition system [3,18], followed by dialysis for 24 h against 10 mM Tris-HCl (pH 7) with three buffer changes.

Specific SH labeling of the detergent solubilized Na^+/phosphate cotransporter was performed in SH reaction media + 0.1% NOG according to the protocols in Tables 1 and 2. NEM, PCMB, PCMBS, or iodoacetamide pretreatment was performed for 10 min at 23°C. The reaction was terminated by dilution and protein separated from unreacted SH reagent by 1 kDa partition centrifugation and dialysis as described above. Removal of PCMB and PCMBS with DTT was performed as described above. Labeling of the pretreated Na^+/phosphate cotransporter with fluorescent SH reagents was performed as described for uniform SH labeling.

Experiments examining the role of SH-labeled peptides in Na^+/phosphate cotransporter function using DTT reversal of PCMB and PCMBS labeling were checked for completeness of organic mercurial removal as follows. Following fluorescent SH reagent labeling of the cotransporter and DTT treatment, an aliquot of cotransporter was labeled with IAF or Fmal (fluorescein maleimide) as described above. Cotransporter was subsequently digested with papain as described below and the SH-labeled peptides examined by HPLC using the Protein Pak 125 column as described below. The effectiveness of DTT reversal of organic mercurial binding to the cotransporter was determined by comparison to direct IAF or Fmal labeling of the cotransporter per milligram fluorescent peptide. DTT treatment resulted in greater than 90% removal of organic mercurial.

DTNB labeling of the Na^+/phosphate cotransporter was performed following protein solubilization in 100 mM Tris-HCl (pH 8), 10 mM EDTA, and 0.1% NOG (non-reducing conditions) or following protein solubilization in 10 mM Tris-HCl (pH 8), 1% SDS, 50 mM β-mercaptoethanol and heating at 95°C for 3 min. DTNB determinations were performed using 1.5 mg of protein, 500 μM DTNB, and 50 mM phosphate buffer (pH 7). The change in absorbance at 412 nm was determined as a function of time using a Gilford 260 spectrophotometer corrected for DTNB reduction (protein blank) and protein absorbance (DTNB blank) [19].

Proteolytic digestion

200 μg of cotransporter or SH reagent treated cotransporter was resuspended in 100 mM NH_4HCO_3 (pH 8), 0.1% SDS, and 15 mM β-mercaptoethanol and heated for 3 min at 95°C. Protein was digested at 1:100, or 1:25 proteinase/protein ratio (w/w) for varying times (1 h to 6 h) at 37°C. The reaction was stopped by addition of a 20-fold excess of soybean trypsin inhibitor (trypsin), TPCK (chymotrypsin), iodoacetamide (papain), or difluoroisopropyl phosphate (V-8 proteinase). Protein and peptides were concentrated by lyophilization, and dialyzed against 10 mM Tris-HCl (pH 7) using 1 kDa cut-off dialysis tubing.

Table 2
SH labeling procedures

Reaction conditions	SH 18	SH 23	SH 31	SH 38	SH 43	SH 51
1. NEM + PCMB		X		–	X	
2. DTT						
3. IAEDANS				X		
4. IAEDANS + PCMB + PCMBS		–		–	X	
1. NEM + PCMBS		–		X	X	
2. DTT						
3. IAEDANS				X		
1. NEM		X		X	X	
2. Acrylodan + PCMB	X		–			–
1. Iodoacetamide + PCMBS	X	–	X	X		X
2. DTT						
3. Acrylodan + PCMB		X				–
1. NEM		X		X	X	
2. Iodoacetamide + PCMB	X		–			–
3. DTT						
4. Acrylodan + PCMBS			X			
1. NEM + PCMB		X		–	X	
2. Iodoacetamide + PCMB	X		X	–		–
3. DTT						
4. Acrylodan + PCMBS				X		–
1. NEM		X		X	X	
2. Iodoacetamide + PCMBS	X		X			–
3. DTT						
4. Acrylodan						X

X indicates labeled with fluorescent derivative, no mark indicates did not react, and – indicates protected from labeling.

Peptide fragments resolved by HPLC (Protein Pak 125 fractions and RP C_4 column fractions) were dialyzed against 10 mM Tris-HCl (pH 7) using 1 kDa cut-off dialysis tubing to reduce SDS concentration. Following dialysis, peptides were concentrated by lyophilization and resuspended in the appropriate media.

CNBr hydrolysis

HPLC fractions or Na^+/phosphate cotransporter were resuspended in 70% formic acid and treated with an equal amount (w/w) of CNBr in 70% formic acid for 18 hours in the dark under N_2. The reaction was terminated by a 20-fold dilution with 10 mM Tris-HCl (pH 7), and the mixture lyophilized. This step was repeated twice to ensure complete removal of CNBr.

HPLC purification of peptides

Peptide fragments were initially resolved on a 7.8 mm × 30 cm Waters Protein Pak 125 column with 50 mM Tris-HCl (pH 7), and 0.1% SDS at a flow rate of 0.4 ml/min. Protein was monitored at 215 nm using a Waters Lambda Max 481 variable wavelength UV/Vis detector. Fluorescence was monitored using a Kratos Spectroflow 980 fluorescence detector. Fluorescence was excited at 290 nm and monitored using band pass filters (fluorescein, above 500 nm; acrylodan, above 450 nm; and IAEDANS, above 400 nm).

