Mapping Conformational Changes of a Type IIb Na\(^+\)/P\(_i\) Cotransporter by Voltage Clamp Fluorometry*

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The fluorescence of a fluorophore depends on its environment, and if attached to a protein it may report on conformational changes. We have combined two-electrode voltage clamp with simultaneous fluorescence measurements to detect conformational changes in a type IIb Na\(^+\)/P\(_i\) cotransporter expressed in *Xenopus* oocytes. Four novel Cys, labeled with a fluorescent probe, yielded voltage- and substrate-dependent changes in fluorescence (F). Neither Cys substitution nor labeling significantly altered the mutant electrogenic properties. Different F responses to voltage and substrate were recorded at the four sites. S155C, located in an intracellular re-entrant loop in the first half of the protein, and E451C, located in an extracellular re-entrant loop in the second half of the protein, both showed Na\(^+\), Li\(^+\), and P\(_i\)-dependent F signals. S226C and Q319C, located at opposite ends of a large extracellular loop in the middle of the protein, mainly responded to changes in Na\(^+\) and Li\(^+\). Hyperpolarization increased F for S155C and S226C but decreased F for Q319C and E451C. The labeling and F response of S155C, confirmed that the intracellular loop containing Ser-155 is re-entrant as it is accessible from the extracellular milieu. The behavior of S155C and E451C indicates a strong involvement of the two re-entrant loops in conformational changes during the transport cycle. Moreover, the data for S226C and Q319C suggest that also the large extracellular loop is associated with transport function. Finally, the reciprocal voltage dependencies of the S155C-E451C and S226C-Q319C pairs suggest reciprocal conformational changes during the transport cycle for their respective local environments.

The type II sodium-phosphate cotransporters (encoded by the solute carrier SLC34A gene family) are polytopic membrane proteins that mediate thermodynamically coupled transport of inorganic P\(_i\) and Na\(^+\) across the cell membrane. Their physiological role is to facilitate cellular uptake of P\(_i\) by coupling it to the transmembrane electrochemical gradient. Three subtypes have been currently identified: NaPi-IIa and IIb are electronegic and operate with a 3:1 Na\(^+\):HPO\(_4^{2-}\) stoichiometry and translocate one net positive charge per transport cycle (for review, see Refs. 1 and 2), whereas NaPi-IIc is electroneutral and operates with a 2:1 Na\(^+\):HPO\(_4^{2-}\) stoichiometry (3).

The transport kinetics of electrogenic type II Na\(^+\)/P\(_i\) cotransporters have been studied earlier by electrophysiology and uptake assays in the *Xenopus* oocyte expression system (reviewed in Ref. 1). Recently, we established the method of voltage clamp fluorometry (VCF)\(^3\) to gain new insights into putative conformational changes occurring during the transport cycle (4). VCF relies on the property that the fluorescence of a fluorophore is sensitive to its local environment, and thus changes in fluorescence recorded from a fluorophore attached to a particular site in a protein can be interpreted as conformational changes affecting the environment of the fluorophore. VCF was first implemented in the study of gating-related conformational changes in K\(^+\) channels (5, 6). Since then, VCF has been applied to several membrane transporter systems, including the glucose transporter SGLT1 (7, 8), the glutamate transporter EAAT3 (9), the GABA transporter GAT1 (10), the serotonin transporter SERT (11), and the Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases (12, 13).

In our first study (4) we introduced a novel Cys residue in the putative re-entrant loop between transmembrane domains (TMDs) 5–6 (S448C, marked with an open triangle in Fig. 1) of the flounder NaPi-IIb isoform. Labeling S448C with a fluorescent probe allowed us to detect voltage- and substrate-dependent changes in fluorescence (F). Using the new fluorescence data we modified our earlier kinetic model of the electrogenic NaPi-II transport cycle that we derived from the interpretation of steady state and presteady state substrate-dependent currents. In the new model, two Na\(^+\) ions (instead of one) bind on the external side before a divalent HPO\(_4^{2-}\), followed by one more Na\(^+\) ion. Subsequently the fully loaded carrier translocates to the internal side. After unloading, the empty carrier returns to an outside facing configuration via an electrogenic partial reaction, and each complete forward transport cycle transfers one positive charge to the intracellular medium (14–17).

In the S448C mutant the cotransport function is blocked after labeling, however analysis of current and fluorescence data indicates that the protein is still able to bind substrate.

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3 The abbreviations used are: VCF, voltage clamp fluorometry; WT, wild type; ANOVA, analysis of variance; TMD, transmembrane domain; MTSEA, (2-aminoethyl)methane thiosulfonate; MTS-TMR, 2-((5 (6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate.
Conformational Changes in NaPi-IIb

Although the absence of a full cotransport cycle may have facilitated the analysis of the data (the protein could only occupy a subset of possible conformations), it is important to validate and extend those observations to a fully functional transporter. In the present study we mutated ten new residues individually to Cys (see Fig. 1), all of which are located in predicted linker regions that face, or are accessible to, the extracellular milieu. For each mutant, we determined whether we could measure voltage-dependent fluoride signals (ΔF) after labeling with a fluorophore. We found that four of the novel Cys mutants gave measurable ΔF signals. In contrast to the S448C mutant described above, for the four new mutants, neither the Cys mutation itself nor the subsequent labeling resulted in a significant alteration in their cotransport kinetics, which closely resembled the WT transport. Our findings therefore offer a first possible glimpse of time-resolved movement of the type II Na+/Pi cotransporter. In the present study we mutated ten new residues individually to Cys (see Fig. 1), all of which are located in predicted linker regions that face, or are accessible to, the extracellular milieu. For each mutant, we determined whether we could measure voltage-dependent fluorescence changes (ΔF) after labeling with a fluorophore. We found that four of the novel Cys mutants gave measurable ΔF signals. In contrast to the S448C mutant described above, for the four new mutants, neither the Cys mutation itself nor the subsequent labeling resulted in a significant alteration in their cotransport kinetics, which closely resembled the WT kinetic fingerprint. This finding has allowed us to interpret the voltage and substrate-induced changes in fluorescence as reflecting the associated conformational changes at different sites of the electrogenic type II Na+/Pi cotransporter during the complete transport cycle. Our findings therefore offer a first possible glimpse of time-resolved movement of the type II Na+/Pi cotransporter.

EXPERIMENTAL PROCEDURES

Reagents and Solutions—(2-Aminoethyl)methane thiosul- fonate hydrobromide (MTSEA) was from Toronto Research Chemicals. 2-((5(6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate (MTS-TMR) was from Biotium. All other reagents were from Sigma or Fluka.

The standard experimental solution (ND100) contained (mm): 100 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4 (adjusted using Tris). In Na⁺ replacement experiments, NaCl was replaced equimolar with choline Cl or LiCl. In the iodide experiments we replaced 50 mM NaCl with 50 mM NaI or sodium gluconate (the latter serving as a Cl replacement control). Solutions containing the required concentrations of Pi were prepared by adding K₂HPO₄/KH₂PO₄ (pH 7.4). Modified Barth’s solution for storing oocytes contains (mm): 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 NaHCO₃, 2 Ca(NO₃)₂, 7.5 HEPES, pH 7.5 adjusted with Tris and supplemented with 5 mg/liter doxycyclin.

Oocyte Expression and Molecular Biology—Point mutations were generated in WT flounder NaPi-IIb (GenBank Accession No. AAB16821) using QuikChange site-directed mutagenesis (Stratagene) with primers from Microsynth and verification by sequencing (Microsynth). Complementary capped RNA was synthesized using the T3 Message Machine kit (Ambion). Oocytes from Xenopus laevis were prepared as previously described (4), injected with 50 nl of cRNA (0.2 μg/μl), and experiments performed 3–7 days after injection. The oocytes were exposed immediately before fluorescence measurements to 0.4 mM MTS-TMR in ND100 solution for 5 min in the dark.

