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Interaction of substrates with the intestinal brush border membrane Na/phosphate cotransporter

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Abstract

The interaction of Na^+ and phosphate with the intestinal brush border membrane Na^+/phosphate cotransporter was examined using stopped-flow tryptophan fluorescence and ion-exchange Dowex columns coupled to a light-activated microsecond timer (LAM timer) which measures exchange kinetics between protein-bound ions and the external medium. Na^+ or $Na^+ + H_2PO_4^-$ induced tryptophan fluorescence quenching with apparent rate constants of 35 s^{-1} and 13 s^{-1}, respectively. Dilution of substrate-bound cotransporter resulted in tryptophan fluorescence recovery consistent with cotransporter return to the substrate-free conformation. Recovery of the substrate-free conformation was slow (1.6 s^{-1}) in the absence of phosphate, was accelerated by $H_2PO_4^-$ (7 s^{-1}) and was inhibited by HPO_4^{2-} (1.1 s^{-1}). The effects of substrates on tryptophan fluorescence were sensitive to substrate site blockers consistent with trpytophan fluorescence monitoring cotransporter conformations and substrate-induced changes in conformation. Equivalent experiments using the LAM timer and either $[^{22}Na^+)$ or $Na^+ + [^{32}P)$ phosphate verified the rate constants for the substrate-induced quenching of tryptophan fluorescence, suggested that 2 Na^+'s were occluded by the cotransporter as part of the Na^+-induced conformational change and that $H_2PO_4^-$ accelerated deocclusion of Na^+. The association of phosphate with the cotransporter was also examined. Although cotransporter-bound phosphate was medium anion-insensitive, a cotransporter conformational change preceding the release of phosphate from the cotransporter was not observed. However, three lines of evidence suggest that release of phosphate from the cotransporter involved a unique cotransporter conformation which may suggest that phosphate was also occluded by the intestinal brush border Na^+/phosphate cotransporter.

Keywords: Intestinal transport; Brush border membrane; Phosphate transport; Sodium ion/phosphate cotransporter

1. Introduction

Phosphate uptake at the intestinal brush border membrane is the result of Na^+-activation of phosphate transport mediated by the Na^+/phosphate cotransporter [1,2]. The interaction of Na^+ with the cotransporter is thought to involve a conformational change resulting in increased cotransporter affinity for phosphate [3–5]. The interaction of phosphate with the cotransporter results in a second conformational change which yields a transport-competent cotransporter conformation [6] as has been suggested for the Na^+/glucose cotransporter [7–9].

The nature of these conformational changes is not understood. Substrate ion-induced conformational changes on the Na^+/phosphate cotransporter have been examined using tryptophan quench reagents. At

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this low level of structural resolution, Na^+ or (Na^+ + phosphate) resulted in two different cotransporter conformations differing in tryptophan quench reagent accessibility [6], differing in apparent pK_a's [6], and differing in SH-reagent sensitivity [10].

Substrate ion association with the ion transporting ATPases have been reported to involve enzyme intermediates in which the ion is protected from the external medium. The ion is thus 'occluded' within the enzyme. Release of occluded ion from the protein requires a conformational change or a change in the enzyme: substrate state [11,12]. Operationally, in the absence of a conformational change, the rate of release of the occluded ion ranges from 0.1 s^{-1} for Rb deocclusion from the Na^+/K^+ ATPase [11] to 1 h^{-1} for the Ca^{2+} ATPase [13,14] and H^+/K^+ ATPase [15] in the presence of Cr-ATP and vanadate, respectively.

Recently, the Na^+/phosphate cotransporter release of Na^+ and phosphate was shown to be independent of external ions [16]. Although the rates of substrate ion release were 1–2 orders of magnitude faster than that seen with the ion transporting ATPases, ion binding and release were sufficiently slow to suggest a role in transport. In addition, the ion-bound cotransporter states displayed characteristics consistent with Na^+-dependent phosphate uptake, including: Na^+ dependence, substrate stoichiometry, phosphate valence state dependence, Na^+ (n-acetylimidazole, NAI) and phosphate (phenylglyoxal, PG) site blocker sensitivity, and a Na^+:cotransporter association rate constant similar to the rate constant for the Na^+-induced conformational change.

The studies summarized here are consistent with Na^+ occlusion by the Na^+/phosphate cotransporter. The association of Na^+ with the cotransporter was NAI-sensitive, involved 2 Na^+'s per monovalent phosphate, and had a rate constant for Na^+ occlusion similar to the rate constant for Na^+-specific quenching of tryptophan fluorescence. In the absence of monovalent phosphate, Na^+ release from the cotransporter was slow, and was accelerated by a factor of 20 by the addition of phosphate. Phosphate acceleration of Na^+ release appeared to require the phosphate-induced conformational change, in that phosphate acceleration of Na^+ release had a similar rate constant to phosphate binding, and was phenylglyoxal-sensitive.

The association of phosphate with the cotransporter was also examined. Phosphate binding required an accessible phosphate site, i.e., phosphate binding was PG-sensitive, and required prior Na^+ binding. Release of phosphate from the cotransporter was medium ion-independent (identical rates of phosphate release in the presence of monovalent phosphate or sulfate). A conformational change prior to phosphate release was not seen. However, since both Na^+'s were released prior to phosphate a unique cotransporter conformation involving the cotransporter and $H_2PO_4^-$ is predicted. This conformation differs from that seen in the absence of prior Na^+ and phosphate binding, suggesting that this conformation is unique to the quaternary complex.

2. Materials and methods

2.1. Na^+/phosphate cotransporter purification

The Na^+/phosphate cotransporter was purified from Ca^{2+}-BBMV (Ca^{2+}-precipitated brush border membrane vesicles) as previously described [16,17]. Chromatofocusing chromatography was performed as previously described using 1 g of SDS-BBMV (SDS treated brush border membrane vesicles, 17), 4 g of BBMV protein, or 1.5 g of BBMV protein eluted through 3-ml Sephadex G-25 columns (15–20 mg of Ca^{2+}-BBMV protein/3 ml Sephadex column) equilibrated with 0.1 mM DTT, 10 mM Hepes/Tris (pH 7.5), 2 mM EDTA, and 150 mM potassium gluconate by centrifugation at $2500 \times g$ for 15 min [18,19]. In all cases the final protein concentration applied to the chromatofocusing column was approx. 1 mg/ml. Fractions eluting between pH 4.4 and pH 4.2 (SDS-BBMV) or pH 4.8 and pH 4.3 (Ca-BBMV, and Sephadex washed BBMV) were collected, dialyzed for 48 h against 4 changes of 10 mM piperazine-HCl (pH 6), and buffer exchanged through a 10 cm Sephadex G-25 equilibrated with 0.1% CHAPS and 20 mM piperazine-HCl (pH 6).