SH-labeled peptide fragments were collected, dialyzed, and resuspended in 0.1% TFA. Peptide fragments were resolved on a Waters C_4 column using a 60 min linear gradient of 0.1% TFA and 0.1% TFA + 70% CH_3CN at a flow rate of 0.5 ml/min.

Secondary peptide fragments were generated from the primary (V-8 proteinase or papain) SH-labeled fragments following two C_4 column purifications. Peptide fragments were resuspended in 100 mM NH_4HCO_3 (pH 8), 0.1% SDS, and 15 mM β-mercaptoethanol and digested with chymotrypsin (V-8 proteinase primary peptide fragments) or V-8 proteinase (primary papain fragments) for 4 h at 37°C at a peptide/proteinase ratio of 25:1. Peptides were lyophilized and dialyzed as described above. Secondary peptide fragments were resolved on the C_4 column using a linear gradient of 0.1% TFA and 0.1% TFA/70% CH_3CN as described above. SH 23 and SH 18 peptides were further resolved on a Waters RP CN column using a 60 min linear gradient of 20% 1-propanol and 80% 1-propanol at a flow rate of 0.4 ml/min.

Fluorescence spectroscopy

All fluorescence experiments were performed on an SLM SPF 500 C spectrofluorometer at 23°C set in the ratio mode. Acrylodan fluorescence was excited at 390 nm and emission recorded as a function of wavelength from 400 nm to 600 nm. IAEDANS fluorescence was excited at 337 nm and recorded as a function of wavelength from 350 nm to 600 nm.

The effect of substrates on acrylodan and IAEDANS fluorescence was performed in 500 mM KCl, 0.1% NOG, and 50 mM Tris-HCl (pH 7). The fluorescence was recorded and NaCl was then added to a final concentration of 100 mM from a 3 M stock and the fluorescence recorded. Monofluorophosphate (final concentration 25 μM) or difluorophosphate (final concentration 200 μM) were then added and the fluorescence recorded. The results are presented as change in fluorescence, $\Delta F/F$ (change in fluorescence/initial fluorescence).

Experiments examining the Na^+-induced conformational change using FITC-PG fluorescence quenching were performed as previously described [17,20]. 50 μg of protein was labeled with FITC-PG in 50 mM Tris-HCl (pH 7) and 0.1% NOG as previously described [17,20]. FITC-PG fluorescence quenching was examined using 10 μg of protein, 0.1% NOG, 0.5 M KCl, and 50 mM Tris-HCl (pH 7). Where indicated 100 mM KCl or 100 mM NaCl were added from 3 M stock solutions.

Inner filter effects were minimized by maintaining protein concentrations below 0.02 O.D. units. Light scatter was corrected using deproteinated proteoliposomes as previously described [21,22]. Dilution artefacts were corrected by comparison of the effects of NaCl and KCl on fluorescence emission and minimized by using concentrated stock solutions.

Tryptophan fluorescence

Tryptophan fluorescence experiments were performed on an SLM SPF 500 C at 23°C set in the ratio mode. Tryptophan fluorescence was examined using 15 μg of protein, in 0.5 M KCl, 0.1% NOG (n-octyl glucoside), and 50 mM Tris-HCl (pH 7). The excitation wavelength was set at 290 nm and emission at 350 nm recorded. In some experiments emission was recorded as a function of wavelength from 300 nm to 400 nm. Excitation and emission slit widths were 2.5 nm.

The effect of substrates on tryptophan fluorescence were examined by addition from concentrated stocks to minimize dilution artefacts. Results were analyzed using a non-linear regression fit of the fluorescence results. Experiments examining the effect of phosphate, fluorophosphates, or arsenate were performed in the presence of 150 mM NaCl.

SDS-PAGE electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Laemmli [23] using 7.5% slab gels, or 10% to 22% linear gradient gels. In experiments using linear gradient gels fluorescein sulfonate was substituted for Bromophenol blue as the tracking dye. Fluorescein sulfonate runs with the ion front, allowing resolution of small (< 3 kDa) peptides.

In some experiments gel lanes were excised and analyzed by scanning densitometry using a Gilford spectrophotometer with the gel scanner. IAF or Fmal absorbance

was monitored at 470 nm, and Coomassie blue absorbance was monitored at 550 nm.

Protein assays

SDS-BBMV protein and Ca^{2+}-BBMV protein were assayed by the method of Bradford using bovine IgG as standard [24]. All chromatography fractions and proteoliposome reconstituted protein were assayed using the SDS-micro Lowry assay with BSA as standard [25].

Chemical synthesis

FITC-PG was synthesized as previously described [20]. FITC-PG was purified using a Sepharose-polylysine column, eluted with 50 mM potassium tetraborate buffer (pH 7.4), and a silica gel column developed with butanol/acetic acid/water (50:40:10, v/v) as previously described [20].

Potassium monofluorophosphate and potassium difluorophosphate were prepared as previously described [14,26].

