Identification of functionally important sites in the first intracellular loop of the NaPi-IIa cotransporter

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The reabsorption of Pi in the renal proximal tubule is achieved by a secondary, active, electroneutral Na\(^+\)-coupled cotransport system that is mediated by the type IIa Na\(^+\)/Pi cotransport protein (NaPi-IIa) located at the brush-border membrane (21). As expressed in *Xenopus laevis* oocytes, the kinetic properties of this protein have been described in detail by study of the \(^{32}\)P\(_i\) uptake or measurement of the P\(_i\)-induced inward current under voltage clamp. This current can be explained in terms of a 3:1 Na\(^+\)/HPO\(_4\)\(^2\)\(^-\) stoichiometry (1, 4–6). NaPi-IIa also exhibits a Na\(^+\) leak (slippage) in the absence of external P\(_i\) (stoichiometry = 1 Na\(^+\)) that is inhibited by phosphonoformic acid (PFA; Ref. 4).

The rat NaPi-IIa isoform is a 637-amino acid glycoprotein with a glycosylated molecular mass of 90–100 kDa (8, 20, 21). The topological model predicted from hydrophobicity data (20) and confirmed by epitope studies (18) exhibits a secondary structure with eight transmembrane domains, a large extracellular loop with two N-glycosylation sites (8), and intracellular NH\(_2\) and COOH termini (Fig. 1B). Intrasequence comparison of the protein revealed that part of the first intracellular loop of the NH\(_2\)-terminal half (ICL-1) shows a high degree of similarity to part of the third extracellular (ECL-3) in the COOH-terminal half (15). To identify regions of functional importance (12), we used the substituted-cysteine accessibility method (SCAM). We could show that cysteine substitutions in the putative ECL-3 loop yield functional constructs that are readily inhibited by external application of methanethiosulfonate (MTS) reagents. This led to the proposal that part of the ECL-3 region contributes to both the cotransport mode and slippage mode pathways of NaPi-IIa (16, 17). Based on the intrasequence similarity between the ICL-1 and ECL-3 regions, we hypothesized that functionally important residues should also exist in the NH\(_2\)-terminal region.

We therefore mutated eight amino acids, one by one, within the identified stretch in the ICL-1 site to a cysteine. These corresponded to sites already identified in the ECL-3 site to yield transporters that were of functional importance based on kinetic properties before and after MTS treatment (17). After the mutations were expressed in *Xenopus laevis* oocytes, the cotransport function of the mutants was assayed electrophysiologically before and after exposure to MTS reagents (as a means of identifying functionally important sites). Two mutants (N199C and V202C) showed a complete loss of cotransport function after incubation in MTS-ethylammonium (MTSEA), which suggested that these sites were accessible and functionally important; the others were only partially or insignificantly inhibited after incubation. Incubation with impermeant MTS-ethyltrimethylammonium (MTSET) did not invoke a significant inhibition of the P\(_i\)-induced current (\(I_{P_i}\)) for any of the eight mutants. Furthermore, kinetic characterization of the mutants revealed two
with significantly altered steady-state kinetics compared with the wild-type (WT). Mutant A203C showed an increased transport rate for the Na$^+$ leak, and mutant N199C exhibited decreased apparent substrate affinities. Further amino acid substitutions at site 199 resulted in mutants that displayed either WT-like co-transport function (with reduced substrate affinity) or slippage mode only. This confirmed that this site is critical for establishing the transport mode of NaPi-IIa.

Taken together with our recent report (14) that documents NaPi-IIa to be a functional monomer, these new findings extend our previous observations (16, 17) and are consistent with NaPi-IIa having a single trans-port pathway that involves associated parts of the ECL-3 and ICL-1 loops.

**EXPERIMENTAL PROCEDURES**

**Reagents and chemicals.** All restriction enzymes were obtained from Pharmacia Biotech or Biofexx. Oligonucleotide primers were obtained from Microsynth (Balgach, Switzerland), and a mutagenesis kit was purchased from Stratagene. MTS reagents were obtained from Toronto Research Chemicals (Toronto, Canada). Other reagents were obtained from Fluka (Buchs, Switzerland).