The cotransporter-enriched fraction was run through a second chromatofocusing chromatography column over the pH range between pH 6 and pH 4, as previously described, substituting CHAPS for *n*-octylglucoside [17]. Fractions eluting between pH 4.6 and pH 4.4 were collected and dialyzed against 10

mM Tris-Cl (pH 7) as described above. The resultant protein was buffer and detergent exchanged through a Sephadex G-25 column equilibrated with 10 mM Tris-Cl (pH 7), 0.1 N NaCl, and 0.1% CHAPS.

Na^+/phosphate cotransporter was run through a S200 Sephacryl column equilibrated with 0.1 N NaCl, 0.1% CHAPS, and 10 mM Tris-Cl (pH 7), and eluted with the same solution. Protein eluting immediately after the void volume was collected, dialyzed against 10 mM Tris-Cl (pH 7) as above, and lyophilized. Lyophilized protein was resuspended in 10% glycerol and 10 mM Tris-Cl (pH 7), and stored at liquid N_2 temperatures until needed.

2.2. Proteoliposome reconstitution

Na^+/Phosphate cotransporter-enriched chromatography fractions were reconstituted in phosphatidylcholine: cholesterol proteoliposomes (80:20, wt:wt) and assayed for Na^+-dependent [^{32}P]phosphate uptake as previously described [17]. Na^+-dependent uptake was defined as uptake in the presence of 100 mM cis NaCl minus uptake in the presence of 100 mM cis TMACl. Na^+-dependent phosphate uptake varied between 11 and 14.5 nmol/mg protein/s for the final Sephacryl column fractions used during the course of these studies ($n = 6$).

2.3. Stopped-flow fluorescence

Fluorescence studies were performed on an SLM SPF 500 c spectrofluorometer equipped with a rapid mixing, stopped-flow attachment. Tryptophan fluorescence emission was measured at either 325 nm or 350 nm, following excitation at 290 nm in the single channel mode, with a gain of 10 compared to the reference channel. Slit widths were maintained at 2–4 nm to yield a fluorescence signal of 2–6 AU's.

2.4. Time course of the Na^+-induced conformational change

Protein solubilized in 0.5 M KCl, 50 mM Tris-Cl (pH 7), and 0.1% CHAPS at a final protein concentration of 2 mg/ml was diluted 1:1 with 200 mM NaCl and 300 mM KCl or 500 mM KCl, 50 mM Tris-Cl (pH 7), and 0.1% CHAPS. Tryptophan fluorescence at either 325 nm or 350 nm was recorded as a function of time following mixing (3–500 ms). Na^+-dependent quenching of tryptophan fluorescence was defined as quenching in the presence of Na^+ minus quenching due to dilution (KCl). Dead-time of the instrument was determined using acid quenching of FITC bound to lysine and was 2–3 ms.

In some experiments the effect of the Na^+ site blocker n-acetylimidazole (NAI) on Na^+-dependent tryptophan fluorescence quenching was examined. Protein was labeled with 2 mM NAI in the presence and absence of 150 mM NaCl as previously described [20,21]. Free NAI was removed from labeled protein by centrifugation through centricon columns fitted with a 30 kDa cut-off filter, and washed twice with 0.5 M KCl, 50 mM Tris-Cl (pH 7), and 0.1% CHAPS.

2.5. Time course of phosphate-induced conformational changes

Cotransporter solubilized in 0.1% CHAPS, 0.5 M KCl, and 50 mM Tris-Cl pH 7 was diluted 1:1 with 0.1% CHAPS, 0.2 M NaCl or 0.2 M KCl and 50 mM Tris-Cl pH 7, and incubated at 4°C for 10 min. Pre-equilibrated cotransporter was mixed with 0.5 M KCl, potassium difluorophosphate (10 μM to 2 mM) and/or potassium monofluorophosphate (5 μM to 500 μM), 50 mM Tris-Cl (pH 7), and 0.1% CHAPS in the stopped-flow (1:1 dilution) and tryptophan fluorescence measured as a function of time after mixing (3–500 ms).

Experiments examining the effect of MFP on the fully-loaded (Na^+ + DiFP) cotransporter were performed using protein pre-equilibrated with 200 mM NaCl, 0.1% CHAPS, 50 mM Tris-Cl (pH 7), and 200 μM DiFP for 10 min at 4°C. Pre-equilibrated cotransporter was diluted 1:1 with 0.1% CHAPS, 50 mM Tris-Cl (pH 7), and MFP (5 μM to 1 mM) in the stopped-flow apparatus. Tryptophan fluorescence was followed as a function of time following mixing (3 ms–2 s).

In some experiments the effect of phenylglyoxal (PG) on the (Na^+ + phosphate)-induced conformational change was examined by pre-labeling the cotransporter with PG. PG labeling was performed as previously described [21,22].

Release of substrates from the cotransporter was measured using Na-equilibrated cotransporter, (Na +

phosphate)-equilibrated cotransporter, (Na^+ + DiFP)-equilibrated cotransporter, or (Na^+ + DiFP + MFP)-equilibrated cotransporter following a 20-fold dilution of equilibrated protein in the stopped-flow apparatus. Protein was diluted into Na^+-free (500 mM KCl) or Na^+-containing media (100 mM NaCl), 0.4 M KCl, 50 mM Tris-Cl (pH 7), and 0.1% CHAPS. Release of substrates from (Na^+ + DiFP)-equilibrated protein (100 mM NaCl, 0.1 mM DiFP, 50 mM Tris-Cl (pH 7), and 0.1% CHAPS) was measured following dilution into 100 mM NaCl or 100 mM TMACl, 0.4 M KCl, 0.1 mM DiFP or 0.1 mM K_2SO_4, 50 mM Tris-Cl, and 0.1% CHAPS. In some experiments 200 μM MFP was added to the dilution media.

2.6. Light-activated microsecond (LAM) timer studies

The association of Na^+ and phosphate with the Na^+/phosphate cotransporter was examined using the LAM timer (BEACF, Galveston, TX). The LAM timer consists of 3 sensors interfaced to 2-μs timers. The sensors are attached to a gantry containing a 1-ml disposable syringe packed with Dowex ion-exchange resin (deocclusion mode) or isotope equilibrated-Sephadex + Dowex ion-exchange resin (occlusion mode) as previously described [23]. LAM timer sensors, attached to the gantry, were fitted to the column. These sensors 'see' flow through the column resins as changes in resin color. In the ion occlusion mode, sensor 1 was placed at the sand:Sephadex interface (top of the column), and sensor 2 was placed at the Sephadex:sand interface. Sensor 3 was placed at the Dowex:plug interface (bottom of the column). Sensor 1 was interfaced to a microsecond timer such that sensor 1 activated the timer (timer 1), and sensor 2 inactivated timer 1. In this configuration, timer 1 measured Sephadex layer residence time. In addition to stopping timer 1, sensor 2 activated timer 2. The third sensor, located at the Dowex:plug interface inactivated timer 2. In this configuration, timer 2 measured Dowex layer residence time.