3. Results

3.1. SH reagent labeling of the Na^+/phosphate cotransporter

Na^+-dependent phosphate uptake by the intestinal Na^+/phosphate cotransporter has been reported to be sen-

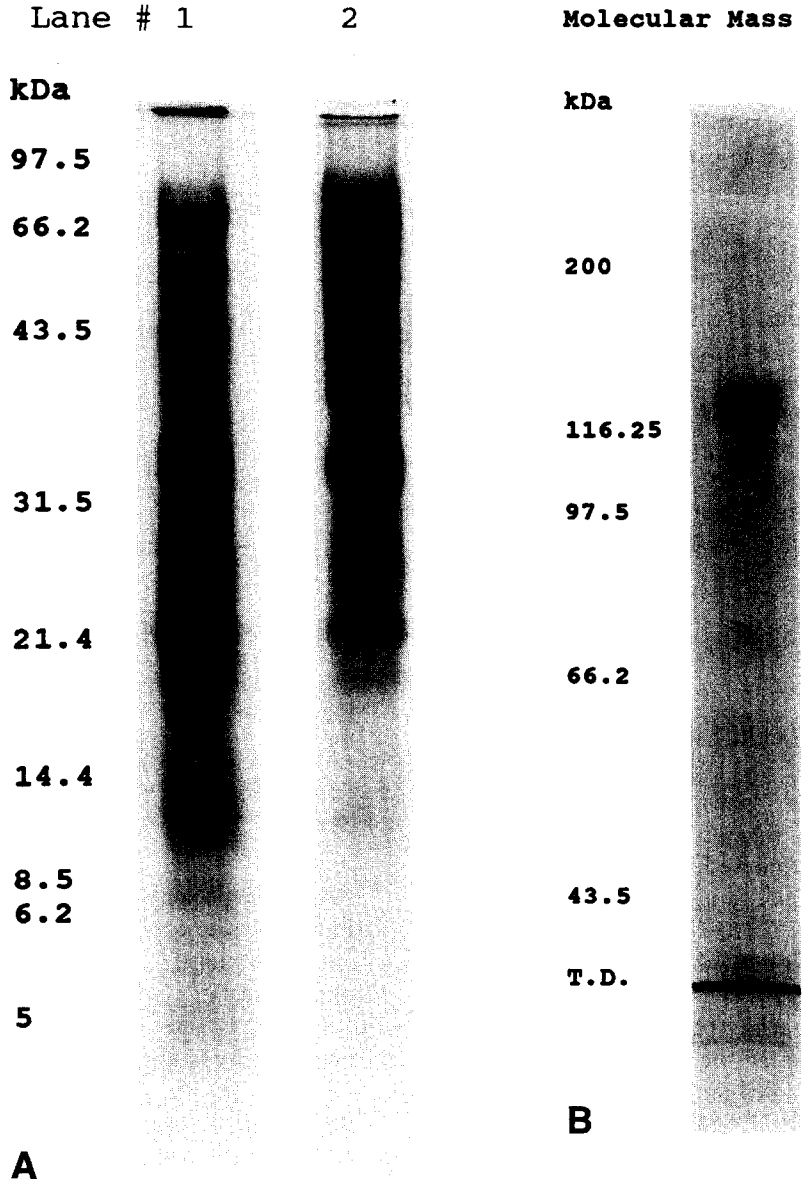

Fig. 1. SDS-PAGE of primary papain and primary V-8 proteinase digestion of Na^+/phosphate cotransporter. (A) 100 μg of Na^+/phosphate cotransporter was digested with papain (lane 1) or V-8 proteinase (lane 2) at a protein/proteinase ratio of 100:1 (w/w) for 4 h at 37°C in 100 mM NH_4HCO_3 (pH 8) and 0.1% SDS. The reaction was quenched as described in Methods. 25 μg was resolved on a 10–22% linear gradient gel. Results are from a single experiment and representative of five separate experiments. (B) 25 μg of Na^+/phosphate cotransporter was run on a 7.5% SDS-PAGE gel and stained with Coomassie blue as described in Methods. Results are from a single cotransporter preparation and representative of four preparations.

sitive to the SH reagents, iodoacetamide and organic mercurials (PCMB and PCMBS) but not to NEM [3]. To examine this difference in SH reagent sensitivity we examined SH labeling of the cotransporter. DTNB labeling of the Na^+/phosphate cotransporter was consistent with 13 SH residues (12.6 ± 0.6, $n = 3$) on the reduced, denatured Na^+/phosphate cotransporter, and seven SH residues (6.6 ± 0.3, $n = 3$) on the non-reduced, non-denatured Na^+/phosphate cotransporter being accessible to DTNB labeling. SH labeling by iodoacetamide and NEM of the intestinal brush-border membrane Na^+/phosphate cotransporter was examined using proteolytic digestion of labeled cotransporter. The results are summarized in Figs. 1–3.

Fig. 1A is a Coomassie blue stained 10% to 22% linear gradient SDS-PAGE gel of the peptide fragments generated by proteolytic digestion of the Na^+/phosphate cotransporter with papain (lane 1) or V-8 proteinase (lane 2). Multiple peptide fragments were generated by both proteinases ranging from 60 kDa to 6 kDa. For comparison, the undigested Na^+/phosphate cotransporter is shown in Fig. 1B.

The Na^+/phosphate cotransporter labeled with IAF (iodoacetamidofluorescein) was also digested with papain (Fig. 2 top), V-8 proteinase (Fig. 2 middle), or chemically hydrolyzed with CNBr (Fig. 2 bottom). IAF-labeled peptides (broken lines) were determined by fluorescein absorbance prior to Coomassie blue staining (solid line). The results are consistent with five IAF-labeled peptides on the non-denatured, non-reduced Na^+/phosphate cotransporter. Similar experiments with IAEDANS-labeled cotransporter resulted in three peptides being labeled with this SH reagent, one of which appeared to be unique to IAEDANS (results not shown).

