Sodium Dependence of the Na\(^+\)-H\(^+\) Exchanger in the Pre-steady State

IMPLICATIONS FOR THE EXCHANGE MECHANISM*

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The pre-steady state time course of amiloride-sensitive Na\(^+\) uptake by the Na\(^+\)-H\(^+\) exchanger in renal brush border membrane vesicles (BBMV) exhibits a burst phase at 0°C which corresponds to the initial turnover of the exchanger (Otsu, K., Kinsella, J. L., Sacktor, B. S., and Froehlich, J. P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4818–4822). Investigation of the Na\(^+\) dependence of the Na\(^+\)-H\(^+\) exchanger between 1 and 10 mM Na\(^+\) revealed that activation of the burst phase involves at least two Na\(^+\) transport sites interacting with positive cooperativity. In this study, characterization of the Na\(^+\) transport sites contributing to the burst phase was extended to include Na\(^+\) concentrations below 1 mM. Between 0.1 and 1 mM Na\(^+\), the amplitude of the burst phase in acid-loaded BBMV (pHi 7.7; pH \(_{e}\) 7.7) exhibited a sigmoidal dependence on [Na\(^+\)]\(_{e}\), consistent with the presence of a second class of high affinity Na\(^+\) transport sites with cooperative binding characteristics. In contrast, steady state Na\(^+\) uptake obeyed Michaelis-Menten kinetics, similar to the behavior observed previously at higher (1–10 mM) Na\(^+\) concentrations. Treatment of the vesicles with carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone, which induced the formation of an inside-negative membrane potential, increased the burst amplitude but had no effect on the steady state uptake velocity. Experiments performed with alkaline-loaded BBMV (pHi 7.7; pH \(_{e}\) 7.7), which permit only a single turnover of the exchanger, gave a simple hyperbolic dependence of the burst amplitude on [Na\(^+\)]\(_{e}\) (0.5–5 mM). We propose that the change in multiplicity of Na\(^+\) transport sites and membrane potential sensitivity that occurs in the transition between the pre-steady state and the steady state of Na\(^+\) uptake in acid-loaded vesicles reflects the presence of an oligomer which operates according to a "flip-flop" mechanism. The minimum subunit composition inferred from the biphasic [Na\(^+\)]\(_{e}\) dependence of the burst amplitude is a dimer at low (<1 mM) Na\(^+\) levels and a tetramer at high [Na\(^+\)]\(_{e}\). Communication between the subunits producing the complex [Na\(^+\)]\(_{e}\), dependence is controlled by the intravesicular (cytoplasmic) H\(^+\) modifier site. Under alkaline conditions (pHi 7.7), where this site is unoccupied, the subunits behave as independent units and cease operation after the first turnover. Occupation of the H\(^+\) modifier site activates a conformational interaction between the subunits that leads to cooperat

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1 The abbreviations used are: BBMV, brush border membrane vesicles; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; MES, 2-(N-morpholino)ethanesulfonic acid.

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1 Unless otherwise indicated, Na\(^+\) refers to Na\(^+\)\(_{e}\) (external Na\(^+\)) and [Na\(^+\)] to [Na\(^+\)]\(_{e}\) (external Na\(^+\) concentration). A similar convention applies to H\(^+\) (\(=\) H\(^+_i\); internal H\(^+\)) and [H\(^+\)] (\(=\) [H\(^+\)]\(_i\); internal H\(^+\) concentration).

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2 The plasma membrane Na\(^+\)-H\(^+\) exchanger mediates a one for one electroneutral exchange of extracellular Na\(^+\) for intracellular H\(^+\) (1–5). The exchanger is involved in a number of cellular physiological activities, including pH homeostasis, transepithelial solute efflux, cell volume regulation, and pH-mediated signaling phenomena associated with differentiation and proliferation (6–8).

Brush border membrane vesicles (BBMV)\(^2\) isolated from renal proximal tubule cells have been extensively used as an experimental model for characterizing the steady state kinetic properties of the Na\(^+\)-H\(^+\) exchanger. Those studies have demonstrated that the exchanger has an external transport site for Na\(^+\) and internal H\(^+\) binding sites which are involved in H\(^+\) transport and allosteric regulation (1, 2, 4, 5, 9, 10). There is also evidence that BBMV have a separate extracellular modifier site for monovalent cations (11). In the presence of chloride, the activity of the Na\(^+\)-H\(^+\) exchanger is competitively blocked by amiloride (12), which locks the exchanger in a conformation preventing Na\(^+\) efflux and further uptake.

At 0°C, time-resolved measurements of amiloride-sensitive Na\(^+\) uptake in acid-loaded BBMV revealed a multiphasic time course consisting of a lag phase, a monoeponential burst phase, and a linear or steady state phase of transport activity (13, 14). Na\(^+\) uptake in each of these phases was enhanced by raising the [Na\(^+\)] and [H\(^+\)] but competitively blocked by lowering the external pH (14). To explain these results, we proposed the following transport model for the Na\(^+\)-H\(^+\) exchanger (Mechanism I) (14), where i and o refer to the intra-and extravesicular compartments, respectively. Sodium translocation (step 5), which is fast compared with the overall rate of accumulation, is responsible for the burst phase and is...
preceded by a conformational transition (step 4) which accounts for the presence of the initial lag. The linear phase of activity, which follows the burst, is presumed to correspond to H+ translocation (step 1), because it is absent in alkaline-loaded vesicles (14) where the internal H+ binding sites are unoccupied. The fact that these vesicles are still able to generate an amiloride-sensitive burst phase (14) implies that the exchanger obeys ping-pong kinetics, because the alkaline pH conditions are unfavorable for H+ counterion transport required by simultaneous binding models.

Although Mechanism I is able to account for the pre-steady state time dependence of the exchanger at a given Na+ level, a more complex scheme is needed to explain the kinetic behavior when the \([Na^+]\) is varied. An analysis of the results obtained at different Na+ levels suggested that at least two Na+ transport sites interacting with positive cooperativity are responsible for activation of the burst phase and that a third high affinity site may modulate this activity at lower Na+ concentrations (13). In contrast to the burst amplitude, the steady state \([Na^+]\) dependence obeys Michaelis-Menten kinetics. To explain this complexity, a "flip-flop" transport scheme was proposed (13) in which the Na+ transport sites reside on adjacent subunits of an oligomer and communicate by means of ligand-induced conformational interations. Disregarding the effects of the additional Na+ binding site below 1 mM, the minimal subunit composition consistent with the kinetic evidence is a dimer.

