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Effect of substrates and pH on the intestinal Na^+/phosphate cotransporter: evidence for an intervesicular divalent phosphate allosteric regulatory site

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Abstract

Intervesicular divalent phosphate-induced inhibition of the intestinal brush-border membrane Na^+/phosphate cotransporter was examined using Na^+-dependent phosphate uptake, substrate-induced tryptophan fluorescence quenching, and the apparent pK_a values for substrate-induced conformational changes. In right-side-out (RSO) reconstituted proteoliposomes, only monovalent phosphate inhibited Na^+-dependent phosphate uptake in the absence of pre-equilibration. Addition of divalent phosphate to inside-out (ISO) proteoliposomes resulted in $80 \pm 5\%$ inhibition of Na^+-dependent phosphate uptake in the absence of pre-equilibration. The nature of divalent phosphate-induced inhibition of cotransporter function was examined using cotransporter partial reaction assays based on substrate-induced conformational changes reported as changes in tryptophan fluorescence. Na^+ but not K^+ induced a quenching of tryptophan fluorescence with a $K_{0.5}$ of 25 mM and an apparent Hill coefficient of 1.8. Monovalent phosphate (difluorophosphate) induced a further quenching of tryptophan fluorescence with a $K_{0.5}$ of 53 μM. Divalent phosphate (monofluorophosphate) had no effect on tryptophan fluorescence, but inhibited the difluorophosphate-induced quenching of tryptophan fluorescence. The Na^+ to Na^+ + divalent phosphate (monofluorophosphate) conformation and the Na^+ to Na^+ + monovalent phosphate (difluorophosphate) conformations were compared using tryptophan quench reagents. These transitions had different apparent pK_a values and different phenylglyoxal sensitivities consistent with monovalent phosphate and divalent phosphate interacting with the cotransporter at separate sites.

Keywords: Sodium ion/phosphate cotransporter; Phosphate transport; pH effect; Substrate effect; Allosteric regulatory site; Divalent phosphate, intervesicular; Brush-border membrane; (Intestine)

1. Introduction

The intestinal brush-border membrane Na^+/phosphate cotransporter discriminates between phosphate valence states during its transport cycle. Based upon transport kinetics [1,2]; inhibition of transport [2–4]; protection against transport inhibition [3,4]; and thermodynamic constraints [1], the cotransporter appears to transport 2 Na^+ per $H_2PO_4^-$. The apparent affinity for monovalent phosphate increased with increasing external Na^+ and decreasing external pH. It is not clear if the effect of decreasing pH is related to increased $H_2PO_4^-$ concentration or increased Na^+ affinity.

Intervesicular HPO_4^{2-} has been shown to inhibit Na^+-dependent phosphate uptake based on monofluorophosphate inhibition from the intervesicular but not external side in brush-border membrane vesicles (BBMV). The apparent affinity for monofluorophosphate was 5-times higher than the apparent affinity for phosphate transport [2,3]. The interaction of divalent phosphate with the cotransporter apparently involved a single Na^+ [2,3].

The molecular mechanism responsible for divalent phosphate inhibition of transport may be modeled in terms of two general mechanisms. The first model may be thought of as locking the cotransporter in a Na^+ conformation at the internal face. By analogy to the effect of phlorizin on the Na^+/glucose cotransporter this mechanism would involve HPO_4^{2-} competing with $H_2PO_4^-$ for an internal site [5]. Alternatively, HPO_4^{2-} could bind to a second non-substrate site resulting in inhibition of Na^+-de-

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pendent phosphate uptake by delayed substrate release, protein conformational changes, or delayed substrate site recycling.

We have examined the molecular mechanism of divalent phosphate-induced inhibition of the intestinal Na^+/phosphate cotransporter. Monofluorophosphate (divalent phosphate analogue) and difluorophosphate (monovalent phosphate analogue) were used as fixed valence state analogues of phosphate [6]. Using tryptophan fluorescence and tryptophan fluorescence quench reagents, four cotransporter conformations were defined. The Na^+ + monofluorophosphate conformation did not appear to be a 'locked' Na^+ conformation based on tryptophan quench reagent sensitivities. Blocking the phosphate site with phenylglyoxal appeared to prevent the Na^+ to Na^+ + monovalent phosphate (difluorophosphate) conformational change but not the Na^+ to Na^+ + divalent phosphate (monofluorophosphate) conformational change. Finally, the Na^+ to Na^+ + monofluorophosphate conformational change had a different pK_a than the Na^+ to Na^+ + difluorophosphate conformational change. These results indicate that monovalent and divalent phosphate induced different conformational changes in the intestinal Na^+/phosphate cotransporter by binding to different sites on the cotransporter, and suggest that divalent phosphate binds to an intervesicular regulatory/non-transport binding site.

2. Materials and methods

2.1. Materials

Chromatofocusing supplies were purchased from Pharmacia, Piscataway, NJ. Phosphatidylcholine, cholesterol, proteolytic enzymes, proteolytic enzyme inhibitors, and buffers were purchased from Sigma, St. Louis, MO. Tryptophan quench reagents and reagents used in the synthesis of the fluorophosphates were purchased from Aldrich, Milwaukee, WI. [^{32}P]Phosphate was purchased from NEN/DuPont, Boston, MA. FITC (fluorescein isothiocyanate), anti-fluorescein IgG, and carboxyfluorescein diacetate were purchased from Molecular Probes, Eugene, OR. All other chemicals were purchased from Fisher and were reagent grade or better.

2.2. Purification of the intestinal Na^+/phosphate cotransporter

The intestinal Na^+/phosphate cotransporter was purified from SDS-BBMV protein by a three-step chromatographic procedure as previously described [2,4]. The resultant protein was proteoliposome reconstituted [5], and examined for Na^+-dependent phosphate uptake [2,4,5]. Using an initial rate of uptake (5–10 seconds) in 100 mM cis Na^+ minus 100 mM TMA^+, reconstituted protein had 13 ± 2.4 nmol/mg protein per s during the course of these studies ($n = 8$ preparations).