In the deocclusion mode, the first 2 sensors and timer 1 were active. Sensor 1 was placed at the Dowex:sand interface, and sensor 2 was placed at the Dowex:plug interface. Sensor 1 activated the timer and sensor 2 stopped the timer. In this configuration the timer measures Dowex resin residence time or ion exchange time.

Cotransporter association with Na^+ was measured with the LAM timer in the occlusion mode [16] and columns containing [^{22}Na]-equilibrated Sephadex G-25 and Dowex 50 W (200–400 mesh). Sephadex was pre-equilibrated with 0.1 mM [^{22}Na]Cl (10^7 CPM/ml), 10 mM Tris-Cl (pH 7) and 0.1% CHAPS by incubation for 12 h at 4°C. Dowex was pre-equilibrated with 1–5 mM NaCl or 1–5 mM KCl, 10 mM Tris-Cl (pH 7), and 0.1% CHAPS by incubation for 12 h at 4°C. A layer of acid washed sand was added to the Dowex:Sephadex interface, and above the Sephadex layer. Disposable syringes (1 ml) were plugged with sterilized aquarium filter packing, and loaded with Dowex (0.5 ml), sand, and variable amounts of [^{22}Na)-equilibrated Sephadex. A second layer of sand was added to the top of the Sephadex layer [23].

Cotransporter association with phosphate was measured using the identical timer configuration as for Na^+ association. In experiments measuring phosphate binding, protein was pre-equilibrated with 10 mM NaCl, 0.1% CHAPS, and 10 mM Tris-Cl (pH 7) by incubation for 20 min at 4°C. Sephadex was pre-equilibrated with 1 mM NaCl, 0.1 mM [^{32}P] (6×10^6 CPM/ml) difluorophosphate, 10 mM Tris-Cl (pH 7), and 0.1% CHAPS by incubation for 12 h at 4°C. Dowex 1X-8 was pre-equilibrated with 1 mM sulfate, difluorophosphate, or monofluorophosphate, 10 mM Tris-Cl (pH 7), and 0.1% CHAPS as described above.

Release of substrate ions from the cotransporter was performed using [^{22}Na]- or Na + [^{32}P]phosphate equilibrated protein, the deocclusion configuration of the LAM timer, and Dowex columns [16]. Protein was pre-equilibrated with 0.1 mM [^{22}Na], 10 mM Tris-Cl (pH 7), and 0.1% CHAPS. Release of Na^+ from the cotransporter was performed following a 20-fold dilution into 10 mM NaCl or 10 mM KCl, 10 mM Tris-Cl (pH 7), and 0.1% CHAPS.

Protein blanks were run using the same media as for the particular binding or release experiment, substituting an equivalent volume of 10 mM Tris-Cl (pH 7) for protein. Appropriate column lengths and vacuum were used to give comparable column residence times as in the experimental determinations. A standard curve of counts eluting from the columns versus

time was generated for subtraction from experimental counts eluting from the column in the presence of protein. Protein-associated counts were defined as counts eluting from the column in the presence of protein minus counts eluting from the column in the absence of protein for the same column residence time. Protein eluting from the column was determined using the method of Lowry [24] after precipitation with 10% TCA as previously described [25].

Residence times ranging between 20 ms and 1 s were fitted to a single exponential using the non-linear regression program (ENZFITTER). Standard curves including residence times of less than 20 ms required two exponentials to fit the counts eluting from the columns in the absence of protein.

Na^+ and phosphate eluting from the columns in the absence of protein were inversely proportional to column residence time. In the absence of protein (protein blanks), [^{22}Na] washing through the columns ranged from $7.8 \pm 0.2\%$ ($n = 7$) of the applied radioactivity at a column residence time of 20 ms to less than $0.15 \pm 0.025\%$ ($n = 7$) at residence times above 600 ms. [^{32}P]Phosphate eluting from the column in the absence of protein varied between $5.6 \pm 0.4\%$ ($n = 5$) of the applied counts at column residence times of 17–23 ms and $0.10 \pm 0.03\%$ ($n = 5$) at column residence times of more than 600 ms.

Reproducibility of column residence time was examined by comparing residence time of the same column in 4 successive runs following careful resuspension of the solid matrix (Dowex). Reproducibility was directly related to column length. The error in 4 successive runs of the same column varied between $1 \pm 0.1\%$ ($n = 12$) at column lengths of 1.5 cm to less than 0.1% ($0.85 \pm 0.11\%$, $n = 8$) at column lengths of 4 cm or longer.

The effect of cotransporter purification on the amount of [^{22}Na] binding to the cotransporter was examined using the LAM timer, the occlusion configuration ([^{22}Na]-equilibrated Sephadex G-25 and Na^+-equilibrated Dowex 50W) and the 3 Na^+/phosphate cotransporter-enriched chromatography fractions (preparative-scale chromatofocused fraction, LCC {17}, small scale chromatofocused fraction, SCC {17}, and S-200 Sephacryl gel filtration fraction, S-200 {17}). The amount of cotransporter was estimated from the relative Coomassie blue staining of the 120 kDa polypeptide (percent of total Coomassie blue staining as determined by area under the 120 kDa peak/area under all peaks) of 7.5% SDS-PAGE gels of these fractions [26], and the amount of Na^+-dependent [^{32}P]phosphate uptake following proteoliposome reconstitution [17].