In this study, Na+ uptake experiments were conducted over a range of Na+ concentrations below 1 mM in an attempt to identify the transport sites displaying high affinity Na+ binding in acid-loaded vesicles. In addition, uptake experiments were conducted at alkaline pH, in the absence of a H+ gradient to evaluate the mechanism of Na+ transport when the linear or steady state phase of accumulation is absent. The results obtained with acid-loaded vesicles are compatible with a tetrameric transport scheme in which the events comprising the first turnover are unique and are activated by the occupation of the cytoplasmic H+ regulatory site. The behavior predicted by this scheme provides a plausible explanation for the sensitivity of pre-steady state Na+ uptake to alterations in the membrane potential induced by a proton ionophore. These results have appeared previously in abstract form (15, 16).

**Experimental Procedures**

**Materials—**[2Na]NaCl was supplied as the carrier-free isotope by Du Pont-New England Nuclear at a specific activity of 3.7-37 GBq/ mg Na+. Amiloride hydrochloride was obtained form Merck Sharp & Dohme and carbonyl cyanide-p-(trifluoromethoxy)phenylhydrazone (FCCP) from Chemical Dynamics Corp. The computer program used for simulating the time course of Na+ accumulation (MLAB) is available from Civilized Software, Inc. (Bethesda, MD).

**Membrane Isolation—**BBMV were isolated from rabbit kidney cortex by the Mg2+ aggregation method described previously (17, 18). Virtually 100% of the membrane vesicles prepared by this method are in the right-side-out configuration. The final membrane pellet was washed in Mg2+-free buffer containing 150 mM KCl, 10 mM Tris, 16 mM HEPES, pH 7.5. The freshly prepared vesicles (10-50 mg/ml) were incubated for 90 min at 20 °C in the same buffer adjusted with MES to the final pH (5.7 or 7.7). Protein concentration was determined by the method of Lowry et al. (19) with bovine serum albumin as standard.

**Na+ Uptake Assay—**Each experiment was performed at least three times using separate fresh membrane preparations. Na+ uptake was measured at 0 °C in an incubation volume of 50 μl containing 250-500 μg of protein and 3.7-7.4 MBq of [2Na]NaCl. 25 μl of the preincubated vesicles (pH 5.7 or 7.7) were placed on the side of an alumin-coated glass tube (13 x 100 mm). The bottom of the tube held 25 μl of the reaction medium which contained 150 mM (Na+ plus K+)-Cl, 16 mM HEPES, and sufficient Tris to adjust the pH of the incubation medium to 7.7 after mixing the solutions. To maintain the incubation temperature at 0 °C, the reaction tube was placed inside of a double-walled insulating tube which contained 2 ml of ice-cold water as described previously (13). Uptake of [2Na]Na+ was initiated by agitating the tubes with a vortex mixer and terminated after a brief interval by the rapid manual addition of 3 ml of an ice-cold quench solution consisting of 150 mM KCl, 16 mM HEPES, and 0.1 mM amiloride hydrochloride. The incubation period, which was allowed to vary between 0.5 and 15 s, was timed with a metronome. The membrane vesicles were immediately harvested on a Millipore filter (0.65 μm) and rinsed with 9 ml of the cold quench solution. The amiloride-insensitive Na+ uptake was subtracted from each sample. Amiloride-insensitive [2Na]Na+ uptake, representing Na+ uptake by passive diffusion, was determined in a separate experiment in which 1 mM amiloride was present in the incubation medium. Amiloride-sensitive [2Na]Na+ uptake due to the Na+-H+ exchanger was obtained by subtracting the linear amiloride-insensitive component from the total [2Na]Na+ uptake measured in the absence of amiloride (13, 14).

In experiments measuring the effects of membrane potential on Na+-H+ exchange, the assay conditions were the same as described above except that 80 μM FCCP in ethanol was added to the membrane suspension. When acid-loaded vesicles (pH 5.7) treated with this H+ ionophore were added to a medium adjusted to a final pH of 7.7, the resulting loss of H+ will rapidly generate an inside-negative membrane potential (1, 2, 18, 20). As a control for these experiments, BBMV were incubated with ethanol alone at a concentration of 2%.

**Computer Simulations—**The differential equation solving program and curve-fitting routines contained in MLAB (21) were used to simulate the time course of Na+ accumulation by the Na+-H+ exchanger. This involved selecting a model and an initial set of kinetic parameters defining the transport site density [C], and rate constants for the forward (k+) and reverse (k-) reactions in the transport cycle. In selecting a model, we assumed that the burst phase represents the initial turnover of the exchanger and that the countertransport mechanism obeys ping-pong kinetics (14). Mechanism I will accommodate the multiphasic time dependence of Na+ accumulation but contains too many undefined variables to be of practical use in modeling the time-dependent behavior. Consequently, a simplified scheme (Mechanism II) was chosen which includes the necessary features for simulating uptake in the pre-steady state.

**Mechanism II**

Parentheses around the ligands indicate occluded states. The approximations needed to construct a four-state model of this mechanism are embodied under "Results." Rate constants for the partial reactions were chosen assuming that Na+ binding (step 2) is a rapid equilibrium and that H+ deocclusion (step 1) and H+ translocation (step 4) control the kinetics of Na+ uptake in the burst and linear phases, respectively. Accordingly, the rate constant for the forward reaction in step 1, κ+, was evaluated from the slope of the semilogarithmic plot of the burst phase (inset, Fig. 1A), whereas κ− was obtained by dividing the steady state velocity by the approximate site density evaluated from the amplitude of the burst phase (Bo). Step 4 is a composite reaction that includes Na+ release, H+ binding, and H+ translocation. Because BBMV actively accumulating Na+ at 0 °C show no evidence of isotope backflux (14), we were able to treat Na+ translocation and Na+ release as irreversible reactions. A similar approximation was applied to the H+ translocation step on the grounds that the outwardly directed pH gradient and alkaline pH of the external medium (pH 7.7) favor proton extrusion and dissociation over re-association and re-uptake. Na+, release and H+, binding are fast reactions and therefore do not contribute to the rate limitation imposed by step 4, which determines the overall velocity of the exchanger.