3.2. HPLC resolution Na^+/phosphate cotransporter fragments

Polypeptides resulting from papain or V-8 proteinase proteolytic digestion of the IAF-labeled, acrylodan-labeled, and IAEDANS-labeled Na^+/phosphate cotransporter were also examined by HPLC using an initial separation by the Protein Pak 125 column followed by C_4 column purification of the SH-labeled peptides using a linear gradient of 0.1% TFA and 0.1% TFA/70% CH_3CN. The peptide fragments containing SH-labeled residues were subsequently digested with chymotrypsin (V-8 proteinase I° fragments), or V-8 proteinase (papain digestion I° fragments) and then separated on the C_4 column. Four peptides, SH 18 and SH 23 from the papain/V-8 proteinase digestion protocol, and SH 19 and SH 21 from the V-8 proteinase/chymotrypsin digestion protocol were hydrolyzed with CNBr (SH 18, and SH 23) and resolved on a RP CN column, or pepsin digested (SH 19 and SH 21) and resolved on the C_4 column to ensure that these peptides were not incomplete digestion products of each other. The results of these studies are summarized in Fig. 3.

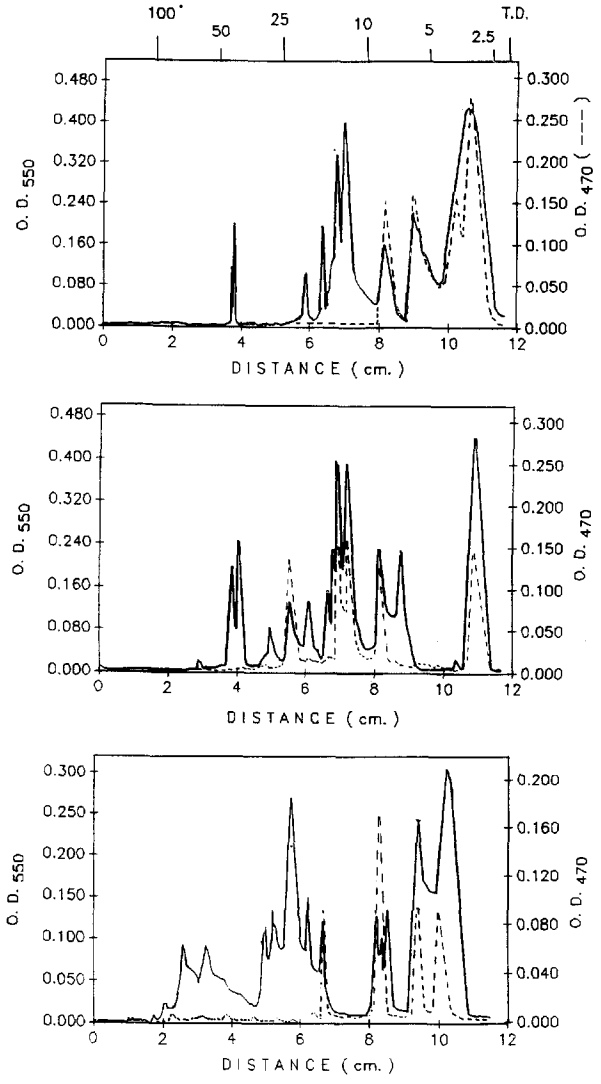

Fig. 2. SDS-PAGE resolution of CNBr, papain, and V-8 proteinase pepetide fragments. 125 μg of Na^+/phosphate cotransporter was uniformly labeled with IAF as described in Methods. Labeled cotransporter was hydrolyzed with CNBr for 12 h at 23°C (top), digested with papain (middle), or V-8 proteinase (bottom) was resolved on a 10% to 22% linear gradient gel, examined for IAF fluorescence (broken line) and then stained with Coomassie blue (solid line). Results are representative for three separate experiments.

Using the papain/V-8 proteinase digestion protocol and HPLC analysis, labeling methods specific for IAF-labeled peptides and IAEDANS-labeled peptides were developed. Identical labeling patterns were seen with IAF labeling and acrylodan labeling. The specific SH-containing peptides labeling methods are summarized in Methods.

3.3. Specific SH labeling

Acrylodan labeling of the Na^+/phosphate cotransporter resulted in two broad fluorescence emission peaks (results not shown). A peak centered near 470 nm corresponding to acrylodan-labeled residues in hydrophobic environments (fluorescence emission of acrylodan in acetonitrile is at

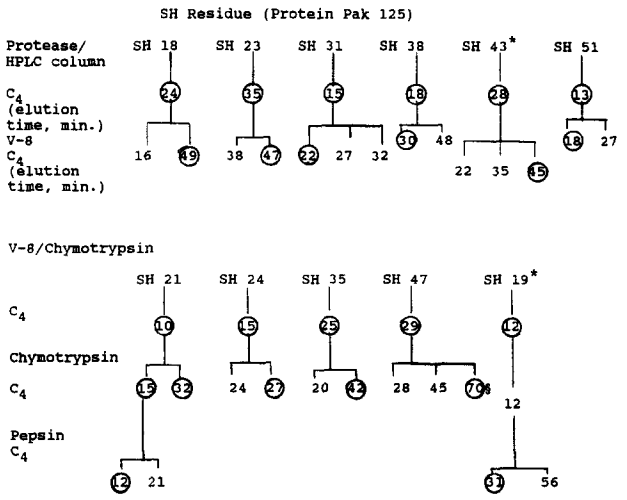

Fig. 3. Primary and secondary digestion fragments of the Na^+/phosphate cotransporter. IAF- or IAEDANS-labeled cotransporter was digested with papain or V-8 proteinase as described in Methods. Peptide fragments were resolved on a Waters Protein Pak 125 HPLC column as described in Methods. Fluorescent peptide fragments were further resolved on a Waters RP C_4 HPLC column, and subjected to secondary digestion (papain fragments with V-8 proteinase, and V-8 proteinase fragments with chymotrypsin). Peptide fragments were resolved on the C_4 column. Numbers represent column elution time and circled fragments represent fluorescent fragments. Results summarize a total of 35 HPLC experiments. * IAEDANS labeled. § following 45-min linear gradient of 70% CH_3CN, a 30-min linear gradient of 70% CH_3CN/ 20% THF was run. This fragment eluted at 10% THF.