Following the preparative-scale chromatofocusing column purification step, the Na^+/phosphate cotransporter represented approximately 25% ($30 \pm 5\%$, $n = 4$) of the protein eluting between pH 4.8 and pH 4.3 (LCC 4.6), and 25% ($25 \pm 7\%$, $n = 2$) of the protein eluting between pH 4.4 and pH 4.2 (LCC 4.4). [^{22}Na]-binding to LCC 4.6 and LCC 4.4 fractions were similar. LCC 4.6 bound 5 ± 1 nmol [^{22}Na]/mg protein, and LCC 4.4 fractions bound 4.6 ± 0.6 nmol [^{22}Na]/mg protein. No differences were seen in the 3 preparations following the second chromatofocusing column (SCC 4.5) or the Sephacryl column (S-200). SCC 4.5 fractions bound 11.5 ± 1 nmol [^{22}Na]/mg protein. The 120 kDa polypeptide was estimated to comprise $60 \pm 8\%$ of the protein in this fraction. The S-200 fractions bound 14.7 ± 0.8 nmol [^{22}Na]/mg protein. The 120 kDa polypeptide was the only band staining with Coomassie blue following purification on the Sephacryl column and comprised $95 \pm 5\%$ of the total stain. The co-enrichment in 120 kDa polypeptide, Na^+-dependent phosphate uptake [17], and NAI-sensitive [^{22}Na]-binding indicate that substrate binding (Na^+ or phosphate) represents a measure of cotransporter activity.

3. Results

3.1. Association of Na^+ and phosphate with the Na^+/phosphate cotransporter using stopped-flow fluorescence measurements

The effect of the addition of Na^+ (trace A) or K^+ (trace B) on tryptophan fluorescence is shown in Fig. 1. Na^+ but not K^+ resulted in a $25 \pm 3\%$ ($n = 24$) quenching of tryptophan fluorescence with an apparent $K_{0.5}$ (Na^+ concentration resulting in 50% tryptophan fluorescence quenching) of 20 ± 3 mM ($n = 5$), and a Hill coefficient of 2.1 ± 0.2 ($n = 5$). The decrease in fluorescence was fitted to a single exponential with a rate constant of 38 ± 3 s^{-1} (trace A). These results are similar to previous reports of the effect of Na^+ on FITC-PG (fluorescein isothio-

cyanatophenylglyoxal) fluorescence bound at or near the cotransporter phosphate site [5,16], the effect of Na^+ on tryptophan fluorescence [6], and Na^+ activation of [^{32}P]phosphate uptake [2,17,20,27]. Pretreatment with the putative Na^+-site blocker, NAI, eliminated the Na^+-induced tryptophan fluorescence quenching ($2 \pm 0.5\%$ tryptophan fluorescence quenching, $n = 8$), consistent with previous results [20,28].

In the presence of Na^+ but not K^+, the addition of phosphate induced a second quenching of tryptophan fluorescence, consistent with a (Na^+ + phosphate)-induced conformational change. Fig. 2 shows the time course of the (Na^+ + phosphate)-induced conformational change. The effect of phosphate on tryptophan fluorescence emission at 350 nm was specific for monovalent phosphate (DiFP, potassium difluorophosphate, trace A). Divalent phosphate (MFP, monofluorophosphate, trace B) did not alter tryptophan fluorescence emission at 350 nm. These results are consistent with previous reports that $H_2PO_4^{2-}$ is the transport-competent phosphate valence state [27,28].

The time course of the (Na^+ + DiFP)-induced conformational change was fitted to a single exponential decay with a rate constant of $13 \pm 2\ s^{-1}$ ($n = 18$). The apparent $K_{0.5}$ for monovalent phosphate in the presence of 100 mM NaCl at pH 7 was $60 \pm 8\ \mu M$ ($n = 5$) similar to previous results for Na^+-dependent phosphate uptake [27,28] and the apparent $K_{0.5}$ for steady-state tryptophan fluorescence studies [6].

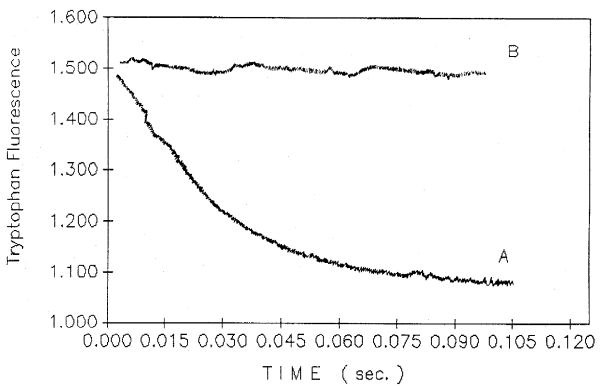

Fig. 1. Effect of monovalent ions on cotransporter tryptophan fluorescence. 10 μg of protein in 0.1% CHAPS, 0.5 M KCl, and 50 mM Tris-Cl (pH 7) was diluted 1:1 in the stopped-flow apparatus with 0.1 M NaCl, 0.4 M KCl, 0.1% CHAPS, and 10 mM Tris-Cl (pH 7) (trace A), or 0.5 M KCl, 0.1% CHAPS, and 50 mM Tris-Cl pH 7 (trace B). Tryptophan fluorescence emission at 350 nm was continously recorded as a function of time. Results are from a single experiment and representative of 24 determinations.

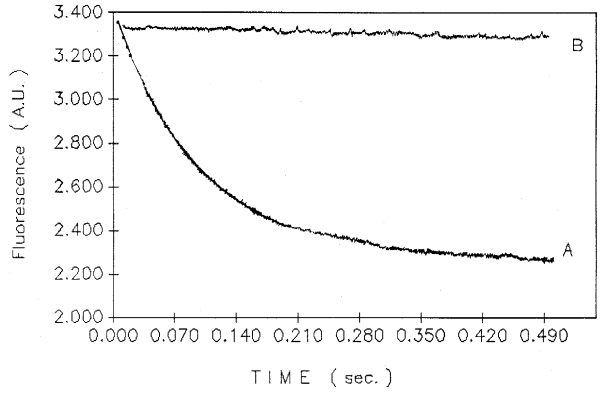

Fig. 2. Effect of phosphate on cotransporter tryptophan fluorescence. 10 μg of protein in 0.1% CHAPS, 0.4 M KCl, 0.1 M NaCl, and 50 mM Tris-Cl (pH 7) was diluted 1:1 in the stopped-flow apparatus with 0.1% CHAPS, 0.4 M KCl, 0.1 M NaCl, 50 mM Tris-Cl (pH 7), and 1 mM potassium difluorophosphate (trace A), or 0.5 mM potassium monofluorophosphate (trace B). Tryptophan fluorescence emission at 350 nm was recorded continously as a function of time. Results are from a single determination and representative of 18 experiments.