2.3. Proteoliposome reconstitution

Purified protein was proteoliposome reconstituted into phosphatidylcholine/cholesterol liposomes as previously described [2,5]. Protein samples were collected from the Amicon MMC following detergent removal, diluted with 50 ml of reconstitution buffer without detergent (150 mM potassium gluconate, 10 mM Hepes-Tris (pH 7.5), 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, and collected by centrifugation at $100\,000 \times g$ for 60 min. Proteoliposomes were washed once with 100 mM TMA gluconate, 50 mM potassium gluconate and 10 mM Hepes-Tris (pH 7.5), collected by centrifugation, and stored at liquid N_2 until needed.

In experiments examining the sidedness of Na^+/phosphate cotransporter reconstitution using FNAI-labeled Na^+/phosphate cotransporter 25 mM borate-KOH buffer (pH 7.5) was substituted for Hepes-Tris during detergent removal, and proteoliposome washes.

Proteoliposome reconstituted cotransporter was orientation selected by purification on a WGA column (wheat germ lectin coupled to Sepharose 6 MB). Proteoliposomes were incubated at 8°C for 12 h in 0.5 M NaCl and 100 mM Tris-HCl (pH 7) (WGA loading buffer). The equilibrated proteoliposomes were then added to a 2–4-fold excess of WGA beads, and reacted with beads for 60 min at 8°C with gentle agitation. A column was poured in a 5 ml disposable syringe stoppered with sterilized aquarium filter material, the column washed with 10 column volumes of WGA loading buffer, and 2-ml fractions collected (WGA 1). The column was then washed with WGA loading buffer plus 0.2 M α-methyl mannopyranoside, and 2-ml fractions collected (WGA 2). Column fractions were assayed for protein ($O.D._{280}$, optical density at 280 nm). Fractions corresponding to WGA 1 and 2 were washed twice with 100 mM TMA gluconate, 50 mM potassium gluconate, and 25 mM Hepes-Tris (pH 7.4) (TMA buffer) or 25 mM borate-KOH buffer (pH 7.5) (TMA-borate buffer), collected by centrifugation, and resuspended in TMA buffer or TMA-borate buffer. Proteoliposomes were washed once with 0.8 M KSCN and 25 mM Hepes-Tris (pH 7.5) or 25 mM borate-KOH buffer (pH 7.5) for 20 min at 4°C, collected by centrifugation, and resuspended in TMA or TMA-borate buffer.

Orientation of reconstituted cotransporter was examined using substrate site accessibility to impermeant reagents and Na^+-dependent phosphate transport. Following proteoliposome reconstitution, protein was labeled at or near the cotransporter Na^+ site with FNAI (fluorescein *N*-acetylimidazole) or cotransporter phosphate site with FITC-PG (fluorescein isothiocyanatophenylgloxal) as previously described [7–9]. Cotransporter orientation was determined from the amount of fluorescein released from labeled

cotransporter by papain or V-8 proteinase digestion [10] for 6 h at 37°C at a 1:25 proteinase/protein (w/w) ratio. The reaction was stopped by addition of a 10-fold excess of iodoacetamide (papain) or 25 mM diisopropyl fluorophosphate (V-8 proteinase). Proteoliposomes were collected by centrifugation, and the amount of fluorescein released into the supernatant per mg protein determined using an SLM SPF 500 C spectrofluorometer set in the ratio mode. Fluorescein fluorescence was excited at 490 nm and emission at 522 nm recorded. Light scattering was corrected using unlabeled proteoliposomes.

Cotransporter orientation was also determined using fluorescein labeled cotransporter and fluorescein fluorescence quenching by an anti-fluorescein mAb. Fluorescein fluorescence of WGA protein was determined in 150 mM KCl, and 25 mM Hepes-Tris (pH 7.5). Anti-fluorescein IgG was added and fluorescein fluorescence quenching determined as a function of IgG added. Quenching was analyzed using the Lehrer modified Stern–Volmer equation as described for tryptophan fluorescence.

Verification of cotransporter sidedness following WGA column purification also included Na^+-dependent phosphate uptake and the Na^+ site label, FNAI. Prior to reconstitution, CHAPS solubilized cotransporter was labeled with FNAI in the presence and absence of Na^+ as previously described [9,11]. FNAI-labeled cotransporter was proteoliposome reconstituted substituting KOH-borate buffer (pH 7.5) for Hepes-Tris (pH 7.5). *N*-Acetylimidazole bound to protein has greater stability in borate buffer compared to Hepes-Tris [12]. Proteoliposomes were washed and collected as described above. An aliquot of the unselected proteoliposome reconstituted, FNAI-labeled cotransporter was saved. The remainder was WGA column resolved into WGA 1 and WGA 2 as described above.

FNAI-labeled, WGA column resolved proteoliposomes were examined for Na^+-dependent phosphate uptake, FNAI/mg protein bound, and FNAI released into the supernatant by NH_2OH treatment. NAI bound to tyrosine has been shown to be sensitive to NH_2OH [11]. Na^+-dependent phosphate uptake was determined as described below.

2.4. 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 buffer (Tris-HCl (pH 9.5 to pH 8), Hepes-Tris (pH 7.5 and pH 7), Pipes-Tris (pH 6.5), and Mes-Tris (pH 6 to pH 5)). The excitation wavelength was set at 290 nm and emission at 350 nm or recorded as a function of wavelength from 300 nm to 400 nm. Excitation and emission slit widths were 2.5 nm.

The effects 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.

Experiments examining the effect of tryptophan quench reagents on cotransporter conformation were analyzed in terms of the modified Stern–Volmer equation [13–15]:

$$F_o/\Delta F = 1/(f_a K_s[Q]) + 1/f_a$$

where F_o is the fluorescence in the absence of quench reagent, Q, ΔF is the change in fluorescence upon addition of quencher, f_a is the fraction of accessible fluorophores, and K_s is the apparent Stern–Volmer quench constant. Plots of $F_o/\Delta F$ versus $1/[Q]$ were linear between 10 mM and 150 mM quench reagent with a *y*-intercept of $1/f_a$ and a slope of $(f_a K_s)^{-1}$. Experiments examining the effect of I^- on tryptophan fluorescence included 1 mM Na_2SO_3 in the iodide stock solution to minimize I_3^- formation. Absorbance at 290 nm by acrylamide was corrected by the method of Parker [16]. Inner filter effects were minimized by using absorbances of less than 0.02 absorbance units. Background fluorescence was corrected by the method of McClure and Edelman [17]. Light scatter was corrected by blanking against buffer in the absence of protein. Fluorescence results are reported as lamp corrected emission spectra.