450 nm), and a second peak centered at 515 nm corresponding to acrylodan in a hydrophilic environment (acrylodan emission in water is at 530 nm).

Acrylodan emission of SH residues labeled at specific residues of the Na^+/phosphate cotransporter based on proteolytic polypeptide fragment analysis is shown in Fig. 4. Three general emission regions were seen. SH 18 (open dots) and SH 23 (solid dots) had fluorescence emission maxima near 460 nm and were classified as being located in hydrophobic sectors of the Na^+/phosphate cotransporter. SH 51 (solid line) and SH 31 (dashed line) had emission maxima near 530 nm and were classified as being located in hydrophilic regions of the cotransporter. Intermediate between these two extremes, SH 38 had an emission maxima near 510 nm.

The fluorescence emission of IAEDANS-labeled peptides (SH 23, SH 38, and SH 43) are shown in Fig. 5. In agreement with our analysis of acrylodan-labeled peptides, IAEDANS-labeled SH 23 had a fluorescent emission consistent with a hydrophobic environment (IAEDANS emission at 360 nm) and IAEDANS-labeled SH 38 had an hydrophilic emission (470 nm). Labeling of SH 43 was unique to IAEDANS. SH 43 appeared to reside in an environment more hydrophobic than SH 38, but more hydrophilic than SH 23 (IAEDANS emission at 380 nm).

3.4. SH polypeptide fragments involved in Na^+-dependent phosphate uptake

The selective SH labeling procedures summarized in the Methods were used to identify the SH residue(s) involved in non-competitive inhibition of Na^+-dependent phosphate uptake. Three assays of cotransporter function were used to examine cotransporter function: (1) Na^+-induced FITC-PG fluorescence quenching, (2) substrate-induced tryptophan fluorescence quenching, and (3) Na^+-dependent phosphate uptake. The results are summarized in Figs. 6–8.

Fig. 6 shows the effect of NEM or iodoacetamide labeling of the SH-containing peptides on Na^+-dependent

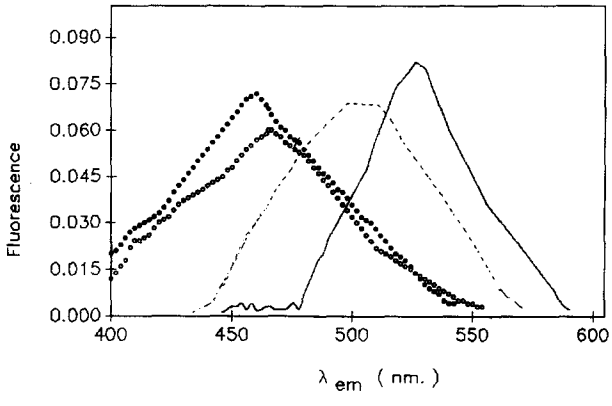

Fig. 4. Acrylodan fluorescence emission of Na^+/phosphate cotransporter. 50-μg aliquots of cotransporter were labeled with acrylodan at the specified SH residue(s) as described in Table 1. Acrylodan fluorescence was determined using 15 μg of labeled cotransporter in 500 mM KCl, 20 mM Tris-HCl (pH 7), and 0.1% NOG. Acrylodan fluorescence was excited at 390 nm and emission recorded between 400 nm and 600 nm. Results are from a single experiment and representative of five experiments. Solid line, SH 51; solid dots, SH 23; dashed line, SH 31; and open dots, SH 18.

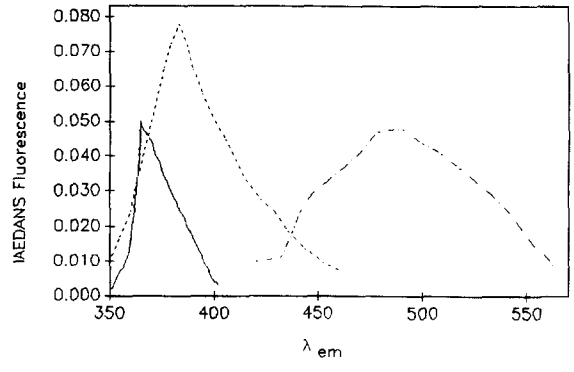

Fig. 5. IAEDANS fluorescence emission of Na^+/phosphate cotransporter. 50 μg of Na^+/phosphate cotransporter was labeled with IAEDANS at the specified SH residue(s) as described in Table 1. IAEDANS fluorescence was determined using 15 μg of labeled protein in 500 mM KCl, 20 mM Tris-HCl (pH 7), and 0,1% NOG. IAEDANS fluorescence was excited at 337 nm and the emission recorded from 350 nm to 500 nm. Results are from a single experiment and representative of four experiments. Solid line, SH 23; dashed-dotted line, SH 38; dashed line, SH 43.