**Molecular biology.** Mutations were introduced in accordance with the instructions in the manual accompanying the QuikChange site-directed mutagenesis kit (Stratagene) as previously described (16). The sequence was verified by sequencing. All constructs were cloned in pSport1 (GIBCO-BRL). The in vitro synthesis and capping of cRNAs were performed according to instructions in the Ambion MEGAscript TM T7 kit manual (16).

**Solutions.** The solutions for the electrophysiological assays were composed as follows. Control superfusate ND100 contained (in mM) 100 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES adjusted to pH 7.4 with Tris. Control superfusate ND0 composition was as for ND100 but with N-methyl-$	ext{D}$-glucamine used to replace Na$^+$, and solutions were adjusted to pH 7.4 with HCl. Solutions with intermediate Na$^+$ concentrations were prepared by mixing ND0 and ND100 in the appropriate proportions. For the substrate test solutions, Pi was added to ND100 from a 1 M K$_2$HPO$_4$/K$_2$PO$_4$ stock that was preadjusted to pH 7.4, and the Na$^+$-Pi, cotransport inhibitor PFA was added to ND100 from frozen stock (in H$_2$O) to yield a final concentration of 3 mM. MTSEA and MTSET were prepared in DMSO, frozen in aliquots at 1 M and 10 M, and freshly diluted in ND100 from the stock for each oocyte immediately before use. The final concentration of DMSO did not exceed 0.2%. At this concentration, the kinetic characteristics of the expressed constructs were unaltered.

**Xenopus laevis oocyte expression.** The procedures for oocyte preparation and cRNA injection have been described in detail elsewhere (29). Oocytes were injected with either 50 nl of water or 50 nl of water containing 10 ng of cRNA. Oocytes were incubated in modified Barth’s solution and the experiments were performed 3–4 days after injection. All pooled data were generated with oocytes from at least two different donor frogs.

**Functional assays and data analysis.** The procedure used for the $^{32}$P-uptake assay has been described in detail elsewhere (29). $^{32}$P uptake was measured 3 days after injection of both water- and cRNA-injected oocytes ($n = 8$).

The standard two-electrode voltage-clamp technique was used as previously described (4). The steady-state response of an oocyte to P$_i$ and PFA was always measured at a holding potential ($V_H$) of $-50$ mV in ND100 except where otherwise noted. Data were acquired on-line using Digidata 1200 hardware and compatible pClamp8 software (Axon Instruments, Foster City, CA). Recorded currents were prefiltered with a bandwidth less than twice the sampling rate.

The P$_i$ and Na$^+$ steady-state activation protocols were performed as previously described (4), and apparent affinity constants were obtained by fitting the modified Hill equation to the dose-response data (4). For the incubation with MTS reagents, MTSEA or MTSET was applied to the chamber with gravity feed via a 0.5-mm-diameter cannula positioned near the cell. Reagents were applied in the presence of ND100. Incubation time was 3 min and was followed by a 1-min washout period. The apparent second-order reaction
rate constant \( (k) \) for the Cys modification was estimated by fitting a single exponential to the MTS concentration dependency expressed as

\[
P_{\text{co}}/P_0 = \exp(-ckt)
\]

(1)

where \( P_{\text{co}} \) is the cotransport mode current after MTS incubation at concentration \( c \) for \( t \) seconds, and \( P_0 \) is the control response at \( t = 0 \). Pre-steady-state relaxations were recorded using voltage steps from \( V_b = -60 \text{ mV} \) to voltages in the range of \(-160 \) to \(+80 \text{ mV}\). Endogenous capacitive currents were eliminated by subtracting the response to 3 mM PFA as previously described (3). The charge was quantitated by numerical integration of the relaxations, and the steady-state charge distribution was fit with the Boltzmann relation using the nonlinear regression algorithm

\[
Q = Q_{\text{hyp}} + Q_{\text{max}} / [1 + \exp[-ze(V - V_{0.5})/kT]]
\]

(2)

where \( Q_{\text{max}} \) is the maximum charge translocated, \( Q_{\text{hyp}} \) is the steady-state charge at the hyperpolarizing limit and depends on \( V_{0.5} \), \( V_{\text{hyp}} \) is the voltage at which the charge is distributed equally between the two states, \( z \) is the apparent valency per cotransporter, \( e \) is the electronic charge, \( k \) is Boltzmann’s constant, and \( T \) is the absolute temperature.