Conventional Two-electrode Voltage Clamp—The procedure for standard two-electrode voltage clamp has been described in detail previously, as have the protocols (4, 16). Briefly, steady state Pᵢ activation was determined by varying the Pᵢ concentration in the presence of ND100 and subtracting the respective currents in ND100 from those in ND100 + Pᵢ; steady state Na⁺ activation was similarly determined by subtracting the respective responses in NDX from those in NDX + Pᵢ (1 mM), where X is the test Na⁺ concentration (in mM). Steady state Pᵢ-induced currents (Iᵢₚᵢ) were fit with a form of the modified Hill Equation 1,

\[ I_{i,p} = I_{i,p,\text{max}} \left[ \frac{[S]^{n}}{[S]^{n} + (K_m)^{n}} \right] + K \]  \hspace{1cm} (Eq. 1)

where [S] is the concentration of the variable substrate (Na⁺ or Pᵢ), Iᵢₚᵢ, max is the maximum electrogenic activity, Kᵢₚᵢ values the apparent substrate affinity for substrate S; H, the Hill coefficient, and K is a constant that takes account of uncoupled leak effects (17, 18). For Pᵢ activation, H = 1 and Equation 1 reduces to a Michaelian form.

Presteady state relaxations were quantitated by fitting with a two-component exponential function. The faster component, which represents endogenous linear capacitive charging of the oocyte membrane, was subtracted from the total relaxation to yield the NaPi-II-dependent component. This was numerically integrated to obtain the charge Q moved for a step from the holding potential (Vᵢₚᵢ) to the test potential (Vᵢ), as previously described (e.g. (17, 18). The Q-V data were fit with a Boltzmann function of the form in Equation 2,

\[ Q = Q_{\text{hyp}} + Q_{\text{max}} \left[ 1 + \exp \left( \frac{ze(V_{0.5} - V)/kT}{kT} \right) \right] \]  \hspace{1cm} (Eq. 2)

where V₀.₅ is the voltage at which the charge is distributed equally between two hypothetical states, z is the apparent valency of an equivalent charge that moves through the whole of the membrane field, Qᵢₚᵢ, max is the total charge available to move, Qᵢₚᵢ, hyp is the charge at the hyperpolarizing limit and is a function of Vᵢₚᵢ and e, k, and T have their usual meanings.

Apparatus for Simultaneous Voltage Clamp and Fluorometry—The apparatus for simultaneous VCF has been described in detail previously (4) and consisted of a conventional two-electrode voltage clamp with a laboratory-built fluorescence microscope. The latter comprised a ×10 fluorescence objective (CFI S Fluor, 0.5 N.A., 1.2 mm W.D., Nikon) and a filter set (XF33 cube, comprising a 535DF35 excitation filter, 570DRLP dichroic mirror and 605DF50 emission filter; Omega Optical Inc). Emitted light was measured using a silicon photodiode (S1336-18BQ, Hamamatsu) connected directly to the input of an integrating headstage (CV 201, Molecular Devices) and the headstage signal was processed by a patch clamp amplifier (Axopatch 200A, Molecular Devices). The scaled output of the Axopatch 200A was further processed by a differential amplifier/filter unit (LPF-8, Warner Instruments) before digitization.

Experimental Protocols—Voltage-dependent changes in fluorescence (ΔF) were determined using a voltage-step protocol. The membrane voltage was stepped from Vᵢₚᵢ = −60 mV to test potentials ranging between −200 mV and +200 mV in 40-mV increments for a duration of 100–200 ms (the protocol was adjusted for each NaPi-IIB mutant to obtain saturation of F at extreme potentials, but avoid unnecessary stress to the oocyte), and averaged over 10–15 sweeps. F data were acquired at 20k samples s⁻¹ and filtered at 70 Hz. The effect of substrate on F was determined by changing the bath solution. To facilitate comparison between conditions and oocytes, one data set was
always acquired in ND100 solution for each oocyte. All data were corrected for fluorescence rundown as described previously (4).

Data Analysis—First, each F-V recording was adjusted to zero at $V_h = -60 \text{ mV}$ to obtain $\Delta F$ relative to $-60 \text{ mV}$. After correction for photobleaching, the F-V data were fitted with a Boltzmann equation (Equation 2, where $F$ is substituted for $Q$).

Data were pooled from several oocytes after first normalizing all data to $F_{\text{max}}$, obtained in ND100 for that oocyte. For display purposes the F-V data were shifted (by $F_{\text{hyp}}$) to the same $F_{\text{max}}$ as the ND100 condition. For statistical analysis using a paired Student’s $t$ test or ANOVA, we compared data obtained by fitting each oocyte individually, whereas slopes of regression lines were compared between pooled data. Statistical analysis was made using GraphPad Prism version 3.02 for Windows, GraphPad Software. In the graphs, data are shown as mean ± S.E.

RESULTS
Identification of Sites that Exhibit Voltage-dependent $F$ using Cys Scanning

We introduced novel Cys in each of the predicted external linkers and in the first intracellular loop (predicted to be re-entrant and therefore accessible to the extracellular medium) in NaPi-IIb (Fig. 1A). We characterized each mutant by electrophysiology and compared its behavior to that of the WT. All mutants were functional as evidenced by their robust Pi-induced currents ($I_{\text{Pi}}$) at $-50 \text{ mV}$, $1 \text{ mM Pi}$, which confirmed that the Cys substitution was well tolerated (data not shown). The corresponding current-voltage ($I$-$V$) relations were similar to WT (shown in Fig. 1, B and C), with the exception of S520C. We then exposed the mutants to $1 \text{ mM MTSEA}$ to see if this procedure would block $I_{\text{Pi}}$, as we had previously observed with the S448C mutant (4). Apart from S520C (see below), no major changes were seen in the $I_{\text{Pi}}$ of any of the mutants (data not shown).

S520C, located in the fourth extracellular loop, was the only mutant that showed an altered voltage dependence of $I_{\text{Pi}}$. After MTS treatment the voltage dependence became more like that of the WT. All mutants were functional as evidenced by their robust Pi-induced currents ($I_{\text{Pi}}$) at $-50 \text{ mV}$, $1 \text{ mM Pi}$, which confirmed that the Cys substitution was well tolerated (data not shown). The corresponding current-voltage ($I$-$V$) relations were similar to WT (shown in Fig. 1, B and C), with the exception of S520C. We then exposed the mutants to $1 \text{ mM MTSEA}$ to see if this procedure would block $I_{\text{Pi}}$, as we had previously observed with the S448C mutant (4). Apart from S520C (see below), no major changes were seen in the $I_{\text{Pi}}$ of any of the mutants (data not shown).

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icant ΔF in response to changes in \(V_h\) after labeling with MTS-TMR, and only these mutants were studied further. The lack of voltage-dependent \(F\) for the other mutants (K110C, Q154C, A156C, T220C, S410C, and S520C) may indicate that these novel Cys were not accessible for labeling. Another explanation is that the attached fluorophore did not sense a change in its environment under the experimental conditions applied (e.g. S520C).