Although divalent phosphate (MFP) had no effect on tryptophan fluorescence emissions at 350 nm, MFP enhanced tryptophan emission at 325 nm in the presence of Na^+ and DiFP. Tryptophan emission at 325 nm is shown in Fig. 3. The addition of MFP to the (Na^+ + DiFP)-bound cotransporter (trace A) resulted in a large increase in tryptophan fluorescence with an apparent $K_{0.5}$ of $12 \pm 4\ \mu M$ ($n = 4$). The time course of the increase in tryptophan fluorescence was fitted to a single exponential with a rate constant of $8 \pm 1.5\ s^{-1}$ ($n = 12$). The association of MFP with the cotransporter in the absence of monovalent phosphate (Na^+-conformation) is also shown in Fig. 3 (trace B). In the absence of DiFP, the rate of MFP enhancement of tryptophan fluorescence was 65% slower. In the absence of DiFP the tryptophan fluorescence enhancement was fitted to a single exponential with a rate constant of $2.8 \pm 0.2\ s^{-1}$.

Pre-incubation of the Na^+ conformation with 0.1 mM MFP did not alter the time course of the (Na^+ + phosphate)-induced conformational change measured at 350 nm. The (Na^+ + MFP)-bound cotransporter

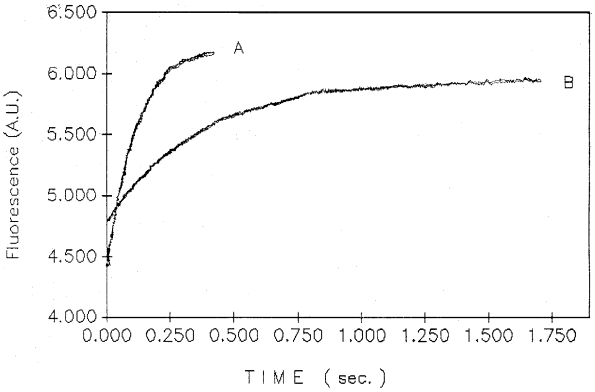

Fig. 3. Effect of divalent phosphate on tryptophan fluorescence. 10 μg of protein in 0.1% CHAPS, 0.4 M KCl, 0.1 M NaCl, 50 mM Tris-Cl (pH 7) and in the presence (trace A) or absence (trace B) of 0.2 mM potassium difluorophosphate was diluted 1:1 in the stopped-flow apparatus with 0.4 M KCl, 0.1 M NaCl, 0.1% CHAPS, 50 mM Tris-Cl (pH 7), and 0.2 mM potassium monofluorophosphate. Tryptophan fluorescence emission at 325 nm was continuously recorded as a function of time. Results are from a single experiment and representative of 16 determinations without DiFP, and 14 determinations with DiFP.

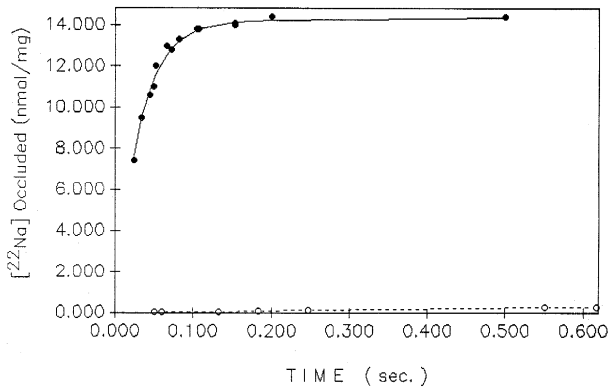

Fig. 4. [^{22}Na] Occlusion measurements using the LAM timer. 10 μg of protein (solid circles) or 10 μg of NAI-treated protein (open circles) in 0.1% CHAPS and 10 mM Tris-Cl (pH 7) in 100 μl was added to 1 ml disposable syringes containing 3 cm of Dowex 50 W equilibrated with 0.1% CHAPS, 1 mM NaCl, and 10 mM Tris-Cl (pH 7), and 1 cm to 3 cm of Sephadex G-25 equilibrated with 0.1 mM [^{22}Na]Cl, 0.1% CHAPS, and 10 mM Tris-Cl (pH 7). Resin residence times, protein blanks, and protein eluting from the columns were determined as described in Section 2. Results are from a single experiment and representative of 7 experiments.

displayed similar tryptophan fluorescence quenching ($15 \pm 2\%$, $n = 10$) and rate constants (12 ± 1 s^{-1}, $n = 10$) upon addition of 0.25 mM phosphate to the Na^+ + MFP equilibrated cotransporter compared to the (Na^+ + phosphate)-induced conformational change seen in the absence of MFP (Fig. 2).

3.2. Association of Na^+ and phosphate with the Na^+/phosphate cotransporter using LAM timer measurements

The binding of Na^+ to the cotransporter was determined using CHAPS-solubilized cotransporter, [^{22}Na]-equilibrated Sephadex, Dowex cation exchange resin columns [16,23], and the LAM timer [16]. A typical result is shown in Fig. 4.

In the absence of phosphate, Na^+ bound rapidly to the CHAPS-solubilized cotransporter. Binding of Na^+ was fitted by a single exponential with a rate constant of 32 ± 2 s^{-1} ($n = 10$) similar to previous results for Na^+ binding [16], Na^+-induced quenching of FITC-PG fluorescence [16], and Na^+-induced quenching of tryptophan fluorescence (see Fig. 1). Based upon a calculated molecular mass of 120 kDa from SDS-PAGE gels [5,17,28], 2 Na^+'s bound per cotransporter (14.6 ± 1.1 nmol of Na^+/mg cotransporter,

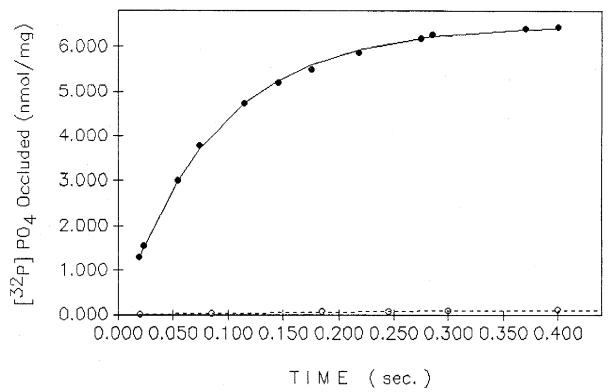

Fig. 5. [^{32}P]Phosphate binding measurements using the LAM timer. 10 μg of protein in 0.1% CHAPS, 10 mM Tris-Cl (pH 7), and 1 mM NaCl (solid circles) or 1 mM KCl (open circles) was added to 1 ml disposable syringes containing 3 cm of Dowex 1X-8 equilibrated with 1 mM NaCl (closed circles) or 1 mM KCl (open circles), 0.2 mM potassium difluorophosphate, 10 mM Tris-Cl (pH 7), and 0.1% CHAPS, and 1 to 3 cm of Sephadex G-25 equilibrated with 25 μM [^{32}P]difluorophosphate, 1 mM NaCl (solid circles) or 1 mM KCl (open circles), 10 mM Tris-Cl (pH 7), and 0.1% CHAPS. Resin residence times, protein blanks, and protein concentrations were determined as described in Section 2. Results are from a single experiment and representative of 6 experiments.