**RESULTS**

Identification of similar regions and expression of mutants in oocytes. Figure 1A shows the result of an intrasequence comparison of the NaPi-IIa cotransporter protein. This revealed two stretches of amino acids from 414 to 464 in the ECL-3 site of the COOH-terminal half and from 160 to 210 in the ICL-1 of the NH2-terminal half that have a high degree of similarity (80%). The location of these stretches is indicated on the current topological model for NaPi-IIa (Fig. 1B). As indicated in Fig. 1C, the aligned amino acid sequences include two regions of five and six residues, respectively, that show exact identity. We applied SCAM to eight selected sites in the ICL-1 region (Fig. 1C) that were chosen to correspond to those sites identified in the ECL-3 site as being functionally important within the corresponding stretch in the ECL-3 site (17).

Cys mutants were expressed in *Xenopus* oocytes and characterized in terms of expression (via Western blot analysis) and transport function by measuring the \( I_P \) under voltage clamp. As shown in Fig. 2A, all eight mutants were expressed in comparable amounts and with the same expression pattern as the WT NaPi-IIa protein. All constructs were expressed at the membrane as evidenced by \( I_P > 10\text{-fold} \) higher than the endogenous response (data not shown). Electrogenic responses varied between 40 and 80% of the WT activity found in oocytes from the same donor frog (data not shown). Mutant N199C was notable for displaying \( I_P \)s that were typically only 20% of the WT response. Such variation in activity could be due to the induction of altered kinetics by Cys mutagenesis (see Kinetic characterization of Cys mutants) and/or reduced surface expression due to improper membrane targeting. Because our primary interest in this study was construct function, the latter effect was not investigated in detail here.

Effect of MTS reagents on transport function of mutants. The NaPi-IIa cotransporter exhibits two transport modes that can be assayed by electrophysiology: a \( \text{Na}^+ \)-dependent leakage or slippage mode in the absence of external \( \text{P} \) (stoichiometry = 1 \( \text{Na}^+ \)) that is inhibited by the competitive inhibitor PFA and a cotransport mode (assumed \( \text{Na}^+/\text{HPO}_4^2- \) stoichiometry = 3:1; Ref. 4). We implicitly assumed that 3 mM PFA was sufficient to fully block the \( \text{Na}^+ \) leakage so that the current level attained during PFA application (i.e., corresponding to the endogenous oocyte leakage) would indicate the NaPi-IIa zero-transport condition at a given \( V_b \). The leak current is then given by \(-I_{\text{PFA}}\), where \( I_{\text{PFA}} \) is the PFA-transport condition. To quantify the cotransport mode activity, we assumed that the \( \text{Na}^+ \) leak is uncoupled from the cotransport mode. As the \( I_P \) was measured relative to the holding current, this must be adjusted by \(-I_{\text{PFA}}\) to obtain the true cotransport current relative to the zero-transport level [i.e., the cotransport activity is then given by \( I_P + I_{\text{PFA}} \) as previously described (17)].

To determine whether the mutated sites were functionally important, we tested the mutant function before and after exposure to cysteine-modifying reagents MTS and MTS. We assumed that a change in either the \( P_{\text{f}} \) or PFA electrogenic responses was evidence for the association of the modified site with the respective transport mode. Figure 2B shows representative recordings from oocytes that expressed the WT, N199C, and A203C constructs before and after incubation for 3 min in 10 mM MTS. None of the mutants showed a significant change in \( I_{\text{PFA}} \) after MTS exposure, which indicates that the slippage mode was not affected. As previously reported, the WT showed only marginal loss of cotransport function (17). In contrast, mutant N199C showed no inward current in response to \( \text{P} \) after MTS treatment, whereas A203C showed only a partial reduction in \( I_P \) even after repeated application of MTS at 10 mM for the same incubation time (data not shown). As summarized in Fig. 2C (open bars), under these application conditions, compared with the WT, the cotransport mode of two mutants (N199C and V202C) was fully inhibited, whereas the others showed partial or little inhibition after MTS treatment.