Steady State and Presteady State Kinetics of WT and Mutants

For the four mutants that showed voltage-dependent \(F\), we analyzed steady state and presteady state currents to determine if the transport kinetics had been altered by the mutation or by labeling. Fig. 1C shows the voltage dependence of the \(P_i\)-dependent current (\(I_{Pi}\)) in (unlabeled) oocytes expressing WT, S155C, S226C, Q319C or E451C. Each shows qualitatively similar current-voltage (\(I-V\)) dependences and they only differ according to the absolute amount of current generated for any given oocyte. Taken together, all constructs yielded approximately similar \(I_{Pi}\) when averaged over batches and individual oocytes; however, there was considerable variation in \(I_{Pi}\) between individual oocytes, most likely because of variations in the expression levels of transporters at the cell membrane. We then measured the apparent \(P_i\) affinity (\(K_{\text{app}}(P_i)\)) at voltages ranging between −140 and +20 mV for WT and the four mutants. As shown in Fig. 1D, for S226C, Q319C, and E451C, \(K_{\text{app}}(P_i)\) was similar to that of the WT. For S155C, \(K_{\text{app}}(P_i)\) was increased (i.e. the apparent \(P_i\) affinity was decreased) significantly compared with that of the WT, which indicated that the substitution at this site caused a moderate alteration of the cotransport kinetics.

Next, we compared the steady state and presteady state currents before and after labeling with MTS-TMR (WT, S155C, S226C, E451C) or MTS-SEA (WT, S155C, S226C, Q319C). For the WT, no difference was observed in either \(I_{Pi}\) (not shown) or presteady state current recordings before and after incubating with MTS-TMR (Fig. 2A–C) or MTS-SEA (not shown), which is consistent with the assumption that none of the 19 native Cys of the flounder NaPi-IIb protein are accessible with this reagent. Similar results were obtained with MTS-SEA (not shown). The results agree with previous work, which showed that the WT rat NaPi-IIa isoform could not be labeled by Cys-reactive biotinylating reagents (19) and that WT flounder NaPi-IIb did not show any voltage-dependent ΔF after incubation with MTS-TMR (4). Thus, we can conclude that the WT NaPi-IIb protein is not functionally modified by the Cys-reactive reagents used in this study. Fig. 2C shows the Boltzmann fit parameters obtained from presteady state current recordings acquired over a range of \(Na^+\) concentrations. The results are similar to those obtained previously (14), but in the present study more \(Na^+\) concentrations were included. As depicted in Fig. 2C, the predicted maximum amount of charge available for translocation (\(Q_{\text{max}}\)) increased with increasing \(Na^+\). Fitting the \(Q_{\text{max}}\) data with the Michaelis-Menten equation (Equation 1, H constrained to 1) yielded an apparent \(K_{\text{m}}(Na)\) of 8 ± 1 mM for the \(Na^+\)-dependent component of \(Q_{\text{max}}\), compared with 4 ± 2 mM (S155C), 24 ± 3 mM (S226C), 11 ± 3 mM (Q319C), and 24 ± 5 (E451C) (not shown).

In addition to \(Q_{\text{max}}\), the apparent valency (\(z\)) and the midpoint of the Boltzmann distribution (\(V_{0.5}\)) were also \(Na^+\)-dependent (Fig. 2C). \(z\) varied between 0.3 and 0.6, depending on the \(Na^+\) concentration. For \(V_{0.5}\), the most negative value was obtained with 25 mM \(Na^+\), and increasing or decreasing \(Na^+\) from this value led to more positive \(V_{0.5}\) values. The \(V_{0.5}\) data obtained in the 25–125 mM range were fitted with a straight line to yield a slope of 113 ± 11 mV/log[Na] that was comparable to the values obtained for the mutants (S155C: 104 ± 12, S226C: 91 ± 4, Q319C: 105 ± 8 and E451C: 129 ± 5). The complex \(Na^+\) dependence of the parameters obtained from the Boltzmann fit most likely results from the contributions of more than two voltage-dependent partial reactions to the overall charge distribution (recall that Equation 2 describes a two-state model; for a detailed discussion, see e.g. Refs. 16 and 17).

For the three mutants S155S, S226C, and Q319C, we documented some minor alterations in the kinetics after labeling. \(I_{Pi}\) was 10–15% reduced after labeling for S155C, Q319C, and E451C in the voltage range −140 to +20 mV. For S226C, labeling resulting in an apparent reduction in the voltage dependence of \(P_i\) cotransport, as the slope of the \(I-V\) relationship decreased by −50% (not shown). These changes in electrogenic steady state \(P_i\) cotransport were paralleled by minor changes in the \(Na^+\) dependences of the presteady state charge distributions before and after labeling for S155C and S226C, but not for Q319C or E451C (Fig. 2D). In ND0, labeling did not affect \(V_{0.5}\) for any of the mutants (not shown). However, in the presence of \(Na^+\), MTS-TMR treatment shifted \(V_{0.5}\) for S155C to more negative potentials, whereas the opposite effect was seen for S226C (Fig. 2D). This indicated that labeling had introduced a small shift in the preferred occupancy of \(Na^+\)-dependent conformational states for these mutants. On the other hand, the slope \(V_{0.5}/\log[Na]\) was not changed for any of the mutants, which suggested that the depth of the hypothetical ion binding well for \(Na^+\) was unchanged by fluorophore labeling (4, 20, 21). Labeling did not change the total amount of mobile charge (\(Q_{\text{max}}\)) nor the apparent \(K_{\text{m}}(Na)\) measured by fitting the \(Q_{\text{max}}\) data with Equation 1 for any of these four mutants (not shown).

Finally, we investigated the effect of complete replacement of external \(Na^+\) by \(Li^+\) on the \(Q-V\) distribution of the WT NaPi-IIa protein. \(Li^+\) does not substitute for \(Na^+\) as the driving cation for \(P_i\) cotransport (not shown), yet evidence for an interaction of \(Li^+\) ions with the NaPi-II protein was obtained in our previous VCF study (4) using the S448C mutant. Replacing \(Na^+\) with \(Li^+\) still resulted in significant voltage-dependent \(F\) for this mutant whereas no ΔF was observed in choline replacement. To examine if \(Li^+\) affected the presteady state kinetics of the WT protein, we recorded presteady state currents in \(Na^+\) (ND100), choline (ND0), and \(Li^+\) (LD100) solutions. The Boltzmann fit data are summarized in Table 1. Substituting \(Na^+\) with \(Li^+\) or choline reduced the estimated \(Q_{\text{max}}\) to the same level (we attribute this residual charge movement to the movement of intrinsic charge of the empty carrier). Also the apparent valency \(z\) measured in LD100 was statistically
indistinguishable from the ND0 condition, whereas $z$ increased significantly in ND100 solution. In contrast, both Na$^+$ and Li$^+$ shifted $V_{0.5}$ in a statistically significant manner to more positive potentials, compared with choline. These results indicate that Li$^+$ ions can also interact with the WT protein and alter its conformational distribution, but without making a substantial contribution to the amount of detectable mobile charge.

**TABLE 1**

|        | $V_{0.5}$ | $Q_{\text{max}}$ | $z$ |
|--------|-----------|-------------------|-----|
| ND0    | $-41.1 \pm 2.5^*$ | $4.4 \pm 0.9$ | $4.44 \pm 0.04$ |
| ND100  | $38 \pm 6^*$       | $9.8 \pm 0.7^*$  | $0.66 \pm 0.02^*$ |
| LD100  | $-4.1 \pm 2.5^*$   | $4.6 \pm 0.7$    | $0.51 \pm 0.01$  |

*Statistically significant difference to the ND0 condition using repeated-measures ANOVA with Tukey’s post-test ($p < 0.05$). Means $\pm$ S.E., $n = 6$. 