$n = 12$). Pre-treatment of the cotransporter with the Na^+ site blocker, NAI reduced Na^+ binding $97 \pm 4\%$ ($n = 10$).

The amount of Na^+ bound per mg cotransporter was unaffected by the cation used to equilibrate the Dowex cation exchange resin. With Na^+-equilibrated Dowex, 14.4 ± 1 nmol [$^{22}Na^+$] bound/mg protein ($n = 8$) compared to 14.7 ± 0.8 nmol [$^{22}Na^+$] bound/mg protein ($n = 6$) using K^+-equilibrated Dowex. These results suggest that bound Na^+ was sheltered from exchange with cations in the external media, since the cotransporter readily distinguishes between Na^+ and K^+ (see Figs. 1 and 2) and there was a 10- to 50-fold ion-gradient favoring release of label (100 μM [^{22}Na] compared 1–5 mM Na^+ in the Dowex cation exchange resin).

Binding of [^{32}P]phosphate to the cotransporter in the presence and absence of Na^+ is shown in Fig. 5. A single phosphate (7.1 ± 0.6 nmol [^{32}P]/mg cotransporter) bound to the cotransporter with a rate constant of 11.5 ± 1 s^{-1} ($n = 8$) in the presence of Na^+ (closed circles). In the absence of Na^+ (open circles) or following pre-treatment with the phosphate site blocker, phenylglyoxal (PG, results not shown), less than 0.3 nmol of [^{32}P]phosphate bound/mg protein. The amount of phosphate bound was independent of the medium anion (salt used to equilibrate Dowex anion exchange resin). Sulfate-equilibrated resin bound identical amounts of phosphate (6.9 ± 0.6 nmol [^{32}P]phosphate/mg protein, $n = 4$).

3.3. Na^+ and phosphate release from the cotransporter

Release of Na^+ from the Na^+/phosphate cotransporter in the presence and absence of phosphate was examined using stopped-flow fluorescence and the LAM timer. Na^+-equilibrated protein was diluted 20-fold into radioisotope-free media (LAM Timer) or Na^+-free media (stopped-flow fluorescence). Dilution of Na^+-equilibrated protein into Na^+-free media resulted in an enhancement of tryptophan fluorescence

Table 1
Effect of substrates on Na^+ and phosphate release from the Na^+/phosphate cortansporter

Conditions	Tryptophan fluorescence		LAM timer	
	Rate (s^{-1})	$\Delta F/F$ (%)	Rate (s^{-1})	Ion bound (nmol/mg)
Na^+-bound diluted into Na-free media	1.6 ± 0.1 ($n = 17$)	12 ± 1 ($n = 17$)	1.44 ± 0.1 ($n = 7$)	13.9 ± 1 ($n = 7$)
Na^+-bound diluted into Na^+ media	1.6 ± 0.1 ($n = 21$)	11 ± 1.3 ($n = 21$)	1.5 ± 0.1 ($n = 7$)	14.4 ± 1.2 ($n = 7$)
(Na^+ + DiFP)-bound diluted into substrate-free media	6.8 ± 0.4 ($n = 15$)	21 ± 4 ($n = 15$)	[12 ± 1.8 ($n = 9$)] { 6.2 ± 0.8 ($n = 9$)}	[13.7 ± 0.9 ($n = 9$)] { 7.5 ± 0.5 ($n = 9$)}
(Na^+ + P_i)-bound diluted into substrate-free media	3.4 ± 0.3 ($n = 16$)	28 ± 5 ($n = 16$)	[$13. \pm 1.1$ ($n = 6$)] [3 ± 0.3 ($n = 6$)] { 3.4 ± 0.4 ($n = 6$)}	[14.6 ± 1 ($n = 6$)] { 7.4 ± 0.4 ($n = 6$)}
(Na^+ + DiFP)-bound + MFP diluted into substrate-free media	0.9 ± 0.1 ($n = 14$)	14 ± 2 ($n = 14$)	[13 ± 0.5 ($n = 6$)] [1.9 ± 0.2 ($n = 6$)] { 1.1 ± 0.3 ($n = 6$)}	[14.8 ± 1 ($n = 6$)] { 7.5 ± 0.3 ($n = 6$)}
(Na^+ + DiFP)-bound diluted into Na^+ media	6.2 ± 0.3 ($n = 15$)	16 ± 3 ($n = 15$)		
(Na^+ + MFP)-bound diluted into substrate-free media			1.6 ± 0.2 ($n = 4$)	13.9 ± 0.9 ($n = 4$)

n = number of experiments.
LAM timer results in [brackets] refer to [^{22}Na] and LAM timer results in {parentheses} refer to [^{32}P] phosphate. Results are means ± S.E. of n, number of experiments.
The amount of [^{22}Na] bound by the NAI-labeled cotransporter was 0.24 ± 0.08 nmol/mg protein ($n = 7$).
The amount of [^{32}P]phosphate bound in the absence of Na^+ was 0.17 ± 0.07 nmol/mg protein ($n = 5$).
The amount of [^{32}P]phosphate bound by the PG-labeled cotransporter was 0.031 ± 0.02 nmol/mg protein ($n = 8$).

consistent with release of Na^+ and cotransporter return to the substrate-free conformation. The tryptophan fluorescence enhancement was fitted to a single exponential with a rate constant of 1.6 s^{-1} (Table 1). Similar experiments using [^{22}Na]-equilibrated protein, a 20-fold dilution into Na^+-free media, and the LAM Timer were fitted to a single exponential decay with a rate constant of 1.4 s^{-1} (Table 1).

The effect of DiFP, MFP, and phosphate on tryptophan fluorescence recovery was also examined. A typical experiment is shown in Fig. 6. DiFP (trace A) and phosphate (trace C) accelerated the rate of tryptophan fluorescence enhancement 4.5 and 2 times, respectively compared to the Na^+ conformation (see Table 1). Tryptophan fluorescence recovery of the (Na^+ + DiFP)conformation was fitted to a single exponential with a rate constant of 7 s^{-1}. Tryptophan fluorescence enhancement of the (Na^+ + phosphate)-conformation was fitted to a single exponential with a rate constant of 0.9 s^{-1} following dilution of the quaternary complex into monofluorophosphate (see Table 1).