Because the ICL-1 site was predicted to be intracellular, substituted cysteine residues there were not expected to be very accessible with extracellularly applied MTS and MTSET. We assumed that a change in either the \( P_{\text{f}} \) or PFA electrogenic responses was evidence for the association of the modified site with the respective transport mode. Figure 2B shows representative recordings from oocytes that expressed the WT, N199C, and A203C constructs before and after incubation for 3 min in 10 mM MTS. None of the mutants showed a significant change in \( I_{\text{PFA}} \) after MTS exposure, which indicates that the slippage mode was not affected. As previously reported, the WT showed only marginal loss of cotransport function (17). In contrast, mutant N199C showed no inward current in response to \( \text{P} \) after MTS treatment, whereas A203C showed only a partial reduction in \( I_P \) even after repeated application of MTS at 10 mM for the same incubation time (data not shown). As summarized in Fig. 2C (open bars), under these application conditions, compared with the WT, the cotransport mode of two mutants (N199C and V202C) was fully inhibited, whereas the others showed partial or little inhibition after MTS treatment.

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conditions. This suggests that these novel cysteines were inaccessible from the extracellular medium. Moreover, the loss of cotransport function documented for MTSEA most likely resulted from membrane permeation and subsequent Cys modification by MTSEA from the cytosol (9, 12). With the exception of A203C, L204C, and M205C ($P < 0.01$, unpaired $t$-test), the other mutants showed no statistically significant deviation from WT behavior.

Mutants N199C and V202C showed a progressive loss of cotransport function on exposure to increasing MTSEA concentrations over a fixed time (3 min) that eventually reached zero (Fig. 2D). This decay was described analytically by fitting a single decaying exponential (Eq. 1) to these data. This behavior would be expected if the reaction of MTSEA with cysteine followed second-order kinetics (12, 22). Setting $t = 180$ s, the reaction rates were $2.3 \times 10^{-6} \mu M^{-1} s^{-1}$ for N199C and $2.2 \times 10^{-6} \mu M^{-1} s^{-1}$ for V202C. We also examined the time dependence of loss of function at a fixed concentration (1 mM) of MTS reagent. Similar exponential decays were obtained (data not shown) in support of the model assumed for Cys modification (12). For other mutants that showed only partial loss of cotransport function (Fig. 2C), this quantification was not performed because of uncertainties regarding the specificity of this effect and fitting errors.

**Kinetic characterization of Cys mutants.** As previously reported (16, 17), Cys mutagenesis alone can lead to altered transport kinetics for NaPi-IIa. We therefore investigated the slippage and cotransport mode characteristics of the new mutants. First, the Na+ leak ($-I_{PFA}$) was determined for each mutant and quantified relative to the cotransport mode current as a function of time. Continuous line is a fit with a single exponential function (see text). Each data point represents mean ± SE ($n = 3$); points without error bars have SE smaller than symbol size.