The rate constants for the reactions controlling Na+ binding (κ+) and release (κ−) were assigned values based on the following reaction producing half-saturation of the burst amplitude, Bo. Between 0.1 and 1 mM Na+, saturation of the burst is half-maximal at 0.65 mM Na+ (Fig. 2A). To a first approximation \( K_{d1} = k_+ (1 + \text{Bo}) / k_− \) so that \( k_− = 650 \text{ s}^{-1} \) when \( \text{Bo} = 1 \times 10^3 \text{ molecules s}^{-1} \). In the range between 1 and 10 mM Na+, \( K_{d1} = 5 \text{ mM} (14) \) requiring that \( k_+ \) be assigned a larger value of 5000 s−1. Because the Na+ concentration in the incubation medium exceeds that of the exchanger, it will remain constant during the assay, allowing Na+ binding to be treated as a pseudofirst-order reaction. Using this approximation the rate con-
stant for Na+ binding becomes k0[Na+], which at 1 mM Na+ is 1000 s⁻¹. The forward rate constant for Na+ translocation, k0, is inversely related to the duration of the lag phase (see "Results") and was evaluated by fitting Mechanism II to the pre-steady state time course of amiloride-sensitive Na+ uptake. The transport site density was evaluated from the burst amplitude (Bb). The latter, which was obtained by extrapolation of steady state Na+ uptake to zero time, approximates the concentration of active exchangers participating in the initial turnover event.

Optimization of the model parameters was achieved by application of the curve-fitting routines in MLAB and by visual inspection of the simulations. The visual approach was used to establish an initial set of values for the kinetic parameters which were subsequently refined through application of the Marquardt-Levenberg curve-fitting algorithm in MLAB (21). In making the final adjustments, Na+ binding and dissociation were assigned constant values, while allowing the remaining rate constants and transport site density to vary within preselected ranges. Convergence of the fit was evaluated by comparing the sum of the squared errors between the simulated and experimental data points in consecutive curve-fitting operations.

RESULTS

Time Course of 22Na+ Accumulation—For the determination of the kinetics of 22Na+ uptake in BBMV, the temperature of the incubation medium was maintained at 0°C. Under these conditions, pre-steady state 22Na+ uptake by the system is slow enough to be measured by hand using a metronome to time the incubation. Quenching was accomplished by the addition of 100 μM amiloride, which prevents further Na+ uptake as well as Na+ efflux (13). Transport via the Na+-H+ exchanger was expressed as amiloride-sensitive Na+ uptake, which was obtained by subtracting the amiloride-insensitive activity from the total Na+ uptake measured in the absence of amiloride (see "Experimental Procedures").

The characteristic time dependence of 22Na+ accumulation by BBMV at 0.1 mM Na+ is shown in Fig. 1A. In this experiment, the membrane vesicles were preincubated in pH 5.7 medium to allow the equilibration of protons in the intracellular compartments. The vesicles were then diluted into a medium containing 22Na+ adjusted so that the final pH after mixing was 7.7. The time course of Na+ uptake exhibited a lag phase (<1 s duration) followed by a rapid phase of accumulation referred to as the burst. After 4–5 s, there appeared a slower linear phase of activity representing steady state Na+ accumulation. Extrapolation of this linear phase to zero reaction time gave a positive intercept on the y axis defined as the burst amplitude, Bb (Fig. 1A). This parameter was used to estimate the amount of Na+ uptake occurring during the burst phase. When Na+ uptake in the burst phase was subtracted from the extrapolated steady state, a semilogarithmic plot of the remaining time points fell on a straight line after an initial downward deflection (inset, Fig. 1A). This initial behavior is due to the presence of a lag phase which precedes rapid Na+ uptake during the monoexponential burst phase. The slope of the line following the deflection gives the apparent rate of the burst phase, which in this experiment was 0.7 s⁻¹. In a separate experiment, amiloride-insensitive 22Na+ uptake measured in the presence of 1 mM amiloride (not shown) displayed a linear increase with time during the 15-s incubation interval. Typically, the amiloride-insensitive component represented less than 10% of the total activity measured in the steady state.

For comparison, Fig. 1B shows a similar uptake experiment using the same preparation but at a Na+ concentration of 1 mM. The 10-fold increase in [Na+] gave a proportional increase in the burst amplitude (0.006 versus 0.07 nmol of Na+/mg of protein) and steady state velocity (0.0096 versus 0.095 nmol of Na+/mg/s), whereas the lag duration and apparent burst rate did not appear to change (inset, Fig. 1A). These effects of Na+ are identical to those reported previously at higher (1–5 mM) Na+ concentrations and indicate that the transport reactions controlling the lag and burst phases obey first-order kinetics.
Effect of [Na+] on Burst Amplitude and Steady State Velocity—Our previous studies demonstrated that the burst amplitude has a sigmoidal dependence on [Na+] in the 1–10 mM range (13). When the plot of burst amplitude versus [Na+] is examined carefully, extrapolation of the curve to zero [Na+] yields a positive intercept on the y axis (cf. Fig. 6B), suggesting that the exchanger has a second class of Na+ binding sites with a K0 less than 1 mM. To investigate this possibility, we performed a series of experiments between 0.1 and 1 mM Na+ in which the time course of amiloride-sensitive *Na+ accumulation was measured in acid-loaded vesicles (pH 5.7) diluted into an alkaline medium (final pH, 7.7). The burst amplitude and steady state velocity were evaluated at each Na+ concentration from the time course of amiloride-sensitive Na+ uptake during the linear phase of activity. The kinetic data gathered from three separate experiments using different membrane preparations are presented in Figs. 2, A and B. To facilitate comparison of these results, the burst amplitudes were normalized to the maximum value in each data set. In response to raising [Na+], the burst amplitude increased sigmoidally (Fig. 2A), beginning at the origin and reaching half-saturation at about 0.65 mM. The maximum value of Bo, which varied between 0.06 and 0.08 nmol/mg of protein, was approximately 16% of the maximum value obtained between 1 and 10 mM Na+ (13). The sigmoidal pattern of Na+ activation in Fig. 2 suggests that this preparation contains at least two high affinity Na+ binding sites in addition to the low affinity (K0,5 = 4–5 mM) sites reported previously (13). However, additional evidence is needed to confirm this hypothesis (see below), because a sigmoidal dependence of Bo on [Na+] can also occur in the case where only a single Na+ binding site is present (22).

Fig. 2B shows a double-reciprocal transformation of the steady state velocity of Na+ uptake versus [Na+] taken from the same set of experiments plotted in Fig. 2A. The linearity of this plot is consistent with binding of Na+ to a single saturable transport site, in agreement with the Michaelis-Menten behavior detected at higher Na+ levels (13). The apparent Km and Vmax for Na+, calculated from the x and y axis intercepts of this linear transformation, were 6.5 mM and 0.5 nmol/mg/s, respectively. These values are not appreciably different from those obtained at higher Na+ concentrations (K0,5 = 6 mM; Vmax = 0.4 nmol/mg/s; Ref. 13), indicating that the exchangers corresponding to the high and low affinity Na+ binding sites have similar steady state properties.