**FIGURE 2.** Presteady state current analysis of the effect of introducing single Cys and labeling with MTS-TMR. A, original current recordings of the presteady state current response after a step change in membrane potential from $-60 \text{ mV}$ to target potentials ($-160 \text{ to } +60 \text{ mV}$ in 20-mV increments) obtained from an oocyte expressing WT NaPi-IIb in ND100 (left) and ND0 (right) solution. Dotted lines show the single exponential fitted component corresponding to the presteady state current attributed to NaPi-IIb. B, charge-voltage relationships of WT NaPi-IIb before (left) and after (right) exposure to MTS-TMR. The charge was obtained by integrating the area under the fitted curve in recordings similar to A, acquired at different Na$^+$ concentrations. The data were fit with the Boltzmann equation (Equation 2; solid lines). C, Boltzmann fit parameters obtained for WT NaPi-IIb from data similar to B before (filled squares) and after (empty squares) MTS-TMR treatment. Left, the $Q_{\text{max}}$ data were plotted as a function of Na$^+$ concentration and fitted with the Michaelis-Menten equation with $H = 1$ and yielded an apparent $K_m$ (Na) of $8 \pm 1 \text{ mM}$. Mid-left, apparent valency $z$ plotted as a function of the Na$^+$ concentration. Mid-right, The $V_{0.5}$ data were plotted as a function of the Na$^+$ concentration (left) and then replotted as a function of log[Na] (right). The data points between 25 and 125 mM Na$^+$ were fitted with a linear regression line with the slope $113 \pm 11 \text{ mV/log[Na]}$. D, presteady state current analysis of the four mutants before and after exposure to MTS-TMR. The $Q-V$ distributions were very similar to WT and only $V_{0.5}$/log[Na] is shown for each mutant. The slopes of linear regression lines fitted to the data were as follows (control, MTS-TMR-treated): S155C: $104 \pm 12, 101 \pm 14$; S226C: $91 \pm 7, 102 \pm 9$; Q319C: $105 \pm 8, 102 \pm 9$; E451C: $129 \pm 5, 131 \pm 4 \text{ mV/log[Na]}$. In C and D we show means $\pm$ S.E., $n = 3–4$. 

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Voltage- and Substrate-dependent Fluorescence Signals Recorded from S155C, S226C, Q319C, and E451C Mutant S155C

**Na\(^+\) Dependence**—Labeling S155C with MTS-TMR resulted in a construct for which robust voltage-dependent \(F\) (total \(\Delta F/F \sim 3\)–6\%) was observed in response to changes in membrane voltage. Fig. 3A shows an original fluorescence trace recorded in ND100 and ND0 solution. Stepping the membrane potential from \(V_h = -60\) mV to more negative potentials decreased \(F\), whereas it increased for more positive potentials. Because \(F\) saturated at both hyper- and hypopolarizing voltages, we could satisfactorily fit the \(F\)-\(V\) distribution with a Boltzmann function (Equation 2; solid lines in Fig. 3B). The \(F\)-\(V\) distribution was strongly dependent on \(Na^+\). Reducing the external \(Na^+\) concentration shifted the midpoint of the Boltzmann curve (\(V_{0.5}\)) to the left (Fig. 3E), and the maximum amount of fluorescence change (\(F_{max}\)) predicted from the fit was essentially constant between 125 and 50 mM \(Na^+\). At very low \(Na^+\), we were no longer able to obtain saturation of \(F\) at the hyperpolarizing limit; however by constraining \(F_{max}\) to be the same at all \(Na^+\) concentrations, we could improve the quality of the fit significantly. We found a linear dependence of \(V_{0.5}\) on \(\log[Na]\) in the 10 to 125 mM range, with a slope of 140 mV \(\pm\) 5/log[Na] (Fig. 3E). Furthermore the steepness of the slope of the Boltzmann distribution (\(z\)) was reduced with decreasing \(Na^+\) (Fig. 3F).

**Li\(^+\) Dependence**—To investigate if \(F\) was also influenced by external Li\(^+\) ions as we previously reported for the S448C mutant (4), we repeated the above ion replacement protocol.

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**FIGURE 3.** Voltage-and substrate-dependent changes in fluorescence (\(F\)) recorded in S155C-expressing oocytes. A, original \(F\) traces recorded in ND100 (left) and ND0 (right) solution. B, \(F\)-\(V\) data were acquired at different \(Na^+\) concentrations and fitted with Equation 2 (solid lines). C, \(F\)-\(V\)-data were acquired at different Li\(^+\) concentrations and in 100 mM Li\(^+\) with 1 mM P\(_i\). D, \(F\)-\(V\)-data were acquired in ND100 solution with different amounts of P\(_i\) added. E, \(V_{0.5}\) obtained from the fit of Equation 2 to B and C plotted as a function of \(\log[Na]\) or \(\log[Li]\). The data were fit with linear regression lines with slopes of 140 \(\pm\) 5 mV/log[Na] and 154 \(\pm\) 4 mV/log[Li]. F, \(z\) obtained from the fit of Equation 2 to B and C plotted as a function of [Na\(^+\)] or [Li\(^+\)]. G, \(F_{max}\) obtained from the fit of Equation 2 to D, plotted as a function of P\(_i\) and fit with Equation 1 (\(H\) constrained to 1). The apparent \(K_m(P_i)\) from the fit was 99 \(\pm\) 17 \(\mu\)M. Means \(\pm\) S.E., \(n = 4\).
parameters in a statistically significant manner (see Fig. 3C), which indicated that although Li+ may interact with the protein, it does not support P_i binding. Moreover, adding 1 mM P_i to ND0 solution did not alter the F-V distribution, as compared with ND0 alone, which further underscored the dependence of P_i interaction on Na+ (not shown).

Mutant S226C

Na+ Dependence—After labeling the S226C mutant with MTS-TMR, we observed large voltage-dependent changes in F (total ΔF/F 4–8% in ND100; Fig. 4A). As with the S155C mutant, hyperpolarization decreased F, and saturation was observed at both voltage extremes. We obtained F-V data at different Na+ concentrations and fitted these data with Equation 2 (solid lines in Fig. 4B). F_max obtained from the fit was reasonably constant over the entire Na+ range (not shown). Compared with S155C, the F-V distribution was shifted to more positive voltages for all Na+ concentrations tested: in ND100 V_0.5 was 32 ± 2 mV, compared with −34 ± 1 mV for S155C, and the shift in V_0.5 toward more negative voltages with reducing Na+ was less pronounced than for S155C. The relationship between V_0.5 and log[Na] was linear for Na+ concentrations ≥25 mM, which yielded a slope of 58 ± 8 mV/log[Na] using linear regression (Fig. 4D). Also z was dependent on the Na+ concentration (Fig. 4E).

Li+ Dependence—The effect of Li+ on the F-V distribution was similar to that of Na+ (Fig. 4C). The main difference was that the F-V distribution was shifted to more positive voltages compared with the equivalent Na+ concentrations, so that at 100 mM Li+, V_0.5 was 73 ± 7 mV, compared with 32 ± 3 mV in 100 mM Na+. Like Na+, in Li+ the relationship between V_0.5 and log[Li] was linear for Li+ concentrations ≥25 mM, and the slope 59 ± 5 mV/log[Li] from the linear regression was statistically indistinguishable from that of Na+ (58 ± 8 mV/log[Na]) (Fig. 4D). Moreover the Na+ and Li+–dependencies of z were similar (Fig. 4E), which indicated that for this mutant, Li+ induces similar changes in the fluorophore environment to Na+.