2.5. [^{32}P]Phosphate uptake

Proteoliposome reconstituted cotransporter was incubated for 12 h at 4°C with 25 μM potassium monofluorophosphate or 200 μM potassium difluorophosphate, 100 mM NaCl or 100 mM TMACl, 50 mM KCl, and 25 mM Hepes-Tris (pH 7.5) or 25 mM borate-KOH (pH 7.5). In some experiments the effect of monovalent and divalent phosphates from the cis side were examined without preincubation. Na^+-dependent [^{32}P]phosphate uptake was determined using a rapid mixing/rapid sampling system and 0.22 μm filters. Initial rates of uptake were determined using 10-s uptakes and 10 μg of protein. Uptake media consisted of 100 mM NaCl or 100 mM TMACl, 50 mM KCl, 25 mM Hepes-Tris (pH 7.5), 25 μM [^{32}P]phosphate, and 1 μg valinomycin. Na^+-dependent uptake was defined as uptake in the presence of Na^+ minus uptake in the presence of TMA^+. Uptakes are reported as nmol/mg protein per s and are means ± S.E. of triplicate determinations.

2.6. Reagent synthesis

Fluorescent derivatives of phenylglyoxal, and *N*-acetylimidazole were synthesized as previously described [7,11]. Monofluorophosphate and difluorophosphate were synthesized by the method of Lange [18] as previously described [3,7].

2.7. Enzyme assays

Ca^{2+}-BBMV (Ca^{2+}-precipitated brush-border membrane vesicles) protein and SDS-BBMV (SDS-treated Ca^{2+}-BBMV protein) protein were assayed using the brush-border enzyme markers sucrase [19], and alkaline phosphatase [20]. Protein was assayed using the Bio-Rad reagent with IgG as standard (Ca^{2+}-BBMV or SDS-BBMV) or the SDS micro-Lowry method [21] with bovine serum albumin as standard (chromatography fractions, and proteoliposome reconstituted protein).

3. Results

3.1. Monofluorophosphate and difluorophosphate inhibition of Na^+-dependent phosphate uptake in oriented proteoliposomes

The orientation of proteoliposome reconstituted Na^+/phosphate cotransporter using the substrate sites as extraliposomal side markers is shown in Table 1. Prior to WGA column separation, the proteoliposomes were 35% RSO and 40% ISO. The remaining 25% was judged to have an adhered or random orientation based on its removal from the pelletable material by treatment with 0.8 M KSCN [22,23]. Following WGA column purification, WGA fraction 1 was > 97% ISO, and WGA fraction 2 was > 95% RSO based on proteinase release of fluorescent substrate site labels or anti-fluorescein mAb fluorescence quenching.

WGA column resolution of RSO- and ISO-oriented proteoliposomes was also examined using FNAI inhibition of Na^+-dependent phosphate uptake and relief of inhibition by treatment with the impermeant reagent NH_2OH. The results of these experiments are shown in Fig. 1.

Na^+/phosphate cotransporter labeled with FNAI prior to reconstitution was proteoliposome reconstituted and WGA column resolved into WGA fraction 1, and WGA fraction 2. The WGA column fractions were examined for Na^+-dependent phosphate uptake (open bars), FNAI labeling (solid bars), and NH_2OH reversal of FNAI labeling

Table 1
Orientation of proteoliposome reconstituted Na^+/phosphate cotransporter

	Substrate site accessibility			
	proteolytic digestion		anti-fluorescein mAb	
	FNAI (%)	FITC-PG (%)	FNAI (f_a)	FITC-PG (f_a)
Pre-column	–	–	35 ± 5	40 ± 4
WGA 1	2 ± 1	4 ± 1	5 ± 2	3 ± 1
WGA 2	96 ± 3	95 ± 3	98 ± 3	95 ± 2

Results are means ± S.E. of triplicate determinations and four separate column separations.

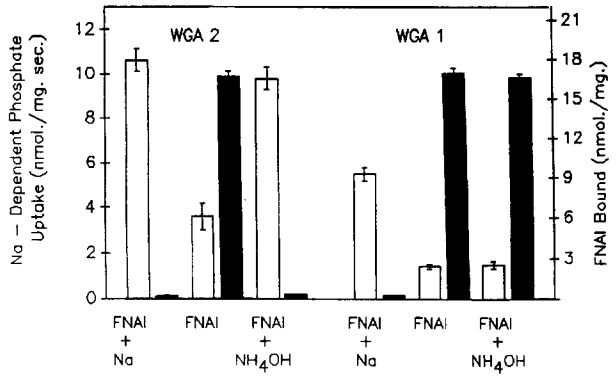

Fig. 1. Effect of FNAI and NH_2OH on Na^+-dependent phosphate uptake by proteoliposome reconstituted cotransporter. 250 μg of Na^+/phosphate cotransporter was labeled with 15 μM FNAI in 150 mM KCl or 150 mM NaCl, 1% CHAPS, and 25 mM KOH-borate buffer (pH 7.5) as described in Methods. Protein was proteoliposome reconstituted, and WGA column resolved into WGA fraction 1, and WGA fraction 2 as described in Methods. An aliquot of each fraction was treated with 0.2 M NH_2OH as described in Methods. Na^+-dependent phosphate uptake and the amount of FNAI bound was determined as described in Methods. Open bars, Na^+-dependent [^{32}P]phosphate uptake; and solid bars, FNAI bound per mg protein. Results are means ± S.E. of duplicate determinations and three column separations.

and inhibition of transport activity. NAI labeling of tyrosine has been shown to be sensitive to NH_2OH [11].