Effect of [Na+] on Na+ Uptake at Alkaline pH—In addition to acting as substrate, H+ can activate Na+-H+ exchange by binding to a modifier site on the cytoplasmic (internal) surface of the transport protein (6, 10). At alkaline pH (pH 7.7), this site becomes unoccupied, resulting in the time course of amiloride-sensitive Na+ uptake shown in the inset to Fig. 3. Compared with the behavior seen in acid-loaded vesicles (cf. Fig. 1B), Na+ uptake in the steady state was completely abolished, whereas the lag duration (~0.5 s) and the apparent burst rate (0.5 s–1) did not appear to be significantly altered. Na+ accumulation during the burst phase can be eliminated by treatment with gramicidin (13, 14), excluding binding to external sites on the membrane as a possible explanation for the burst. Taken together, these results suggest that desaturation of the internal H+ modifier site leads to termination of Na+ uptake after the exchanger has completed the first turnover (14).

Our previous analysis of the effects of [Na+] on the activity of the Na+-H+ exchanger in acid-loaded vesicles indicated that more than one Na+ transport site is occupied during the burst phase (13). To determine whether the internal H+ modifier site has any effect on this behavior, we re-investigated the Na+ dependence of the burst phase under alkaline loading conditions, restricting our measurements to the range of Na+ concentrations >0.5 mM. As seen in Fig. 3, a double-reciprocal transformation of the change in burst amplitude versus [Na+] was linear, indicative of a simple (hyperbolic) dependence of the burst amplitude on [Na+]. The Na+ concentration yielding 50% saturation of the burst amplitude evaluated from this plot was 6.13 mM, which is similar to the value reported previously in acid-loaded vesicles (2, 4, 5, 13). In contrast, the transport site density as reflected in the burst amplitude at saturating Na+ was 0.47 nmol/mg of protein, which is approximately one-half the saturation value expressed in acid-loaded BBMV (1 nmol/mg of protein). These results suggest that occupation of the internal H+ modifier site is essential for both the cooperative binding interaction between the Na+ transport sites and the optimal expression of those sites in the pre-steady state phase of Na+ uptake in acid-loaded BBMV.
Effect of Membrane Potential on Na⁺ Uptake—Evidence that Na⁺-H⁺ exchange in renal brush border membranes is electroneutral is based on experiments showing that steady state Na⁺ uptake at 20 °C is insensitive to an imposed membrane potential (1, 2, 23). At 0 °C, activation of the burst phase involves more than one Na⁺ transport site (13), implying that the stoichiometry of the exchanger is greater than 1:1 during the initial turnover. If protons are unable to fully compensate for Na⁺ movement during the pre-steady state, this could lead to the accumulation of positive charge inside the vesicle. To investigate this possibility, we examined the effect of membrane potential on the transport activity of the exchanger by treating the vesicles with the proton ionophore FCCP. In the presence of this ionophore, the outwardly directed pH gradient present during Na⁺ uptake will generate a membrane potential which is negative on the inside. Fig. 4 shows an experiment conducted at 0 °C in which FCCP-treated vesicles equilibrated with pH 5.7 buffer were diluted into a medium containing 1 mM [³²⁵Na⁺] at pH 7.7 (final pH). Compared with a control experiment in which FCCP was absent, the ionophore-treated vesicles showed an enhanced level of Na⁺ uptake during the burst phase (0.215 versus 0.11 nmol Na⁺/mg). Notably, no further increase over the control activity was detected during the subsequent linear phase, in agreement with the electroneutral behavior reported at 20 °C (1, 2, 21). In addition, no significant changes were observed in either the lag duration or the apparent burst rate (inset, Fig. 4). The unique electrical behavior demonstrated by these experiments point to a charge imbalance during the first turnover of the exchanger, which may result from unequal pre-steady state fluxes of Na⁺ and H⁺.

Computer Simulation of Na⁺ Uptake—The time dependence of Na⁺ uptake exhibits two relaxation events, a lag and a burst phase, which implies that there are at least three slow steps in the exchange cycle (24). Mechanism I satisfies this criterion, because it includes a slow Na⁺-induced conformational transition (step 4) in addition to the slow translocation reaction (steps 1 and 5), which control the burst rate and overall velocity, respectively. A disadvantage of this scheme is that several of the rate constants are unknown, limiting its usefulness in modeling Na⁺ uptake by the exchanger. Therefore, we resorted to a simplified scheme (Mechanism II) which reproduced the basic features of the time course of amiloride-sensitive Na⁺ uptake. Mechanism II predicts a simple competitive relationship between Na⁺ and H⁺ binding at the external membrane surface but differs from Mechanism I in placing the slow transition controlling the burst rate (step 1) before Na⁺ binding. This slow transition represents H⁺ release from an occluded conformation, which occurs in two steps (deocclusion and dissociation), but is treated as a single irreversible slow event. Further simplification was achieved by condensing internal Na⁺ release, H⁺ binding and translocation into a single irreversible reaction (step 4), which includes the rate-limiting step in the exchange cycle. Reversal of this step is prevented by the acidic pH of the intravesicular compartment, which favors H⁺ binding following Na⁺ release.

Initial values for the parameters used in these simulations were evaluated from the pre-steady state and steady state kinetic data as described under “Experimental Procedures.” At the time Na⁺ is added, the exchanger is poised in C₁(H), which accumulates as a result of the acidic conditions (pH 5.7) on both sides of the membrane and occupation of the internal modifier site by H⁺ (14). Dilution of the vesicles into an alkaline medium (pH 7.7) will induce H⁺ dissociation and allow Na⁺ to bind, activating the exchange cycle in the clockwise direction. When amiloride is added, it binds to C₂, preventing further uptake by forming a dead-end complex. Na⁺ sequestered by the exchanger equals the Na⁺ trapped inside the lumen plus whatever additional Na⁺ is released from carrier intermediate states after quenching. This includes Na⁺ bound to C₃Na, which is quantitatively released to the inside because of the irreversible nature of Na⁺ translocation under our experimental conditions (14). Na⁺ associated with C₁Na, on the other hand, will be released to the outside after quenching, because this complex binds Na⁺ weakly (Kₘ = 5 mM) and, by implication, has a large dissociation rate constant.