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**Fig. 2.** A: protein expression of mutant constructs in *Xenopus laevis* oocytes. Western blot of whole cell lysate from 3 oocytes pooled for each construct injected with cRNA coding for the indicated mutants as well as the wild-type (WT) is shown. Water-injected oocytes were used as a control. NaPi-IIa was visualized using an antibody raised against the rat NaPi-IIa NH$_2$ terminus. B: representative current recordings from oocytes that expressed the WT and mutants N199C and A203C before (left) and after (right) incubation for 3 min with 10 mM methanethiosulfonate ethylammonium (MTSEA). Each oocyte was tested with 1 mM Pi (open bar) and 3 mM phosphonoformic acid (PFA, solid bar) for the period indicated. Continuous line, current reached during the initial PFA application; dashed line, baseline current in ND100 solution. Note that for each cell the recording baseline levels were not adjusted before and after MTSEA exposure. C: pooled data for mutants and WT showing inhibition of the electrogenic cotransport mode response after incubation with 10 mM MTSEA (open bars) and methanethiosulfonate ethyltrimethylammonium (MTSET; solid bars) expressed as percentage of the initial response. Data are means ± SE; $n = 8$. Significance determined using unpaired $t$-test relative to the WT data: *$0.01 < P < 0.05$, significantly different from WT; **$0.001 < P < 0.01$, very significantly different; and ***$P < 0.001$, extremely significantly different. Unmarked bars were not significantly different from WT. D: loss of cotransport function for mutants N199C and V202C. Oocytes expressing mutants were incubated for a fixed time (3 min) with increasingly higher concentrations of MTSEA (○) or MTSET (●), and the electrogenic cotransport mode response was measured. Continuous line is a fit with a single exponential function (see text). Each data point represents mean ± SE ($n = 3$); points without error bars have SE smaller than symbol size.
slippage index (17): \( I_{PFA}(I_{PFA} + I_P) \). \( I_{PFA} \) and \( I_P \) were
determined using the standard test concentrations (1 mM Pi, and 3 mM PFA) at \( V_h = -50 \) mV. All mutants showed a WT-like slippage index except N199C, which showed little detectable Na\(^+\) leak and A203C, which showed a fivefold larger index (Fig. 3A; see also Fig. 2B). These findings indicated that Cys substitution at sites 199 and 203 had led to differentially altered kinetics for the slippage mode. Second, for the cotransport mode, we assessed the behavior of the mutants using the indices for Pi, and Na\(^+\) activation (17). These indices were obtained from the ratio of \( I_P \) at 0.1 and 1 mM Pi (100 mM Na\(^+\)) for Pi activation and the ratio of \( I_P \) at 50 and 100 mM Na\(^+\) (1 mM Pi) for Na\(^+\) activation at \( V_h = -50 \) mV. All mutants except N199C showed Pi and Na\(^+\) activation indices that were within the expected WT tolerance (Fig. 3B).

Taken together, these basic functional assays revealed that Cys substitution had altered fundamental functional characteristics of two mutants. Because the slippage and Na\(^+\)/Pi; activation indices alone do not reveal details of the possible mechanisms responsible for these changes (e.g., identification of specific transitions in the transport cycle), we next investigated these two mutants in more detail.

**Slippage mode behavior of mutant A203C.** The current scheme for NaPi-IIa slippage mode kinetics involves an empty carrier-voltage-dependent transition, binding and debinding of one Na\(^+\) ion, and an electro-neutral translocation by Na\(^+\) (4, 16). To identify which of these transitions could be influenced by cysteine-203, we characterized the steady-state behavior of A203C in the slippage mode. Na\(^+\)-induced currents were measured at different Na\(^+\) concentrations in the absence of Pi, relative to the response in 0 mM Na\(^+\). As shown in Fig. 4A, A203C showed an increase in Na\(^+\) leak at all concentrations over and above the WT response. Endogenous effects accounted for <10% of these currents as confirmed with noninjected control oocytes (data not shown). These data could be described analytically using a Michaelis-Menten relationship, which was in agreement with our earlier finding (4) and also confirmed that Cys modification had not altered the stoichiometry of the Na\(^+\) interaction with the transporter. The fit indicated that the predicted apparent affinity for Na\(^+\) (\( K_{Na} \)) remained essentially the same within the limits of the fit error (WT, 147 ± 20 mM; A203C, 178 ± 18 mM; \( n = 4 \)). This suggested that the apparently increased Na\(^+\) leak for the A203C mutant was not due to an increase in the apparent Na\(^+\) binding affinity.

A slippage index that exceeds the WT value could also be accounted for by a slower cotransport rate, because this index was determined relative to the cotransport mode rate. To resolve this issue, we estimated the cotransport turnover rate from pre-steady-state kinetics (4, 6). Figure 4B (inset) shows representative pre-steady-state relaxations recorded from an oocyte expressing mutant A203C with the endogenous membrane-charging component removed by subtraction of the PFA response (3). These relaxations were indistinguishable in form and time course from those recorded from WT-expressing oocytes (data not shown). A single Boltzmann function (Eq. 2) was fit to the corresponding \( Q-V \) data (Fig. 4B) to give the apparent valency (\( z \)), midpoint potential (\( V_{0.5} \)), and maximum available charge (\( Q_{max} \)). The estimates of \( z \) (0.81 ± 0.05; \( n = 4 \)) and \( V_{0.5} \) (−56 ± 5 mV; \( n = 4 \)) did not differ significantly from previously reported values for the WT [e.g., \( V_{0.5} = -50 \) mV and \( z = 0.6 \) (16)]. The turnover rate in the cotransport mode at \( V_h = -50 \) mV can be estimated from