WGA fraction 1, not labeled with FNAI had 55 ± 5% ($n = 3$) of the transport activity of WGA fraction 2. FNAI-labeled WGA fraction 1 was 74 ± 3% ($n = 3$) inhibited by FNAI. Addition of 0.2 M hydroxylamine did not alter FNAI inhibition of Na^+-dependent phosphate uptake, nor FNAI labeling. FNAI-labeled WGA fraction 2 was 65 ± 3% inhibited by FNAI, and addition of 0.2 M NH_2OH resulted in a 89% reversal of FNAI inhibition and loss of 88 ± 4% of the proteoliposome associated FNAI labeling. FNAI labeling seen in WGA fraction 2 following hydroxylamine was similar to the amount of labeling seen in proteoliposomes labeled with FNAI in the presence of 150 mM Na^+.

Fig. 2 shows the effect of monovalent phosphate (difluorophosphate, left- and right-hatched bars) and divalent phosphate (solid and crossed-hatched bars) on RSO and ISO proteoliposomes with (solid bars) and without (cross-hatched) preincubation with the indicated phosphate analogue. In the absence of preincubation with monofluorophosphate, divalent phosphate weakly inhibited (7 ± 1.5%, $n = 3$) Na^+-dependent phosphate uptake (cross-hatched bars) by right-side-out (RSO) proteoliposomes. Incubation with 25 μM monofluorophosphate resulted in 81 ± 3% inhibition (solid bars) of phosphate uptake.

ISO (inside-out) proteoliposomes had similar monofluorophosphate inhibition of Na^+-dependent phosphate uptake with (solid bars) and without (hatched bars) preincubation. Na^+-dependent phosphate uptake was 75% inhibited with or without preincubation. It should be noted that ISO proteoliposomes had approximately 55% of the Na^+-de-

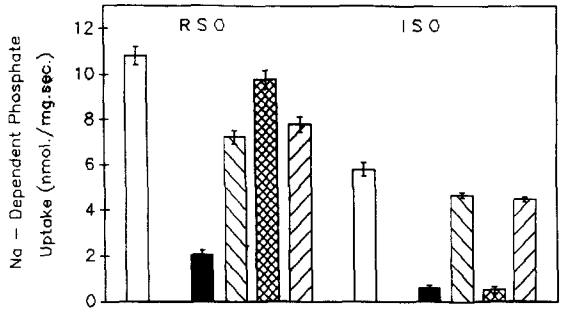

Fig. 2. Effect of monofluorophosphate and difluorophosphate on Na^+-dependent phosphate uptake. WGA column resolved proteolipsomes were examined for Na^+-dependent [^{32}P]phosphate uptake as described in Methods. Open bars, phosphate uptake without added phosphate analogue. Solid bars, phosphate uptake following a 12 h 8°C preincubation with 25 μM potassium monofluorophosphate. Cross-hatched bars, 25 μM potassium monofluorophosphate added directly to the phosphate uptake media (no preincubation). Left-hatched bars, phosphate uptake following a 12 h 8°C preincubation with 100 μM potassium difluorophosphate. Right-hatched bars, 100 μM potassium difluorophosphate added directly to the uptake media (no preincubation). Results are means ± S.E. of triplicate determinations and representative of three determinations.

pendent phosphate uptake activity seen in RSO proteliposomes.

Monovalent phosphate inhibited Na^+-dependent phosphate uptake identically with or without preincubation. The apparent inhibition of 34 ± 4% ($n = 4$) in RSO proteliposomes and 24 ± 3% in ISO proteoliposomes is consistent with an apparent $K_{0.5}$ of 116 μM for inhibition of Na^+-dependent phosphate uptake in Ca^{2+}-BBMV [3,7] and the observation that Ca^{2+}-BBMV are 90% or greater right-side-out [24].

3.2. Effect of substrates on Na^+/phosphate cotransporter tryptophan fluorescence

With FITC-PG bound at or near the phosphate site, the Na^+/phosphate cotransporter has been shown to undergo a Na^+-induced conformational change [7]. Using tryptophan fluorescence (excitation at 290 nm) a similar Na^+-induced conformational change was observed (Fig. 3). Fig. 3A shows a 25 ± 3% ($n = 8$) quenching of tryptophan fluorescence by 150 mM NaCl (broken line) compared to 0.5 M KCl (solid line) and a slight red shift (5 nm). Addition of 150 μM difluorophosphate resulted in an additional 12 ± 3% ($n = 6$) quenching of tryptophan fluorescence and a slight blue shift back to near the substrate-free emission.

Monofluorophosphate did not quench tryptophan fluorescence in the presence of Na^+ (Fig. 3B, dotted/dashed line). In addition, there was a large 17% increase in tryptophan fluorescence emission in the 320 nm to 340 nm range not seen upon addition of difluorophosphate (compare with Fig. 3A).