The simulated time courses for CNa formation at 0.1 and 1.0 mM Na⁺ (curve 1) together with the corresponding time courses for Na⁺ uptake occurring subsequent to the first turnover (curve 2) are shown in Fig. 1A and B. The simulated activities combine to yield the measured Na⁺, uptake curve with its characteristic triphasic time dependence. The initial lag in Na⁺ uptake was sensitive to changes in the rate of Na⁺ translocation; increasing k₁ from 10 to 100 s⁻¹ shortened the duration of the lag so that it became undetectable (inset, Fig. 1B). A similar effect could be achieved by simply raising the [Na⁺] (not shown), which increases the apparent rate of Na⁺ translocation by removing the kinetic restriction imposed by Na⁺ binding in the preceding step. These results serve to identify the lag phase with the Na⁺ translocation reaction. The subsequent relaxation event, the burst, is determined by the kinetics of the H⁺ deocclusion reaction (step 1), which is only slightly faster (0.8 s⁻¹) than the step controlling the linear phase of uptake (0.5–0.7 s⁻¹). If k₂ is allowed to become much larger than k₃, the lag remains, but the burst disappears (not shown), because C₁Na breaks down much more rapidly than it is formed. Under these conditions, the transport cycle includes only two slow steps (k₁ and k₂), confirming that a minimum of three slow reactions are required to generate two relaxation events. Reducing k₃ relative to k₁ had the opposite effect of making the burst more prominent; when k₃ = 0, steady state Na⁺ uptake was completely abolished, resembling the situation in alkaline-loading vesicles (inset, Fig. 3).

We tested whether Mechanism II is able to reproduce the Na⁺ concentration dependence of the Na⁺-H⁺ exchanger by...
attempting to simulate the uptake curves shown in Fig. 1A and B (0.1 and 1 mM Na\(^+\)). Initially we tried varying just the pseudofirst-order rate constant for Na\(^+\) binding, \(k_0[Na\(^+\)]\), but found that the lag disappeared as the [Na\(^+\)] was increased from 0.1 to 1 mM (see above). In addition, the site concentration had to be changed while varying the [Na\(^+\)] in order to simulate Na\(^+\) accumulation during the burst phase. Fig. 5A shows simulations of Na\(^+\) uptake at 0.1 and 1 mM in which the transport site density was held constant. To ensure that there would be an adequate response to varying the [Na\(^+\)], the rate constant for Na\(^+\) dissociation, \(k_2\), had to be assigned a large value (2 \(\times\) 10\(^8\) s\(^{-1}\)), which satisfies the \(K_a\) for this reaction scheme (0.65 mM). Additional changes involved increasing \(k_3\) from 0.8 to 2 s\(^{-1}\) and decreasing \(k_4\) from 0.7 to 0.43 s\(^{-1}\). These adjustments gave a reasonably close fit to the time-dependent data at 1 mM Na\(^+\) but failed at the lower concentration (lower curve, Fig. 5A). The simulation at 0.1 mM Na\(^+\) could be improved in the linear phase of the uptake curve by artificially lowering the transport site density (Fig. 5B); however, the simulated burst was too slow. Further adjustments in \(k_2\), alone or in combination with the other rate constants (but maintaining a constant site density), were unable to overcome this difficulty. These results show that a single choice of rate constants could not be found for Mechanism II which would allow both the time and the [Na\(^+\)] dependence of the burst phase to be simulated.

It is apparent that Mechanism II will not accommodate the [Na\(^+\)] dependence of the exchanger without further modification. However, as demonstrated by the simulations in Fig. 1A and B, Mechanism II can be used to simulate the formation of the burst phase intermediate at different Na\(^+\) concentrations if the transport site density is allowed to vary. This adjustment is artificial because the site density should remain constant under all conditions. However, this approach for evaluating the Na\(^+\) stoichiometry of the burst phase is as valid as one based on a scheme in which the [Na\(^+\)] is varied at a constant site density. This is because the concentration of the intermediate(s) contributing to the burst phase at each point in time is uniquely defined by the temporal characteristics of the lag, burst, and steady state phases of Na\(^+\) uptake and is, therefore, independent of the model chosen.

The Na\(^+\) stoichiometry of the burst phase was determined by simulation of the burst phase intermediate, which accumulates to a maximum level defined by the number of Na\(^+\) transport sites and their strength of interaction (25). For a system with \(n\) interdependent and rapidly equilibrating Na\(^+\) binding sites, the steady state concentration of the burst phase intermediate, [C\(_i\)Na\(^+\)], is related to the Na\(^+\) concentration, [Na\(^+\)], by the following equation.

\[
[C_{iNa}] = A_1 \cdot [Na^+] / (1 + A_2 \cdot [Na^+] + \ldots + A_{n+} \cdot [Na^+]^n) \quad \text{(Eq. 1)}
\]

The \(A_i\) are constant terms that are functions of the specific rate constants used in the model. This derivation assumes that only the fully-ligated species is able to produce an active translocation complex and that pump reversal due to Na\(^+\) accumulation can be neglected. For \(n > 1\), this equation predicts a complex (nonhyperbolic) dependence of [C\(_i\)Na] on [Na\(^+\)]. It can be easily shown that a similar dependence on [Na\(^+\)] is predicted for the case in which more than one intermediate species contributes to the burst phase.

The formation of the burst phase intermediate, C\(_i\)Na, was evaluated at each of the Na\(^+\) concentrations in Fig. 2 by simulation of the time course of Na\(^+\) uptake according to Mechanism II. In fitting the data, the transport site density was allowed to vary in conjunction with \(k_0[Na^+]\) (reflecting the change in [Na\(^+\)]). These constraints gave a fairly close approximation to the time- and concentration-dependent behavior; however, a much better fit was obtained by allowing \(k_4\) to also vary, but inversely with the [Na\(^+\)]. The simulated burst phase intermediate levels, which are plotted in Fig. 6A, reveal that [C\(_i\)Na], like the burst amplitude, \(B_0\), is sigmoidally dependent on [Na\(^+\)]. Fig. 6B, which includes data between 1 and 10 mM Na\(^+\), shows the [Na\(^+\)] dependence of [C\(_i\)Na] and \(B_0\) plotted over a 50-fold concentration range. Both curves are biphasic, rising initially between 0.2 and 1 mM Na\(^+\) to an intermediate plateau and then increasing again between 2 and 10 mM Na\(^+\). By fitting [C\(_i\)Na] to a two-term Hill equation with Na\(^+\) as the independent variable, we were able to estimate the number of Na\(^+\) binding sites contributing to each phase. The results of this fit, which are summarized in Table I, reveal that there are multiple Na\(^+\) binding sites in each class and

\[K_a = (k_3-k_4)/k_3 = k_2/k_3 = k_4/k_3 + k_3/k_4 = k_4/k_3 \quad \text{(Eq. F1)}\]

For \(k_3 = 0.8 \text{ s}^{-1}\), \(k_2 = 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\), \(k_4 = 500 \text{ s}^{-1}\), \(k_0 = 10 \text{ s}^{-1}\), and \(k_4 = 0.7 \text{ s}^{-1}\), \(K_a = 0.018 \text{ M}\). By increasing \(k_4\) from 500 to 20,000 s\(^{-1}\), \(k_3\) from 0.8 to 2 s\(^{-1}\), and decreasing \(k_0\) from 0.7 to 0.4 s\(^{-1}\), then \(K_a = 0.645 \text{ M}\).