\[
\phi = \frac{I_{P_{50}}}{Q_{max}} \tag{3}
\]

where \( I_{P_{50}} \) is the cotransport current at \( V_h = -50 \) mV to yield 26 ± 6 s\(^{-1} \) (\( n = 6 \)). The estimate of \( \phi \) obtained from Eq. 3 was similar to previously reported estimates in the range of 12–23 s\(^{-1} \) for the NaPi-IIa cotransporter (4, 16) and indicated that this parameter had not been significantly altered by the Cys mutation. Taken together, these findings suggest that the Cys mutation at site 203 had increased the transport rate only in the slippage mode.
Cotransport mode behavior of mutant N199C. The deviation from WT behavior of the activation indices for mutant N199C suggested alterations in the respective substrate affinities. We therefore generated dose-response curves for this mutant for Pi activation (at 100 mM Na\(^+\)) and Na\(^+\) activation (at 1 mM Pi) as shown in Fig. 4, C and D, respectively. Unlike the WT, the Pi activation for N199C at 100 mM Na\(^+\) (V\(_h\) = −50 mV) did not saturate even at 4 mM Pi, although the fit using the Hill equation indicated a Michaelian relationship (predicted Hill coefficient, n\(_H\) = 0.8). The estimated apparent affinity constant for Pi (\(K_{m}^{\text{Pi}}\)) was ~0.06 mM for the WT as we have previously reported (4), whereas for N199C, \(K_{m}^{\text{Pi}}\) increased to 3.56 mM (Fig. 4C). The fits to the Pi dose-response data (Fig. 4C) suggested that the predicted maximum transport rate (\(V_{\text{max}}\)) for N199C (\(V_{\text{max}}\)) was comparable to that of the WT (\(V_{\text{WT}}\)). The lack of saturation for the N199C data made estimation of \(V_{\text{max}}\) more prone to error. We therefore repeated these assays and compared the ratio of \(V_{\text{max}}^{\text{N199C}}\) to \(V_{\text{max}}^{\text{WT}}\) in each case for groups of oocytes (n = 4) from different donor frogs (n = 3) to yield \(V_{\text{max}}^{\text{N199C}}/V_{\text{max}}^{\text{WT}}\) = 0.8 ± 0.2. This result suggested that the maximum transport rate was only marginally affected by Cys mutagenesis (see DISCUSSION).

For Na\(^+\) activation (Fig. 4D), mutant N199C also showed a significantly altered response. For up to 125 mM Na\(^+\) (the maximum concentration used), the data remained within a zone of initial positive inflexion that typifies a cooperative binding process. A fit of the Hill equation to these data with \(n_{H}\) constrained to 3 assumed maximum cooperativity for Na\(^+\) binding and a 3:1 stoichiometry (5) predicted an apparent \(K_{m}^{\text{Na}}\) > 2 M for N199C, which is >40-fold greater than the \(K_{m}^{\text{Na}}\) = 64 mM predicted from the fit to the WT data [which is also in agreement with the previously reported estimates, e.g., 52.0 mM (4)].

Mutations at site 199 lead to functional and dysfunctional mutants. To explore further the role of site 199 in conferring substrate affinity, we made other amino acid substitutions at this site by including conservative and nonconservative substitutions with different side-
chain lengths and/or charges. The six mutant constructs (N199A, N199D, N199Q, N199R, N199H, and N199T) were assayed for expression in Western blot analysis of whole oocyte lysate (Fig. 5A). This confirmed that all mutants were expressed with the same molecular weight and similar protein amounts as the WT transporter.