The effect of substrate concentration on tryptophan

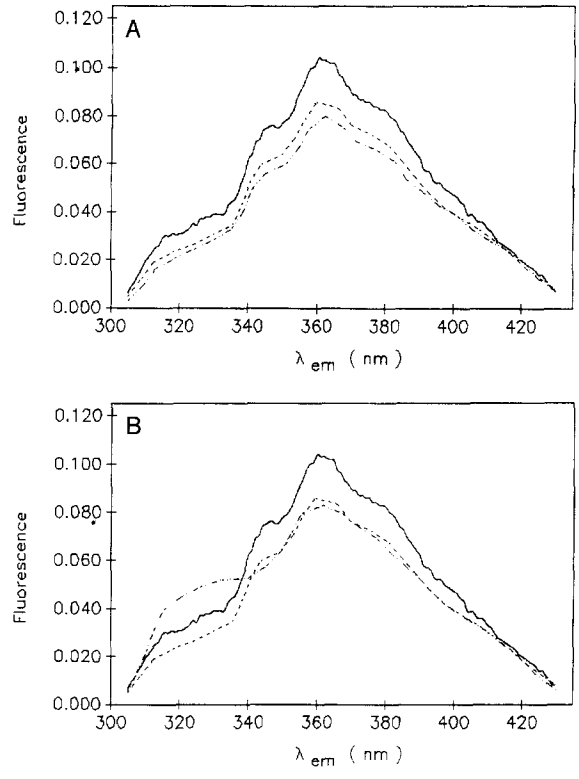

Fig. 3. Tryptophan fluorescence emission of the Na^+/phosphate cotransporter. (A) 20 μg of protein in 500 mM KCl, 0.1% NOG, and 25 mM Tris-HCl (pH 7) was excited at 290 nm and its fluorescence emission recorded as a function of wavelength (solid line). Tryptophan fluorescence was then recorded following the addition of 100 mM NaCl (dashed line) or 100 mM NaCl and 200 μM potassium difluorophosphate (dashed-dotted line). (B) Tryptophan fluorescence of 20 μg of protein in 500 mM KCl, 25 mM Tris-HCl (pH 7), 0.1% NOG (solid line) was recorded in the presence of 100 mM NaCl (dashed line), or 100 mM NaCl and 50 μM potassium monofluorophosphate (dashed-dotted line).

fluorescence is shown in Figs. 4 and 5. Fig. 4 shows the effect of increasing Na^+ concentration on tryptophan fluorescence. Fluorescence quenching was a saturable function

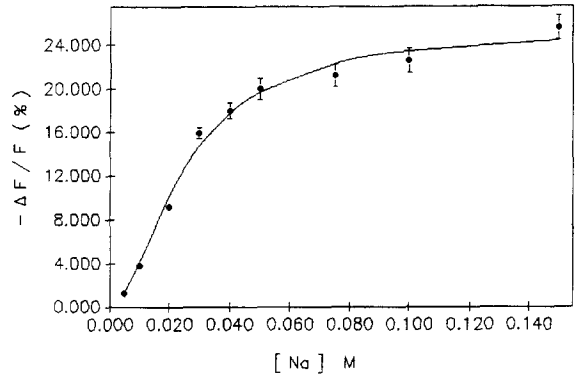

Fig. 4. Effect of Na^+ concentration on tryptophan fluorescence. Tryptophan fluorescence of 20 μg of protein in 500 mM KCl, 0.1% NOG, and 25 mM Tris-HCl (pH 7) was excited at 290 nm and its emission recorded at 350 nm. NaCl was added and the fluorescence emission at 350 nm recorded. In a parallel experiment TMACl was added to an identical aliquot. Results are means ± S.E. of triplicate determinations and representative of six experiments.

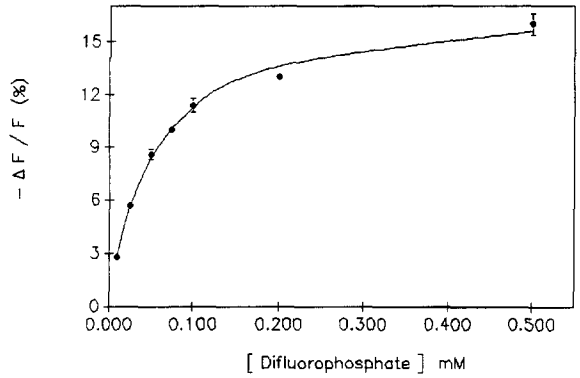

Fig. 5. Effect of difluorophosphate on tryptophan fluorescence. Tryptophan fluorescence emission at 350 nm was recorded from 20 μg of protein in 0.1% NOG, 500 mM KCl, 100 mM NaCl, and 25 mM Tris-HCl (pH 7). Potassium difluorophosphate was added from a 10 mM stock. Results are means ± S.E. of triplicate determinations and representative of four experiments.

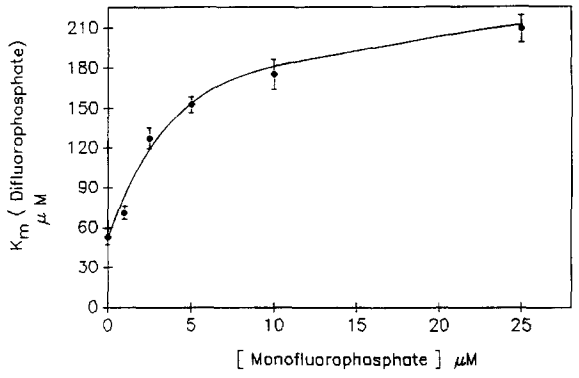

Fig. 6. Effect of monofluorophosphate on apparent K_m for the Na^+ + difluorophosphate-induced tryptophan fluorescence quenching. Tryptophan fluorescence quenching was determined as described in the legend to Fig. 3. Prior to addition of difluorophosphate (10 μM to 500 μM) the indicated final concentration of potassium monofluorophosphate was added. The apparent K_m for difluorophosphate was determined by non-linear regression fit of the results using the software package ENZFITTER. Results shown are from a single experiment performed in triplicate and representative of three separate experiments.

of $[Na^+]$ with an apparent $K_{0.5}$ of 25 ± 3 mM ($n = 5$) at pH 7.4. The apparent Hill coefficient was 1.8 ± 0.2 ($n = 5$) suggesting that 2 Na^+ bound per cotransporter. These results are in excellent agreement with previous studies examining the effect of Na^+ on activation of phosphate uptake in Ca^{2+}-BBM vesicles [1,3,25] and proteoliposomes [2,4].

The effect of phosphate on tryptophan fluorescence is shown in Fig. 5. In the presence of Na^+, difluorophosphate quenched tryptophan fluorescence with an apparent $K_{0.5}$ of 53 ± 3 μM ($n = 5$). This value is similar to that reported for Na^+-dependent phosphate uptake in brush border membrane vesicles [1,25], phosphate affinity in proteoliposome reconstituted protein, and Fig. 2.

Monofluorophosphate has been suggested to inhibit Na^+-dependent phosphate uptake at an intervesicular site ([3], and Fig. 2). The nature of this inhibition was examined using tryptophan fluorescence quenching in the presence of Na^+ and difluorophosphate. Fig. 6 indicates that monofluorophosphate increased the apparent $K_{0.5}$ for the Na^+ + difluorophosphate-induced tryptophan fluorescence quenching. The apparent $K_{0.5}$ for monofluorophosphate inhibition of the Na^+ + difluorophosphate-induced conformational change was 4 ± 1 μM ($n = 3$).