**Figure 5. Kinetic modeling of Na\(^+\) uptake by BBMV at 0.1 and 1 mM Na\(^+\).** A, the data are re-plotted from Fig. 1, A (O) and B (●). Smooth curves through the data points are simulations corresponding to the sum of C\(_i\)Na and Na\(^+\), in Mechanism II. Rate constants were assigned the following values (in s\(^{-1}\) M\(^{-1}\)): \(k_1/k_2 = 2/0\); \(k_1[Na^+]k_2 = 100/20,000\) (at 0.1 mM Na\(^+\)) or 1000/20,000 (at 1 mM Na\(^+\)); \(k_4/k_3 = 10/0\); \(k_4/k_3 = 9.43/0.018\) M. Initially, [C\(_i\)H] = 0.47 nmol/mg of protein, and all other intermediate concentrations were equal to zero. B, the data are re-plotted from Fig. 1A (0.1 mM Na\(^+\)). Kinetic parameters were the same as above except that [C\(_i\)K] = 0.245 nmol/mg of protein and \(k_4 = 0.25 \text{ s}^{-1}\). Curves 1 and 2 and the curve approximating the data points are simulations corresponding to the formation of C\(_i\)Na and Na\(^+\), and their sum, respectively.
Sodium Dependence of the Na\(^+\)-H\(^+\) Exchanger

**FIG. 6.** Sodium ion concentration dependence of the burst amplitude \((B_0)\) and the burst phase intermediate \([C,Na]\). \(A, \text{[Na}\(^+\)]\) range: 0.2-1.0 mM. The data are re-plotted from Fig. 2A using the average value for \(B_0\) (O), \([C,Na]\) (●) was evaluated at each Na\(^+\) concentration by simulation of amiloride-sensitive Na\(^+\) uptake according to Mechanism II. The transport site density (0.034-0.22 nmol/mg of protein), \(K_{Na}^0\) (200-1000 s\(^{-1}\)) and \(k_{t} (0.72-0.341 s\(^{-1}\))\) were allowed to vary. Other rate constants were assigned the following values (in s\(^{-1}\)): \(k_1 = 0.8, k_{-2} = 0, k_{-2} = 650, k_5 = 12.5, k_3 = 0\). \(B, \text{[Na}\(^+\)]\) range: 0.2-10 mM. Different membrane preparations were used to generate the results in the concentration ranges 0.2-1 mM and 1-10 mM Na\(^+\). To construct the composite curve, data points between 0.2 and 1 mM Na\(^+\) were re-plotted from \(A\) after multiplying \(B_0\) and \([C,Na]\) by a factor of 1.23 to correct for the difference in uptake activities at 1 mM Na\(^+\). \([C,Na]\) was evaluated at each \([Na]\) between 1 and 10 mM as described in \(A\) and by allowing the transport site density (0.16-1.03 nmol/mg of protein), \(K_{Na}^0\) (1000-10,000 s\(^{-1}\)) and \(k_5 (0.49-0.26 s\(^{-1}\))\) to vary. Other rate constants were assigned the following values (in s\(^{-1}\)): \(k_1 = 0.8, k_{-1} = 0, k_{-2} = 5000, k_{-2} = 10, k_{-3} = 0\).

**TABLE 1**

Stoichiometry of the Na\(^+\) transport sites

The stoichiometry of low and high affinity Na\(^+\) transport sites was determined by modeling the Na\(^+\) dependence of \([C,Na]\) in Fig. 6 with a two-term Hill equation using MLAB: \(A\cdot[Na]^n\cdot\text{[K}_{Na}^0]^{-m} + B\cdot[Na]^n\cdot\text{[L}_{Na}^0]^{-m}\cdot[Na]^n\cdot\text{[E]}^{-m}\). \(n\) and \(m\) represent the minimum number of Na\(^+\) binding sites in the low and high affinity classes, respectively. Initial constraints used in fitting the data were as follows: \(A = 0.05-0.2 \text{ nmol/mg of protein}; B = 0.5-1.5 \text{ nmol/mg protein}; K_{Na}^0 = 0.2-1.0 \text{ mM}; L_{Na}^0 = 1-10 \text{ mM}; n = 1-5; m = 1-5\). The solution was allowed to converge to a constant minimum value of the best sum of weighted squares \(S\). Parameters given on the second line of the table were obtained by fitting the data in the 0.2-1 mM Na\(^+\) range using a one-term Hill equation without constraints.

| \([\text{A}]\) | \([\text{B}]\) | \(K_{Na}^0\) | \(L_{Na}^0\) | \(n\) | \(m\) | \(S\) |
|---|---|---|---|---|---|---|
| \(\text{nmol/mg protein}\) | \(\text{mM}\) | \(\times 10^4\) | | | | |
| 0.14 | 1.09 | 0.59 | 5.64 | 4.59 | 2.45 | 2.61 |
| 0.42 | 1.23 | 2.13 | 2.13 | 0.33 | | |

that both their transport activities and apparent Na\(^+\) affinities differ by a factor of 10. There appeared to be more sites in the high affinity class (\(n = 4-5\)) than in the class with lower affinity (\(n = 2-3\)); however, this could be due to a complexity in the Na\(^+\) binding mechanism not included in the model. One possibility is that both classes of sites originate on a single molecular species and that inhibitory interactions produced by specific patterns of Na\(^+\) binding terminate the single molecular species and that inhibitory interactions prevent the full expression of the high affinity sites, creating an artificially steep Na\(^+\) dependence (see "Discussion").

**DISCUSSION**

Previous investigations of the mechanism of the renal brush border Na\(^+\)-H\(^+\) exchanger have focused on the steady state kinetic behavior measured at room temperature (4, 9-11). In this study, the reaction temperature was reduced to 0 °C, enabling us to resolve the pre-steady state time course by means of a manual mixing technique. In acid-loaded (pH 5.7) BBMV, Na\(^+\) uptake during this initial phase consisted of a brief lag followed by a monoeponential burst phase, which corresponds to the initial cycle of Na\(^+\) translocation (14). Incrementally raising the Na\(^+\) concentration over a 100-fold range (0.1-10 mM) produced a biphasic increase in the burst amplitude (Fig. 6), reflecting the presence of two separate classes of Na\(^+\) transport sites, but had no effect on the lag duration or apparent burst rate (inset, Fig. 1A). Kinetic modeling of the time and concentration dependence of Na\(^+\) uptake during the burst phase revealed that each class contained multiple Na\(^+\) binding sites that interact cooperatively. In contrast, the linear phase of Na\(^+\) uptake following the burst obeyed simple Michaelis-Menten kinetics with evidence for only a single class of Na\(^+\) transport sites (\(K_m = 6 \text{ mM}\)). Another unique characteristic of the burst phase was its sensitivity to an inside-negative membrane potential, which enhanced Na\(^+\) uptake (Fig. 4). Na\(^+\) accumulation subsequent to the burst phase was unaffected by changing the membrane potential, in agreement with the electroneutral behavior observed at 20 °C (1, 2, 20, 23). The unique Na\(^+\) dependence and potential sensitivity manifested by the burst phase at 0 °C are difficult to reconcile with current models of the Na\(^+\)-H\(^+\) exchanger based on steady state kinetic measurements conducted at higher temperatures (4, 9-11). The transition from a complex to a simple Na\(^+\) dependence is, however, strongly reminiscent of the kinetic behavior associated with multimeric flip-flop enzymes (26, 27), suggesting that there may be a common structural basis for these effects.