P_i Dependence—To determine whether the F-V distribution was affected by P_i, we measured F in ND100, ND0, and LD100 with 1 mM P_i added in each case (Fig. 4A, B and C). In ND100, P_i affected neither F_max nor V_0.5. However, the apparent valency was decreased from 0.45 ± 0.02 to 0.27 ± 0.04. When we added P_i to LD100, we observed a small, but statistically significant decrease in z from 0.42 ± 0.03 to

with Li+ as the variable cation (Fig. 3C). The maximum voltage-induced change in F was similar in LD100 and ND100, and we fitted the F-V distribution with a Boltzmann function (Equation 2). Reducing Li+ also shifted V_0.5 to the left, however at low Li+ (=10 mM) we could no longer fit the data reliably with Equation 2 because of the lack of saturation at the hyperpolarizing limit. As for Na+, F_max reported by the fit remained reasonably constant between 100 and 10 mM Li+ and we therefore fixed this parameter to improve the quality of the fit. We found a linear dependence of V_0.5 with log[Li] in the 10–100 mM range, with a slope of 154 ± 8 mV/log[Li] (Fig. 3E). However, in contrast to Na+, z was not altered between 10 and 100 mM Li+ (Fig. 3F), which indicated that there are dissimilarities in the interaction of Na+ and Li+ with the NaPi-IIb protein.

P_i Dependence—Finally, we measured the effect of increasing the P_i concentration on the F-V distribution while keeping Na+ constant at 100 mM. Increasing P_i markedly reduced F_max (Fig. 3G), but had little effect on V_0.5 or the apparent valency z (not shown). We plotted F_max as a function of P_i concentration and fitted the data with the Michaelis-Menten equation (Equation 1 with H constrained to 1). The fit yielded an apparent affinity for the P_i-dependent decrease in F_max of 99 ± 17 μM. This value is similar to K_m(P_i) determined from the P_i-induced currents in Fig. 1D.

The effect of P_i on F appears to have an absolute requirement for Na+. In LD100, P_i did not affect any of the Boltzmann fit parameters in a statistically significant manner (see Fig. 3C), which indicated that although Li+ may interact with the protein, it does not support P_i binding. Moreover, adding 1 mM P_i to ND0 solution did not alter the F-V distribution, as compared with ND0 alone, which further underscored the dependence of P_i interaction on Na+] (not shown).
Conformational Changes in NaPi-IIb

**Li⁺ Dependence**—The effect of Li⁺ on the F-V distribution of Q319C (Fig. 5C) was quite similar to that of Na⁺, and thus this mutant phenotype mirrors that of S226C. Like S226C, the F-V distribution of Q319C was shifted to more positive voltages in Li⁺ solutions than in Na⁺. In LD100, V_{max} was 64 ± 4 mV, compared with only 36 ± 2 mV in ND100. In Li⁺ the relationship between V_{0.5} and log[Li] was linear for Li⁺ concentrations ≥ 25 mM (Fig. 5D), and the slope 64 ± 1 mV/log[Li] from the linear regression was slightly (and statistically significantly) higher compared with Na⁺ (55 ± 3 mV/log[Na]). The Na⁺ and Li⁺ dependence of z were similar (Fig. 5E), although Li⁺ tended to have a smaller effect on z than Na⁺. Finally, as for Na⁺, F_{max} was unaffected by varying the Li⁺ concentration (not shown).

**P, Dependence**—Adding 1 mM Pi to LD100 produced statistically significant changes in some of the Boltzmann fit parameters for Q319C. We observed a decrease of ~14% in F_{max} and the apparent valency z decreased from 0.63 ± 0.04 to 0.54 ± 0.05, whereas V_{0.5} remained unchanged. In contrast, adding 1 mM Pi to LD100 or ND0 did not change any of the measured parameters significantly. These results show that a fluorophore attached to Q319C partially senses the conformational changes effected by Pi, in the presence of Na⁺, and thus this mutant differs in this respect from its counterpart, S226C. Our finding that Pi does not affect the F-V distribution in LD100 or ND0 further supports the notion that Pi interaction requires Na⁺ and neither Li⁺ nor choline can substitute for Na⁺.

**Mutant E451C**

Na⁺ Dependence—After labeling with MTS-TMR, E451C-expressing oocytes showed a small (total ΔF/F < 1%) but measurable change in F in response to membrane voltage (Fig. 6). As for Q319C, F decreased at depolarizing potentials. F saturated at both hypo- and hyperpolarizing limits for Na⁺ concentrations between 50 and 125 mM and we fit the data with Equation 2 (solid lines in Fig. 6B). V_{0.5} was about 12 ± 7 mV in ND100, and there was a linear dependence of V_{0.5} on log[Na] for Na⁺ ≥ 50 mM with a slope of 99 ± 2 mV/log[Na] (Fig. 6E). For this Na⁺ concentration range F_{max} was constant. The apparent valency z decreases with decreasing Na⁺ (Fig. 6F), so that at Na⁺ ≤ 25 mM z approached zero, F_{max} became very large and the F-V distribution could not be reliably fit with Equation 2. We therefore plotted the F-V distribution obtained for Na⁺ concentrations between 0 and 25 mM without offsetting for F_{max} (inset in Fig. 6B).

![Figure 5. Voltage-and substrate-dependent changes in fluorescence (F) recorded in Q319C-expressing oocytes.](image)

A, original F traces recorded in ND100 (top) and ND0 (bottom) solution. B, F-V data were acquired at different Na⁺ concentrations and in 100 mM Na⁺ with 1 mM P_i, and fitted with Equation 2 (solid lines). Only data obtained in 0 and 100 mM Na⁺ and 100 mM Na⁺ + 1 mM P_i are shown. C, F-V data were acquired at different Li⁺ concentrations (0 and 100 mM are shown) and in 100 mM Li⁺ with 1 mM P_i. D, V_{max} obtained from the fit of Equation 2 to B and C plotted as a function of log[Na] or log[Li]. E, z obtained from the fit of Equation 2 to B and C plotted as a function of Na⁺ or Li⁺. Means ± S.E., n = 4–5.

0.36 ± 0.03. As for ND100, no other Boltzmann fit parameters were significantly affected by adding P_i to LD100. Finally, in ND0 solution, we observed no effect on the F-V distribution after adding P_i. These findings indicate that the S226C site mainly senses conformational changes associated with the empty carrier and the Na⁺ (Li⁺) binding steps that precede P_i binding.

**Mutant Q319C**

Na⁺ Dependence—Labeling Q319C-expressing oocytes with MTS-TMR yielded voltage-dependent fluorescence (total ΔF/F ~1–3%) which, in contrast to the two mutants described above, decreased at depolarizing potentials. The F-V distributions for Na⁺ and Li⁺ dependence (shown in Fig. 5, B and C, respectively) resembled mirror images of those for S226C (Fig. 4, B and C). F recorded from Q319C saturated at both the hyper- and hypopolarizing limits. Like the other mutants, we obtained F-V data at different Na⁺ concentrations and fitted it with Equation 2 (solid lines in Fig. 5B). V_{0.5} in ND100 was 36 ± 2 mV, which is similar to that of S226C (32 ± 3 mV). There was a linear dependence of V_{0.5} on log[Na] for Na⁺ ≥ 50 mM, and linear regression (Fig. 4D) yielded a slope of 55 ± 3 mV/log[Na], which was also similar to the value obtained for S226C (58 ± 8 mV/log[Na]). Furthermore, z decreased with decreasing Na⁺ (Fig. 4E), whereas F_{max} remained constant. Thus, Q319C strongly resembles S226C in its response to voltage and Na⁺, although it presents a mirror image with respect to the effect of membrane voltage on F.
Li⁺ Dependence—The Li⁺ dependence of F recorded from E451C is shown in Fig. 6C (solid lines represent the fit with Equation 2). As for Na⁺, Fₘₐₓ remained constant in the range 50–100 mM, but was slightly smaller (~10%) compared with Na⁺. In addition, z measured in Li⁺ was smaller than in Na⁺, and was reasonably constant over the range of Li⁺ concentrations used. For Li⁺ ≈ 25 mM (not shown) the data could no longer be reliably fit with Equation 2. The F-V distribution was shifted to more positive voltages in Li⁺ than in Na⁺, as illustrated by the V₀.₅ data (Fig. 6E), but unlike for Na⁺, the Li⁺ data did not show a linear variation with log [Li⁺]. In general, the poor signal-to-noise ratio of the data made interpretation for this mutant difficult, and the results should therefore be treated with some caution.