3.3. Examination of Na^+/phosphate cotransporter conformations using tryptophan fluorescence quench reagents

The nature of the apparent competitive inhibition of monofluorophosphate and difluorophosphate for the Na^+ + difluorophosphate-induced conformational change was further examined using cotransporter conformational change partial reaction assays. Conformations of the Na^+/phosphate cotransporter were examined using tryptophan fluorescence and the conformational changes were defined with respect to tryptophan quench reagents. The results from these studies were analyzed using the Lehrer modified Stern–Volmer equation as described in Methods and are summarized in Table 2.

The major effects of Na^+ binding was a decrease in the fraction of tryptophan residues accessible (f_a) to acryl-

Table 2
Effect of substrates on tryptophan fluorescence

Conditions	Quench reagent					
	Cs^+		I^-		acrylamide	
	f_a (%)	K_s (M^{-1})	f_a (%)	K_s (M^{-1})	f_a (%)	K_s (M^{-1})
KCl	65 ± 3	20 ± 2	78 ± 4	17 ± 3	94 ± 3	12 ± 3
NaCl	67 ± 4	22 ± 3	52 ± 4	30 ± 4	67 ± 6	18 ± 2
$Na^+ + PO_4^{3-}$	58 ± 3	17 ± 2	48 ± 5	20 ± 2	77 ± 7	20 ± 3
Na^+ + MFP	46 ± 2	37 ± 3	54 ± 5	28 ± 2	60 ± 4	26 ± 3
Na^+ + DFP	64 ± 3	38 ± 2	77 ± 3	28 ± 4	54 ± 2	24 ± 2
$Na^+ + AsO_4^{3-}$	78 ± 5	19 ± 4	68 ± 8	19 ± 4	79 ± 2	29 ± 3

Results shown for all conditions except arsenate are from six experiments performed in duplicate. Results with arsenate are from four experiments performed in duplicate. MFP, monofluorophosphate; DFP, difluorophosphate.

amide and I^-. Acrylamide quenching decreased 29% and I quenching decreased 33% following the addition of Na^+. In the presence of Na^+, difluorophosphate further decreased acrylamide quenching 19% and increased I^- quenching 44%. Cs^+ quenching was unaffected by addition of Na^+, or Na^+ and difluorophosphate.

Monofluorophosphate had little effect on acrylamide (10% decrease compared to the Na^+ conformation), or I^- (4% increase compared to the Na^+ conformation) quenching, but decreased Cs^+ quenching 31% compared to the Na^+ form. Monofluorophosphate did not appear to 'lock' the cotransporter in the Na^+ conformation based on the decreased Cs^+ quenching of the Na^+ + monofluorophosphate conformation, nor enhance a return to the substrate-free conformation based on the failure of I^- and acrylamide quenching to return to the K^+ levels.

The effect of monofluorophosphate or difluorophosphate on the conformation of the Na^+/phosphate cotransporter was examined using FITC-PG-labeled cotransporter. FITC-PG is thought to bind at or near the cotransporter phosphate site inhibiting Na^+-dependent phosphate uptake but not the Na^+-induced conformational change [3,7,9]. The effects of substrates on the FITC-PG-labeled Na^+/phosphate cotransporter are shown in Table 3.

Compared to the unlabeled cotransporter, the substrate-free conformation of the FITC-PG-labeled cotransporter had reduced Cs^+ quenching. I^- and acrylamide quenching were not affected. The Na^+ conformation of the FITC-PG-labeled cotransporter was nearly identical to the Na^+ conformation of the unlabeled cotransporter. Acrylamide quenching decreased 37% and I^- quenching decreased 28% in the presence of Na^+ compared to K^+.

Unlike the unlabeled cotransporter, the FITC-PG-labeled cotransporter did not respond to difluorophosphate. I^- quenching of the FITC-PG-labeled cotransporter decreased 10% in Na^+ and difluorophosphate compared to a 48% increase for the unlabeled cotransporter under identical conditions. Acrylamide quenching of the FITC-PG-labeled cotransporter was also reduced. Acrylamide quenching of the FITC-PG-labeled cotransporter was 6.7% compared to 19% for the unlabeled cotransporter. These results suggest that FITC-PG labeling blocks the Na^+ + difluorophosphate-induced conformational change.

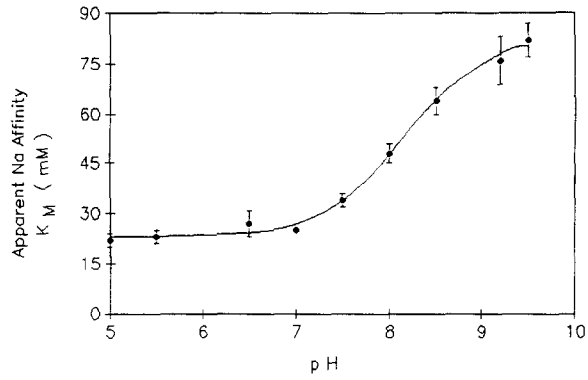

Fig. 7. Effect of pH on the Na^+-induced conformational change. Tryptophan fluorescence was determined as described in the legend to Fig. 3. pH of the medium was varied between pH 5 and pH 9.2. Results are means ± S.E. of triplicate determinations and representative of four experiments.

The FITC-PG-labeled cotransporter did appear to assume a Na^+ + monofluorophosphate conformation. The FITC-PG-labeled cotransporter had a similar decrease in Cs^+ quenching (27% compared to 31% for the unlabeled cotransporter, and a similar decrease in acrylamide quenching (17% compared to 19% for the unlabeled cotransporter) in Na^+ + monofluorophosphate compared to Na^+ alone. These results are consistent with previous reports that FITC-PG binds at or near the cotransporter phosphate site [3,9], and that difluorophosphate competes with phosphate for transport. These results suggest that monofluorophosphate and difluorophosphate bind to separate sites, that the Na^+ and Na^+ + monofluorophosphate conformations are distinct cotransporter forms, and are consistent with four distinct conformations of the intestinal Na^+/phosphate cotransporter.