Our unsuccessful attempts to simulate Na\(^+\) uptake at different Ne\(^+\) concentrations using a simple four-state model which reproduced the time-dependent features of Na\(^+\)-H\(^+\) exchange (Mechanism II) uncovered behavior requiring modification of our previous scheme (Mechanism I). First, the order in which Na\(^+\) binding occurs in relation to the reactions responsible for the lag and burst phases was critical in deter-
mining the simulated response to variations in the [Na\(^+\)]. Placing Na\(^+\) binding just before the lag-producing step (Na\(^+\) transport) resulted in the disappearance of the lag at high [Na\(^+\)] (inset, Fig. 1B). In contrast to the lag, the apparent burst rate was independent of Na\(^+\) over the same concentration range, ostensibly because the rate of the burst-controlling step (H\(^+\) deocclusion) is much slower than the kinetics of Na\(^+\) binding. Because the experimentally determined lag duration and burst rate showed no tendency to vary with the [Na\(^+\)] (inset, Fig. 1A), it is apparent that neither of the reactions controlling these events is immediately preceded by Na\(^+\) binding. The sequence in Mechanism I is incompatible with this requirement, because it places the first-order transition corresponding to the lag (step 4) just after the Na\(^+\) binding step. Since Na\(^+\) and H\(^+\) directly compete for the free carrier (14), it would appear that the lag-producing step, as well as that controlling the burst phase, are situated upstream from Na\(^+\) binding in the H\(^+\) translocation loop of the exchange cycle.

An additional feature of the exchange mechanism uncovered by our simulations was the failure of Mechanism II to reproduce the behavior of the burst phase over a 10-fold Na\(^+\) concentration range (Fig. 5). By allowing the transport site density to vary together with the rate constant controlling the overall velocity (k\(_b\)), we were able to use Mechanism II to evaluate the [Na\(^+\)] dependence of the burst phase intermediate, C\(_{Na}\), and show that its steady state level is sigmoidally dependent on [Na\(^+\)] (Fig. 6). This nonhyperbolic dependence on Na\(^+\) implies that more than one Na\(^+\) transport site has to be occupied in order to activate the first cycle of Na\(^+\) accumulation by the exchanger (25). This sigmoidal relationship was observed at both the high (K\(_{Na} = 0.65 \text{ mM}\)) and low affinity (K\(_{Na} = 6 \text{ mM}\)) Na\(^+\) transport sites but was absent from the overall velocity, which shows a simple (hyperbolic) dependence on [Na\(^+\)] (Fig. 2B). Within the context of Mechanism II, the absence of sigmoidicity in the steady state velocity can be attributed to a Na\(^+\)-dependent shift in k\(_b\), which occurs in a direction opposite to the change in the steady state level of C\(_{Na}\). As discussed below, this may result from alternation of the transport sites in a polymeric exchanger as opposed to a true decline in the turnover rate.

Explanations for the complex Na\(^+\) dependence of the burst phase involving hysteresis, binding to a separate allosteric site, and random bi-substrate addition have been discussed elsewhere and excluded on the grounds of incompatibility with the observed kinetic properties of the exchanger (13, 14). An important clue is the mechanism underlying the complex behavior of the initial turnover is provided by the paradoxical disappearance of these effects in subsequent transport cycles. In an earlier report (13) we proposed that this discrepancy might be explained by a flip-flop scheme in which the functional transport unit is a dimeric protein comprised of identical subunits that alternate between Na\(^+\) and H\(^+\) transport. The sequence of events representing the initial turnover of the Na\(^+\)-H\(^+\) exchanger behaving according to a flip-flop mechanism is shown in Mechanism III.

The central features of this scheme are as follows. 1) The subunits communicate by means of conformational coupling; 2) Na\(^+\) binding at one subunit induces a conformational change that enhances the affinity of Na\(^+\) binding at the second subunit; 3) binding of the second Na\(^+\) ion triggers the translocation of Na\(^+\) bound to the first subunit; 4) carrier recycling couples H\(^+\) translocation in the first subunit to Na\(^+\) translocation in the second subunit. This initial sequence leads to the uptake of two Na\(^+\) ions and the loss of one H\(^+\), with a net gain of one positive charge. In subsequent cycles, the sites alternate in an out-of-phase fashion (steps 7 and 8) so that the Na\(^+\) and H\(^+\) movements exactly balance each other.

\[
\text{C}_\text{Na} + \text{H}^+ \rightarrow \text{C}_\text{Na}^+ + \text{H}_\text{Na}\]

(7)

\[
\text{C}_\text{Na}^+ + \text{H}^+ \rightarrow \text{C} + \text{H}_\text{Na}\]

(8)

**Mechanism III**

As a consequence, electroneutrality is maintained, and the Na\(^+\) dependence of the overall velocity obeys Michaelis-Menten kinetics. It should be noted that the conformational constraint which limits the expression of subunits to alternate cycles can appear as a decline in the turnover number when modeling the time-dependent kinetic data with a monomeric scheme. This is explained by the fact that the overall velocity is proportional to the concentration of simultaneously available active sites as well as the turnover rate per exchanger.

The biphasic distribution of Na\(^+\) binding sites in Fig. 6 suggests that our preparation may contain more than one active species. That this behavior might be due to a mixed population of inside-out and right-side-out vesicles combined with asymmetric Na\(^+\) binding can be excluded on the grounds that the amiloride binding site inhibiting Na\(^+\) flux is located exclusively on the extracellular surface of the brush border membrane (9, 28). An intracellular amiloride binding site has recently been identified in BBMV (29); however, amiloride binding to this site has no effect on Na\(^+\)-H\(^+\) exchange activity.