The electrogenic activities of the mutants were examined, and the mutants were divided into two categories based on responses to Pi and PFA (Fig. 5B). Mutants N199A and N199T showed an inward I_{Pi} similar to the WT but smaller in magnitude. Thus N199A and N199T were able to function in the cotransport mode but possibly with reduced substrate affinities like N199C. Furthermore, the responses to PFA were both similar to the WT (an upward deflection, indicative of block of the Na^+ leak), and the slippage indices were within the WT range previously reported (17). This confirmed that the slippage mode was also intact. In contrast, mutants N199D, N199Q, N199R, and N199H did not show a WT-like inwardly directed I_{Pi} but instead showed I_{Pi} superimposed on I_{PFA}. This finding suggested that, for these mutants, the cotransport mode was inhibited, but the slippage mode was still intact. To confirm this conclusion, we performed ^{32}Pi-uptake experiments on the mutants at 1 and 3 mM Pi (Fig. 5C). Mutants N199D, N199Q, N199R, and N199H showed ^{32}Pi uptake that was only marginally greater than for water-injected control oocytes. Mutants N199A and N199T did show a significant ^{32}Pi uptake but only at 3 mM Pi. As expected, the WT showed only a marginal difference in uptake for these Pi concentrations, which was in agreement with electrophysiological results (see Fig. 4C). This behavior suggested that, like N199C, mutants N199A and N199T also had diminished substrate affinities. This was confirmed in the Pi-activation assays (see Fig. 5C, inset) in which both constructs gave dramatically altered Pi-activation curves with no evidence of saturation at 3 mM Pi. The low transport activity and a lack of saturation at the highest applied Pi concentration precluded estimation of the kinetic parameters for these mutants for both Pi and Na^+ activation.

**DISCUSSION**

We have identified two sites, 199 and 202, in the putative ICL-1 of the NaPi-IIa protein that are accessible to the Cys-modifying reagent MTSEA when the native residues are replaced by cysteines. Cys modification with externally applied MTSEA led to full suppression of the cotransport function. In contrast, the membrane-impermeant reagent MTSET, which is of comparable size and is positively charged like MTSEA (11, 12), did not significantly alter cotransport function. In agreement with other evidence, this suggests that MTSEA can permeate the lipid bilayer (9, 12) and that Cys residues substituted at sites 199 and 202 were accessed from the cytosol. It is also possible that MTSEA could access these sites via the lipid phase; however, the intact oocyte preparation does not allow...
us to easily distinguish this case from direct cytosolic access. Additional indirect evidence in support of this sidedness of Cys accessibility is that, unlike our previous cysteine-scanning studies of ECL-3 regions, in which concentrations of MTs reagents in the micromolar range were generally sufficient to alter cotransport (16, 17), it was necessary to use concentrations in the millimolar range to effect the same change of function. Furthermore, the estimated reaction-rate constants were three orders of magnitude slower than we have previously reported for Cys mutants in ECL-3 loops (17). These slower rates may reflect the influences of transmembrane diffusion rate and Cys scavenging in the oocyte cytosol as the rate-limiting steps in the overall MTSEA-Cys reaction kinetics. Exposure to MT SEA for mutants with Cys residues substituted at sites other than site 199 led to marginal loss of cotransport function. This suggests that either the corresponding cysteine residues were even less accessible or, if modification did occur, these sites are not functionally important.

Residue at site 199 is a critical determinant of transport mode. A significant finding in this study was that one site, 199, was a strong determinant of steady-state substrate-activation kinetics and the transport mode. Substitution of the native Asp with Cys, Ala, or Thr resulted in constructs that displayed inward $I_{\text{Pis}}$, which are indicative of intact cotransport function (and were confirmed by $^{32}\text{P}$ uptake) albeit with a significantly reduced apparent substrate affinity. Extending the size of the native Asp to a CH$_2$ group, as for Glu, or substituting charged residues (such as His, Arg, or Glu) led to an electrogenic response to Pi (either inward cotransport current or suppression of slippage) consistent with the lower apparent substrate affinities. For example, a change in the apparent affinity for one substrate can result from a change in the equilibrium binding constant of the cosubstrate. To distinguish between a specific alteration in Pi- or Na$^+$-binding kinetics would require kinetic characterization of the mutants that were precluded by the small electrogeneric responses and lack of saturation of $P_i$ and Na$^+$ activation.