Pᵢ Dependence—The Pᵢ dependence (measured in ND100) of the F-V distribution of E451C is shown in Fig. 6D. F saturated at hyper- and hypopolarizing limits at all Pᵢ concentrations used, and the data were fit with Equation 2 (solid lines in Fig. 6D). The effect of Pᵢ on the F-V distribution closely mirrors that of S155C (Fig. 3G), whereby the effects of voltage on F were opposite. As Pᵢ concentration increased, Fₘₐₓ reported by the fit decreased. By plotting the Fₘₐₓ data as a function of Pᵢ (Fig. 6G) and fitting with the Michaelis-Menten equation we obtained a Kᵢ(Pᵢ) of 81 ± 34 μM, which was very similar to the value obtained for S155C (99 ± 17 μM, Fig. 3G). Another similarity to S155C was the finding that both V₀.₅ and z were only slightly affected by Pᵢ (not shown).
Conformational Changes in NaPi-IIb

Effect of Iodide on F

We used iodide, a collisional quencher, to gain insight into whether the changes in fluorescence observed in the four mutants involve a change in the accessibility of the tethered fluorophore to the external medium. If the fluorescence decrease is caused by movement of the fluorophore into a more polar environment, then the decrease should be enhanced in iodide solution (5, 22). Fig. 7 shows F recorded in oocytes expressing S155C, S226C, Q319C, or E451C in ND100 solution and in 50 mM iodide solution. With the exception of S155C, three mutants responded to iodide by a decrease in Fmax (Table 2). The largest decrease occurred in E451C, where Fmax was reduced by ~60% and V0.5 was right-shifted by 80 mV. Replacing 50 mM NaCl with 50 mM sodium glucuronate had no effect on any of the Boltzmann fit parameters for any of the mutants (not shown), indicating that the effects seen are specific to iodide. Thus, the result that iodide decreased, rather than enhanced, the voltage-dependent fluorescence changes for three of the four mutants would argue against the hypothesis that the fluorescence decrease is caused by movement of the fluorophore into a more polar extracellular medium.

The data do not allow us to determine whether the iodide effect is stronger at depolarizing or hyperpolarizing potentials (note that the F-V curves in Fig. 7 are shifted to Fmax for display purposes only). However, for E451C the right-shift in V0.5 indicates that iodide suppresses F mainly at hyperpolarizing potentials. Because iodide is negatively charged, this effect is not because of the membrane potential alone. It is possible that hyperpolarizing potentials increase the accessibility of the fluorophore tethered to E451C to the external medium, thus blunting the fluorescence increase.

DISCUSSION

In a previous study we established the methodology of VCF to investigate conformational changes in the type IIb Na/Pi cotransporter (4). We showed that after labeling the protein at a site in the large re-entrant loop in the second half of the protein (S448C; see Fig. 1A) with a fluorophore, we could measure substrate- and voltage-dependent changes in fluorescence. These changes are evidence of conformational changes that occur in the environment sensed by the fluorophore. In this present study we have used VCF to map regions in NaPi-IIb where conformational changes occur during the transport cycle. The new findings serve to deepen our understanding of the partial kinetic steps occurring during transport. We identified four sites which, when mutated to cysteines, could be labeled with a fluorophore and which showed voltage- and substrate-dependent changes in fluorescence. Unlike the previously characterized S448C mutant, whose cotransport activity was blocked after labeling (4), all four mutants identified in the present study remained fully functional after labeling with relatively small changes in their basic kinetics. Thus, the changes in fluorescence associated with each mutant measured under the same experimental conditions could reflect conformational changes that occur in four well-defined locales in the wild-type protein during its transport cycle.

S226C and Q319C—Unlike all other extracellular loop regions in the NaPi-II protein, the large extracellular glycosylated loop (ECL-2) between putative TMDs 3 and 4 (Fig. 8B) has not previously been subjected to cysteine scanning accessibility studies. Thus, we had no preconceptions about the role (if any) of this large loop in the transport cycle of NaPi-Iib. The mutants S226C and Q319C are located at opposite ends of the loop. Both mutants showed voltage-dependent F in the absence of Na+, suggesting that both of them report on conformational changes that occur in response to changes in voltage in the absence of substrate (empty carrier, transition 1–8 in Fig. 8A). Because in our model the empty carrier is assumed to possess one intrinsic negative charge (see Refs. 4, 14, 16), depolarizing potentials are predicted to increase the probability of a conformation in which the substrate binding sites face inward (state 8). Thus interpreting the F-V data for S226C and Q319C in Figs.

![Figure 7](image)

**FIGURE 7.** Effect of iodide on fluorescence (F). F-V data were acquired in ND100 solution and in solution where 50 mM NaCl was replaced with NaI. Data were fitted with Equation 2 and shifted to Fmax. The fit parameters are given in Table 2. Means ± S.E., n = 5.

**TABLE 2**

| Mutant | Control | Iodide | V0.5 | Fmax |
|--------|---------|--------|------|------|
| S155C  | 1.00 ± 0.07 | 0.45 ± 0.06 | 58 ± 5 |
| S226C  | 1.00 ± 0.04 | 0.47 ± 0.03 | 25 ± 4 |
| Q319C  | 0.99 ± 0.05 | 0.57 ± 0.05 | 25 ± 4 |
| E451C  | 0.99 ± 0.04 | 0.46 ± 0.04 | 13 ± 5 |

*Statistically significant difference to the control condition using repeated-measures ANOVA with Tukey’s post-test (p < 0.05). Means ± S.E., n = 5.*
4 and 5 suggests that state 8 (empty carrier with inward-facing binding sites) is associated with high fluorescence for S226C and low fluorescence for Q319C.

Because presteady state charge movements are associated with the empty carrier transition for all electrogenic NaPi-II isoforms (14, 16, 17), we can compare the data obtained for the charge-voltage (Q-V) and fluorescence-voltage (F-V) distributions. A comparison of the Boltzmann fit parameters obtained for both methods from experiments done in the absence of Na (ND0), showed that the midpoint (V_{0.5}) for the F-V data were shifted toward more positive potentials, compared with the Q-V data: from the F-V data we obtained 33 ± 33 mV for S226C and 15 ± 5 mV for Q319C, whereas from the Q-V distribution, we obtained −45 ± 3 mV and −54 ± 4 mV, respectively. If both parameters reported on the same hypothetical empty carrier transition (1–8), we would expect the Boltzmann fit parameters to be the same. This discrepancy is not a consequence of labeling5) the transporter with MTS-TMR, since as shown in Fig. 2D, the change in V_{0.5} for the empty carrier after labeling is marginal. Instead, the discrepancy most likely arises because the Q-V distribution reports on the global distribution of charge in the membrane electrical field, whereas F reports on a local event.