The effects of substrates on tryptophan fluorescence suggest that the cotransporter has altered its

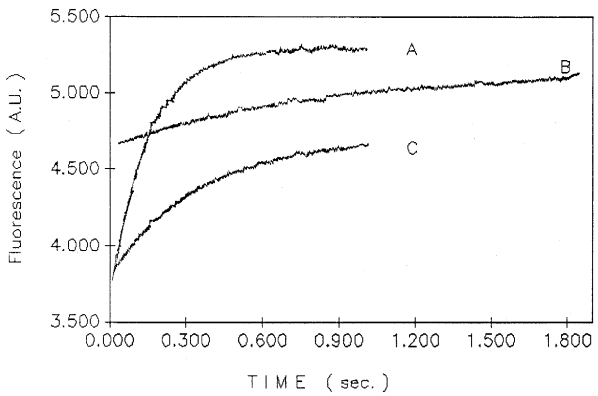

Fig. 6. Effect of divalent phosphate on tryptophan fluorescence of the (Na^+ + phosphate)-conformation. 20 μg of protein in 0.1 M NaCl, 0.4 M KCl, 50 mM Tris-Cl (pH 7), 0.1% CHAPS, and 0.1 mM potassium difluorophosphate (traces A and B) or 0.1 mM potassium phosphate (trace C) were diluted 1:20 into 0.5 M KCl, 50 mM Tris-Cl (pH 7), 0.1% CHAPS (traces A and C), and 0.1 mM potassium monofluorophosphate (trace B) in the stopped-flow aparatus. Tryptophan fluorescence at 350 nm was recorded as a function of time as described in Section 2. Results are from a single determination and representative of 14 determinations for the (Na + DiFP)-conformation, and 12 determinations for the (Na + phosphate)-conformation.

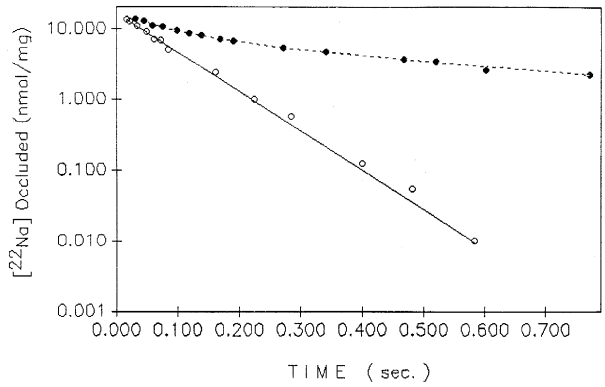

Fig. 7. Release of [$^{22}Na^+$] from the (Na^+ + DiFP)-conformation. 5 μg of [^{22}Na]-equilibrated protein in 0.1% CHAPS and 10 mM Tris-Cl (pH 7) was added to 1 ml Dowex 50 W syringes equilibrated with 10 mM Tris-Cl pH 7, 0.1% CHAPS, and 1 mM potassium difluorophosphate, DiFP (open circles), or 1 mM DiFP + 1 mM potassium monofluorophosphate (solid circles). Residence times, protein blanks, and protein concentration were determined as described in Section 2. Results are from a single experiment and representative of 6 experiments.

conformation. However, changes in tryptophan fluorescence cannot define the nature of the new conformation or which substrates were released. A 20-fold dilution of the quaternary complex into substrate-free media would be expected to result in the release of both Na^+ and phosphate since the dilution will result in a final substrate concentrations well below their apparent K_m's. However, while the tryptophan fluorescence enhancements following 20-fold dilution of the quaternary complex into substrate-free media are consistent with the cotransporter's return to its substrate-free conformation, fluorescence changes alone cannot define the cotransporter conformation. The LAM timer can provide information concerning the nature of this conformational change by defining the rates of substrate loss. LAM timer studies of the release of Na^+ and phosphate from the cotransporter are shown in Figs. 7 and 8, respectively, and summarized in Table 1.

Fig. 7 shows cotransporter release of [$^{22}Na^+$] from the ($2Na^+$ + DiFP)-conformation, and Fig. 8 shows cotransporter release of [^{32}P] DiFP from the ($2Na^+$ + DiFP)-conformation in the presence (open circles) and absence (closed circles) of MFP.

Fig. 7 shows that in the absence of MFP dilution of the (Na^+ + DiFP)-conformation into substrate-free media results in the release of both Na^+'s with

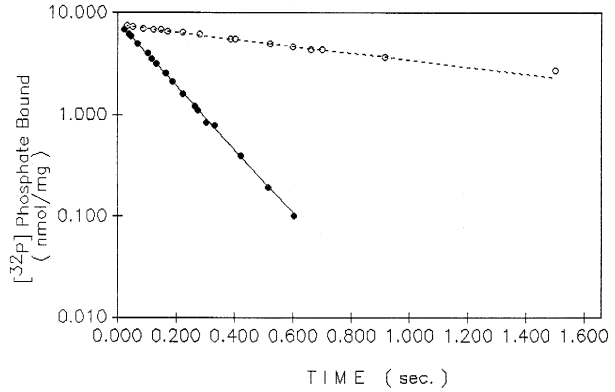

Fig. 8. Release of [^{32}P] from the (Na^+ + DiFP)-conformation. 5 μg of protein equilibrated with 1 mM Na^+, 25 μM [^{32}P]difluorophosphate (closed circles) or 25 μM [^{32}P]potassium difluorophosphate + 25 μM potassium monofluorophosphate (open circles), 0.1% CHAPS, and 10 mM Tris-Cl (pH 7), was run through variable length Dowex 1X-8 columns equilibrated with 1 mM NaCl, 0.1 mM DiFP (closed circles) or 0.1 mM DiFP + MFP (open circles), 0.1% CHAPS, and 10 mM Tris-Cl (pH 7). Residence times, protein concentrations, and protein blanks were determined as described in Section 2. Results are from a single experiments and representative of 5 experiments.

similar or identical rate constants (12 s^{-1}, Table 1). The addition of 0.1 mM MFP resulted in delayed Na^+ release of one Na^+ from the cotransporter (Na^+ + DiFP)-conformation. One Na^+ was released with a rate constant similar to that seen in the absence of MFP (13 s^{-1}), but the second Na^+ was released with a rate constant of 1 s^{-1}.