Because the overall transport rate is in general a function of all transition rates, the documented changes in apparent substrate affinities do not necessarily reflect a corresponding change in the actual substrate affinity. For example, a change in the apparent affinity for one substrate can result from a change in the equilibrium binding constant of the cosubstrate. The Na$^+$-binding kinetics would require kinetic characterization of the mutants that were precluded by the small electrogeneric responses and lack of saturation of $P_i$ and Na$^+$ activation. Under the standard test conditions (1 mM Pi and 100 mM Na$^+$), the $I_{P_i}$ for mutant N199C was $<-20\%$ of the WT when oocytes from the same donor frog were compared, but the extrapolated Michaelis fit to the dose-response data predicted a $V_{\text{max}}$ comparable to the WT. $V_{\text{max}}$ is proportional to the number of active cotransporters in the membrane and maximum transporter turnover. However, unlike for A203C, we were unable to reliably resolve pre-steady-state charge movements for N199C to test whether the Cys mutagenesis had altered its maximum turnover rate.

Cys substitutions modify slippage mode properties. Two mutants showed contrasting slippage indices that resulted from the Cys substitution. For mutant A203C, the Na$^+$-leak turnover rate increased, whereas a Cys substitution at site 199 resulted in a mutant with a significantly smaller index compared with the WT. A lower slippage rate for mutant N199C would also be consistent with the lower apparent substrate affinities.

![Image](http://ajprenal.physiology.org/)

**Fig. 6.** Scheme showing how a common Pi transport pathway might be formed based on sequence comparison, accessibility, and functional importance of residues in ICL-1 (this study) and ECL-3 (17). An $\alpha$-helix motif identified in ECL-3 is also indicated. The associated transmembrane domains are numbered to correspond with the scheme in Fig. 1B.
documented; however, we were unable to further characterize the slippage mode for this mutant because of the limited resolution of the Na⁺-leak currents. Nevertheless, these findings point to the functional importance of these sites in determining (directly or indirectly) the Na⁺ leak. Whereas mutant A203C was only marginally modified by MTSEA exposure, which suggests that this site was not readily accessible to the aqueous environment, the cotransport function of N199C was fully inhibited. Interestingly, when a Cys was substituted between sites 199 and 203 (V202C), full inhibition of cotransport after MTSEA exposure occurred, and this mutant displayed normal slippage behavior. This underscores the different roles that neighboring residues can play in conferring specific transport properties.

Structure-function implications. The present challenges of structure-function studies on multisubstrate transport systems include identifying the structural elements that contribute to the transport pathway(s) and distinguishing between multiple [as proposed for the bacterial lac permease (10)] and common pathways (e.g., Refs. 26, 27) for the co- and driven-substrate translocations. What are the implications of our new findings with respect to NaPi-IIa?

Our present findings do not allow us to make a distinction between separate and common slippage/cotransport pathways. It is tempting to speculate that the altered slippage mode behavior through Cys substitution at sites 199 and 203 might indicate that these sites are directly associated with the Na⁺-leak pathway. However, MTSEA exposure for all constructs left the slippage mode unchanged, independently of whether the cotransport function was suppressed. The robustness of the Na⁺ leak for all constructs tested in ICL-1 contrasts with our finding that MTS modification of some sites in ECL-3 led to transporters with a significantly increased Na⁺ leak as well as a fully suppressed cotransport mode (17) after external application of MTS reagents. We therefore conclude that sites in ICL-1 that directly contribute to the Na⁺-leak pathway still remain to be identified.

On the other hand, our present findings together with previous kinetic and structure-function studies do allow us to speculate on the identity of the P₇-translocation pathway. First, given that NaPi-IIa is a functional monomer (14) and that one HPO₄²⁻ molecule is translocated per transport cycle (1, 4–6), these properties are at least consistent with there being one P₇-translocation pathway within the NaPi-IIa protein. Second, in common with the results from our previous SCAM studies in the ECL-3 region (16, 17), the present study identifies two functionally important sites in ICL-1 which, as cysteines 1) show a sidedness with respect to modification by hydrophilic MTS reagents, and 2) confer complete inhibition of cotransport mode on the protein after MTS exposure. Taken together, these findings offer strong evidence that sites widely separated in the primary amino acid sequence are associated with one and the same P₇ translocation pathway. Moreover, that both COOH- and NH₂-termin-

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