When we compare the effect of substrate on S226C and Q319C, we see that the two mutants behave in a similar manner. Addition of Na to the ratio of the Q-V distribution (V_{0.5}) and its steepness (z). In both mutants V_{0.5} was shifted to more positive potentials with increasing Na + with a slope of 55–60 mV/log[Na]. This is significantly shallower than the ~110 mV/log[Na] measured from the Q-V distribution. When we substituted Na + with Li + we observed that Li + shifted V_{0.5} to even more positive potentials than Na +. Because Li + also shifts V_{0.5} of the Q-V distribution measured on the WT protein, these results provide strong evidence that Li + is able to interact with the protein. However, unlike Na +, Li + did not increase the maximum amount of charge available for translocation (Q_{max}) above that observed in ND0. This supports the conclusion reached in our preceding study (4) that Li + is able to interact with the transporter, but that the binding of Li + is voltage-independent.

S155C and E451C—S155C lies in a predicted intracellular re-entrant loop (ICL-1) between TMDs 2–3 (see Fig. 8B). We postulated that this residue could be accessed from the extracellular medium, because in the rat NaPi-II isoform, the residue at the equivalent position (Ser-183), when mutated to Cys could be modified by externally applied MTS reagents.5 When labeled with MTS-TMR, S155C showed a robust ΔF signal (thus confirming that it is accessible to the external medium), whereas both its neighbors (Q154C and A156C) were silent. It is likely that these latter mutants are inaccessible for labeling, because the equivalent of A156C in rat NaPi-IIa (A184C) was also inaccessible.6

E451C lies in the predicted extracellular re-entrant loop (ECL-3) between TMDs 5–6 (Fig. 8B). Interestingly, there is considerable intrasequence homology between ECL-3 and ICL-1, where S155C lies. Previous cysteine scanning work (19, 23, 24, 25) has shown that both regions are very important for transport function. Significantly, E451C lies very close to S448C, which also gives a strong F signal after labeling, but whose transport function is blocked by labeling (4). Unfortunately, a lack of saturation precluded a Boltzmann analysis of the fluorescence signal from S448C, but the data were consistent with P_{i} causing a profound reduction in F_{max} when applied in Na + solution. S155C and E451C both respond to P_{i} by decreasing F_{max} with a K_{i} of ~90–100 μM, which is similar to the K_{i} value of ~100 μM previously reported for S448C (4). Marked P_{i}-induced reduction of F_{max} is thus a common feature of S155C, E451C and S448C, and sets them apart from S226C and Q319C. While the latter appear to be limited to sensing only transitions 8-1-2a(b) (see Fig. 8A), S155C and E451C sense at least 8-1-2a-2b-3, or possibly the whole transport cycle. Taken together, the results point to a similar and significant involvement of these two re-entrant loops in the transport process of NaPi-Ib.

The midpoint voltages (V_{0.5}) of the F-V distribution of S155C and E451C were both shifted to more negative potentials, compared with V_{0.5} measured from the Q-V distribution, at all Na + concentrations used (in ND100 the shift was ~45 mV for S155C and ~10 mV for E451C, unfortunately in ND0 the F-V data could not be reliably fit with a Boltzmann function). This finding contrasts with the behavior of S226C and Q319C, the F-V distributions of which were shifted to more positive potentials than that of the Q-V distribution. At this stage we can only speculate that these differences arise because of the localized nature of the F signals measured for each site.

Conformational Topology Map—Using the information obtained in this study on the response of mobile elements in the NaPi-II protein we can begin to construct a topology map that shows conformational changes and their relationship to substrate binding and translocation. Fig. 8A shows a kinetic scheme for the transport cycle of electrogenic Na/P _i -II transporters, which is based on results from earlier electrophysiology and fluorescence studies (4, 14, 16, 17). On the extracellular side, binding of two Na + ions (transitions 1-2a-2b) precede the binding of one HPO_{4}^{2−} (2b-3), after which one more Na + ion can bind (3–4). The fully loaded carrier then translocates the bound substrates to the intracellular side in an electroneutral step (4–5) and releases the bound substrates (5–6–7–8). The empty carrier then returns to an outward-facing configuration

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4 We do not know if all transporters in the membrane are labeled with the reagent, or if significant amounts of unlabeled protein remains. This is of no consequence for the fluorescence measurement, because F is only measured from labeled transporters and thus represents a homogenous population. However, when measuring presteady state currents, both labeled and unlabeled cotransporters contribute to the measurements, and since for both S155C and S226C labeling tended to shift the V_{0.5} values of the Q-V distribution towards those of the F-V distribution, the question arises whether the remaining difference results from contamination of the Q-V distribution with unlabeled protein. However, because we observed qualitatively similar results using MTSEA, which has a higher reactivity than MTS-TMR and therefore could be expected to react with a larger number of transporters, we assume that the contribution of unlabeled transporters to the Q-V distribution is negligible. Furthermore, the change in the Boltzmann parameters determined from presteady state currents before and after labeling with MTSEA was much smaller (Fig. 2) than the differences we documented between the Q-V and F-V data.

5 Radanovic, T., Gisler, S. M., Biber, J., and Murer, H. (2006) J. Membr. Biol., in press.

6 T. Radanovic, unpublished observation.
Conformational Changes in NaPi-IIb

FIGURE 8. Modeling the conformational changes occurring during the transport cycle of NaPi-II. A, kinetic scheme for the transport cycle of NaPi-II. Transitions that are influenced by voltage are indicated by blue arrows and include transition of the empty carrier (1 ↔ 8) and the second Na\(^{+}\) binding (2a ↔ 2b). No information is available on the internal substrate interaction transitions, but they are depicted to mirror those taking place on the external side and are indicated with gray arrows. B, topology model showing the transmembrane domains and associated loops. The position of the four mutants from which significant ΔF could be measured after labeling with MTS-TMR are indicated. Different colors were used to distinguish the two halves of the protein. C, cartoon schematic of conformational changes that could cause the substrate-and voltage-dependent changes in fluorescence recorded for the four mutants. The two halves of the protein are depicted with the same colors as in B, and the states are numbered according to the model in A. State 1 (outward-facing) predominates at extreme hyperpolarizing potentials in the absence of substrate and is associated with low fluorescence for S155C and S226C and high fluorescence for Q319C and E451C. Binding of two Na\(^{+}\) ions exposes the binding sites for HPO\(_4\)\(^{2-}\) and the third Na\(^{+}\) ion (state 2b). After binding of HPO\(_4\)\(^{2-}\) and the third Na\(^{+}\), the transporter adopts a conformation where the fluorescence of S155C and E451C is not suppressed by extreme potentials, i.e. hyperpolarizing potentials for S155C (state 3) or depolarizing potentials for E451C (state 5). After releasing the substrates on the intracellular side, the transporter reverts to a conformation (state 8) where maximal changes in fluorescence are induced by extreme potentials for all mutants. State 8 represents the inward-facing conformation of the empty carrier that predominates at extreme depolarizing potentials.

The assignment of the binding sites for each substrate to a specific half of the protein is arbitrary. To the protein in the absence of P\(_i\), the Li\(^{+}\) interaction showed no cooperativity and we postulate that Li\(^{+}\) interacts with only one Na\(^{+}\) binding site. Because Li\(^{+}\) did not contribute any charge movement in addition to that measured for the empty carrier, we hypothesize that the voltage dependence of the first Na\(^{+}\)/Li\(^{+}\) binding step is minimal (for a detailed discussion of this model, see Ref. 4).