A second possibility involves contamination of BBMV by basolateral membranes which could lead to co-expression of the renal brush border and housekeeping Na\(^+\)-H\(^+\) exchangers (30, 31). Although these transporters demonstrate dissimilar apparent affinities for Na\(^+\) (32, 33), the housekeeper typically shows activation at relatively high Na\(^+\) concentrations (K\(_s\) = 25 mM) and a sensitivity toward amiloride in the nanomolar range, which we failed to detect in our preparation. Different isoforms of the Na\(^+\)-H\(^+\) exchanger might also account for the biphasic pattern of Na\(^+\) activation in the pre-steady state, assuming that each isoform is a multimeric protein with interdependent Na\(^+\) binding sites. If so, this kinetic heterogeneity should also appear in the steady state [Na\(^+\)] dependence, whereas we found similar values for the K\(_s\) and V\(_{max}\) at both the high (0.1–1 mM) and low affinity (1–10 mM) Na\(^+\).

*Evidence that these binding sites are exclusively involved in Na\(^+\) transport, as opposed to representing both transport and allosteric binding sites, derives from experiments with gramicidin (14), showing that the ionophore completely abolishes Na\(^+\) uptake in the burst phase.*
binding sites. This argues in favor of a single active oligomeric species, since it would be unlikely to have two multimeric isoforms with identical steady state behavior.

Analysis of the number of Na⁺ binding sites in each class by means of the Hill equation suggested that each functional transport unit is comprised of four to eight protomers (Table 1). The upper limit reflects the larger number of transport sites in the high affinity class, which exceeded those in the low affinity class by more than two to one. Although this difference could be real, it could also result from failure of the model to include inhibition at low Na⁺ levels, which could terminate the activation of Na⁺ transport at the high affinity sites. In an oligomer, the conformational coupling mechanism may impose physical constraints which require a specific pattern of transport site loading to generate an active state. An example of this might be a tetramer in which only the diagonally adjacent Na⁺ binding sites are able to form an active dimeric complex, giving rise to the activity observed at low Na⁺ concentrations. Formation of the inactive complexes, which statistically outnumber the active species, would tend to limit the rise in Na⁺ uptake, producing a titration curve with an artificially steep Na⁺ dependence. In agreement with this, when we allowed the maximal transport capacity of the high affinity sites to increase without constraint (equivalent to removing the inhibition caused by inactive complexes), the estimated number of high affinity transport sites fell from five to two (Table 1). Additional evidence in support of this model derives from radiation inactivation studies by Beliveau et al. (34), who estimated the functional molecular size of the Na⁺-H⁺ exchanger to be 321 kDa. Assuming that the polypeptide chains for the housekeeper and brush border exchangers have similar molecular weights (29, 30), their inactivation results indicate a subunit composition compatible with a tetramer.

A plausible molecular scheme for a tetrameric exchanger based on the alternation-of-sites concept is depicted in Fig. 7. In this representation, the exchanger is a homotetramer comprised of four distinct, conformationally coupled states. During steady state operation, only two of the four subunits are simultaneously engaged in Na⁺ and H⁺ translocation, whereas the remaining two are poised for ligand binding. This arrangement ensures that the ion fluxes maintain electroneutrality during each cycle and that the ligand binding stoichiometry is limited to one Na⁺ (or H⁺) per tetramer. Activation of the exchanger involves two essential steps: 1) occupation of the internal H⁺ modifier site and functional oligomerization (see below) and 2) sequential Na⁺ binding to the four inter-active subunits. At low (<1 mM) Na⁺ levels, only two of these subunits are occupied leading to the formation of a partially active dimer representing about 10% of the total activity (Table I). Saturation of the subunits generates the fully active tetramer and activates the stepwise accumulation of four Na⁺ ions. The coupling rules stipulate that only one proton is pumped in the opposite direction during this initial phase, resulting in the accumulation of three positive charges. An important distinction between this model and the dimer (e.g. Mechanism III) involves the ratio of Na⁺ ions accumulated to protons ejected, which should be accessible to time-resolved studies of the initial H⁺ fluxes at 0 °C. Both models predict a pre-steady state H⁺ burst, but only in the case of the tetramer is an initial transport ratio of three Na⁺ to one H⁺ expected.

The tetrameric model for Na⁺-H⁺ exchange represents the behavior in acid-loaded vesicles in which the internal H⁺ modifier site is fully occupied (14). Na⁺ uptake under alkaline loading conditions is characterized by the absence of continuous cycling and a simple (Michaelis-Menten) dependence on [Na⁺] (Fig. 3). These characteristics suggest that the subunit interactions inferred from the kinetic behavior in acid-loaded vesicles are absent under alkaline conditions. The suggestion in Fig. 7 that the subunits are completely independent, structurally as well as functionally, may be oversimplified, because only half of the Na⁺ transport sites are expressed in the burst phase at saturating [Na⁺] when the internal pH is alkaline. By mass action, Na⁺ binding should activate the expression of all of the sites, and its failure to do so implies that some residual oligomeric structure is present when the internal H⁺ modifier site is unoccupied. By comparing the kinetics of Na⁺ uptake at alkaline pH with the behavior observed in acid-loaded vesicles, it is possible to identify specific features of the Na⁺-H⁺ exchange mechanism that are dependent upon the presence of quarternary protein interactions. Our results indicate, for example, that such interactions are not obligatory for Na⁺ transport, because the exchanger is able to complete one cycle of Na⁺ binding, translocation, and release before ceasing operation at alkaline pH (inset, Fig. 3). On the other hand, subunit-subunit interactions may indeed regulate the availability of the Na⁺ transport sites, because, as noted above, only half of the sites are expressed during the initial turnover in alkaline-loaded vesicles. Upon acidification of the vesicle interior, the linear phase of Na⁺ uptake occurring subsequent to the burst is restored together with the apparent cooperativity of Na⁺ binding in the pre-steady state. This suggests that communication between the polypeptide chains is essential for repetitive (steady state) cycling of the system. Because of the probable association of the linear phase of Na⁺ uptake with H⁺ efflux (14), it is likely that quarternary interactions are also required for the activation of H⁺ translocation. The switch controlling the functional aggregation of the system is the internal H⁺ modifier site (10), which pre-
sumably acts by inducing a conformational rearrangement in a pre-existing oligomer. Growth hormone (35, 36) and a variety of other agents (7) have been shown to influence the activity of the exchanger by producing a protein kinase C-dependent acid shift in the $pK_a$ for H$^+$ binding at the modifier site. It is tempting to speculate that activation of the exchanger resulting from this shift in $pK_a$ is mediated by a H$^+$-induced conformational interaction between adjacent subunits in an oligomer and that these interactions are involved in some crucial aspect of energy transduction.

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