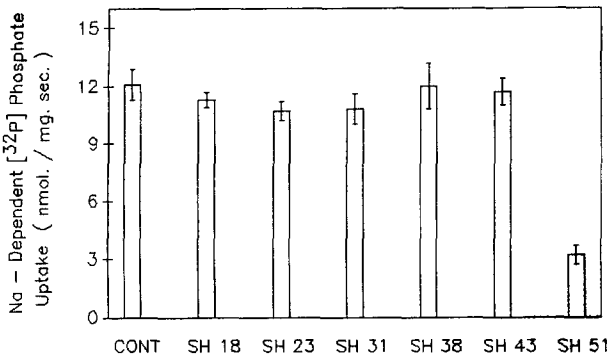

Fig. 6. Effect of labeling of SH-containing peptides on Na^+-dependent phosphate uptake. 75 μg of proteoliposome reconstituted Na^+/phosphate cotransporter was labeled with iodoacetamide or NEM at the indicated SH residue(s) as described in Tables 1 and 2. Na^+-dependent phosphate uptake was determined as described in Methods. Results are means ± S.E. of triplicate determinations and are representative of four experiments.

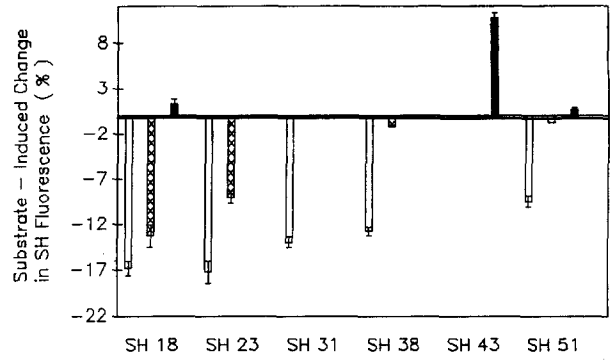

Fig. 8. Effect of substrates on acrylodan- and IAEDANS-labeled Na^+/phosphate cotransporter. Na^+/phosphate cotransporter was labeled with acrylodan or IAEDANS as described in Methods. Acrylodan and IAEDANS fluorescence was determined as described in Methods. Effect of substrates on acrylodan or IAEDANS fluorescence was determined as described in the legend to Fig. 7. Open bars, 100 mM NaCl; hatched bars, 100 mM NaCl + 200 μM potassium difluorophosphate; and solid bars, Na^+ + 50 μM potassium monofluorophosphate. Results are means ± S.E. of triplicate determinations and four experiments.

phosphate uptake. Na^+-dependent phosphate uptake was inhibited by iodoacetamide labeling of SH peptide 51. Labeling of SH 18, 23, 31, 38 or 43 (NEM) had no effect on Na^+-dependent phosphate uptake.

SH labeling of SH-containing peptides 18, 23, 31, 38, or 43 had no effect on the Na^+-induced conformational change measured by Na^+-induced FITC-PG fluorescence quenching (results not shown) or Na^+-induced tryptophan fluorescence quenching (Fig. 7, open bars). Iodoacetamide labeling of SH 51 resulted in a slight decrease in the magnitude of the Na^+-induced conformational change (33 ± 3%, $n = 4$).

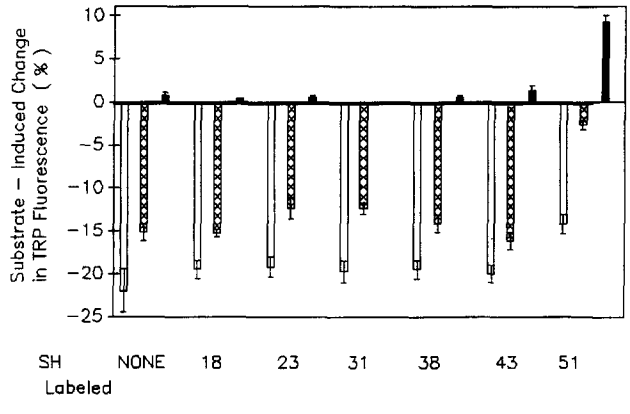

Fig. 7. Effect of substrates on tryptophan fluorescence of iodoacetamide- and NEM-labeled cotransporter. 75 μg of Na^+/phosphate cotransporter was labeled with iodoacetamide or NEM at the indicated SH residue(s) as described in Methods. Tryptophan fluorescence was determined using 15 μg of protein, 500 mM KCl, 20 mM Tris-HCl (pH 7), and 0.1% NOG. Tryptophan fluorescence was excited at 290 nm and emission at 350 nm recorded. The change in fluorescence was determined following addition of 100 mM NaCl and recorded as $(F_K - F_{Na})/F_K$ (open bars), following addition of 200 μM potassium difluorophosphate and recorded as $(F_{Na} - F_{Na+DFP})/F_{Na}$ (hatched bars), or Na^+ + 50 μM potassium monofluorophosphate and recorded as $(F_{Na} - F_{Na+MFP})/F_{Na}$ (solid bars). Results are means ± S.E. of triplicate determinations and five experiments.

The apparent $K_{0.5}$ values for Na^+ were unaffected by SH labeling of the SH-containing peptides by iodoacetamide (parent compound of IAF), NEM (parent compound of IAEDANS), or PCMB (results not shown). These results are consistent non-competitive inhibition of Na^+-dependent phosphate uptake by SH reagents [3,4].

Fig. 7 (hatched bars) shows that the tryptophan fluorescence quenching upon addition of phosphate (difluorophosphate) in the presence of Na^+ (Na^+ + phosphate-induced conformational change) was inhibited by iodoacetamide or organic mercurial labeling of SH 51. Iodoacetamide or NEM labeling of the other SH residue containing peptides had no effect on the Na^+ + phosphate-induced conformational change. Similar results were seen with Na^+ + monofluorophosphate (Fig. 7, solid bars). Labeling SH 51 resulted in a fluorescence enhancement of tryptophan fluorescence not seen in untreated controls nor following SH labeling of the other SH-containing peptides.