3.4. Effect of pH on Na^+/phosphate cotransporter conformation

The Na^+/phosphate cotransporter is thought to be sensitive to pH with respect to cotransporter affinity for Na^+ [25] and possibly phosphate [25]. The effect of pH on the Na^+-induced conformational change was examined. The results are shown in Fig. 7.

Table 3
Effect of substrates on tryptophan fluorescence of the phenylglyoxal-treated Na^+/phosphate cotransporter

Conditions	Quench reagent					
	Cs^+		I^-		acrylamide	
	f_a (%)	K_s (M^{-1})	f_a (%)	K_s (M^{-1})	f_a (%)	K_s (M^{-1})
KCl	44 ± 3	16 ± 2	67 ± 3	17 ± 1	95 ± 3	12 ± 3
NaCl	42 ± 2	17 ± 2	48 ± 4	14 ± 3	60 ± 4	16 ± 3
Na^+ + MFP	32 ± 2	22 ± 3	44 ± 5	18 ± 2	50 ± 4	18 ± 3
Na^+ + DFP	44 ± 3	15 ± 2	43 ± 3	14 ± 4	56 ± 2	16 ± 2

Results are means ± S.E. from four experiments performed in duplicate. MFP, monofluorophosphate; DFP, difluorophosphate.

Fig. 7 shows the Na^+ concentration required for 50% tryptophan fluorescence quenching, $K_{0.5}$, as a function of pH. Increasing pH decreased cotransporter affinity for Na^+ from 23 mM between pH 5.5 and pH 7.4 to 80 mM at pH 9.2. The calculated pK_a for the Na^+-induced conformational change was pH 8.3 ± 0.2 ($n = 4$).

The Na^+-induced conformational change was also examined using tryptophan quench reagent sensitivity. Table 2 indicates that compared to the K^+ conformation (substrate-free), the Na^+ conformation had reduced I^- and acrylamide quenching. I^- and acrylamide quenching were used to examine the pH sensitivity of the substrate-free to Na^+ conformation. The results are summarized in Fig. 8.

Fig. 8 shows the difference between the substrate-free and Na^+ conformations using I^- (solid line)- and acrylamide (broken line)-sensitive tryptophans. The apparent pK_a for the Na^+-induced conformational change was pH 6.6 ± 0.15 ($n = 4$) using the decrease in I^- sensitive tryptophan quenching, and pH 9.3 ± 0.2 ($n = 4$) using the acrylamide-sensitive tryptophans. The different apparent pK_a values for the I^--sensitive and acrylamide-sensitive tryptophans suggest that these two quench reagents reported different tryptophan subclasses. This interpretation is consistent with previous studies [26].

The effects of pH on the (Na^+ + phosphate analogue)-induced conformational changes were also examined. The results are summarized in Fig. 9. The results of the substrate-free to Na^+ conformation studies (Fig. 7) suggest that acrylamide and I^- monitor different tryptophan subpopulations, and that comparison of the monovalent phosphate (Na^+ + difluorophosphate) and divalent phosphate (Na^+ + monofluorophosphate) conformations, must compare the same quench reagents. The transitions from the

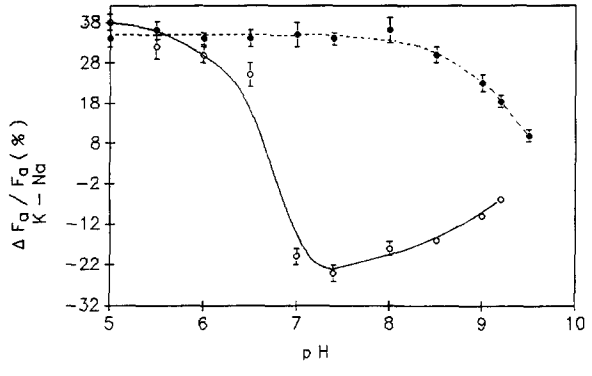

Fig. 8. Effect of pH on the Na^+-induced conformational change using tryptophan quench reagents. Tryptophan fluorescence emission was determined as described in Methods in the presence and absence of 100 mM NaCl. I^- or acrylamide were added (10 mM to 150 mM) and the fluorescence emission recorded. The fraction of tryptophan accessible to each reagent was determined using the Lehrer modified Stern–Volmer equation [12,13] as described in Methods. The difference between the fraction of tryptophan residues accessible to I^- (solid line) and acrylamide (dashed line) in the Na^+ conformation minus the K^+ conformation is shown as a function of medium pH. Results are means from a single experiment and representative of five experiments.

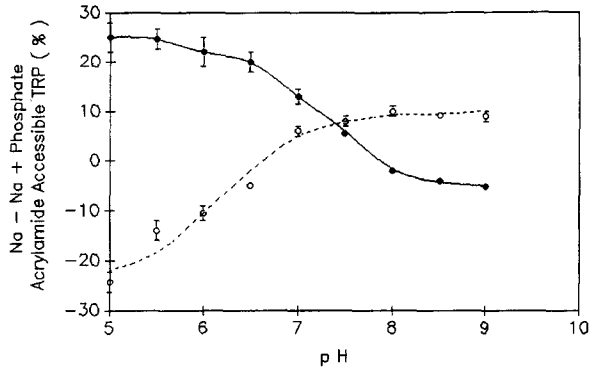

Fig. 9. Effect of pH on the Na^+ + phosphate induced conformational change. Tryptophan fluorescence was determined as described in Methods in the presence of 500 mM KCl, 100 mM NaCl, 0.1% NOG, and 25 mM buffer (pH 5 to pH 9). Acrylamide quenching was determined as described in Methods. In a parallel experiment Na^+ + monofluorophosphate (50 μM, dashed line) or difluorophosphate (200 μM, solid line) was added and the acrylamide quenching of tryptophan fluorescence determined as described in Methods. Results are means ± S.E. of triplicate determinations and representative of four experiments.