In Fig. 8B we have redrawn the topology model of NaPi-II to show the locations of the four residues from which we could record voltage- and substrate-dependent fluorescence when labeled with MTS-TMR. The protein is divided into two halves (separated by the large extracellular loop) that are known to show considerable intrasequence homology (1, 24). We have assigned the two halves different colors that correspond to those in cartoon representations shown in Fig. 8C, which depicts a scenario of conformational states adopted by the protein during its cycling through the transport cycle of Fig. 8A. The cartoons attempt to explain the differential effects that voltage and substrate have on the fluorescence changes measured at the four sites. Note that we do not know the physical mechanism causing the fluorescence change. The iodide experiments suggest that the fluorescence decrease is not caused by movement of the fluorophore into the more polar extracellular environment, leaving other mechanisms such as quench by a nearby side chain residue, as a more likely option.

State 1 represents the outward-facing conformation of the empty carrier. Because the empty carrier is assumed to carry one negative charge, this conformation predominates at very negative potentials. In this state, S155C and S226C both show minimal fluorescence, whereas fluorescence is maximal for Q319C and E451C. At very positive potentials the inward-facing configuration predominates (state 8), and the fluorescence intensities for the four sites are reversed.

The transition 1-2b represents binding of the first two Na\(^{+}\) ions. This step is associated with a shift in the midpoint voltage (V_0.5) to more positive potentials and an increase in the slope (z) of the F-V relationships for all four sites (see Figs. 3–6). The
shift in $V_{0.5}$ indicates that at a given voltage, an increase in external Na$^+$ increases the probability of the protein adopting an outward-facing configuration, whereas the increase in $z$ indicates that an increase occurs in the apparent valency of the charges that move in the electrical field, presumably contributed by the Na$^+$ ions. For S226C and Q319C, $z$ was less dependent on Na$^+$ compared with S155C and E451C, which suggested that the voltage-dependent fluorescence changes reported by the former pair do not strongly reflect Na$^+$-induced conformational changes. Note that although the $F$-$V$ data were fitted with a single Boltzmann, which implies a model where only two transitional changes. Note that although the $F$-$V$ distribution recorded at the two sites. This indicates that conformational changes relating to P$_i$ binding and substrate translocation do not significantly perturb the environment of these two sites. Whether or not the transporter undergoes the full transport cycle has only minor effects on the fluorescence recorded at these sites. For simplicity, the cartoons show that all outward-facing conformations decrease the fluorescence of S226C, and all inward-facing conformations decrease fluorescence of Q319C.

In contrast, for S155C and E451C, application of P$_i$ (in the presence of Na$^+$) caused a concentration-dependent reduction in $F_{\text{max}}$. This implies that in the presence of both substrates, the protein adopts conformations (states 4–5) where the fluorescence can no longer be effectively decreased by extreme hyper- or depolarizing potentials, respectively. Because the protein now is able to undergo a full transport cycle, we do not know which conformational states contribute the most to these fluorescence measurements, because the measured fluorescence arises from all possible states weighted by their respective occupancy probabilities and fluorescence intensities. We have drawn the cartoons to show that after binding of P$_i$ and the third Na$^+$ ion, the conformation of the protein is altered so that the fluorescence of S155C and E451C does not decrease at hyper- and depolarizing potentials, respectively. The data do not, however, allow us to identify the contribution to the fluorescence of individual states when both substrates are present.

CONCLUSIONS

We have identified four sites in the flounder Na/P$_i$-IIB protein which, when labeled with a fluorescent probe, give rise to voltage- and substrate-dependent fluorescence signals. Mutating the residues to cysteines and subsequent labeling with a Cys-reactive fluorescent probe caused only minor changes in the transport kinetics of the protein, indicating that the fluorescence reported on conformational changes of a minimally perturbed protein. The results show that at least a part of the large glycosylated loop between TMDs 3 and 4 is associated with conformational changes, and that these are mainly related to the transition of the empty carrier and the first Na$^+$ binding step(s). In contrast, the two re-entrant loops located between TMDs 2–3 and 5–6 participate in conformational changes that additionally are influenced by P$_i$ binding and/or substrate translocation. The results represent a first step in mapping time-resolved conformational changes of type II Na/P$_i$ cotransporters.

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REFERENCES

1. Forster, I. C., Kohler, K., Biber, J., and Murer, H. (2002) Prog. Biophys. Mol. Biol. 80, 69–108
2. Murer, H., Hernando, N., Forster, I., and Biber, J. (2000) Physiol. Rev. 80, 1373–1409
3. Bacconi, A., Virkki, L. V., Biber, J., Murer, H., and Forster, I. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 12606–12611
4. Virkki, L. V., Murer, H., and Forster, I. C. (2006) J. Gen. Physiol. 127, 539–555
5. Mannuzzu, L. M., Moronne, M. M., and Isacoff, E. Y. (1996) Science 271, 213–216
6. Cha, A., and Bezanilla, F. (1997) Neuron 19, 1127–1140
7. Meinild, A. K., Hirayama, B. A., Wright, E. M., and Loo, D. D. (2002) Biochemistry 41, 1250–1258
8. Loo, D. D., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E., and Wright, E. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7789–7794
9. Larsson, H. P., Tzingounis, A. V., Koch, H. P., and Kavanaugh, M. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3951–3956
10. Li, M., Farley, R. A., and Lester, H. A. (2000) J. Gen. Physiol. 115, 491–508
11. Li, M., and Lester, H. A. (2002) Biochem. J. 383, 135–139
12. Geibel, S., Kaplan, J. H., Bamberg, E., and Friedrich, T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 964–969
13. Geibel, S., Zimmermann, D., Zifarelli, G., Becker, A., Koenderink, J. B., Hu, Y. K., Kaplan, J. H., Friedrich, T., and Bamberg, E. (2003) Ann. N. Y. Acad. Sci. 986, 31–38
14. Forster, I. C., Wagner, C. A., Busch, A. E., Lang, F., Biber, J., Hernando, N., Murer, H., and Werner, A. (1997) J. Membr. Biol. 160, 9–25
15. Forster, I. C., Biber, J., and Murer, H. (2000) Biochem. J. 79, 215–230
16. Forster, I., Hernando, N., Biber, J., and Murer, H. (1998) J. Gen. Physiol. 112, 1–18
17. Virkki, L. V., Forster, I. C., Biber, J., and Murer, H. (2005) Am. J. Physiol. 288, F699–F697
18. Ehnis, C., Forster, I. C., Bacconi, A., Kohler, K., Biber, J., and Murer, H. (2004) J. Gen. Physiol. 124, 489–503
19. Lambert, G., Forster, I. C., Stange, G., Biber, J., and Murer, H. (1999) J. Gen. Physiol. 114, 637–652
20. Mager, S., Cao, Y., and Lester, H. A. (1998) Methods Enzymol. 296, 551–566
21. Mager, S., Kleinberger-Doron, N., Keshet, G. I., Davidson, N., Kann, B. L., and Lester, H. A. (1996) J. Neurosci. 16, 5405–5414
22. Cha, A., and Bezanilla, F. (1998) J. Gen. Physiol. 112, 391–408
23. Lambert, G., Forster, I. C., Stange, G., Kohler, K., Biber, J., and Murer, H. (2001) J. Gen. Physiol. 117, 533–546
24. Kohler, K., Forster, I. C., Stange, G., Biber, J., and Murer, H. (2002) Am. J. Physiol. 282, F687–F696
25. Kohler, K., Forster, I. C., Stange, G., Biber, J., and Murer, H. (2002) J. Gen. Physiol. 120, 693–703