A similar effect of MFP on phosphate release was also seen. In the absence of MFP, phosphate release from the ($2Na^+$ + DiFP)-conformation (Fig. 8, closed circles) or ($2Na^+$ + phosphate)conformation (Table 1) was fitted to single exponentials with rate constants of 6.2 s^{-1} and 3.4 s^{-1}, respectively. MFP inhibited the rate of phosphate release from the ($2Na^+$ + DiFP)-conformation 82% consistent (Fig. 8, open circles) with previous results of the effect of MFP on Na^+-dependent phosphate uptake [28]. The LAM timer results suggest that MFP may alter the sequence of substrate release from the fully-loaded ($2Na^+$ + phosphate)-conformation. In the absence of MFP both Na^+'s were released prior to phosphate (Table 1, conditions 3 and 4). MFP slows or perhaps uncouples the release of the 2 Na^+'s and may allow phosphate release prior to the second Na^+. The LAM timer results also suggest that the changes in tryptophan fluorescence following dilution of the ($2Na^+$ + DiFP)-conformation and the ($2Na^+$ + phosphate)-conformation into substrate-free media (Fig. 6 and Table 1) described rate constants for the cotransporter return to the substrate-free conformation (compare fluorescence and LAM timer results for conditions 3–5 in Table 1).

4. Discussion

The molecular mechanism of ion transport across biological membranes appears to involve some method of sequestering the hydrated ion from its immediate environment, i.e., the hydrophobic cell membrane. Ion transporting ATPases have been shown to 'occlude' substrate ions as part of their reaction cycle [29]. Ion occlusion has been operationally defined in terms of the rate of ion loss from its transport protein and in terms of medium ion insensitivity. In terms of the mechanisms and structures involved in ion 'occlusion', little is known except that putative membrane spanning domains appear to be involved in both ion 'occlusion' and ion 'deocclusion' [30]. With respect to ion transport proteins other than ATPases, occlusion/deocclusion cycles have not been reported for secondary active transporters, ion exchange proteins, or facilitated diffusion carriers, although similar ion seclusion requirements might be expected.

The Na^+/phosphate cotransporter has been shown to undergo an Na^+-dependent conformational change which results in high affinity phosphate binding [17,26]. This Na^+-induced conformational change has also been observed using tryptophan fluorescence [6] and extrinsic probes bound at or near the cotransporter phosphate site [20] or at selected SH residues [10]. The Na^+-induced conformational change did not appear to require a free phosphate site, suggesting that the interaction of Na^+ with the cotransporter is initially independent of phosphate [15,20]. Stopped-flow fluorescence studies have shown that the Na^+-induced conformational change is rapid (33 s^{-1} {16} to 38 s^{-1}, Fig. 1), sensitive to the Na^+ site blocker, n-acetylimidazole (NAI), and involves 2 Na^+'s per cotransporter [16].

Experiments performed with the LAM timer suggest the interaction of Na^+ with the cotransporter

results in a Na^+-protected conformation. In the absence of phosphate, release of Na^+ from the cotransporter was slow (1.4–1.6 s^{-1}) or more than 20 times slower than Na^+ binding to the cotransporter as measured by the LAM timer or Na^+-induced quenching of tryptophan fluorescence. In addition, release of Na^+ from the Na^+:cotransporter conformation was independent of medium cations. Equal rates of Na^+ release from the Na^+:cotransporter conformation were seen with Na^+ or K^+ in the external medium as measured as [^{22}Na] release from detergent-solubilized cotransporter using the LAM timer, or measured by the recovery of tryptophan fluorescence using stopped-flow fluorescence.

In the presence of Na^+ but not K^+, phosphate addition induced a cotransporter conformational change with a rate constant of 13 s^{-1} (Fig. 2), and accelerated Na^+ release with an identical rate constant (12–13 s^{-1}, Figs. 6 and 7). Evidence that the effect of phosphate was cotransporter phosphate site-related was seen with the phosphate site blocker, phenylglyoxal (PG). PG blocked phosphate stimulation of Na^+ release. In agreement with the interpretation that phosphate stimulation of Na^+ release from the cotransporter was phosphate-site dependent was the requirement for the transport-competent phosphate species, $H_2PO_4^-$, and the effect of divalent phosphate, MFP, on Na^+ release. The effect of phosphate valence state on Na^+ release suggests that the phosphate-induced conformational change was required for accelerated Na^+ release. These results are consistent with the occlusion of 2 Na^+'s by the intestinal Na^+/phosphate cotransporter.

Evidence for an occluded phosphate intermediate state is less certain. There is good evidence that the (2 Na^+ + phosphate)-conformation differs from the Na^+-conformation, the (Na^+ + MFP)-conformation, the (2 Na^+ + DiFP)-conformation, or the proposed ($Na^+ + H_2PO_4^- + HPO_4^{2-}$)-conformation with respect to tryptophan fluorescence [6], SH reagent sensitivity [10], and substrate release rates. In addition, the reaction with MFP was conformation-sensitive (Fig. 3, Table 1). However, since both phosphate valence states apparently interact with the quaternary complex ({$2Na^+ + H_2PO_4^-$}-conformation) the existence of a (2 $Na^+ + H_2PO_4^-$)-conformation in vivo seems unlikely. This implies that in vivo, HPO_4^{2-} slows $H_2PO_4^-$ release and return to the substrate-free conformation. This leads to 2 possible roles for divalent phosphate. The first possibility is that HPO_4^{2-} occludes $H_2PO_4^-$ and it is HPO_4^{2-} release which precedes a conformational change deoccluding $H_2PO_4^-$. The second possibility is that HPO_4^{2-} stabilizes the $H_2PO_4^-$ occluded state. Direct examination of the rates of monovalent and divalent phosphate release using [^{32}P]MFP and [^{32}P]DiFP would be required to differentiate between these 2 possibilities. Unfortunately, the low synthetic yields of these phosphate derivatives precludes such studies.

HPO_4-induced $H_2PO_4^-$ occlusion implies that in the absence of HPO_4^{2-}, Na^+-dependent phosphate transport occurs without $H_2PO_4^-$ occlusion. Regardless of the low probability of this (2 $Na^+ + H_2PO_4^-$)-conformation occurring in vivo, HPO_4^{2-}-induced occlusion requires 2 transport mechanisms and 2 substrate release sequences. The intermediate nature of the (2 Na^+ + phosphate)-conformation rate of release of Na^+ and phosphate suggests that not all of the cotransporter responded to divalent phosphate. The possibility that the cotransporter may overcome divalent phosphate inhibition implies that HPO_4^{2-} inhibition is subject to regulation. One possibility is that Na^+ or pH may stimulate loss of HPO_4^{2-} and cotransporter return to the substrate-free conformation. Regardless of the role of HPO_4^{2-}, the conformational change resulting from HPO_4^{2-} binding to the quaternary complex which precedes $H_2PO_4^-$ release and the medium anion insensitivity of $H_2PO_4^-$ release are consistent with $H_2PO_4^-$ occlusion by the Na^+/phosphate cotransporter.

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