Acrylodan and IAEDANS fluorescence has been shown to be environment sensitive [27,28]. The effect of substrates on acrylodan and IAEDANS fluorescence bound to the Na^+/phosphate cotransporter at specific SH-containing peptides was examined to determine the role of the SH-containing peptides in substrate-induced conformational changes. The results are summarized in Fig. 8.

The fluorescence of five SH-containing peptides was affected by the addition of Na^+ (Fig. 8, open bars). The response to Na^+ was SH-containing peptide dependent varying from 20% (SH 23) to 12% (SH 51). Only SH 43 did not respond to Na^+. SH 18 and SH 23 responded to Na^+ + difluorophosphate (Fig. 8, hatched bars). Addition of Na^+ and difluorophosphate resulted in a 13% quenching of SH 18 fluorescence and a 9% quenching of SH 23 acrylodan fluorescence. SH 51 and SH 38 did not respond to Na^+ + difluorophosphate. SH 43 did not respond to

Na^+ or Na^+ + difluorophosphate. Only SH 43 had a significant response to Na^+ + monofluorophosphate (12% fluorescence enhancement).

3.5. Effect of labeling SH 51 on Na^+/phosphate cotransporter conformation

The inhibition of Na^+-dependent phosphate uptake (Fig. 6), and the inhibition of tryptophan fluorescence quenching (Fig. 7) following iodoacetamide labeling of SH 51 are consistent with this SH-containing peptide being involved in Na^+-dependent phosphate uptake. The effect of labeling SH 51 with iodoacetamide, on the remaining SH residues and tryptophan fluorescence quenching in response to substrates is shown in Fig. 9.

Labeling of SH 51 with iodoacetamide inhibited the Na^+ + difluorophosphate-induced conformational change and resulted in a tryptophan fluorescence enhancement in the presence of Na^+ + monofluorophosphate. Identical effects were seen following labeling of the other SH-containing peptides (Fig. 9).

Fig. 10 compares the effect of iodoacetamide labeling of peptide 51 on the substrate-induced quenching of the other acrylodan- and IAEDANS-labeled peptides. As previously reported [9] SH labeling did not alter SH-labeled peptide quenching by Na^+. The response to Na^+ + phosphate was altered. SH 18 and SH 23 retained SH reagent fluorescence quenching in the presence of Na^+ and difluorophosphate similar to the quenching seen prior to SH 51 labeling. SH 18 quenching was 30% reduced, and SH 23 quenching was 35% to 45% reduced following SH 51 labeling. SH 38 which had little fluorescence quenching in the presence of Na^+ + difluorophosphate, and

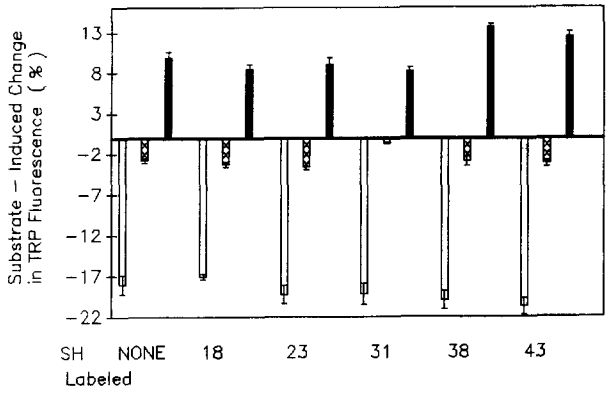

Fig. 9. Effect of substrates on tryptophan fluorescence of the Na^+/phosphate cotransporter labeled at SH 51 with iodoacetamide. Na^+/phosphate cotransporter was labeled with iodoacetamide at SH 51 as described in Methods. Cotransporter was then labeled with either iodoacetamide or NEM at the indicated SH residue(s) as described in Methods. Tryptophan fluorescence and the effect of substrates on tryptophan fluorescence was determined as described in Methods and the legend to Fig. 7. Open bars, Na^+; hatched bars, Na^+ + difluorophosphate; and solid bars, Na^+ + monofluorophosphate. Results are means ± S.E. of triplicate determinations and four experiments.

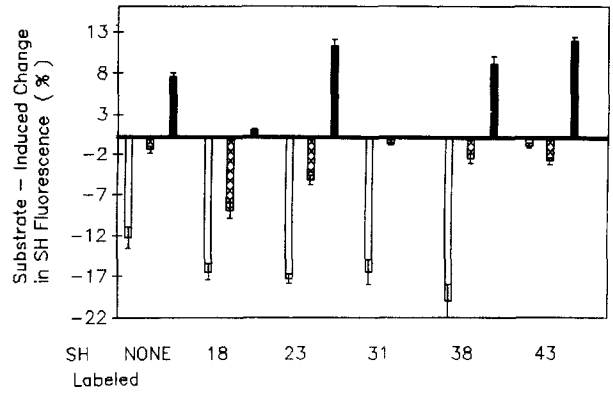

Fig. 10. Effect of substrates on acrylodan and IAEDANS fluorescence of SH 51 labeled Na^+/phosphate cotransporter. Na^+/phosphate cotransporter was labeled with iodoacetamide at SH 51 as described in Methods. Labeled cotransporter was then labeled with acrylodan or IAEDANS at the indicated SH residue(s). Effect of substrates on acrylodan or IAEDANS fluorescence was determined as described in Methods. Open bar, Na^+; hatched bar, Na^+ + difluorophosphate; and solid bar, Na^+ + monofluorophosphate. Results are means ± S.E. of triplicate determinations and five experiments.