Na^+ conformation to the Na^+ + difluorophosphate conformation (solid line), and the Na^+ conformation to the Na^+ + monofluorophosphate (dashed line) conformation were monitored using acrylamide. The transition from the Na^+ conformation to the Na^+ + difluorophosphate conformation had an apparent pK_a of pH 7.1 ± 0.15 ($n = 4$). The transition from the Na^+ conformation to the Na^+ + monofluorophosphate conformation had a pK_a of pH 6 ± 0.1 ($n = 4$). This 1 pH unit difference in apparent pK_a values for these two conformational changes further suggests that these are two distinct conformations of the Na^+/phosphate cotransporter.

4. Discussion

Inhibition of Na^+-dependent phosphate uptake by intervesicular divalent phosphate appears to be the result of divalent phosphate binding to an intervesicular site ([3], Fig. 2). Binding to this site could result in the formation of a transport incompetent ternary complex (cotransporter-Na^+-HPO_4^{2-}, scheme 1). Alternatively, divalent phosphate could bind to a second site, resulting in a conformational change which inhibits transport, release of substrates, or return of empty carrier sites to the external media (scheme 2). These two possible mechanisms are not mutually exclusive nor are they the only two possible mechanisms. The approach used to examine divalent phosphate-induced inhibition of Na^+-dependent phosphate uptake was to define the characteristics of inhibition attempting to highlight differences between the two mechanisms.

Competition between monovalent and divalent phosphate does not distinguish between these two possible mechanisms. Competition between phosphate valence states might be expected regardless of the mechanism. In

scheme 1, dead-end product inhibition, the two phosphate valence states compete for the same intervesicular site. Binding of divalent phosphate, following release of monovalent phosphate with or without Na^+, traps the cotransporter at the intervesicular side. In scheme 2, the two phosphates bind at different sites, and competition between phosphate valence states might result from two mutually exclusive conformations. Similar arguments can be made for different Na^+ + phosphate analogue conformations (see Table 2).

The major points distinguishing the two proposed schemes are: the site of divalent phosphate interaction (scheme 1: divalent and monovalent phosphate compete for the same intervesicular site, and scheme 2: divalent phosphate binds at a second site), and the mechanism of inhibition (scheme 1: monovalent phosphate release from the cotransporter must precede divalent phosphate-induced inhibition, and scheme 2: no inherent kinetic relationship between phosphate valence states). At present, the kinetic relationship between phosphate valence states cannot be assayed since partial reaction assays of substrate release are not available. However, the sites of interaction with the cotransporter can be indirectly examined.

Three lines of evidence suggest that the two phosphate valence state analogues interact with the cotransporter at distinct sites. Detergent-solubilized cotransporter (fully accessible) assumed a Na^+ + phosphate conformation which was not an intermediate between the Na^+ + monofluorophosphate and Na^+ + divalent phosphate forms, nor a fluorophosphate affinity related function of these two conformations. The Na^+ + phosphate conformation had greater acrylamide sensitivity and lower I^- sensitivity than either phosphate analogue. This difference was not related to a higher probability that the Na^+ + phosphate conformation decayed to the substrate-free conformation as the K^+ conformation was more I^- sensitive and less Cs^+ sensitive than the Na^+ + phosphate conformation.

Interaction of divalent phosphate (monofluorophosphate) with the detergent solubilized cotransporter was not affected by labeling the cotransporter with FITC-PG based on tryptophan fluorescence quench reagents. The FITC-PG-labeled cotransporter appeared to assume a Na^+ conformation similar to that of the unlabeled cotransporter. Compared to the substrate-free conformation (K^+ media), the Na^+ conformation of the unlabeled cotransporter was 33% less I^- sensitive, and 29% less acrylamide sensitive. The FITC-PG-labeled cotransporter was 28% less I^- sensitive and 38% less acrylamide sensitive than the substrate-free conformation. Cs^+ sensitive tryptophan residues did not appear to be involved in the Na^+-induced conformational change using unlabeled or FITC-PG-labeled cotransporter. These results suggest that the Na^+/phosphate cotransporter was able to assume a Na^+ conformation similar to the Na^+ conformation of the unlabeled cotransporter.

The Na^+ to Na^+ + monofluorophosphate conformational change of the unlabeled cotransporter was characterized by a 10% decrease in acrylamide quenching and a 31% decrease in Cs^+ quenching (see Table 2). The FITC-PG-labeled cotransporter Na^+ to Na^+ + monofluorophosphate conformational change resulted in a 16% decrease in acrylamide quenching and a 24% decease in Cs^+. I^- quenching of the Na^+ + monofluorophosphate conformation was not altered in unlabeled or FITC-PG-labeled cotransporter.

The Na^+ to Na^+ + difluorophosphate conformational change of the unlabeled cotransporter resulted in a 48% increase in I^- quenching and 19% decrease in acrylamide quenching in the unlabeled cotransporter. The FITC-PG-labeled cotransporter did not show any change in tryptophan fluorescence quench reagent sensitivity, suggesting that the cotransporter remained in the Na^+ conformation and that the difluorophosphate site was blocked by FITC-PG or phenylglyoxal. This conclusion is consistent with previous results suggesting that phenylglyoxal and its derivatives inhibit Na^+-dependent phosphate uptake by binding at or near the cotransporter phosphate site [4,6,8]. Inhibition of the Na^+ + difluorophosphate-induced conformational change but not the Na^+ + monofluorophosphate-induced conformational change is not consistent with the single site model (scheme 1).

The difference in apparent pK_a values for the Na^+ to Na^+ + difluorophosphate and Na^+ to Na^+ + monofluorophosphate conformational changes also suggest a two-site model. Starting at the Na^+ conformation, the transition to the Na^+ + difluorophosphate conformation had an apparent pK_a of pH 7.1 using acrylamide-sensitive tryptophans. In contrast, the acrylamide-sensitive tryptophans involved in the Na^+ to Na^+ + monofluorophosphate conformational change had an apparent pK_a of pH 6. This 1 pH unit difference in the apparent pK_a for the transition from the Na^+ conformation to the Na^+ + phosphate conformation is more readily explained in terms of a two-site model rather than a single-site model.

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