SH 43 which did not respond to Na^+ or difluorophosphate had similar responses to substrates following SH 51 labeling. SH 31 did not respond to Na^+ + difluorophosphate following labeling of SH 51. The major affect of SH 51 labeling appeared to be the response to Na^+ + monofluorophosphate. Prior to labeling SH 51, only IAEDANS bound to SH 43 had a fluorescence enhancement in the presence of Na^+ + monofluorophosphate. Following labeling of SH 51, SH 23 and SH 38 responded to Na^+ + monofluorophosphate. These results are consistent with the major effect of non-competitive inhibitory SH reagents being an altered Na^+ conformation resulting in altered fully loaded conformations.

4. Discussion

The generation of a model of the molecular mechanism of non-competitive inhibition of membrane transport was a major goal of our studies. The first step in the development of this model was to determine the number of SH residues accessible to SH reagents on the native Na^+/phosphate cotransporter and to determine identifying characteristics which could be used to assay selective labeling procedures. The second step was to use transport cycle partial reactions to better define the reaction(s) sensitive to SH reagents.

Four identifying SH characteristics were found. The first characteristic was peptide digestion. Of the seven SH residues labeled with DTNB, six of these peptides were identified by papain or V-8 proteinase digestion, or CNBr hydrolysis. The second characteristic was differential SH reagent labeling. Three SH-containing peptides were labeled with NEM and its derivatives. Five SH-containing peptides were labeled with acrylodan, iodoacetamide, and

iodoacetamide derivatives. The third characteristic was differential SH reagent sensitivity. NEM and its derivatives had no effect on Na^+/phosphate cotransporter function. Iodoacetamide, acrylodan, IAF, and organic mercurials inhibited cotransporter function. The fourth characteristic was SH environment as determined by fluorescence emission of acrylodan-labeled and IAEDANS-labeled peptides. SH 18 and SH 23 were identified as being located in hydrophobic environments. SH 38, SH 31 and SH 51 were identified as being located in hydrophilic environments, and SH 43 was identified as being located in intermediate/hydrophobic environment.

Analysis of the role of SH-containing peptides in cotransporter function involved comparison of the effect of labeling on Na^+-dependent phosphate uptake and substrate-induced conformational changes to the effect of substrates on SH-labeled fluorescence. In this analysis, a SH-containing peptide which did not alter cotransporter function, and did not respond (fluorescence quenching or enhancement) substrates was considered not to be involved in cotransporter function. SH-containing peptides which did not alter cotransporter function, but whose fluorescence was substrate sensitive were considered to participate indirectly and not have an active role in cotransporter function. SH-containing peptides whose labeling altered cotransporter function and whose fluorescence was substrate sensitive were thought to be active participants in cotransporter function. Using these criteria, SH 18, 23, 31, 38, and 51 'sense' the Na^+-induced conformational change, and SH 51 has an active role in this conformational change. SH 18, 23, and 31 'sense' the Na^+ + difluorophosphate-induced conformational change, and SH 51 has an active role in this conformational change. SH 43 has no role in substrate transport, but does 'sense' the inhibitory Na^+ + monofluorophosphate-induced conformational change.

Labeling SH-containing peptide SH 51 resulted in a slight (33%) inhibition of Na^+-induced tryptophan fluorescence quenching, and a 83% inhibition of the Na^+ + difluorophosphate-induced conformational change. These results are consistent with previous studies suggesting that SH reagent inhibition of Na^+-dependent phosphate uptake was the result of an altered Na^+ conformation resulting in an altered Na^+ + difluorophosphate conformation [9]. The results in Figs. 9 and 10 indicate that one result of SH-containing peptide SH 51 labeling was to alter the cotransporter response to monofluorophosphate.

Monofluorophosphate is thought to bind to an intercellular/intervesicular site on the cotransporter resulting in inhibition of Na^+-dependent phosphate uptake. Monofluorophosphate competes with difluorophosphate for two conformations, but apparently does not compete for difluorophosphate (substrate) binding site [9]. The alternative is also true. Difluorophosphate does not compete with monofluorophosphate for its binding site [29]. The observation that SH 51 alkylation results in a Na^+ + monofluorophosphate sensitive conformation is likely a manifestation of SH 51 labeling. From the stand point of the molecular mechanism of SH reagent inhibition of cotransporter function, this monofluorophosphate responsiveness could result in increased sensitivity to intercellular divalent phosphate, increased cotransporter cycling time, or increased competition between the Na^+ + difluorophosphate conformation (transport competent) and the Na^+ + monofluorophosphate conformation (transport inhibited).

Our results also suggest that of the accessible SH residues only those on SH 51 are involved in substrate-induced conformational changes and substrate transport. Alkylation of SH residue(s) on peptide SH 51 resulted in a conformational change. Therefore, while removal of these/this SH residues/residue might alter the cotransporter conformation, it seems unlikely that it would result in the conformation described here. The cotransporter conformation was altered such that the response to substrates by multiple SH-containing peptides was altered. That this conformation did not conform to a typical substrate loaded conformation, for example the Na^+ conformation [9] makes it unlikely that a single residue was responsible for the gross changes in cotransporter behavior reported here.

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